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Cyanobacterial toxins: microcystins

Background document for development of WHO *Guidelines for drinking-water quality* and *Guidelines for safe recreational water environments*

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Preface

Access to safe drinking-water is essential to health, a basic human right and a component of effective policy for health protection. A major World Health Organization (WHO) function to support access to safe drinking-water is the responsibility "to propose ... regulations, and to make recommendations with respect to international health matters ...", including those related to the safety and management of drinking-water.

The first WHO document dealing specifically with public drinking-water quality was published in 1958 as *International standards for drinking-water*. It was revised in 1963 and 1971 under the same title. In 1984–1985, the first edition of the WHO *Guidelines for drinking-water quality* (GDWQ) was published in three volumes: Volume 1, Recommendations; Volume 2, Health criteria and other supporting information; and Volume 3, Surveillance and control of community supplies. Second editions of these volumes were published in 1993, 1996 and 1997, respectively. Addenda to Volumes 1 and 2 of the second edition were published in 1998, addressing selected chemicals. An addendum on microbiological aspects, reviewing selected microorganisms, was published in 2002. The third edition of the GDWQ was published in 2004, the first addendum to the third edition was published in 2006, and the second addendum to the third edition was published in 2008. The fourth edition was published in 2011, and the first addendum to the fourth edition was published in 2017.

The GDWQ are subject to a rolling revision process. Through this process, microbial, chemical and radiological aspects of drinking-water are subject to periodic review, and documentation relating to aspects of protection and control of drinking-water quality is accordingly prepared and updated.

Since the first edition of the GDWQ, WHO has published information on health criteria and other information to support the GDWQ, describing the approaches used in deriving guideline values, and presenting critical reviews and evaluations of the effects on human health of the substances or contaminants of potential health concern in drinking-water. In the first and second editions, these constituted Volume 2 of the GDWQ. Since publication of the third edition, they comprise a series of free-standing monographs, including this one.

For each chemical contaminant or substance considered, a background document evaluating the risks to human health from exposure to that chemical in drinking-water was prepared. The draft health criteria document was submitted to a number of scientific institutions and selected experts for peer review. The draft document was also released to the public domain for comment. Comments were carefully considered and addressed, as appropriate, taking into consideration the processes outlined in the *Policies and procedures used in updating the WHO guidelines for drinking-water quality* and the WHO *Handbook for guideline development*. The revised draft was submitted for final evaluation at expert consultations.

During preparation of background documents and at expert consultations, careful consideration was given to information available in previous risk assessments carried out by the International Programme on Chemical Safety, in its Environmental Health Criteria monographs and Concise International Chemical Assessment Documents; the International Agency for Research on Cancer; the Joint Food and Agriculture Organization of the United Nations (FAO)/WHO Meeting on Pesticide Residues; and the Joint FAO/WHO Expert Committee on Food Additives (which evaluates contaminants such as lead, cadmium, nitrate and nitrite, in addition to food additives).

Further up-to-date information on the GDWQ and the process of their development is available on the WHO website and in the current edition of the GDWQ.

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The work of the following experts was crucial in the development of this document and others in the second addendum to the fourth edition:

Dr M Asami, National Institute of Public Health, Japan
Dr RJ Bevan, independent consultant, United Kingdom
Mr R Carrier, Health Canada, Canada
Dr J Cotruvo, Joseph Cotruvo & Associates and NSF International WHO Collaborating Centre, United States of America
Dr D Cunliffe, South Australian Department of Health, Australia
Dr L D'Anglada, Environmental Protection Agency, United States of America
Dr A Eckhardt, Umweltbundesamt (Federal Environment Agency), Germany
Professor JK Fawell, Cranfield University, United Kingdom
Dr A Hirose, National Institute of Health Sciences of Japan
Dr A Humpage, University of Adelaide (formerly South Australian Water Corporation), Australia
Dr P Marsden, Drinking Water Inspectorate, United Kingdom
Professor Y Matsui, Hokkaido University, Japan
Professor CN Ong, National University of Singapore, Singapore
Dr E Testai, National Institute of Health, Italy

The draft text was discussed at expert consultations held on 14–16 July 2016, 28–30 March 2017 and 13–14 July 2018 on the second addendum to the fourth edition of the GDWQ or *Toxic cyanobacteria in water*. Input from all those who participated in these meetings is gratefully acknowledged. The final version of the document also takes into consideration comments from both peer reviewers and the public, as well as other experts who contributed to the text. These include S Barlow, independent consultant, United Kingdom; L Blaha, Masaryk University, Czech Republic; A Boobis, Imperial College London, United Kingdom; N Chernoff, Environmental Protection Agency, United States of America; I Chorus, formerly Federal Environment Agency of Germany; D Dietrich, University of Konstanz, Germany; I Falconer, University of Adelaide, Australia; J Fastner, Federal Environment Agency of Germany; A Foss, Greenwater Labs, United States of America; L Lawton, Robert Gordon University, Scotland; B Meek, University of Ottawa, Canada; and M Welker, independent consultant, Germany.

The coordinator was Ms J De France, WHO, with support from Dr V Bhat, formerly of NSF International, United States of America. Strategic direction was provided by Mr B Gordon, WHO. Dr A Tritscher, formerly of WHO, and Dr P Verger, WHO, provided liaisons with the Joint FAO/WHO Expert Committee on Food Additives and the Joint FAO/WHO Meeting on Pesticide Residues. Dr R Brown and Ms C Vickers, WHO, provided liaisons with the International Programme on Chemical Safety. Dr M Perez contributed on behalf of the WHO Radiation Programme. Dr Andina Faragher, Biotext, Australia, was responsible for the scientific editing of the document.

Many individuals from various countries contributed to the development of the GDWQ. The efforts of all who contributed to the preparation of this document are greatly appreciated.

Acronyms and abbreviations

Adda	(2*S*,3*S*,4*E*,6*E*,8S,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
ALF	alert level framework
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
bw	body weight
Cys	cysteine
Dhb	dehydrobutyrine
ELISA	enzyme-linked immunosorbent assay
GD	gestation day
GGT	gamma-glutamyl transferase
GSH	glutathione
GST	glutathione *S*-transferase
GV	guideline value
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
i.p.	intraperitoneal
i.v.	intravenous
LC-MS/MS	liquid chromatography tandem mass spectrometry
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LOAEL	lowest-observed-adverse-effect level
MC	microcystin
MC-LA	microcystin–leucine–alanine
MC-LF	microcystin–leucine–phenylalanine
MC-LR	microcystin–leucine–arginine
MC-LW	microcystin–leucine–tryptophan
MC-RR	microcystin–arginine–arginine
MC-YR	microcystin–tyrosine–arginine
Mdha	*N*-methyldehydroalanine
MMPB	2-methyl-3-methoxy-4-phenylbutyric acid
MRP2	multidrug resistance-associated protein 2
NOAEL	no-observed-adverse-effect level
OATP	organic anion transport protein
PoD	point of departure
PP1	protein phosphatase-1
PP2A	protein phosphatase-2A
PP5	protein phosphatase-5
ROS	reactive oxygen species
s.c.	subcutaneous

SOD	superoxide dismutase
TCiW	*Toxic cyanobacteria in water* (WHO guidebook)
TDI	tolerable daily intake
USA	United States of America
WHO	World Health Organization

Contents

Information on cyanobacterial toxins, including microcystins, is comprehensively reviewed in a volume to be published by the World Health Organization, *Toxic cyanobacteria in water* (TCiW; Chorus & Welker, in press). TCiW covers chemical properties of the toxins and information on the cyanobacteria that produce them, as well as guidance on assessing the risks of toxin occurrence, monitoring and management. In contrast, this background document focuses on reviewing toxicological information and other considerations for deriving guideline values for microcystins in water. Sections 1, 2 and 7 are largely summaries of chapters in TCiW, and readers are referred to corresponding chapters in TCiW for further information, including references to original publications.

Executive summary

MC and its variants (MCs) are naturally occurring cyclic peptides produced by strains of various species of cyanobacteria. One of the most common MCs, and the one most studied toxicologically, is MC-LR. MC-producing cyanobacteria are found primarily in freshwater environments, with a wide geographic and ecological distribution. As a result, MCs are the most commonly reported cyanobacterial toxins worldwide. Drinking-water is the most likely route of exposure to MCs. Recreational activities in lakes with cyanobacterial blooms may also be a relevant exposure pathway, potentially to high concentrations of MCs. Limited data suggest that MCs may also accumulate in some food items. Country- or region-specific assessments should take into account whether recreation or food (e.g. fish eaten with viscera or shellfish from bloom-ridden water bodies) may significantly contribute to exposure.

The main driver of high amounts of cyanobacterial biomass is nutrients from anthropogenic sources such as agricultural runoff and wastewater. Hence, control of these sources is the primary long-term management option. Drinking-water can usually be treated to acceptable levels by a well-run conventional treatment plant implementing coagulation, flocculation, filtration and chlorination; if this is not sufficient, ozonation and activated carbon filtration or addition of powdered activated carbon can be effective.

MCs are actively transported into cells by specific organic anion transport proteins (OATPs), leading to a tissue distribution based on the expression of these proteins. Because the liver has high expression of OATPs, MCs are considered to be primarily hepatotoxins, although distribution to other organs and tissues expressing OATPs does occur. Once in the cell, MCs bind with high affinity to certain protein phosphatases that are involved in a wide range of regulatory pathways, including those responsible for cytoskeletal structure, cell replication, stress responses and DNA repair. Acute effects of MC poisoning include intrahepatic haemorrhage as the cytoskeleton within liver cells breaks down, allowing blood to flow between the cells. Chronic effects include a reduced efficacy of proliferation control mechanisms within tumour cells.

The provisional guideline values (GVs) for MCs (lifetime drinking-water GV: 1 µg/L; short-term drinking-water GV: 12 µg/L; recreational water GV: 24 µg/L) are based on a study in mice using MC-LR, corroborated by a similar study in rats. The liver was identified as the most sensitive organ in these studies. Although some recent research has suggested that certain reproductive organs may be affected at lower concentrations, these reports are contradicted by earlier studies and so require corroboration. Although the GVs are based on studies of MC-LR, MCs generally occur as mixtures. Since MC-LR is one of the most common and potent MCs, comparison of the GVs with total MCs as gravimetric or molar equivalents is likely to be protective of public health in most circumstances.

The provisional short-term drinking-water GV is intended to indicate the extent to which the lifetime value can be exceeded for periods of up to 2 weeks until water treatment can be augmented to bring the concentration of MCs back under control. It is not intended to allow for repeated seasonal exceedances of the lifetime value. The short-term value is derived for adults. As a result of their higher water consumption per unit body weight, it is recommended, as a precautionary measure, that bottle-fed infants and small children be provided with an alternative water source if concentrations are greater than 3 µg/L, even for short periods.

1 General description

1.1 Identity

Microcystins (MCs) are cyclic peptides produced by a number of species of cyanobacteria. 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 (2*S*,3*S*,4*E*,6*E*,8S,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Fig. 1.1). The nodularins are a structurally related group of hepatotoxins. Primarily produced by *Nodularia spumigena*, they 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 D-amino acids, usually not found in ribosomally synthesized peptides, and these gave an early indication of a nonribosomal 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 variations exist in all seven amino acids; the most frequent are substitution of L-amino acids at positions 2 and 4, substitution of Mdha by dehydrobutyrine (Dhb) or serine at position 7, and a lack of methylation of amino acids at positions 3 and/or 7 (Fig. 1.1). The structural variations observed in Adda, although not frequent, can be of relevance, since they may affect analytical tests using Adda as a marker. The principle nomenclature of MCs is based on the variable L-amino acids at positions 2 and 4; for example, using the standard one-letter codes for amino acids, MC-LR contains L-leucine (L) at position 2 and L-arginine (R) at position 4. All other variations in the molecule are suffixed to the respective variant; for example, [D-Asp^3]MC-LR lacks the methyl group at position 3.

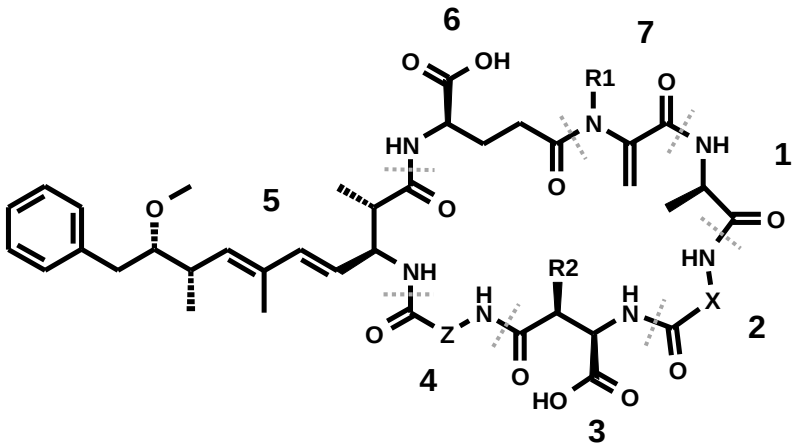

Fig. 1.1. Generic structure of microcystins

Notes: X and Z denote variable L-amino acids at positions 2 and 4. R1, R2, R3 and R4 are sites where common substitutions have been observed. 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.

1.2 Physicochemical properties

Known physicochemical properties of selected MCs and nodularin are summarized in Table 1.1. MCs and nodularins are extremely heat stable. They can have a range of solubilities

at different pH (de Maagd et al., 1999; Liang et al., 2011). Most MCs are hydrophilic. Using the Organisation for Economic Co-operation and Development test guideline no. 117 method, Santori et al. (2020) determined partition coefficients at pH 7 and pH 5, resulting in the following ranking from most to least polar: MC-RR, MC-YR, MC-LR, MC-LW, MC-LF. All structural variants are highly soluble in water, with saturation concentrations several orders of magnitude higher than the World Health Organization (WHO) guideline values (GVs; see section 8.1). Dependence of solubility on pH is relevant for the performance of detection methods. For example, use of an acidic mobile phase (e.g. trifluoroacetic acid) for final preparative high-performance liquid chromatography (HPLC) 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 for MCs purified with trifluoroacetic acid has been shown to form methyl esters (Harada et al., 1996; Foss et al., 2018). These aspects are important when preparing MCs for testing and toxicological investigations.

Table 1.1 Properties of selected microcystins and nodularin for which data on these characteristics are available, in order of decreasing polarity

Property	MC-RR	Nodularin	MC-YR	MC-LR	[Asp^3]MC-LR	[Dha^7]MC-LR	MC-WR	MC-LA	MC-LF
CASRN[a]	111755-37-4	118399-22-7	101064-48-6	101043-37-2	120011-66-7	134842-07	138234-58-9	96180-79-9	154037-70-4
Chemical formula	$C_{49}H_{75}N_{13}O_{12}$	$C_{41}H_{60}N_8O_{10}$	$C_{52}H_{72}N_{10}O_{13}$	$C_{49}H_{74}N_{10}O_{12}$	$C_{48}H_{72}N_{10}O_{12}$	$C_{48}H_{72}N_{10}O_{12}$	$C_{54}H_{73}N_{11}O_{12}$	$C_{46}H_{67}N_7O_{12}$	$C_{51}H_{70}N_7O_{12}$
Molecular weight[a] (g/mol)	1038.224	824.983	1045.213	995.196	981.169	981.16	1068.250	910.087	986.18
Monoisotopic mass (Da)	1037.566	824.443	1044.528	994.549	980.533	980.533	1067.544	909.485	985.516
Colour/physical state	White powder								
K_{ow}[b] at pH 5	2.37	–	2.59	2.85	–	–	–	–	3.84
K_{ow}[b] at pH 7	1.7	–	2.25	2.54	–	–	–	–	3.95
Solubility in other solvents	Dimethyl sulfoxide, methanol, ethanol (note that solvents may alter structures and in vitro processes)								

–: not available; CASRN: Chemical Abstracts Service Registry Number; K_{ow}: octanol–water partition coefficient; NA: not applicable

[a] Average molecular weight calculated based on conventional atomic weights as given in Table 3 of Meija et al. (2016)

[b] From Santori et al. (2020)

Note: For further MCs, see *Toxic cyanobacteria in water*, Fastner & Humpage (in press).

1.3 Organoleptic properties

None of the known cyanobacterial toxins (MCs, cylindrospermopsins, saxitoxins, anatoxins) have been shown to affect the taste or odour of water. However, some cyanobacterial species produce other compounds, such as geosmin and methyl-isoborneol, that do affect taste or odour, indicating the presence of cyanobacteria in raw water. As this applies only to some strains of some cyanobacterial species, the absence of these typical tastes or odours is not a reliable indicator of the absence of cyanotoxins. For an overview of the relationship between

organoleptic properties and toxins, see *Toxic cyanobacteria in water* (TCiW), Kaloudis (in press).

1.4 Major uses and sources

MCs occur naturally, although high concentrations are typical for fresh waters influenced by human activity – for example, by wastewater or runoff from agricultural land that introduces nutrients that fertilize the growth of phototrophic organisms, including cyanobacteria. There are no known commercial applications of MCs. MC-producing strains can be found in all major orders of Cyanobacteria – that is, Chroococcales, Oscillatoriales, Nostocales and Stigonematales. *Microcystis* is the most widely occurring genus with MC-producing species; other widespread ones include *Anabaena* (some species of which are now classified as *Dolichospermum*), *Nostoc* and *Planktothrix* (for more information on the new classification of genera, see TCiW, Vidal et al., in press). Although these are the taxa most often associated with MC production, it is not restricted to these, and strains of some benthic genera (i.e. organisms that grow on sediments or other submerged surfaces, such as *Phormidium*) also include species with MC-producing strains. Nodularins have so far been found largely in strains of the genus *Nodularia*, primarily in *Nodularia spumigena*, which can form blooms in brackish, typically coastal water. Toxigenic strains (containing the required genes for MC synthesis) and nontoxigenic strains of a given species often co-occur. Although many toxigenic strains simultaneously produce several MC variants, usually only 1–3 variants make up the bulk of total MCs. The variants, as well as the amount of MCs occurring in a given bloom, are largely determined by the composition of strains (or genotypes) in the bloom, 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 – by a factor of not more than 2–3 in most studies and occasionally up to a factor of 5. Differences in MC contents between different toxigenic strains can vary much more widely, from trace amounts to more than 10 mg/g dry weight. MC content per cell (i.e. "cell quota") up to 550 fg/cell and toxin content of up to 14 µg/mm³ biovolume have been reported for individual *Microcystis* sp. strains – these values seem to be exceptionally high, and a number of other studies suggest maximum cell quotas or toxin contents around 200 fg/cell or 5 µg/mm³ biovolume, respectively (see discussion in TCiW, Ibelings et al., in press). Similar ranges of MC cell quotas and toxin contents have been reported for *Planktothrix agardhii* and *P. rubescens*, with values for *P. rubescens* reported as high as 850 fg/cell and 20 µg/mm³ biovolume, respectively.

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

For more details on MC-producing organisms, biosynthesis and environmental fate, see TCiW, Fastner & Humpage (in press).

2 Environmental levels and human exposure

2.1 Air

MCs are not volatile, and so exposure via inhalation is possible only through spray carrying cyanobacterial cells or toxins – for example, via overhead irrigation, 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 – that is, >120 000 cells/mL of *Microcystis* sp. (up to 2140 µg/L of total MCs, 0.7–45% of which was extracellular) – were only in the low pg/m^3 range (Wood & Dietrich, 2011).

2.2 Food

MCs and other cyanobacterial toxins have been found in some foods, such as algal dietary supplements, and fish, mussels and shellfish 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. Higher values were reported from fish liver and animals eaten with viscera (which contain cyanobacterial cells), such as whole fish and molluscs (for more details, see Testai et al., 2016). Therefore, exposure to MCs from ingestion of contaminated food will vary greatly depending on 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 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. The risks may be higher for communities that 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 on the frequency and magnitude of contamination, as well as local habits for 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 containing cyanobacterial blooms. Most of the available data refer to experiments irrigating with extreme concentrations of cells and/or toxins, and may therefore be poorly representative of likely realistic scenarios. However, these studies do indicate that accumulation can occur. Cyanobacterial cells containing MCs can also 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 a potential source of exposure to consider when estimating health risks caused by MCs.

MCs have been detected at levels above 1 µg/g in dry weight samples of commercial cyanobacterial dietary supplement products. However, the proportion of positive samples and 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 was harvested. MC-contaminated dietary supplements can be a major source of

exposure to MCs; recommendations for consumption vary widely between products, from 0.5 to 15 g/day, with some products indicating no maximum limit.

MCs were not found in the milk of dairy cattle that were exposed to *Microcystis aeruginosa* via drinking-water, although most of the MCs to which the cattle were exposed were quite hydrophilic.

For more information on MCs in food and dietary supplements see TCiW, Dietrich (in press); Ibelings et al. (in press).

2.3 Water

Since MC-producing cyanobacteria are found primarily in freshwater environments, in many settings the primary waterborne route of human exposure to MCs is the consumption of drinking-water, if it is produced from surface waters that are untreated or insufficiently treated. Another 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. Recreational exposure may be associated with acute intoxication risks.

MCs are the most commonly reported cyanotoxin, with reports from surface waters indicating 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). They can be released from cells by cell lysis that is induced, for example, by adverse growth conditions, phage activity or algicide 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 levels may occur occasionally.

Reported in situ concentrations of total MCs (extracellular plus intracellular) in raw water vary widely, from nondetectable (<0.1 µg/L) to more than 100 mg/L. However, on average (e.g. in integrated water samples), the MC concentration rarely exceeds 10–20 µg/L and is often lower than this. Some toxigenic cyanobacteria can form surface blooms as a result of buoyancy, and such blooms can further accumulate by wind-driven dislocation to form massive scums. Depending on weather conditions and water body morphology, concentration gradients over depths and areas may be very steep and often change rapidly (within less than 1 hour). Elevated MC concentrations are most likely at downwind, near-shore sites with massive scums of *Microcystis* sp. Blooms of *Planktothrix agardhii* occur in well-mixed, shallow water bodies and only rarely form light surface blooms, resulting in more spatially homogeneous MC concentrations. Other MC-producing cyanobacteria (e.g. *Dolichospermum*) show an intermediate tendency to form surface blooms.

A particular pattern of spatial gradients of MC concentrations is caused 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. This species causes maximum MCs not at the surface (although occasionally *P. rubescens* forms surface blooms) but rather in this narrow layer at some metres depth, sometimes coinciding with the depths at which drinking-water is taken from reservoirs.

High, lake-wide concentrations of extracellular MCs are most likely following algicide treatment of dense cyanobacterial blooms, with up to 1800 µg/L reported. Massive release of MCs following natural lysis (e.g. in response to 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 section 7.4). A number of taxonomically diverse heterotrophic bacteria are capable of degrading MCs.

Although 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/L. Such findings are likely to be 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/L in some samples, due to a bloom near the drinking-water off-take in Lake Erie. The advisory was lifted 2 days later after water treatment adjustments reduced cyanotoxin concentrations to nondetectable in most samples and below 0.5 µg/L in the remaining samples.

Recreational activity in surface waters with cyanobacterial blooms can cause exposure to MCs (and other toxins in blooms and scums), mainly through unintentional swallowing of water, but also potentially through intranasal or intratracheal exposure. 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/L range.

Inhalational exposure to MCs in spray may be a relevant pathway for specific recreational activities, such as waterskiing or jet-skiing, and for specific occupational situations involving spraying 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, although in most cases for a limited time.

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

2.4 Estimated total exposure and relative contribution of drinking-water

Where surface water is used as the source for drinking-water, this is the most likely means of exposure to MCs. However, this assumption is a starting point, and country- or region-specific assessments should take other potential pathways into account. Recreational activities in lakes with cyanobacterial blooms may expose individuals to high concentrations of MCs, as described in section 2.3. Food may contribute to exposure – for example, fish eaten with viscera and shellfish from bloom-ridden water bodies. For most situations for the general population, the oral route is the main route of concern.

Patterns and duration of exposure 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 in a given setting. Chapter 5 of TCiW (in press) gives further guidance and background information on assessing routes of exposure.

For specific population groups, exposure may occur via the parenteral route – for example, associated with use of contaminated water for haemodialysis or infusions. Risks are potentially high if water from contaminated surface waters is used for haemodialysis, which was the major

source of exposure (including some lethal exposures) documented in the cases discussed in TCiW, Azevedo (in press).

As noted in section 2.2, contaminated dietary supplements can also be a major exposure source to MCs. However, because of 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.

3 Kinetics and metabolism in laboratory animals and humans

3.1 Absorption

The dose and route of exposure play crucial roles in MC absorption. The lipid–water partition coefficient of many MC variants means that oral cavity and sublingual absorption would not be expected. However, this has not been investigated. Once in the gastrointestinal tract, several factors affect 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 body weight [bw]) suggested that the stomach absorbed the toxin to some degree, with primary absorption through the villi of the small intestine (Ito, Kondo & Harada, 2000). MC-LR absorption into the systemic circulation may have been aided by villi erosion at this high dose. Parenteral administration (e.g. intraperitoneal [i.p.]) bypasses the digestive tract. However, i.p. 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, but not fully elucidated. Available data indicate that the organic acid transporter protein (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, 2010; Svoboda et al., 2011). However, the specific OATP(s) responsible for intestinal absorption of MCs 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 these factors are poorly understood at present (Alam et al., 2018).

Scant data are available about efflux proteins involved in MC transport. Intestinal absorption/permeability studies using Caco-2 cells support facilitated uptake of MC-LR (Zeller, Clément & Fessard, 2011). Henri et al. (2014) reported that, in Caco-2 cell monolayers, MC-LR (at 1, 10, 48 or 75 μM) was rapidly taken into cells from the apical (intestinal lumen) side, but, after 30–45 minutes, the majority of toxin was re-excreted back into the apical compartment, likely by efflux proteins, with only 0.3–1.35% of the toxin reaching the basolateral compartment over 24 hours.

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, allowing contact with 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, Kondo & Harada, 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.

3.2 Distribution

Once MCs are absorbed and in the circulatory system, their distribution to tissues and other body fluids depends on many factors, including 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 considerable uptake of MCs by the liver (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 highly 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) in which specific OATPs were inhibited have shown reduction or elimination of MC-induced liver toxicity, further supporting the role of OATPs 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: studies with human hepatocytes have shown 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, gene regulation and variability in OATP gene expression may further affect distribution (Lee et al., 2005; Alam et al., 2018).

Understanding of MC distribution in vivo has been derived primarily from studies using radiolabelled MCs. The site of MC radiolabelling may affect distribution profiles. Indeed, [^{3}H]-labelling at the Mdha^7 residue alters the ability of MCs to bind covalently with protein phosphatases and other thiols (e.g. glutathione – γ-glutamyl-cysteinyl-glycine [GSH]) (Hilborn et al., 2007; Miles et al., 2016).

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

Studies addressing distribution of unlabelled 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 six 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 and kidneys (in that order); staining was greater in animals that died. Staining in the heart, pancreas and spleen was only seen in the blood plasma (Ito, Kondo & Harada, 2000).

Male Duroc pigs (n = 6) were dosed daily by gavage with MC-LR at 2 µg/kg bw for 35 days, and serum samples were collected weekly. MC-LR and MC-LR-GSH in methanolic extracts, and total Adda by the 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) method, were measured in blood and tissues (Greer, Meneely & Elliott, 2018). MC-LR, GSH conjugate and Adda fragment ion were not detected in the serum of any animal at any time. The livers of three

of six pigs contained MMPB-detectable MC-LR (26.4 µg per liver, about 1.1% of the total administered dose) (Greer, Meneely & Elliott, 2018). However, this study had some weaknesses, especially in quantitative aspects, associated with limitations in the analytical detection methods in tissues and the presence of lasalocid A (Welten et al., 2019), an OATP inhibitor, which could potentially interfere with MC-LR uptake.

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

Most studies of MC distribution have used non-oral routes of exposure. Hepatic accumulation of [^{3}H]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 hour (Nishiwaki et al., 1994). Hepatic content of [^{3}H]-dihydroMC-LR, as a percentage of the administered dose, was about 80-fold lower after oral administration, when most of the radioactivity remained in the gastrointestinal tract (Nishiwaki et al., 1994).

After intravenous (i.v.) injection of MC-LR at 35 µg/kg bw in male VAF/plus CD-1 mice, 67% of the dose of [^{3}H]-dihydroMC-LR was detected in the liver, with a biphasic plasma clearance ($t_{1/2}$ = 0.8 and 6.9 minutes, respectively) (Robinson et al., 1991). These biphasic plasma clearance rates, coupled with rapid uptake in the liver ($t_{1/2}$ = 6.8 minutes), suggest that the hepatic tissue accumulation was a major factor in plasma clearance (Robinson et al., 1991). Partition coefficients (30 minutes) of 206, 4 and 11 for liver, kidney and intestine, respectively, calculated for mice support MC accumulation in organs other than the liver (Robinson et al., 1991).

[^{3}H]dihydroMC-LR administration by the i.v. route 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 ($t_{1/2}$ = 3 and 269 minutes) than the lower dose ($t_{1/2}$ = 3.7 and 134 minutes) (Stotts et al., 1997a). Hepatic damage at the higher dose rate may have resulted in slower clearance. Interspecies differences are likely to account for the longer β-phase $t_{1/2}$ in swine than in mice. When swine were dosed with [^{3}H]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 hours) predominated, with 47–65% of the total dose. Distribution to 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 unlabelled MCs (Wang et al., 2008). It confirmed that unmetabolized MC-LR and MC-RR were detected in kidney, lung, stomach and liver (in that order) (Wang et al., 2008). However, as in many studies using unlabelled MCs, this study only analysed for unmetabolized MC-LR and MC-RR, which limits the interpretations that can be drawn.

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); this warrants further investigation. The available data indicate a much lower uptake, as a percentage of dose, into the liver after oral exposure than after i.p. and i.v. administration, indicating that intestinal uptake is slow and limits systemic exposure.

In summary, 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 through first-pass clearance. However, MCs are also partially distributed to organs beyond the liver. Clearance from the blood is slower at higher doses, presumably due to the liver toxicity and circulatory shock that occur at high doses (Health Canada, 2018). Additional work using appropriate analytical techniques is required to extend knowledge about MC kinetics to real-world exposure scenarios, so that the mechanisms responsible for transport both pre- and post-metabolism can be understood.

3.3 Metabolism

For MC biotransformation, the major reaction is conjugation with thiol compounds – GSH and cysteine (Cys) – which has been described both in vitro and in vivo. Other MC metabolites have also 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 are less toxic than the parent, with 2- to 17-fold higher mouse median lethal dose (LD_{50}) values (Kondo et al., 1992). MC-LR conjugates (GSH, Cys, γ-GluCys) have also been shown to be weaker inhibitors of protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A) in vitro (protein phosphatase inhibition is considered the molecular initiating event), suggesting that at least some of the reduced potency is due to reduced affinity for the protein phosphatase 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 S et al., 2015), likely due to decreased conjugation.

The α,β-unsaturated carbonyl group ($Mdha^7$/Dha^7) is considered to be the main site for thiol conjugation (Kondo et al., 1992). MCs can be conjugated with physiological thiols in vitro (Kondo et al., 1992; Foss et al., 2018), as well as with mercaptoethanol used for derivatization before 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 protein phosphatase–bound MCs (Smith et al., 2010).

GSH conjugation largely occurs via a spontaneous reaction in vivo and under physiological conditions in vitro, but is also catalysed 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 catalyse conjugation of GSH with MC-LR, MC-RR, MC-LW and 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 with higher hydrophilicity, with MC-RR being conjugated at the highest rate. A differential rate of conjugation in response to MC $Mdha^7$ moiety chemistry was also observed using mercaptoethanol as a model thiol: the kinetics of conjugation with $Mdha^7$/Dha^7 has been shown to be much faster ($t_{½}$ = 0.21–0.84 hours) than conjugation with Dhb^7 ($t_{½} \geq 50$ hours). This difference has been applied analytically to distinguish these variants (Miles et al., 2012, 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: higher enzymatic conjugation occurred with rats and mice than with human cytosol for MC-LR and MC-RR (Buratti & Testai, 2015).

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

Although indicating reversibility, the quantitative data on MC detection in matrices other than water should 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 mode, as used by the authors of the two publications involving in vivo studies.

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 in hepatocytes, and a high first-pass clearance occurs following oral exposure. 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 relevant to drinking-water exposure and shows relevant kinetic differences from the oral route.

3.4 Elimination

Primary elimination of both parent and metabolized MCs occurs 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 as a result of 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 after exposure of a canine to a *Microcystis* bloom contained intact, unmetabolized MC-LA (Rankin et al., 2013). However, the unmetabolized MC may have been excreted by the liver to bile. Multidrug resistance-associated protein 2 (MRP2) has been shown to transport MC-LF, MC-LR and MC-RR (in that order), 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 an 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 OATP1B3 (see section 3.2), 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 faster to the bile canaliculus (Kaur et al., 2019).

Faecal elimination has been observed in studies using radiolabelled MCs. After intravascular administration of [^{3}H]dihydroMC-LR in mice, 24% of the administered dose was excreted over a 6-day period: 9% in urine and 15% in faeces (Robinson et al., 1991). This supports 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}H]dihydroMC-LR, despite the site of thiol conjugation being reduced. Similarly, in isolated perfused rat liver, 1.7% of [^{3}H]dihydroMC-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, and the remaining radiolabel was associated with more polar metabolites. Identities of specific metabolites were not reported in these studies.

Following oral gavage of MC-LR, immunostaining techniques showed excretion of MC-LR in the mucus from goblet cells in both the small intestine and large intestine. The kidneys were also stained, indicating a renal route of elimination (Ito, Kondo & Harada, 2000). Following i.v. administration of a crude extract to Wistar rats, MC-RR and MC-LR concentrations were consistently higher in kidney than in liver throughout the experiment, indicating excretion of unmetabolized MCs through the kidney (Wang et al., 2008). Unmetabolized MC-LR (11–33 ng/mL) was also observed in mouse urine 5 hours after 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 after accidental exposure to a *Microcystis* bloom showed that 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). MCs were still detectable (via MMPB) in urine 10–68 days after exposure in three surviving dogs. Bile contained high MCs (5400 ng/mL) on death of one dog, but levels were an order of magnitude higher in urine (41 000 ng/mL), supporting the renal route as 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, Kondo & Harada, 2001). This shows that MC conjugates are excreted from the hepatocytes via the sinusoidal as well as the biliary side. It suggests that the lower toxicity of GSH and Cys conjugates may be partly related to their distribution to excretory organs and elimination of metabolites in vivo.

Li et al. (2018) investigated the excretion patterns of MC-RR-GSH injected i.p. (0.55 µmol/kg bw) in male Sprague–Dawley rats. Analysis of urine and faeces by liquid chromatography tandem mass spectrometry (LC-MS/MS) indicated that excretion was evident by the first time point (0.25 hours) in both excreta and reached a peak 2 hours after dosing, sharply dropping and levelling off at 6 hours, with 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 MCs were detected in patients' serum by enzyme-linked immunosorbent assay (ELISA) for more than 50 days after exposure (Soares et al., 2006; Hilborn et al., 2007).

4 Effects on humans

Cyanobacteria have been reported for more than 100 years to cause animal and human poisoning when present in lakes, ponds and dugouts around the world. It is unclear in most instances which cyanotoxin (if any) was involved because they had not been characterized at the time of the incident or were not analysed for, or multiple cyanotoxins were present. Many of the reported symptoms in historical reports are quite general and cannot be considered in isolation as diagnostic of MC poisoning. 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.

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 sulfate, high copper concentrations may explain symptoms such as diarrhoea, vomiting, stomach cramps and nausea; however, this would require copper concentrations above the concentration of 1–2 mg/L at which it is used as an algicide (see WHO, 2017, for a discussion of copper toxicity and derivation of guideline values for copper).

4.1 Case studies

4.1.1 Drinking-water

Consumption of drinking-water originating from raw surface water that was infested by cyanobacterial blooms producing MCs and was not properly treated gave rise to some outbreaks of illness in the late 20th century (Funari & Testai, 2008; US EPA, 2015). Symptoms ranged 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 1960–1965 was linked to annual blooms of *Microcystis* in the lake used 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 following the flooding of the newly constructed Itaparica Dam reservoir in 1988. Some 2000 gastroenteritis cases were reported over a 42-day period, and 88 people, mostly children, died (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 microorganisms 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. After review of clinical data and water sample tests, 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 gamma-glutamyl transferase (GGT), was seen in people who had drunk water supplied from sources containing blooms of *Microcystis* after treatment with copper sulfate (Malpas Dam, Armidale, Australia) but not in people who had drunk water from another supply (Falconer, Beresford & Runnegar, 1983). MC-YM had been identified in these blooms.

4.1.2 Recreational exposure

Exposure during recreational activity can be associated with three different routes of absorption: oral, due to accidental ingestion of water contaminated with algal cells; dermal, due to direct contact; and inhalation, providing that MCs are aerosolized or cyanobacterial cells are contained in spray. Inhalation of MC-containing aerosols (MC concentration of 0.052–2.89 ng/m^3) in 81 individuals resulted in MC detection in the nasal swabs but not in plasma (limit of detection: 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 using these routes of exposure resulted in both localized tissue damage and liver toxicity similar to i.p. dosing (Fitzgeorge, Clark & Keevil, 1994; Ito, Kondo & Harada, 2001; see section 5.1 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 related to MCs or 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 (Dillenberg & Dehnel, 1960). *Anabaena* (many of which now belong to the genus *Dolichospermum*) 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 *Microcystis 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. MC-LR was identified in the bloom material (Pearson et al., 1990). However, high levels of *Escherichia coli* were also found in the reservoir water 2 weeks later. The authors suggested that exposure to MCs may have had a role in some of the clinical symptoms.

Acute intoxication by an 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 – 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 he was admitted to a medical centre 4 days later. 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 aspartate aminotransferase [AST], alanine aminotransferase [ALT] and GGT, but normal bilirubin and alkaline phosphatase [ALP]). Tests for HIV, Epstein–Barr virus, *Clamidia pneumoniae* and *Mycoplasma* were negative. The patient recovered completely within 20 days.

In Montevideo, Uruguay, a 20-month old child was hospitalized following repeated recreational exposure to *Microcystis* blooms containing MCs at up to 8200 μg/L (Vidal et al., 2017). Faecal coliforms were below the local health alert limit of 1000 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 and 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 MC-LR at 2.4 ng/g and [D-Leu^{1}]MC-LR at 75.4 ng/g.

Numerous reports are available of skin irritations and localised effects associated with recreational contact with MC-producing cyanobacteria in lakes or freshwater basins, but no correlation has been 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 in which 259 chronic rhinitis patients were 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 nontoxic strains identified phycobiliprotein complexes in *Microcystis aeruginosa* as the sensitizing agent (Geh et al., 2015). These findings suggest that the relevant metric for 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. diarrhoea, abdominal pain, nausea, vomiting) with the presence of cyanobacterial cells in a lake affected by MC-producing blooms (Lévesque et al., 2014). Health surveillance data from the USA have also linked outbreaks of illness (e.g. 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 fresh waters (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 responsible for some or all of these effects. For example, the bacterial community associated with cyanobacteria, particularly *Aeromonas* strains, has been suggested to play a role in causing the gastrointestinal symptoms reported (Berg et al., 2009).

4.1.3 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. Of the affected patients, 100 developed acute liver failure and, of these, 76 died (Jochimsen et al., 1998; Carmichael et al., 2001). The methanol–extractable or "free" MC concentrations in serum 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 8–51% 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 used 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). An MC concentration of 0.4 µg/L was detected in the drinking-water supply before on-site treatment. Serum samples were collected from 12 dialysis patients for 8 weeks. MCs in the serum ranged from 0.46 to 0.96 ng/mL (by ELISA), with the highest concentrations occurring 31–38 days after MCs were last detected in water samples. Biochemical markers of hepatic cellular injury and cholestasis (elevations of AST, ALT, bilirubin, ALP and GGT) in serum during all 8 weeks of observation frequently exceeded normal values. 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, so the authors concluded that the patients were not continuously exposed to the toxin; instead, the toxin detected in serum after 8 weeks may have been present in the form of bound toxin in the liver (Soares et al., 2006).

4.2 Epidemiological studies

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

Bearing in mind these limitations, a positive association was found between the risk for HCC and water sourced from surface waters, with estimates of relative risk ranging from 1.5 to 4. Consumption of untreated pond or ditch water was associated with an 8-fold increase in liver cancer incidence compared with well water consumption (Yu, 1995). MC-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 (e.g. pesticides) and biological contaminants would also be expected to be present 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 to MCs of about 0.2 µg per person during the summer months (this exposure is miscalculated in the paper as 1.9 pg/day). Later studies from China have associated slightly higher exposure rates from food and water combined (0.36–2.03 µg/person/day) with detectable serum MCs and increased levels of serum liver enzymes (Chen et al., 2009; Li et al., 2011; see below).

A later case–control study in Haimen, 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 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 2.2–3.9 µg of 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 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. Children in the low-exposure group consumed MCs at an estimated level of 0.36 µg/day, and high-exposure children consumed 2.03 µg/day. Mean serum levels of MCs in the groups were <0.1 (limit of detection), 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 for liver damage associated with MCs was 1.72 (95% confidence interval = 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 epidemiological studies allow causal relationships to be established between MC exposure and liver tumours.

5 Effects on experimental animals and in vitro systems

5.1 Acute exposure

Fatalities in animals have been reported following the consumption of water containing large numbers (>10^6/mL) of cyanobacterial cells (Stewart, Seawright & Shaw, 2008; Mancini et al., 2010; Wood et al., 2010).

Fawell et al. (1999) estimated oral LD_{50} 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 LD_{50} of 10.9 mg/kg bw for purified MC-LR (>95% pure by HPLC) in 6-week old female BALB/c mice; 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, Kondo & Harada, 1997; Rao et al., 2005). Young animals (5–6 weeks old) showed 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 LD_{50} of MC-LR by the i.p. route is approximately 25–150 µg/kg bw in mice (Fawell, James & James, 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. LD_{50} values of several other MCs that have been determined are generally less than a factor of 2 higher than that of MC-LR, although about 5–10-fold higher for some (e.g. MC-RR and MC-M(O)R) (Zurawell et al., 2005).

LD_{50} values for mice treated with MC-LR intratracheally (Ito, Kondo & Harada, 2001) or intranasally (Fitzgeorge, Clark & Keevil, 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} 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, Palovics & Bankine, 2001). The MC content (presumed to be total MC-LR, MC-RR and MC-YR) ranged from 0.1 to 2.21 mg/g. To determine sensitization, guinea pigs were initiated with an intradermal injection followed 7 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 in rabbits.

5.2 Short-term and subchronic exposure

Among the 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 have been carried out with poorly characterized extracts, administered via the i.p. route (which is 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 using a much higher internal dose than 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, Kondo & Harada (2000) treated 7 male BALB/C mice and 39 male ICR mice (aged 24 weeks and 32 weeks, respectively) with a single gavage dose of MC-LR of 500 µg/kg bw at 0, 1, 6, 7, 12 and 13 weeks (i.e. six doses in total). Lethality was 71% in BALB/C mice 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/day for 13 weeks. Thirty-nine organs or tissues from the control and high-dose groups, and lungs, liver and kidneys from the intermediate-dose groups, were examined microscopically. Blood samples from subsets of seven animals were analysed for haematology and serum biochemistry. No treatment-related changes were noted at the lowest dose. At 200 µg/kg bw/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 doses, serum transaminases were significantly elevated, serum GGT 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 males and females was increased by 14% and 20%, respectively, but body weight was 7% lower in males compared with control mice. The no-observed-adverse-affect level (NOAEL) for MC-LR was considered to be 40 µg/kg bw/day (Fawell, James & James, 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 × male BDIX; n = 10 per group) given 0, 50 or 150 µg/kg bw/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 enzymes were analysed, as was histopathology of the liver and kidney. Increased leukocyte counts (38%) were observed in the highest-dose group, and increased LDH (low dose: 84%; high dose: 100%) and ALP (low dose: 34%; high dose: 33%) were seen in both treatment groups. There were no changes in ALT or AST, known markers 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 greater in the high-dose group. Other histological effects, including Kupffer cell activation and PAS staining, showed no dose–response relationship. No effects on the kidneys were observed. The lowest-observed-adverse-affect level (LOAEL) was 50 μg/kg bw/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 second day for 90 days with MC-LR at 0, 40 or 200 μg/kg bw/day (He et al., 2017). Although perturbations in 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 statistical analyses, especially when inflammatory infiltrates and apoptosis were also seen in one of the three 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) – these are additional preferable parameters when evaluating steatosis.

Schaeffer, Malpas & Barton (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/kg bw/day based on the MC-LR content of the lyophilized *Aphanizomenon flos-aquae* (as determined 13 years after the original trial). No effects were seen on body weight; liver, kidney or spleen weight; or histopathology at the highest estimated dose (Schaeffer, Malpas & Barton, 1999). Although this dosing regimen is more similar to human oral exposure, as with other studies using cyanobacterial extracts, the presence of other cyanobacterial components complicates interpretation of the results.

Sedan et al. (2015) administered MC-LR by gavage at 50 or 100 μg/kg bw to male N:NIH mice every second day for a month, and extrapolated the daily dose to 25 μg/kg bw/day, claiming they obtained an effect below the NOAEL identified in the Fawell et al. (1999) 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 relationship (higher at 100 μg/kg bw), whereas oxidized GSH and lipid peroxidation did not.

Huang, Zheng & Xu (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/day of MC-RR (commercial product; purity not reported) via gavage for 7 days, after which animals were euthanazed 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/day and higher was reported. Expression of apoptosis-related proteins BAX and Bcl-2 was also significantly altered at doses of 46 and 23 μg/kg bw/day, respectively; their ratio (BAX/Bcl-2) was significantly increased at all doses greater than 4.6 μg/kg bw/day.

Extract from *Microcystis 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/day, based on the extract LD_{50} value in mice (Falconer et al., 1994). The extract contained at least

seven MC variants, with MC-YR tentatively identified as the major constituent. Liver injury (evident from histopathology and changes in serum enzymes) was 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/day.

Greer, Meneely & Elliott (2018) treated pigs (different breeds in the two trials) by oral gavage with MC-LR (0.04 µg/kg/day for 98 days, $n = 8$; or 2.0 µg/kg/day for 35 days, $n = 6$). They analysed serum and various organs (including liver, kidney, spleen, small and large intestine, and brain) for both free and bound MC-LR. Qualitative analysis for MC-LR-GSH was undertaken in the livers and kidneys from the high-dose group only. As noted above, the quality of the study is limited, especially regarding quantitative aspects, as a result of inaccuracies in the detection methods. No toxicity data are reported in the paper; however, Welten et al. (2019) analysed samples from the high-dose group of Greer, Meneely & Elliott (2018) plus those from another eight pigs dosed by gavage at 8.0 µg/kg/day for 35 days 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 8.0 µg/kg/day group. Multivariate analyses of the various profiles showed no treatment-related effects; nor did any of the plasma indicators of liver damage.

Benson et al. (2005) exposed groups of six male BALB/c mice to monodispersed submicrometre 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/m^3, and doses deposited in the respiratory tract were estimated to be 3, 6 and 12.5 µg/kg bw/day. Control mice were exposed to the aerosolized vehicle (20% ethanol). Clinical chemistry analysis; organ weights; and histopathology of the liver, respiratory tract tissues, adrenal glands, kidney, spleen, thymus, gastrointestinal tract and testes were 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, necrosis and atrophy of olfactory epithelial cells.

5.3 Long-term exposure

5.3.1 Systemic effects

A growing number of long-term studies in rodents 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 provisional GV for MC-LR of 1 µg/L (or multiples of it). For comparison, if murine water consumption of 1.5 mL/10 g bw/day is assumed and uncertainty factors remain unchanged (Chen, Giesy & Xie, 2016), a mouse GV-equivalent for MC-LR of 0.22 µg/L can be calculated from a tolerable daily intake (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 MC-LR at 0 or 20 µg/L daily for up to 18 months (20 mice per dose). Average cumulative MC-LR intake for the 18 months was estimated as 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 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 0, 1, 40 or 80 µg/L (estimated as average intakes of 0, 0.2, 8.0 or 16.0 µg/kg bw/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 and 126 days, respectively, of exposure onward.

Labine, Gong & Minuk (2017) exposed CD-1 mice (20 per group) to drinking-water containing MC-LR at 1 µg/L, MC-LR at 1 µg/L plus thiacetamide (TAA) at 300 mg/L, or TAA only at 300 mg/L for 28 weeks. After this period, serum was analysed for ALT and total bilirubin, and livers were examined by histopathology. Consumption rates of the dose materials were not reported, and so exposure in units of µg/kg/day cannot be calculated. No treatment-related effects were 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 MC-LR at 1, 10 or 40 µg/L for 6 months in drinking-water (estimated by the authors to correspond to 0.1, 1 and 4 µg/kg bw/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 MC-LR at 1, 5, 10, 20 or 40 µg/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 was reported 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 – that is, a total of 16 or 20 weeks, respectively) of MC-LR at a dose of 80 µg/kg bw. A further seven 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 euthanazed 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., 1997).

5.3.2 Neurological effects

Li X-B et al. (2014) administered MC-LR (purity >95%) to groups of eight 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 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 stain; 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 murine OATPs (mOATP1A5 and the known MC-LR transporter mOATP1B2). 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 MC-LR, MC-LW or MC-LF concentrations of 2.5 μmol/L, total protein phosphatase activity was reduced by 25%, 30% and 60%, respectively. MC-LF at 5 μmol/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. All three congeners reduced cell viability (MTT reduction) and neurite length, with MC-LF and MC-LW (particularly MC-LF) being significantly more potent than MC-LR. 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 noted that these analogues do not significantly differ in inhibitory potency towards PP1 and PP2A; they therefore hypothesized that the observed differences related to affinity for OATPs (i.e. uptake kinetics).

5.3.3 Reproductive and developmental toxicity

A number of reproductive studies have been reported in which the toxin was administered by the i.p. route. This route of exposure bathes the reproductive organs in essentially pure test solution, rendering results difficult to interpret in terms of human health risk from oral exposure. 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 days (GDs) 6 to 15 at 0, 200, 600 or 2000 μg/kg bw/day. Nine of the 26 females dosed with 2000 μg/kg bw/day died or were euthanazed. High-dose females had abnormal livers, but body weight 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/day (Fawell, James & James, 1994), equal to the NOAEL for maternal toxicity – both of these are 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 *Microcystis aeruginosa* extract in drinking-water at an estimated dose of 2700 μg/kg bw/day from weaning (17 weeks before mating) through mating. Seven of 73 pups from MC-exposed dams were found to have reduced brain size (compared with 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 subcutaneously (s.c.) to groups of eight CD-1 female mice daily at one of three periods during gestation (GD 7–8, GD 9–10 or GD 11–12). Intraperitoneal doses were 0, 32, 64 and 128 μg/kg bw, and 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 survival or postnatal growth over 4 days of newborns from dams that survived this dose. No effects were seen in fetuses 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 results 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/day; US EPA, 2015). Clinical signs of toxicity were not seen, and the treatment did not affect water consumption, or body or testes weight. 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; and sperm motility was reduced by 25% and 50%, respectively, at 3 months, and by 59% and 70%, respectively, at 6 months. 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 levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) 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 shortcomings that limit its usefulness for human health risk assessment; these include use of methanol as a vehicle for MC-LR, which was not administered to control animals; effects present also in the control group; poorly described analysis of sperm; no report of purity of the test item; no check for MC-LR concentration in the administered drinking-water; and questionable statistical evaluation (US EPA, 2015; Buratti et al., 2017; Health Canada, 2018).

Eighty male mice (strain not specified) were divided into groups of 10 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 in 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 because of potential generation of artefacts (US EPA, 2015).

Groups of 10 male BALB/C mice were provided with MC-LR at 0, 1, 10, 20 or 30 μg/L in drinking-water for 90 or 180 days to investigate effects on the prostate (Pan et al., 2018). Prostate weight as a percentage of 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 mice 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 three highest dose groups after 180 days of exposure.

In a study examining effects of MC-LR on the ovaries, 30 female BALB/C mice per dose group were treated for 3–6 months with drinking-water containing MC-LR (isolated from *Microcystis aeruginosa*; purity ≥95%) at 0, 1, 10 or 40 μg/L; body weight and amount of water consumed were not reported. Ovary weight as a percentage of body weight 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 in the 40 μg/L group at 3 months and in all treatment groups at 6 months). Progesterone was significantly increased in the 10 and 40 μg/L groups at 3 months, but only in 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, questions regarding the statistical evaluation mean that the determination of significant values is questionable: a two-way ANOVA with post-test should have been used rather than a one-way ANOVA; the number of replicates is too low for the number of parameters evaluated; and not all parameters had a normal distribution.

Li X et al. (2015) exposed female Sprague–Dawley rats (28 days old, seven per dose group) to MC-LR at 0, 1.0, 5.0 or 20.0 μg/kg in 0.2% methanol (v/v) by gavage once every 48 hours for 8 weeks. Twenty-four hours 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 female offspring from the 5.0 and 20.0 μg/kg groups, and significantly decreased swimming speed in the female offspring from the 20.0 μg/kg group. Malondialdehyde contents in the hippocampus were significantly increased in the male offspring at 5.0 μg/kg, and in both male and female offspring at 20.0 μg/kg. Total SOD activities were also significantly increased in the hippocampus of the male and female offspring at 20.0 μg/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 of 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 MC-LR at 10 μg/kg/day (presumed to be five dams each, although this was not explicitly stated; Zhao, Li & Chen, 2015). Dosing continued for 28 days, until postnatal day 15. Dams delivered naturally, and pups were euthanazed on postnatal day 15. Free MC-LR content of the pups' pooled brain samples was determined to be 3.75 ± 0.94 ng/g dry weight 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, and GSH content and acetylcholine esterase activity were reduced compared with controls. However, as a result of the unusual route of exposure, and hence different MC kinetics, it is not possible to draw any conclusions for 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).

5.3.4 Genotoxicity and carcinogenicity

Genotoxicity studies of pure MCs have generally given negative results (IARC 2010; Sieroslawska, 2013). Both in vitro and in vivo genotoxicity studies of cyanobacterial extracts have shown positive results for DNA damage induced by formation of reactive oxygen species (ROS), as well as inhibition of repair pathways. The available data on the genotoxicity of cyanobacterial toxins, including MCs, have been reviewed (US EPA, 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 to be due to differences in source of the cyanobacteria and composition of the complex extract mixtures. Cellular DNA damage

observed after in vitro treatment with pure MCs may be due to induction of apoptosis and cytotoxicity rather than direct effects on the DNA (US EPA, 2015).

MCs have been classified as Group 2B – possibly carcinogenic to humans (IARC, 2010) – based on their tumour promotional activity, which is 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. MC-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, Sundh & Mattsson, 2010).

Two long-term oral dosing studies of purified MC-LR as a potential tumour initiator are available. Ito et al. (1997) administered MC-LR at 80 µg/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 MC-LR at 20 µg/kg/day was injected i.p. in mice 100 times over 28 weeks did induce detectable nodules (Ito et al., 1997). MC-LR and its Cys conjugate were detected in the livers of mice dosed by the i.p. route but not by gavage.

In the second study, four groups of adult male CD-1 mice (n = 20 per group) were dosed for 28 weeks with water, water containing MC-LR at 1.0 µg/L, water containing thioacetamide, and water containing both MC-LR and thioacetamide. No tumours were present in the control or MC-LR-alone groups. In the other two groups, four and five mice developed liver tumours, with a similar mean size, Ki-67 staining, number of atypical mitoses and liver cancer gene expression profiles (Labine & 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, *N*-nitrosodiethylamine) 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 GST placental form–positive (GST-P) foci when compared with treatment with the initiator alone. The same was true when the initiator treatment was combined with a partial hepatectomy (to stimulate tissue repair), followed by i.p. exposure to MC-LR (Nishiwaki-Matsushima et al., 1992; Ohta et al., 1994). GST-P-positive foci are regarded as tumour precursors and thus indicators for potential tumour formation.

In a study that examined possible synergism between aflatoxin B_1 (AFB_1) and MC-LR, male Fischer 344 rats were given i.p. injections of diethylnitrosamine (DEN) and/or AFB_1 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 were 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 AFB_1, but not in those treated with a combination of the two.

The results from these studies support the conclusion that 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 nonhepatic tissues, but a direct link with MCs cannot be established. Falconer & Buckley (1989) reported evidence of skin tumour promotion by extracts of *Microcystis* in drinking-water in dimethylbutylamine-initiated mice. Total skin tumour weight was increased, although the number of tumours per mouse was only slightly increased (Falconer & Buckley, 1989). Humpage et al. (2000) administered *M. aeruginosa* extract in drinking-water to mice pretreated 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 pretreated 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).

5.3.5 Immunotoxicity

A few studies have suggested that exposure to MCs (from cyanobacterial extracts) at MC-LReq doses ranging from 4.97 to 50 μg/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 and humoral immune response; and altered plasma leukocyte, 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 MC-LR at 1–1000 nmol/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 5-fold greater IL-8 secretion than MC-RR, although no differences in production of intracellular ROS were observed (Huguet et al., 2013), suggesting that other mechanisms can occur, with patterns that differ depending on the toxin.

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

5.3.6 Hematological effects

As reviewed by others (US EPA, 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. Exposure of human erythrocytes in vitro to MC-LR 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.

5.4 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, 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 OATPs are inhibited (Hermansky, Casey & Stohs, 1990; Hermansky, Wolff & Stohs, 1990; 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, even though biliary and urinary metabolites of MCs have been reported. Recent findings by Kaur et al. (2019) demonstrated that MC-LR is transported by MRP2 but not by the MDR, BSEP or BCRP transporters. Nor was there any MRP2 transport of the MC-LR-GSH conjugate.

The mode of action of MC-LR has been extensively reviewed by Liu & Sun (2015) and Buratti et al. (2017). MCs cause inhibition of protein phosphatases (PP1, PP2A and PP5); PP5 is 2–10-fold less sensitive than PP1 and PP2A for a range of MC congeners (Altaner et al., 2019). Inhibition of protein phosphatases is considered to be the molecular initiating event, leading to loss of coordination between kinase phosphorylation and phosphatase dephosphorylation of cytokeratins, which results in destabilization of the cytoskeleton and microtubuli (Falconer & Yeung, 1992; Feurstein et al., 2011). This event initiates altered cell function, which is followed by cellular apoptosis and necrosis. In the liver, acute and relatively high doses lead to loss of cell morphology and cell-to-cell adhesion, which allow haemorrhage into that organ due to damage of sinusoidal capillaries. At the low doses (below 20 μg/kg bw; Gehringer, 2004) typical of repeated long-term exposure, phosphatase inhibition induces cellular proliferation, hepatic hypertrophy and tumour promotion activity.

The increased protein phosphorylation related to PP2A inhibition and to activation of the protein kinase (MAPK) pathway induces abnormal signalling in multiple pathways, triggering a cascade of events that lead to a series of cellular responses. Most of the information on these intracellular perturbations comes from in vitro studies. Many of the cell lines used in these studies lack metabolic competency, and 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 the following events:

- Modification of cytoskeleton and disruption of actin filaments. 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). It results from hyperphosphorylation of different types of microfilament-associated proteins, such as Tau (Feurstein et al., 2011), vasodilator-stimulated phosphoprotein (VASP) and HSP27 (Sun et al., 2015; Zeng 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, Tu & Xu, 2015).
- Oxidative stress. The increased formation of ROS and/or GSH depletion, leading to oxidative stress, drives cells towards apoptotic cell death, rather than to necrosis. This results from release of apoptotic factors, which is seen both in vitro (Žegura, Lah & Filipič, 2004; Meng et al., 2015) and in vivo (Qin et al., 2015). It is associated with time-dependent alterations in GSH levels in rat liver; significant changes in antioxidant enzymes, including GSH peroxidase and GSH reductase; activation of nuclear factor kappa B (NF-κB); and expression of p53, BAX and Bcl-2 (Chen et al., 2016). 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 pretreatment (Meng et al., 2015; Xue et al., 2015). However, the concentrations of MCs and antioxidants used were extremely high, and it remains questionable whether ROS is a major contributor to the development of acute and, more importantly, chronic toxicity.

- Induction of apoptosis, due to mechanisms other than oxidative stress, including phosphorylation of p53, Bcl-2, BAX, cytochrome c and caspases (Wang et al., 2013; Liu et al., 2016). Rats appear to be more prone to induction of apoptosis than mice or humans, in both of which necrosis has been shown to dominate (Woolbright et al., 2017).
- Reduced DNA repair (Douglas et al., 2001; Kleppe, Herfindal & Døskeland, 2015).
- Cell proliferation leading to tumour promotion, associated with the known tumour suppressor role of PP2A. Alteration in cell proliferation, cell division, signal transduction and gene expression as a result of hyperphosphorylation of transcription factors c-Myc and c-Jun has been reported (Liu et al., 2016). The in vitro activation of NF-κB, the phosphatidylinositol 3-kinase (PI3-K)/AKT–mediated MMP-2/9 hyperexpression observed in vivo (Xu et al., 2013), and the modulation of tumour necrosis factor alpha (TNF-α) support the tumour-promoting activity of MCs through PP2A inhibition (Zhang et al., 2012).

5.4.1 Toxic equivalency

MC-LR is one of the most potent MC variants, with the lowest reported i.p. LD_{50} values: 50–60 μg/kg bw. However, MC-LA has a similar LD_{50} to MC-LR. 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} values have been reported for a range of other variants (Zurawell et al., 2005), and toxicity equivalency factors limited to acute toxicity have been proposed by Wolf & 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 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 information on repeated dose toxicity from MC-LR, the most studied congener, to the whole MC group might be difficult.

6 Overall database and quality of evidence

6.1 Summary of health effects

Acute and subchronic 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 also be affected. Data from animal studies have provided a reasonably detailed understanding of the mechanisms underlying these hepatic effects (see section 5.4). However, the human data are limited by lack of quantitative exposure information, and by potential coexposure 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 (almost exclusively MC-LR)

indicate that MC-LR 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, Štraser & Filipič, 2011; US EPA, 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).

Limited data are available on developmental toxicity. An oral study in mice using dosing during organogenesis did not show any adverse effects on embryofetal survival or development (Fawell, James & James, 1994). 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 these studies, i.p. dosing causes the reproductive tissues to be bathed in test material, leading to much higher exposure concentrations than would be achievable via oral exposure. More recent oral studies (Chen et al., 2011; Wu et al., 2015; Pan et al, 2018; Zhou et al., 2020) that used exposure via drinking-water to very low doses of MC-LR are 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. 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.

Currently, only limited data are available for assessing adverse effects (neurological, immunological, haematological) in other organs or tissues.

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

6.2 Quality of evidence

The available database has a number of deficiencies, including a paucity of studies that used highly purified and well-characterized toxins, and that used the oral route of exposure. Only limited information is available on a number of key end-points, including neurotoxicity, reproductive effects and effects of chronic exposure. Many of the reproductive and developmental studies have significant methodological limitations, as described in section 5.3.3. 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 whose relevance to in vivo exposures is unclear. Available epidemiological studies have lacked adequate exposure assessments and control of potential confounders, as described in section 4.2. Nevertheless, there is general agreement on key processes in MC toxicity. For example, consistent evidence has been obtained 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. As well, the primary biochemical target is recognized as protein phosphatase inhibition, which triggers a cascade of dose-related events that lead to the observed toxic outcomes.

7 Practical considerations

MCs are the most commonly reported and best researched of the cyanotoxins. Where blooms occur, MC concentrations can fluctuate widely as a result of uneven distribution of blooms in a water body, heterogeneity of clones within blooms and variation in the amount of toxin produced by individual clones.

Chapters 7–10 of TCiW (in press) give guidance on multiple barriers to reduce cyanotoxin levels in water, including controlling nutrient loads from the catchment, managing water bodies, optimizing sites for drinking-water off-takes 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 measures used to mitigate cyanotoxin risks by developing a water safety plan (Bartram et al., 2009; TciW, Chorus & McKeown, in press).

7.1 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, they may produce significant concentrations of toxins. Monitoring of source waters should include assessing factors that can affect the growth of cyanobacteria, including total phosphorus, temperature, water residence time and pH (for details, see TCiW, Padisák et al., in press). On-site visual assessment for turbidity with greenish discolouration or scums – for example, using a Secchi disc to measure water transparency – and microscopy are effective, low-cost, direct methods that can trigger increased vigilance if MC-producing cyanobacteria are observed. Monitoring over several seasons can often 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, Foss & Chorus, in press).

Monitoring programmes should be adaptive, so that sampling and analyses are increased when there is evidence of increasing amounts of cyanobacteria. For early warning and to trigger short-term management responses, alert level frameworks (ALFs) are useful both for drinking-water and for recreational water use. To trigger alerts, these frameworks primarily use levels of cyanobacterial biomass (measured as biovolume or chlorophyll *a*; Table 7.1) below which concentrations exceeding the health-based values of cyanotoxins for drinking-water (acute, short-term or lifetime) or recreational water are unlikely.

Table 7.1. Alert levels for cyanobacterial biomass indicators that trigger management responses

	Indicators of cyanobacterial biomass	
Alert level	**Biovolume (mm^3/L)**	**Chlorophyll *a* (with cyanobacteria dominant) (µg/L)**
Alert Level 1 threshold for drinking-water	0.3	1
Alert Level 2 threshold for drinking-water	4	12
Alert level threshold for recreational water use	8	24

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 discolouration; those that do not may be overlooked. Therefore, if the presence of cyanobacteria is suspected, microscopic examination for the presence of cyanobacteria that could potentially produce MCs is important. As blooms develop, monitoring can be expanded to include quantitative measures of cyanobacterial biomass that could indicate potential toxin concentrations, such as cyanobacterial biovolumes or chlorophyll *a*, or direct analyses of MC concentrations.

Wherever possible, particularly when estimates of MCs derived from biomass indicators are in the range of GVs, toxin analyses should be performed. This is because concentrations associated with blooms can vary substantially. The data from toxin analyses may allow restrictions on site use to be avoided or lifted where these were based on biovolume or chlorophyll *a* concentrations.

Template alert level decision trees for monitoring cyanobacteria and responding to exceedances are given in TCiW, Humpage & Cunliffe (in press) for drinking-water and in TCiW, Chorus & Testai (in press) for recreational water exposure.

7.2 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 vary. Techniques range from relatively simple visual inspections; to testing for phosphorus, pH, Secchi disc transparency, cell numbers, species identification, biovolumes and chlorophyll *a*; to toxin analysis. Limits of quantification below 1 µg/L can be achieved with all of the established methods outlined below. For less sensitive detection methods, appropriate sample concentration is required. More sensitive methods generally require less sample preparation, but costs per analysis tend to be higher.

For cell-bound and total (cell-bound plus extracellular) MCs, extraction (e.g. by freeze–thaw cycles) is performed before analysis. Filtration can be used to separate cells so that intracellular and extracellular fractions can be tested separately. More hydrophobic MC variants can be efficiently extracted from cells with aqueous methanol. Samples or crude extracts can be concentrated using evaporation or solid-phase extraction (SPE); the latter also provides sample clean-up. For absorbance detection (ultraviolet [UV], photodiode array [PDA]), pre-concentration and clean-up may be required, and many methods for analysing dissolved MCs require prior concentration of samples from larger volumes of water by SPE to achieve a limit of quantification below 1 µg/L.

The method with the highest specificity and sensitivity is liquid chromatography tandem mass spectrometry (LC-MS/MS). 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, liquid chromatography separation coupled with absorbance detection (UV, PDA) allows 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 of all. This is because the chromophore of MCs is the conjugated double bond of the Adda moiety, which is conserved among all known structural variants; hence, molar extinction coefficients of individual MC variants are similar (ISO, 2005). A caveat is that absorbance detection may overestimate peaks not fully separated from other substances in the matrix, particularly when peak intensity is low.

To account for total MCs, nonspecific methods can be used. One approach allows quantification of total MCs (and nodularin) through the oxidative cleavage of the Adda moiety and subsequent analysis of the product, 2-methyl-3-methoxy-4-phenylbutanoic acid (MMPB). More commonly used commercially available tests include immunological (e.g. ELISA) and biochemical (e.g. protein phosphatase inhibition assay) techniques. Although such tests do not enable identification of specific MC congeners, they are useful for screening.

These methods were developed for analysis of water samples. Their application to more complex matrices (e.g. food, stomach/tissue contents) requires identification of matrix effects, prior clean-up, and determination of recovery rates by spiking samples with known amounts of MC.

Molecular tests have been developed to identify the presence of one gene fragment 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 of MCs (see TCiW, Padisák et al., in press, for further information).

The choice of analytical methods depends on local or regional accessibility, costs and, in particular, the purpose of the analyses. Objectives may include screening for risk assessment, assessing compliance with GVs, and research.

For more information on analytical methods, see TCiW, Lawton et al. (in press).

7.3 Source control

For planktonic toxic cyanobacteria, preventing blooms in source waters is the key to long-term control of the risks they present. The most sustainable approach is to keep concentrations of plant nutrients low. Most cyanobacteria proliferate under eutrophic conditions – that is, elevated concentrations of nutrients, particularly phosphorus – and total phosphorus concentrations below 20–50 µg/L (with the threshold depending on water body characteristics) will often limit the development of cyanobacterial blooms. 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* develops 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).

Other measures can be applied to water bodies to mitigate cyanotoxin occurrence, including artificial water column mixing, nutrient reduction through sediment removal or treatment, and biomanipulation. Success of these measures is highly dependent on the specific conditions in the water body, as discussed in TCiW, Burch, Brookes & Chorus (in press).

Many reservoir off-take structures (towers) can take water from multiple depths to account for vertical heterogeneity. Variable off-takes allow water layers containing the highest concentrations of cyanobacteria to be avoided. 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 in removing cyanobacteria and in biodegrading 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.

7.4 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. Unless blooms are decaying, a high proportion of MCs is cell bound and therefore effectively removable by physical processes. These include coagulation followed by flocculation, clarification and rapid media filtration, as well as slow sand filtration or membrane filtration.

Filtration processes require care to avoid shear stress that may rupture cells. 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 CYN concentrates (e.g. filter backwash, sludges, sludge supernatants) are not allowed to return to the head of the filtration plant during a bloom.

To enhance flocculation, pre-oxidation is sometimes used. Depending on the amounts applied, oxidants can lyse the cells, causing toxin release; at sufficiently high oxidant concentrations, they can also degrade the released toxins (see below). However, elevated cyanotoxin concentrations (including MCs) typically occur during blooms, which cause a high organic load at the treatment plant. Oxidizing this material without prior filtration is likely to cause high concentrations of disinfection by-products, so filtration before oxidation is recommended.

Dissolved MCs can be removed by adsorption onto powdered activated carbon (PAC) or granular activated carbon (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.

Oxidation by chlorine or ozone can be effective in degrading dissolved MCs. However, the type and concentration of organic substances, as well as pH, strongly affect the amount of disinfectant needed. Elevated organic carbon 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 are ineffective against MCs.

The treatment methods discussed above can reduce MC concentrations to below 1 µg/L. However, validation of efficacy under specific local conditions is important: efficacy is highly dependent on water quality and other conditions in the treatment system. Validation may include field trials and laboratory investigations such as jar testing. Toxin removal during blooms should be verified by monitoring of MCs in the finished drinking-water.

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

For further information, see TCiW, Newcombe, Ho & Capelo-Neto (in press).

8 Conclusions

8.1 Derivation of the provisional guideline value

Insufficient data are available to derive a GV for MC variants except MC-LR.

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

- Fawell et al. (1999). Mice of both sexes given MC-LR by gavage at 40 µg/kg bw/day for 13 weeks did not show treatment-related effects in the parameters measured. Only slight hepatic damage was observed at the LOAEL of 200 µg/kg bw/day in a limited number of treated animals. At the highest dose tested (1 mg/kg bw/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 seen in the mean levels of AST or ALT, which are early markers of hepatotoxicity. Some of the histological effects, including Kupffer cell activation and PAS staining, showed no dose–response relationship: all 10 animals at the low and high doses displayed similar degree of damage.

A number of studies have investigated effects of MCs on reproductive organs and on embryofetal development. Some studies did not find 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 MC-LR of 600 µg/kg bw/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/day in drinking-water from weaning (17 weeks before 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), exposure of mice via drinking-water to relatively low doses of MC-LR was reported to cause adverse effects in the testis (reduced sperm count and motility, altered expression of some reproductive hormones, altered testis proteome patterns), prostate (hyperplasia) and ovaries (follicular histology, hormonal changes). Other studies (Li X-B et al., 2014; Li S et al., 2015; Zhao, Li & Chen, 2015) in which MC-LR was administered by gavage or s.c. described effects on learning in pups. However, omission of key

data and some limitations in the study design preclude use of these end-points 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 because of the very low concentrations of aerosolized MCs detected in air above a dense bloom (Wood & 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. During most recreational activities, the oral route (through involuntary intake of contaminated water) is considered more likely to lead to systemic effects.

Ito et al. (1997) found 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/day over 28 weeks. These findings support the NOAEL of 40 µg/kg bw/day 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 in hepatic toxicity biomarkers) at 50 µg/kg bw/day given every 48 hours to mice by gavage for 1 month, can support both the NOAEL 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. Whereas the Heinze study has the advantage of administering 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 more suitable for derivation of a lifetime GV. Some studies have a longer duration than Fawell et al. (1999), but none provide the comprehensive analysis of effects required in a key study.
- There is evidence that there may be fundamental differences in the mechanisms leading to hepatocellular death between rats on one hand and mice and humans on the other (Woolbright et al., 2017).

Although the duration of the Heinze (1999) study was shorter and more applicable to the exposure duration envisaged for application of the short-term GV, the advantages of the Fawell et al. (1999) study mean that an additional uncertainty factor is not needed for extrapolation from a LOAEL to a NOAEL, which would increase the total uncertainty and reduce confidence in the derivation of the short-term GV. For this reason, the NOAEL derived by Fawell et al. (1999) was selected as the basis for the short-term and recreational GVs, as well as the lifetime GV.

The TDI for lifetime exposure includes an uncertainty factor of 1000, consisting of a factor of 10 for each of interspecies differences and intraspecies variability in humans, and a factor of 10 for use of a subchronic study and other database deficiencies, as described in section 6 and further described below.

No uncertainty factor was included for database deficiencies to calculate the provisional short-term and recreational GVs, since the PoD is based on a sufficiently relevant period of exposure, which is short for the envisaged scenarios. Regarding developmental toxicity, an adequate

developmental mouse oral study (Fawell et al., 1999) gave a very high NOAEL of 600 µg/kg bw/day, well above the NOAEL identified for hepatic effects; this was supported by another two studies (Falconer et al., 1988; Chernoff et al., 2002). The results of studies on other possible end-points, such as male reproductive organs, are conflicting. Some studies that reported adverse effects at doses estimated to range from 0.79 to 25 µg/kg bw/day 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; these studies were conducted in 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 exposure. Moreover, Falconer et al. (1988) found no effect on male mouse fertility at doses up to 2700 µg/kg bw/day for 17 weeks via the drinking-water. 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/day. A cancer end-point is not relevant to short-term exposures: 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.

However, the evidence does suggest that MCs are likely to accumulate in the liver with longer-term exposure, potentially reaching harmful concentrations. This long-term process is associated with much greater data uncertainty, which led to the application of a database uncertainty factor of 10 for the derivation of the lifetime GV.

For deriving the lifetime drinking-water GV, the fraction of exposure allocated to drinking-water was 80% (rather than the 20% default) because drinking-water is expected to be the most likely long-term source of exposure. All other default assumptions were applied as described in WHO (2009) for deriving the lifetime as well as the short-term drinking-water GVs, and WHO (2003) for deriving the recreational water GV.

The GVs for MC-LR are considered provisional due to inadequacies in the database as reflected in section 6.2 and the database UF of 1000 for the lifetime GV.

Calculation of provisional TDI for MC-LR:

$$\mathrm{TDI}_{\mathrm{MC,chronic}} = \frac{\mathrm{NOAEL}}{\mathrm{UF}} = \frac{40}{1000}\ \frac{\mu g}{kg}/\mathrm{day} = 0.04\ \frac{\mu g}{kg}/\mathrm{day}$$

where

$\mathrm{TDI}_{\mathrm{MC,chronic}}$ = tolerable daily intake for chronic exposure

NOAEL = no-observed-adverse-effect level (40 µg/kg bw/day, based on Fawell et al., 1999)

UF = uncertainty factor (1000 = 10 for interspecies variation × 10 for intraspecies variation × 10 for database deficiencies, including use of a subchronic study)

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), the TDI would be 0.05 µg/kg/day.

Calculation of provisional lifetime drinking-water GV for MC-LR:

$$GV_{chronic} = \frac{NOAEL * bw * P}{UF * C} = \frac{40 * 60 * 0.8}{1000 * 2} \mu g/L = 0.96 \mu g/L \approx 1 \mu g/L$$

where

$GV_{chronic}$ = guideline value for chronic (lifetime) exposure

NOAEL = no-observed-adverse-effect level (40 µg/kg bw/day, based on Fawell et al., 1999)

bw = body weight (default = 60 kg for an adult)

P = fraction of exposure allocated to drinking-water (80%, as other sources of exposure such as air, food and soil are considered minor for lifetime exposure)

UF = uncertainty factor (1000 = 10 for interspecies variation × 10 for intraspecies variation × 10 for database deficiencies, including use of a subchronic study)

C = daily drinking-water consumption (default = 2 L for an adult).

Calculation of provisional short-term drinking-water GV for MC-LR:

$$GV_{short-term} = \frac{NOAEL * bw * P}{UF * C} = \frac{40 * 60 * 1}{100 * 2} \mu g/L = 12 \mu g/L$$

where

$GV_{short-term}$ = guideline value for short-term exposure

NOAEL = no-observed-adverse-effect level (40 µg/kg bw/day, based on Fawell et al., 1999)

bw = body weight (default = 60 kg for an adult)

P = fraction of exposure allocated to drinking-water (default for short-term exposure = 100%, as drinking-water is expected to be the most significant source of exposure)

UF = uncertainty factor (100 = 10 for interspecies variation × 10 for intraspecies variation)

C = daily drinking-water consumption (default = 2 L for an adult).

Calculation of provisional recreational water GV for MC-LR:

$$\mathrm{GV_{recreation}} = \frac{\mathrm{NOAEL} * \mathrm{bw}}{\mathrm{UF} * \mathrm{C}} = \frac{40 * 15}{100 * 0.25}\,\mu\mathrm{g/L} = 24\ \mu\mathrm{g/L}$$

where

$GV_{recreation}$ = guideline value for recreational exposure

NOAEL = no-observed-adverse-effect level (40 µg/kg bw/day, based on Fawell et al., 1999)

bw = body weight (default = 15 kg for a child)

UF = uncertainty factor (100 = 10 for interspecies variation × 10 for intraspecies variation)

C = daily incidental water consumption (default = 250 mL for a child).

8.2 Considerations in applying the provisional guideline values

The public should be informed about cyanobacterial blooms in source waters when the water is used for recreation or for producing drinking-water. This is particularly important if toxin concentrations in finished drinking-water temporarily exceed the GVs. As well, 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 use of the water is important to avoid people turning to other, less safe sources of water.

For recreational sites with blooms, information and warnings are particularly important. The most common situation is that monitoring cannot occur at sufficiently short time intervals (i.e. daily rather than weekly) to ensure that it captures situations with heavy scums or pronounced greenish turbidity (to the extent that one can barely see one's feet when knee-deep in the water). Site users therefore need information about avoiding contact with, or ingestion of, water under such situations. Temporary closure of sites is an option if blooms contain high toxin concentrations, exceeding the recreational water GV (for further detail, see TCiW, D'Anglada, in press). In determining toxin concentrations that trigger such responses, it is important to consider the actual site of water use (e.g. for raw water abstraction, bathing), since averaged MC concentrations may underestimate the risk at a particular site.

The provisional GVs are based on toxicological data for MC-LR. However, MCs usually occur as mixtures. Since MC-LR is one of the most toxic and common MCs, and in the absence of oral toxicity data for other congeners, for assessing risk it is recommended that total MCs as gravimetric or molar equivalents be evaluated against the GVs. However, the kinetic differences among variants (see section 3) mean that further investigation of the oral toxicity of MC variants other than MC-LR is warranted to reduce this relevant source of uncertainty.

In some regions, other sources of exposure besides drinking-water can be significant (see section 2.4). This includes food from locations where blooms have a long duration and there is high consumption of locally affected food items (e.g. fish eaten with viscera, or shellfish). In such situations, it may be appropriate to consider reducing the allocation factor for the lifetime and short-term drinking-water GVs based on relative exposure data for the population.

The short-term drinking-water GV is intended to provide guidance on how much the lifetime GV 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 repeated seasonal exceedances of the lifetime GV.

The short-term drinking-water GV 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 than for adults), 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.

As described in section 7.2, GVs can be used within the context of ALFs for early warning and to trigger short-term management responses. For further information on MC monitoring relative to GVs in the context of ALFs, see TCiW, Chapter 5 (in press).

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