



The dose makes the poison

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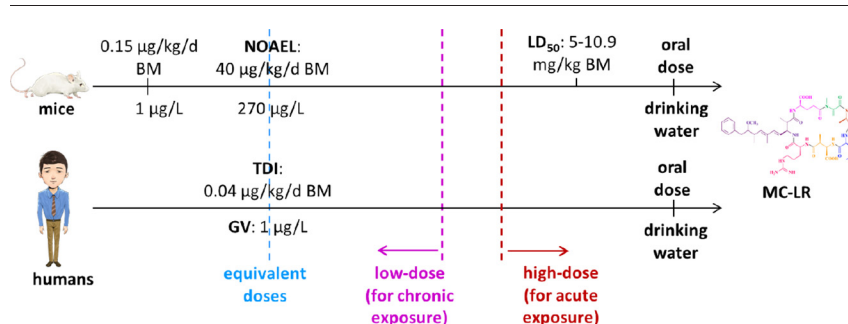
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HIGHLIGHTS

- A critical review of a previous study was performed.
- Exposure to the same concentrations do not result in equivalent doses among species.
- Inter-species variations should be considered in experimental design.
- Ranges of low-dose for effects of microcystins in animals and humans should be defined.
- Chronic toxicity and especially carcinogenicities of microcystins need further studies.

GRAPHICAL ABSTRACT



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ABSTRACT

Some microcystins (MCs) might cause hepatotoxicity in animals and humans. MC-LR is also a tumor promoter and a suspect carcinogen. In 2010, the International Agency for Research on Cancer (IARC) classified MC-LR as a possible human carcinogen (Group 2B). Recently, an article entitled “Long-term, low-dose exposure to microcystin toxin does not increase the risk of liver tumor development or growth in mice” was published in *Hepatology Research* by Meaghan Labine and Gerald Y. Minuk. However, the experimental design was flawed and the conclusion is misleading. 1 µg/L MC-LR in drinking water is the provisional guideline value established by the World Health Organization (WHO) for humans in 1998, based on a tolerable daily intake (TDI) of 0.04 µg/kg body mass (BM). Assuming the mice drink 1.5 mL/10 g BM of water per day, the exposure dose would be 0.15 µg/kg/d BM, about 270-fold less than 40 µg/kg/d, the no-observed-adverse-effect level (NOAEL). Thus, the dose of MC-LR was too small and “unlikely to result in liver tumor development or enhance existing tumor growth”, even with a long-term (28 weeks) exposure. Presumably, they didn’t consider inter-species variations between mice and humans, including toxicokinetics and toxicodynamics. Ranges of “low-dose” MCs for animals and humans should be defined. Also, the authors misunderstood or misrepresented several previous studies. Before drawing final conclusions on the carcinogenicity of MCs, further well-designed experiments are warranted.

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Recently, an article entitled “Long-term, low-dose exposure to microcystin toxin does not increase the risk of liver tumor development or growth in mice” was published in *Hepatology Research*, by Labine and Minuk (2015). Since we have been doing work in the area of algal toxins, we read with interest the paper regarding the carcinogenicity of long-term, low-dose oral exposure to microcystins (MCs). As

interested yet critical readers of the article, we would like to share some understanding and comments on the paper. First, we would like to acknowledge Drs. Labine and Minuk for their effort to report on safety issues of MCs. However, in that paper the authors concluded that MCs are not complete carcinogens (acting as both initiator and promoter) or tumor promoters, which is contrary to results published by other authors who have found that microcystin-LR (MC-LR) is a tumor promoter and a suspect carcinogen (Žegura et al., 2011; Žegura, 2016). Their conclusion is also inconsistent with conclusions drawn by international agencies. For instance, in 2010, the International Agency for Research on Cancer (IARC) classified MC-LR as a possible human carcinogen (Group 2B) (IARC, 2010). We were surprised by the conclusions of Labine and Minuk (2015). Upon studying their article, we have concluded that the experimental design applied was flawed and resulted in data insufficient to support the conclusions drawn and thus making the conclusions made misleading. Specifically, the dose applied to the mice was not relevant for making comparisons with or extrapolating to effects in other species.

Toxicity is a function of both exposure and effect. That is, responses are a function of duration and intensity of exposure and potency, which is an inherent property of the toxicant in a specific species. While the statement “The dose makes the poison” (Latin: “*Sola dosis facit venenum*”), which is attributed to the Swiss physician Paracelsus (Philippus Aureolus Theophrastus Bombastus von Hohenheim) in 1538 (Anon, 1965), is a toxicological maxim that means: “All things are poison and nothing is without poison; only the dose makes a thing not a poison”. However, it can also mean that, if a threshold for effects was not reached, there will not be an observed response or adverse effect. If the dose in the body or at a critical site of action does not reach a sufficient concentration or internal dose, which is sometimes referred to as the critical body burden, then no toxicity would be observed. With this concept as background, we evaluated the doses applied in their study and then drew different conclusions than did Labine and Minuk (2015).

In their study, Labine and Minuk (2015) exposed mice to 1 µg/L MC-LR in drinking water, which is the provisional guideline, established by the World Health Organization (WHO) for protection of humans (WHO, 1998). In their study, male CD-1 mice were exposed to either drinking water alone (water group), drinking water containing 1 µg/L MC-LR (MC-LR group), MC-LR plus thioacetamide (MC-LR/TAA group) or thioacetamide alone (TAA group) (n = 20/group). After 28 weeks of exposure, no tumors were detected in the water or MC-LR alone groups, while 4 mice in the TAA group and 5 in the MC-LR/TAA group developed liver tumors. The mean size of the tumors in the MC-LR/TAA and TAA alone groups were similar as were the results of Ki-67 staining, number of atypical mitoses and liver cancer gene expression profiles. The authors concluded that long-term, low-dose exposure to MCs is unlikely to result in development of tumors in liver, or enhance existing tumor growth.

Assuming mice drink 1.5 mL/10 g body mass (BM) of water per day, the dose, normalized to body mass would be 0.15 µg/kg/d BM, which would be about 270-fold less than the no-observed-adverse-effect level (NOAEL) of 40 µg/kg BM/d (Fawell et al., 1994, 1999; WHO, 1998). Thus, the internal dose delivered to a mouse would have been less than the dose of MC-LR that would have been able to result in development of tumors in the liver or enhance growth of existing tumors, even during longer-term (28 weeks) exposure. Presumably, the authors of that paper didn't consider inter-species, allometric differences between mice and humans, including toxicokinetics and toxicodynamics. Ranges of “low-dose” for effects of MCs on animals and humans should be defined. Also, the authors misunderstood or misrepresented results and/or conclusions of several previous studies. Before drawing their conclusions on carcinogenicities of MCs, further, well designed experiments are warranted. Here, we present some background and analyses that might be useful when designing studies that are more appropriate and rigorous.

1. Background

Cyanotoxins, such as microcystins (MCs), are released by blooms of cyanobacteria that result from eutrophication where excess quantities of nutrients, generally phosphorus (P), are present, but higher temperatures also favor occurrence of blooms. Thus, frequencies, durations and intensities of blooms are expected to worsen with increasing loading of nutrients and warming of the climate, reported worldwide (Carmichael and Boyer, 2016). MCs are the largest and most diverse group of cyanotoxins, with more than 100 structural variants, with molecular masses between 895 and 1115 Da (Niedermeyer, 2013; Chen et al., 2016). Their general structure is cyclo(-D-Ala¹-L-X²-D-erythro-β-methylAsp³-L-Z⁴-Adda⁵-D-Glu⁶-N-methyldehydro-Ala⁷). Although modifications have been reported for all of the amino acids, two L-amino acid residues X and Z are responsible for most of the congeners (Niedermeyer, 2013; Chen et al., 2016). Concentrations of dissolved MCs in a range of 0.1–10 µg/L have been observed in surface waters, while cell-bound concentrations were several orders of magnitude greater (Žegura et al., 2011; Chen et al., 2017). MC-LR is one of the most common and potent variants and it is also the most widely studied MC (Chen and Xie, 2016).

Due to reports of intoxications of both humans and animals and evidence that MCs cause toxicities, such as hepatotoxicity, reproductive toxicity, neurotoxicity, immunotoxicity and disrupt endocrine systems of animals, MCs have received increasing attention, especially as a public health threat (Chen et al., 2016; Hu et al., 2016; Valério et al., 2016; Buratti et al., 2017; Svirčev et al., 2017). MC-LR has been shown to be a promoter of growth of tumors and is suspected to be a carcinogen (Žegura et al., 2011; Žegura, 2016). Exposures of humans to MCs can occur by ingestion of contaminated drinking water, inhalation and dermal contact with toxins during recreation, consumption of cultivated plants, aquatic products including fish and blue-green algae supplements, and via the intravenous route during haemodialysis with contaminated water (Svirčev et al., 2017).

2. In exposures, the same concentrations do not result in equivalent doses among species

Interpretation of results of studies of carcinogenicity is profoundly affected by conditions during exposures, especially by inappropriately selected doses. This is particularly important for interpreting results of studies, where, due to insufficiently large doses, exposures do not result in significant carcinogenicity (USEPA, 2005). In fact, 1 µg/L MC-LR, the dose used in the study by Labine and Minuk (2015), is the provisional guideline value established by the World Health Organization (WHO, 1998) in drinking water for protection of humans. That value was based on a tolerable daily intake (TDI) 0.04 µg/kg BM (Fig. 1) (WHO, 1998; Chorus and Bartram, 1999). Due to limited information from epidemiological studies of humans exposed to MCs, assessments of hazard and risk have relied on extrapolation from toxicological data for animal models, generally mice and rats. However, data on toxicity to animal models is limited, especially for chronic, adverse effects at lesser doses. Results of a 13-week study in which mice were dosed orally with MC-LR are considered the most suitable for derivation of a guideline value (Fawell et al., 1994, 1999; WHO, 1998). In that study, a NOAEL of 40 µg MC-LR/kg BM per day (by gavage), based on pathology of the liver, was determined for both male and female Cr1:CD-1 (ICR)BR mice. A fairly conservative (protective) TDI of 0.04 µg/kg/d BM for humans can be calculated by applying an uncertainty factor (UF) of 1000 (10 for inter-species variation, 10 for intra-species (individual) variation, and 10 for limitations in the database, in particular, lack of data on chronic toxicity and carcinogenicity) to the NOAEL (Eq. (1), WHO, 1998; Chorus and Bartram, 1999; Codd et al., 2005; Dietrich and Hoeger, 2005; Falconer and Humpage, 2005). This TDI is supported by a 44-day study, in which pigs were given extracts of *Microcystis aeruginosa* in their drinking water (Fig. 1, Falconer et al., 1994; WHO,

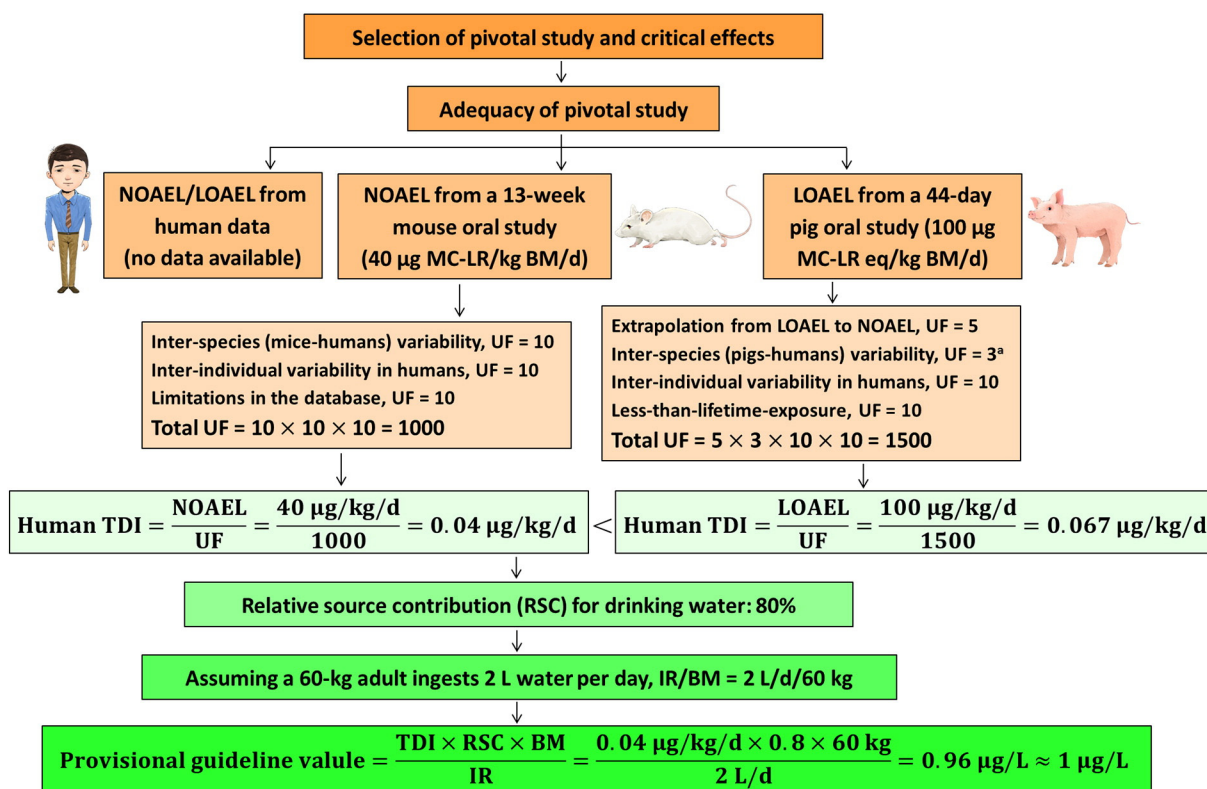


Fig. 1. Procedures for derivation of provisional guideline value of microcystin-LR (MC-LR) in drinking water for humans by World Health Organization (WHO) (adapted from IPCS, 1995; Dietrich and Hoeger, 2005). ^a3 rather than 10 for inter-species variability because pigs physiologically resemble humans more closely than rodents (Chorus and Bartram, 1999). NOAEL: no-observed-adverse-effect level, LOAEL: lowest-observable-adverse-effect level, BM: body weight, UF: uncertainty factor, IR: intake rate, TDI: tolerable daily intake, eq: equivalent.

1998; Chorus and Bartram, 1999). Because there is little assumed exposure from any other source or route, an allocation factor (AF) of 80% (0.8) was used to predict the proportion of daily exposure arising from drinking water (relative source contribution RSC = 0.8). Assuming a 60-kg adult ingests 2 L of water per day (drinking water intake rate/body mass, IR/BM = 2 L/d/60 kg), the provisional guideline for total MC-LR (free plus cell-bound) was determined to be 0.96 µg/L, which when rounded up is 1 µg/L in drinking water (Eq. (2), Fig. 1) (WHO, 1998; Chorus and Bartram, 1999). Due to application of safety factors, this guideline is very protective and exposure to this concentration would not be expected to result in observable adverse effects.

$$\text{TDI} = \frac{\text{NOAEL}}{\text{UF}} = \frac{40 \mu\text{g/kg/d}}{10 \times 10 \times 10} = 0.04 \mu\text{g/kg/d} \quad (1)$$

$$\text{Guideline value} = \frac{\text{TDI} \times \text{RSC} \times \text{BM}}{\text{IR}} = \frac{0.04 \mu\text{g/kg/d} \times 0.8 \times 60 \text{ kg}}{2 \text{ L/d}} = 0.96 \mu\text{g/L} \approx 1 \mu\text{g/L} \quad (2)$$

Due to differences in allometric variables between humans and mice and because the guideline was developed to be protective, rather than predictive, it was inappropriate to use the guideline value for humans, 1 µg/L MC-LR in drinking water, for assessing the carcinogenicity of long-term, low-dose exposure to MCs in CD-1 mice. Labine and Minuk (2015) noted that, the amount of water consumed as opposed to the proportion that was spilt in the animal holding cages could not be determined. Thus, the precise amount of MC-LR exposure was unclear. We agree that an oral dosing exposure route is an appropriate method of exposure during a study of carcinogenicity, but spillage and associated ingested dose uncertainty were likely entirely predictable outcomes at the study design. We argue that gavage dosing would have reduced uncertainty related to dose, and therefore sample sizes used in the published study are likely to be larger than would otherwise be required.

Even if there is uncertainty related to ingested doses in this study, we can estimate the dose. Assuming that mice drink 1.5 mL/10 g BM of water per day (IR/BM = 1.5 mL/10 g/d, AALAS, 2009; Dr. Labine, personal communication from an E-mail received on December 8th, 2015), the actual exposure dose would have been 0.15 µg/kg, BM/d (Eq. (3)). This dose is about 270-fold less than the NOAEL of for mice, which is 40 µg/kg, BM/d (Fig. 2) (Fawell et al., 1994, 1999). Thus, since “the dose makes the poison”, it is not difficult to understand why such a small dose of MC-LR was “unlikely to result in development of tumors in liver or enhance existing tumor growth”, even if a longer-term (28 weeks) exposure had been performed.

However, the statement “long-term, low-dose exposure to microcystin toxin does not increase the risk of liver tumor development or growth” may be true, but we cannot hastily draw the conclusion from results of the study presented by Labine and Minuk (2015), because the accepted NOAEL for CD-1 mice (40 µg/kg/d, about 270 µg/L in drinking water (Fig. 2) was not tested. The NOAEL is certainly within the range of low-dose, which might require additional research. Thus, the question raised by Drs. Labine and Minuk, i.e., whether long-term exposure to low concentration of MCs is “capable of initiating or promoting the growth of liver tumors” has yet to be determined. The dose ranges of “low-dose” for mice and humans should be clearly defined, and selection of dose should be well considered for combined chronic toxicity/carcinogenicity study (OECD, 2011).

$$\begin{aligned} \text{Actual exposure dose} &= \frac{\text{MC-LR Concentration} \times \text{IR}}{\text{BM}} \approx 1 \mu\text{g/L} \times 1.5 \text{ mL}/10 \text{ g/d} \\ &= 1 \mu\text{g/L} \times 1.5 \text{ L}/10 \text{ kg/d} = 0.15 \mu\text{g/kg/d} \quad (3) \end{aligned}$$

Presumably, Drs. Labine and Minuk did not consider inter-species variations (between mice and humans), including toxicokinetics and toxicodynamics, to properly scale the exposure. The maximum safe dose for humans cannot be derived directly by equating to the NOAEL

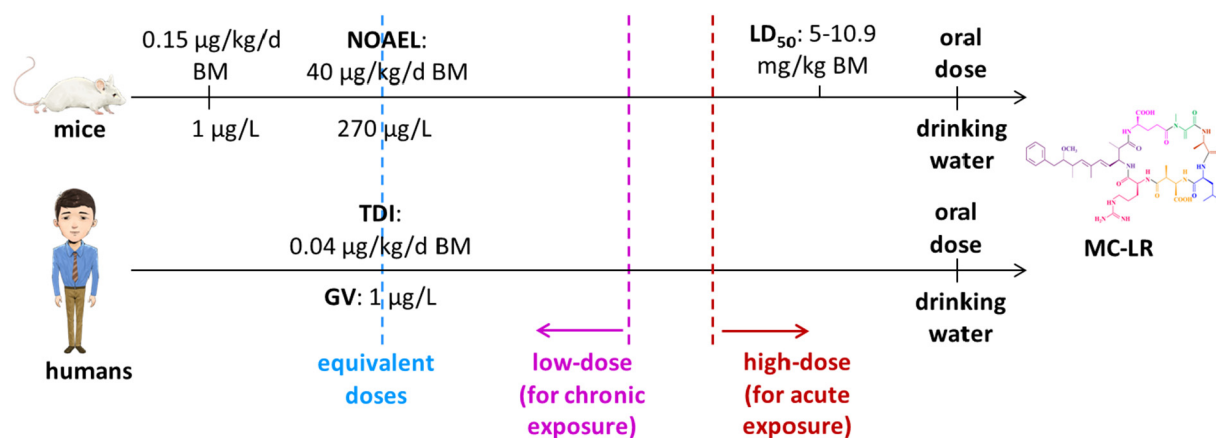


Fig. 2. Oral exposure of mice and humans to microcystin-LR (MC-LR). The values of lethal dose, 50% (median lethal dose, LD₅₀) are cited from the following references: Fawell et al. (1999), Yoshida et al. (1997), and Rao et al. (2005). NOAEL: no-observed-adverse-effect level, BM: body mass, TDI: tolerable daily intake, GV: guideline value.

in µg/kg, BM for animals. Alternatively, in toxicity tests with animals, a provisional guideline value in a µg/L (drinking water) or µg/kg BM (dose) for humans cannot be used directly. In other words, the dose translation/scaling/extrapolation and identification of “toxicologically equivalent” doses among species are important for assessments of risks, especially when the aim is dose-response assessment and setting acceptable levels of human exposure. Although mice and humans are both mammals and have certain similarities, it must be kept in mind that very important differences (species differences) exist and that when dealing with toxicants, these differences frequently are the margin between life and death (Oehme, 1974). There is substantial evidence indicating that animals used in toxicity studies can differ from humans in how they absorb, distribute, metabolize, and/or excrete toxicants (Oehme, 1970; Farrer et al., 2015). Animals might also differ from humans in their ability to repair damage caused by toxicants. Thus, an inter-species (between mice and humans) uncertainty factor of 10 is used to derive the TDI and provisional guideline value of MC-LR in drinking water, accounting for the variations between mice and humans in sensitivity, absorption/uptake, distribution, metabolism, detoxification, and excretion/elimination/clearance of MC-LR (WHO, 1998; Chorus and Bartram, 1999).

Results of several studies have suggested that humans might be more susceptible to adverse effects of MCs than are other mammals. Albumin content, plasma and albumin binding affinities of both MC-LR and MC-RR in humans were significantly greater than those of porcine and bovine models (Zhang et al., 2013). Greater affinities between MCs and plasma proteins including albumin, with relatively long half-lives in serum, might prevent rapid clearance by the kidney and reduce pinocytosis by the vascular epithelium, and prolong the half-life of MCs in humans (Pollaro and Heinis, 2010). Furthermore, for both MC-LR and MC-RR congeners, hepatic cytosol of rodents (both rats and mice) exhibited greater catalytic efficiency (2- to 3-fold greater intrinsic clearance, $Cl_{iapp} = V_{maxapp} / K_{mapp}$) by glutathione transferase (GST) and greater rates of conjugation with GSH than for humans (Buratti and Testai, 2015). This is mainly due to greater affinities for the substrate (MCs) for liver cytosol, with K_{mapp} values being an order of magnitude less for animal models than that of humans. Given the fact that conjugation of MCs to GSH (MC-GSH) is the first step in detoxification of MCs, followed by degradation to the cysteine conjugate (MC-Cys) (Kondo et al., 1996; Pflugmacher et al., 1998; Guo et al., 2015), since both GSH and Cys conjugates exhibit lesser toxic potencies than the parent compound (MCs) (Kondo et al., 1992; Pflugmacher, 2016), it is reasonable to assume that humans are quite sensitive to toxic effects of MCs compared to mice or rats, and an uncertainty should be considered when conducting assessments of risk.

3. Minor comments

3.1. Sub-chronic study

On page 683, 2nd paragraph: “Based largely on the results of acute toxicity studies in pigs...” This is a misrepresentation of the results of the 44-day oral exposure of pigs by Falconer et al. (1994), which was a sub-chronic dosing study, not an acute toxicity study.

3.2. Tumor promoter

In the last paragraph on page 689, the authors state: “It should be noted, however, that not all studies have reported liver tumor development in rats or mice exposed to MC-LR. For example, Nishiwaki-Matsushima et al. and Ohta et al. did not identify liver tumors in Fisher 344 rats exposed to MC-LR (25-50 µg/kg bodyweight) neither did Falconer and Humpage who treated C57 black mice with 1.2-4.2 equivalents/kg bodyweight of MC in their drinking water.” This statement misrepresents previous studies. Results of studies by Nishiwaki-Matsushima et al. (1992) and Ohta et al. (1994) indicated that MC-LR is a tumor promoter, rather than a complete/full carcinogen, which is able to act as both initiator and promoter. No evidence of promotion of lymphoid or duodenal adenoma/adenocarcinoma tumor growth in C57 black mice initiated using *N*-methyl-*N*-nitrosourea, was reported for exposure to extracts of *Microcystis* (Falconer and Humpage, 1996). However, the results that no primary tumors were observed in livers of any group should not be ignored. It was clearly stated that “To progress this area of investigation further, studies of liver tumour growth, initiated by a range of carcinogens, will be undertaken, in strains of mice more prone to this type of tumour.” (Falconer and Humpage, 1996).

4. Concluding remarks

In summary, we find the conclusions of the paper entitled “Long-term, low-dose exposure to microcystin toxin does not increase the risk of liver tumor development or growth in mice” to not be supported by the data. This was caused by use of an inappropriate experimental design, especially for exposures chosen, and the conclusions are therefore misleading. The exposure dose of this study, 0.15 µg/kg, BM/d, was 270-fold less than 40 µg/kg, BM/d, the no-observed-adverse-effect level (NOAEL) for mice. This small dose of MC-LR was “unlikely to result in liver tumor development or enhance existing tumor growth”. Therefore, the study design used by Drs. Labine and Minuk was inappropriate and inadequate for assessing carcinogenicity of long-term, low-dose

oral exposure to MCs. The dose ranges of “low-dose” MCs for animals and humans should be clearly defined. Before any final conclusions can be drawn on the carcinogenicity of MCs, further experiments with a more appropriate design, especially the selection of doses, are warranted. Whether the present WHO provisional guideline value of MC-LR (1 µg/L) for drinking water is protective of humans needs additional study. A more in-depth analysis of risks posed by various MCs, structure–activity relationships for MCs, possible interactive (additive, synergistic, or antagonistic) toxic effects of co-exposure, and comparative toxicology of MCs on humans and animals will be useful for reducing uncertainties related to the WHO provisional tolerable daily intake (TDI), derived for MC-LR only and extrapolated to all other congeners.

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