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Current Opinion in Toxicology

"More toxic than thought!"

R. Fotler and D. R. Dietrich

Climate change enhances the formation and duration of toxin producing cyanobacterial blooms. Although toxins, e.g. Microcystins (MC), and their maximum levels in drinking water (1μg MC-$LR_{equiv.}$/Liter) and foodstuffs are regulated and controlled, the basis for this regulation is questionable. Nearly all governmental regulations rely on WHO guidance values (GV) that were derived from an *in vivo* mouse study using a single MC congener (MC-LR). However cellular uptake (and thus toxicity) is governed by organic anion transporting polypeptides (OATPs), whereby rodents and humans differ drastically with regard to the expression and transport affinity and capacity of OATPs, and thus mice appear less susceptible to MC. Accordingly, the current questionable GVs provided by WHO must be replaced *ad interim* with a Toxicity Equivalence Factor (TEF) approach, whereby as a consequence current GVs need to be lowered by at least a factor 22 as shown here. The latter would result in a GV for drinking water of 0.045μg MC-$LR_{equiv.}$/Liter to ensure safety of humans. As not all MC congeners can be tested, a new assessment approach using modern toxicology methods e.g. *in vitro* and *in silico* tools including artificial intelligence approaches must be undertaken to better characterize risks from exposure to toxic.

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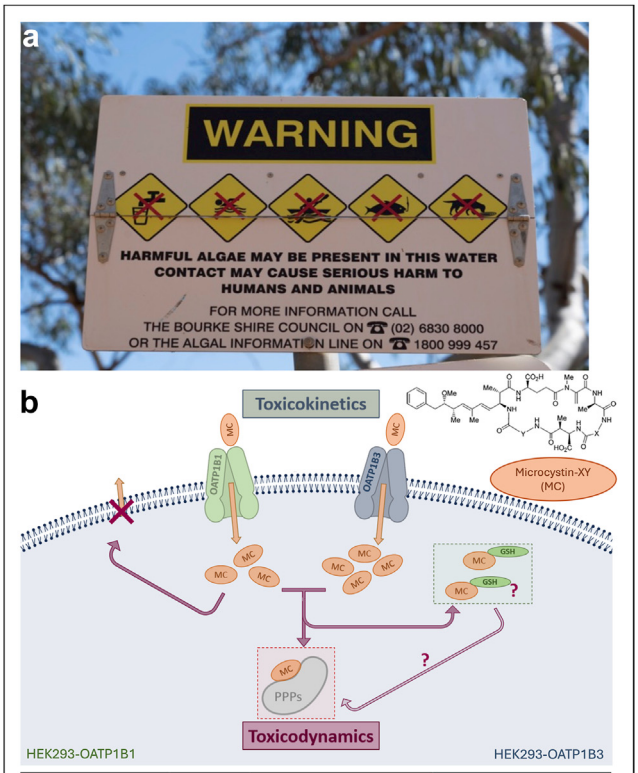
Environmental situation

Nearly two decades ago, Pearl and Huisman [1] suggested that with climate change, and thus globally increasing temperatures, potentially toxic cyanobacterial blooms in surface waters may become more frequent and intense. Recent data demonstrate that this not only occurs in nutrient-rich environments [2] but also in nutrient-poor environments, in fresh and brackish surface waters, and the marine system [3]. Central to the latter appears the continued heating of the water bodies concomitant with the vertical stratification of the water bodies [4]. Stratified water bodies provide optimal niches for massive cyanobacterial blooms, whether at many meters below the surface or at the surface proper. All blooms have the potential to contaminate potable water resources, food and food stuffs (including farmed and/or wild fish, shellfish and crustaceans), and prevent recreational use of surface waters. Cyanobacteria produce potent toxins, known as cyanotoxins, which can affect huge surface waters, e.g. the Darling river system in Australia (Figure 1a), Lake Erie, or large areas of the Baltic Sea in Europe. Cyanotoxins are chemically stable compounds, persisting in aquatic environments for extended periods of time and accumulate in sediments, plants, and aquatic organisms. Among the most hazardous cyanotoxins, Microcystins (MCs) are a group of structurally similar heptapeptide macrocycles consisting of common l-amino acids, but also uncommon and unique d-amino acids. The variable l-amino acid positions, along with various (de)methylation sites, provide for currently >300 known MC congeners [5]. MC concentrations sometimes exceeding 24′000 μg MC-$LR_{equiv.}$/L have been reported during cyanobacterial blooms [6].

Issue with the current risk assessment (RA) and WHO Guidance values (GV)

Despite this plethora of toxins and the high potential for human exposure, little progress has been made with regard to the prediction and assessment of MC toxicity in humans. This is particularly concerning given the reported cases of human intoxications and mortalities [7]. Indeed, the most recent World Health Organization (WHO) Guidance Document on MC for drinking water quality and safe recreational water environments still relies [8] entirely on a single mouse study as a basis for hazard assessment. The aforementioned concern is further exacerbated by the current understanding that the variances in biological characteristics between rodent species and humans render the extrapolation of potential hazards to humans both dubious and unreliable. Furthermore, the mouse study, pivotal to the hazard and risk assessment (RA) conducted by the WHO, was executed utilizing only a single microcystin congener (MC-LR), notwithstanding the fact that MC-LR is improbable to accurately reflect the genuine

Figure 1



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Microcystin	Toxicity Class	Cytotoxicity incl. Toxicokinetics				Toxicodynamics			
		OATP1B1		OATP1B3		PPP1		PPP5	
Congener	Toxicity	EC_{50} [nM]	TEF	EC_{50} [nM]	TEF	IC_{50} [nM]	TEF	IC_{50} [nM]	TEF
[ß-D-Asp^3,Dhb^7]MC-RR	low			511.9	0.03	62.0	0.00	877.1	0.01
MC-RR	low			52.97	0.30	1.50	0.20	11.7	0.44
[ß-D-Asp^3]MC-LR	moderate	23.37	0.72	23.87	0.67	0.90	0.33	10.2	0.50
MC-YR	moderate	115.2	0.15	12.10	1.32	1.20	0.25	5.60	0.91
MC-RY	moderate	580.6	0.03	5.890	2.71	0.50	0.60	4.03	1.27
MC-LR	moderate	16.78	1.00	15.99	1.00	0.30	1.00	5.10	1.00
MC-HtyR	moderate	41.10	0.41	16.08	0.99	0.70	0.43	4.70	1.09
MC-HilR	moderate	15.00	1.12	17.71	0.90	0.60	0.50	4.20	1.21
MC-WR	moderate	22.04	0.76	11.24	1.42	1.30	0.23	5.10	1.00
MC-LA	moderate	6.551	2.56	3.476	4.60	2.00	0.15	4.70	1.09
MC-LY	high	5.317	3.16	2.352	6.80	0.80	0.38	4.10	1.24
MC-LF	high	1.281	13.10	0.921	17.36	1.20	0.25	2.05	2.49
MC-LW	high	1.058	15.86	0.722	22.13	1.90	0.16	6.10	0.84

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Cyanobacterial bloom warning and corresponding toxicity in human cells. (a) Toxic algal bloom warning sign, at the south bank of the Darling River, Bourke (NSW), Australia 2019 (courtesy Falk Schreiber); **(b)** Toxicokinetic and dynamic aspects of microcystin cytotoxicity, as illustrated via OATP1B1 and 1B3-expressing HEK293 cells. GSH: glutathione. **(c)** Cytotoxicity of different MC congeners in HEK293-1B1 and HEK293-1B3 cells (toxicokinetics and dynamics) expressed as half-maximal effective concentration (EC_{50}) and PPP1- and PPP5-enzyme inhibition expressed as half-maximal inhibitory concentrations (IC_{50}). Toxicity Equivalent Factor (TEF) for the individual congener was calculated as the ratio of MC congener-specific EC_{50} and IC_{50} values to EC_{50} and IC_{50} of MC-LR, as suggested earlier [6]. MC, Microcystin; PPPs, Ser/Thr phosphoprotein phosphatases.

hazard posed by all currently identified MC congeners present in cyanobacterial blooms. MC toxicity is governed by the transporter-mediated uptake and excretion (toxicokinetics) of MCs into cells (Figure 1b) and the interaction of MCs with intracellular components (toxicodynamics), specifically those of hepatocytes as after oral exposure, MCs are taken up by intestine and reach the liver. Indeed, hepatotoxicity is the primary cause of human mortality upon intravenous, intraperitoneal, or oral exposure to MCs [7]. Organic anion transporting polypeptides (OATPs) drive cellular uptake of MCs [9] into human primary hepatocytes or HEK293 cells [9,10] expressing OATPs (Figure 1b), whilst MC-mediated inhibition of Ser/Thr phosphoprotein phosphatases (PPPs), and the interaction with glutathione (GSH) and other cellular components (Figure 1b) then leads to cytotoxicity (Figure 1b and c). As mammalian Ser/Thr PPPs (PPP1, 2A, 4, 5, and 6) are evolutionarily highly conserved [11] and tissue transcription patterns of PPP isoforms in rodent and human hepatocytes are highly comparable. Accordingly, it can be safely assumed that the inhibition of PPPs by MC congeners would be very similar if not nearly identical amongst rodents and humans. In contrast, large species differences in the toxicokinetics of MCs are expected as rodents express only one single OATP isoform (Oatp1b2), while humans express two (OATP1B1 and OATP1B3). Indeed, human OATP1B1 and OATP1B3 were shown to transport MC faster and more efficiently [9] than the rodent Oatp1b2, thus underscoring that rodents are poor surrogate models for assessing MC hazards in humans.

Possible ways forward

The aforementioned concern underscores that the prevailing methodology employed by the WHO likely grossly undervalues the toxicity of various MC congeners, contradicting contemporary advancements of modern toxicology. Indeed, major developments in evidence-based toxicological hazard and RA over the past decades have led to ongoing improvements in science-based frameworks e.g. the Adverse Outcome Pathways (AOPs) approach and New new Approach Methods (NAMs) including the use of artificial intelligence (AI) for predicting toxicity [12]. Due to the high structural similarity of MC congeners and the well characterized mechanism of action, i.e. uptake via OATPs and PPP inhibition, a MC-specific AOP could be defined. NAMs, e.g. *in silico* [13] and *in vitro* [10] approaches, could form the basis for a proper quantification of the hazard of each MC congener with higher relevance for humans and lead to AI predictions of MC congener-specific toxicity [14]. For example, using human OATP1B1 and 1B3-expressing HEK293 cells, Fotler [15] compared the cytotoxicity of a number of MC congeners (Figure 1c). Concomitant analysis of PPP inhibition provided a basis to calculate Toxicity Equivalent Factors (TEFs) [6] for both cytotoxicity (encompassing toxicokinetics and dynamics) and PPP inhibition (toxicodynamics). TEFs are calculated by comparing all MC congeners tested (Figure 1c) to the toxicity and PPP inhibition capacity of the most prominent MC

(MC-LR). As shown in Figure 1c, the MC congener PPP1- and PPP5-enzyme inhibitive capacity, when expressed as TEF [13,15], differs amongst MC congeners by a factor ≥100. Generally higher MC concentrations were required to inhibit PPP5 than for PPP1. TEFs also allow to determine the kinetic portion of cytotoxicity (Figure 1c), whereby e.g. MC-LW and -LF are taken up 63 and 87 times faster than MC-LR by OATP1B1, and 89 and 116 times faster than MC-LR by OATP1B3. The latter emphasizes the vast differences in MC congeners toxicokinetics and thus their important contribution to overall cytotoxicity (Figure 1c).

Limitations with current *in vitro* systems and translatability

Obviously, cell systems e.g. OATP-expressing HEK293 cells do not contain transporters for the excretion of MCs into the bile following conjugation with GSH as would be the case in hepatocytes, although it is currently assumed that some of the MC are also being transported back out into the blood via OATPs. Moreover, the GSH-conjugation capacity is much lower in HEK293 cells than would be apparent in human hepatocytes, thereby seemingly providing for a slight overestimation of the overall cell toxicity. However, the comparison with primary hepatocytes from two human donors demonstrated a >70-fold higher susceptibility of human primary hepatocytes to MC-LR, -LW and -LF toxicity than observed in HEK293-1B1 and HEK293-1B3 cells [10]. The latter, highlights the fact that human cell-based surrogate *in vitro* systems are more sensitive than rodent *in vivo* models to MC toxicity, MC-LR is not the lead MC congener to be employed for hazard and RA, and that current WHO Guidance Values (GVs) need to be revised.

Interim approach to decreased human exposure and increased safety

To bridge the latter incongruency in hazard and RA and in view of the fact that MC-LR is the standard MC congener that was also employed to derive GV for all MC congeners in rodent studies [8], an interim phase approach would be to employ the TEF differences observed in the *in vitro* studies [13,15] in the RA of WHO. The latter would mean that the currently employed GV for lifetime drinking water, 1.0 μg MC-$LR_{equiv.}$/L, would have to be corrected, in case of the predominant presence of MC-LW or MC-LF, by a reducing factor of *22* and 17, respectively. It is important to note that while MC-LR denotes one single specific MC congener, the term MC-$LR_{equiv.}$ denotes the sum of all MC congeners present in a sample expressed as if they were all MC-LR. This in the worst case, i.e. predominance of MC-LW in surface water bloom employed for drinking water, would result in a GV for lifetime drinking waterof 0.045 μg MC-$LR_{equiv.}$/L rather than the 1.0 μg MC-$LR_{equiv.}$/L currently considered safe. Moreover, even this GV does not take into account the intra-human differences observed in the expression levels of OATP. Moreover, a large number of single nucleotide polymorphisms (SNPs) and other sequence variations that can affect the function and thus toxicokinetics [16] have been described in the *SLCO1B1* gene (OATP1B1), and their allele frequencies vary markedly among different populations [17–20]. This could mean that upon decreased hepatic uptake of MCs due to OATP SNPs, higher circulating blood levels of MCs could evolve and thus shift primary toxicity from the liver to the kidney and/or central nervous system. In consequence, SNPs of OATPs could dramatically shift the hazard and thus the risk scenario in subpopulations currently not considered in the approach taken by WHO [8].

The question then arises whether or not the aforementioned worst-case scenario of high MC-LW or MC-LF exposures is realistic or not. Indeed, it has been demonstrated that in 32 % of cyanobacterial blooms analyzed in the Netherlands, MC-LW and -LF were present [21], albeit they were not the predominant MC congeners. Given the significant discrepancies in TEF among various MC congeners and their concurrent presence in blooms, an optimal method entails aggregating the individual toxicity contributions of each congener to derive a comprehensive toxicity assessment. In most cases, however, the required analytical capabilities would surpass the analytical means available to the authorities responsible, meaning that individual MC congeners are not determined and quantified. Moreover, TEF values are currently available for only a very small subset of MC congeners [13,15], thereby preventing a summation of TEF's to reach an overall MC hazard value. Consequently, the implementation of expedited detection techniques that quantify total microcystins (MCs) while omitting the identification of specific congeners would facilitate prompt decision-making by regulatory bodies and water providers to optimize water treatment, explore alternative sources, or, in dire circumstances, restrict public access to drinking and recreational water, as previously observed in the USA, Australia, Brazil, and other regions. However, to protect the public from undue exposure to more MC congeners more toxic than MC-LR, an interim phase GV for lifetime drinking water of 0.045 μg MC-$LR_{equiv.}$/L should be adopted.

Conclusions

Continued climate change and intensified use of water resources will augment the formation of cyanobacterial blooms [1–4] and, accordingly, the potential exposure to cyanobacterial toxins e.g. MCs. The latter is especially true in more arid areas of the world (Figure 1a), where access to surface waters will become increasingly difficult and thus results in a high pressure on this key

resource. Accordingly, the current questionable GVs provided by WHO [8] must be replaced *ad interim* with a TEF approach, whereby as a consequence, current GVs need to be lowered by a factor 22 as discussed here. In the near future, an international effort using modern toxicology approaches to improve the hazard and RA is required in order to prevent and protect the public from unwanted risk to cyanobacterial toxins such as MCs. In light of the plethora of MC congeners known, only a NAM approach, including *in silico* predictions [13] using AI capabilities [14] and more complex *in vitro* assays e.g. human liver organoids, would provide a basis to derive some form of a prediction model that then could allow for the development of more reliable GVs for drinking water, recreational waters, and for contaminated food.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Regina Fotler reports financial support was provided by the Arthur and Aenne Feindt Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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