CHAPTER 6

MICROCYSTINS: OCCURRENCE AND HUMAN HEALTH EFFECTS

Franca M. BURATTI[1], Maura MANGANELLI[1], Meriç ALBAY[2], Simona SCARDALA[1], Emanuela TESTAI[1]

[1]Istituto Superiore di Sanità, Department Environment and Health, Rome, Italy
E-mail: (Franca M. Buratti) franca.buratti@iss.it
E-mail: (Maura Manganelli maura.manganelli@iss.it
E-mail: (Simona Scardala) simona.scardala@iss.it
E-mail: (Emanuela Testai) emanuela.testai@iss.it

[2]İstanbul University, Faculty of Aquatic Sciences, Department of Marine and Freshwater Resources Management, İstanbul, Türkiye
E-mail: merbay@istanbul.edu.tr

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Abstract

Microcystins (MCs) are among the most widespread and studied cyanobacterial toxins. More than 300 different MCs congeners have been identified so far, of which MC-LR is the most well-known and is considered as one of the most acutely toxic. Chemically, MCs are cyclic heptapeptides produced through nonribosomal peptide synthases by a number of different cyanobacteria. Microcystin-containing 'blooms' occur worldwide and have caused numerous cases of animals poisoning (many of which were lethal) and a small number of well-evidenced non fatal cases of human poisoning. While acute fatal human intoxication would require ingestion of unlikely high amounts of scum, long-term effects from repeated exposure to low doses (e.g. contained in drinking or recreational waters, in food and feed) can be anticipated. Based on the increasing occurrence of cyanobacteria due to climate change, MCs can be considered an emerging risk for animal, human and the environment and represent a good example of the One Health approach. Here we give a critical review of information on MCs' producing organisms, biosynthesis and genes encoding it, occurrence and toxicological profile, with a focus on kinetics, systemic toxicity, mode of action and human health effects, including the derivation of limit values for their concentration in water to which humans are exposed and recommendations by International Organizations and/ or regulatory limits across the globe.

Keywords: Microcystins, cyanobacteria, toxicological risk assessment

1. Introduction

Cyanobacteria are prokaryotic, autotrophic photosynthetic, gram-negative microorganisms widespread worldwide, especially in freshwater and marine environments, but they can also occupy habitats in extreme environments, such as hot springs or salt lakes or the arid desert, where they can form desert crusts. Although present in the environment since 3.5 billion years, in recent decades in many lakes, rivers and reservoirs used for drinking water, fisheries or recreational activities, they are multiplying excessively, producing visible mass populations (blooms, scums and mats) of increasing density and duration. Causes include eutrophication and climate changes, related to anthropogenic activities, all over the world (Codd et al., 2005; Whitton et al., 2012). Cyanobacteria can produce bioactive secondary metabolites which encompass a wide range of compounds, among which some have been characterized and show specific toxicological profiles, known as cyanotoxins. Although evolutionary principles suggest these substances to have roles in the survival and proliferation of the producing strains, their precise function is yet unclear.

Since the blooms can contain cyanotoxins and cause health impacts where human or animal population are exposed, the phenomenon has attracted attention and created concern of public health and drinking water supply authorities, recreation and tourism organizations, fisheries and other waterbody-users, requiring risk management measures to protect human and animal health.

The toxicity of a bloom depends primarily on the presence of toxic genotypes, i.e. those possessing the genes/gene complex for toxin production, even if this may not be enough for the presence of the toxin. In field populations the ratio between the toxic and non-toxic genotypes may vary greatly (Kurmayer and Christiansen, 2009), in function of various factors, among which the concentration of dissolved CO_2. The production of toxins, in turn, appears to be in some cases constitutive and related to the growth rates, while in other cases it seems to depend on the density of the bloom and other environmental factors (Manganelli, 2016). It is still very difficult to generalise, and make predictive models, since every species behave differently to the various stimuli (Funari et al., 2017; Testai et al., 2016).

Among the known cyanotoxins, microcystins (MCs) are the most frequently detected in various part of the world and the most studied.

2. Cyanobacteria Producing Microcystins and Occurrence

Microcystins are produced by morphologically and physiologically diverse cyanobacteria genera from all orders of Cyanobacteria (Table 1), including *Anabaena, Dolichospermum* (ex

Anabaena), *Aphanizomenon, Geitlerinema, Leptolyngbya, Microcystis, Nostocales, Phormidium*, and *Planktothrix* (Buratti et al., 2017; Turner et al., 2018; Codd et al., 2020; Chorus and Welker, 2021). Of all these, only the planktonic species *Microcystis*, *Planktothrix* and the *Dolichospermum/Anabaena/Aphanizomenon* group produce large blooms (Fastner and Humpage, 2021) (Figure 1). They are chiefly contained within the cyanobacterial cells, and they may be released from senescent, dying or decaying cells. While they are chemically very stable, biological degradation of dissolved microcystins by aquatic bacteria is effective, with half-lives usually in the range of hours to days.

Figure 1. Worldwide detection of planktonic species of cyanobacteria, producing MCs, able to form large blooms. Black dots for *Microcystis*, yellow for *Planktothrix* and red the *Dolichospermum/Anabaena/Aphanizomenon* group

The best known and most widespread is the unicellular colonial genus *Microcystis*, whose blooms have been reported worldwide except from Antarctica (Bouhaddada et al., 2016; Eguzozie et al., 2016; Yu et al., 2014; Sabart et al., 2013; Wood et al., 2012a; Naselli Flores et al., 2007; Harke et al., 2016 and references therein). A wide genetic and physiological plasticity (Dick et al., 2021; Yancey et al., 2022) allows *Microcystis* to dominate phytoplankton communities across a wide range of physicochemical conditions in the different lakes and regions in the world. *Microcystis* is usually found in eutrophic waters but can attain high biomass also at low P concentration due to both its possibility to regulate its buoyancy, using

Table 1. Examples of MCs worldwide detection and producing cyanobacteria					
Taxon	**µg/L (unless other unit is given) or frequency of detection**	**Origin**	**Sampling site**	**Reported co-occurrence with other toxins**	**Reference**
Microcystis	up to $124x10^3$ in bloom	worldwide			Chorus and Welker, 2021
Planktothrix agardhii	up to 100 in bloom	worldwide			Chorus and Welker, 2021
Planktothrix rubescens	<1-10 in water up to 34 x 10^3 in bloom	worldwide			Chorus and Welker, 2021
not reported	up to $2.2x10^{-3}$	Canada	marine environment	DTX, PTX, YTX	Shartau et al., 2023
cyanobacteria associated with macrophytes	detected in 20% of water samples and in 19% of macrophyte samples	Europe	lake	ATX (most frequently detected in macrophyte samples) CYN (most frequently detected in water samples)	Fastner et al., 2023
Microcystis *Anabaena* *Aphanizomenon* *Oscillatoria*	up to 296 up to 4019 up to 5.5 up to 7.1	Great Britain	natural and artificial lakes, reservoir		Turner et al., 2018
not identified, potential downstream transport	accumulated (over approximately monthly intervals) dissolved MC on Solid Phase Adsorption Toxin Tracking (SPATT) up to 803 ng/g resin particulate MCs up to 0.007	USA	lagoonal estuary	domoic acid (co-occurred in 50% of accumulated dissolved MCs samples)	Anderson et al., 2023
Nostocales dominated benthos *Synechococcales* dominated plankton. (low adundance)	no MCs in water 55µg/g dw in biofilms from stony substrates	Asia	Oligotrophic lake		Belykh et al., 2023
Microcoleus autumnalis, *Phormidesmis priestleyi*, *Wilmottia murrayi*	<0.15-1.6	Antarctic mats	freshwater bodies on Ross Island in Victoria Land and from the McMurdo Ice Shelf		Usman et al., 2022

not reported	up to 7 in water samples up to 9.2 ug/kg ww in fish muscle	Canada	River and wild-caught fish	ATX, Homo ATX, anabaeno-peptins A and B	Skafi et al., 2021
Microcystis spp, *Karenia brevis*	up to 2833	USA	estuarine environments	Brevetoxin, BMAA, AEG, DAB	Metcalf et al., 2021
Dolichospermum, *Anabaena, Aphanizomenon, Microcystis* and *Pseudanabaena*	Total MCs (nd-10)x10^{-3}in water samples No MCs (<LOD) but genera identified in $PM_{2.5}$	USA	Water and aerosol ($PM_{2.5}$) eutrophic estuary		Plaas et al., 2022
Aphanizomenon, Aphanocapsa, Cylindrospermopsis, Planktothrix, Microcystis, Limnothrix, Planktolyngbya and *Pseudoanabaena*	up to 5.7 in water up to 45.3 in scum	USA	lake	ATX, CYN, NOD	Howard et al., 2021
	0.005-0.4	Greenland	18 artic lake		Trout-Haney et al., 2016
Oscillatoria	50% of water bodies examined had detectable levels of MCs	Africa	Costal waters		Kadiri et al., 2020
Microcystis aeruginosa complex	up to $3x10^3$	South America	Costal waters		Kruk et al., 2021
Microcystis aeruginosa, flos aquae and *wesenbergii*	ranged from a median of not detected to 0.65 µg/L, a max value of 33 µg/L	USA	Costal waters	PSP, DST	Lehman et al., 2017

nutrients accumulated at the bottom and at the thermocline in deeper lakes (Paerl, 2014) and to a high affinity for dissolved inorganic phosphorous (DIP), which allows it to bloom in low DIP environments (O'Neill et al., 2012). Through gas vesicles *Microcystis* can migrate vertically to optimal light intensity and can form surface scums, thus limiting light availability to other phytoplankton (Komarek, 2003). *Microcystis* can be protected from predators either by producing deterrent chemicals (Van Wichelen et al., 2016) or by forming large colonies, inedible by grazers (Ger et al., 2016). In addition to an efficient CO_2 concentration mechanism, common to other cyanobacteria as well (Shapiro, 1997), *Microcystis* possesses more C_i uptake systems, suited to different concentrations of CO_2, and can thus be a better competitor in waters enriched with CO_2 (Sandrini et al., 2016).

Planktothrix thrives in various habitats, from all the continents (Kurmayer et al., 2016 and references therein). These organisms were originally classified according to morphological traits, like colour and cell size, but also habitat: the two most widespread phenotypes are the red *P. rubescens* that inhabits deep stratified oligo- mesotrophic lakes and the green *P. Agardhii* that attains high density in shallow eutrophic lakes (Suda et al., 2002; Kurmayer et al., 2016; Ostermaier et al., 2012). However molecular analysis showed that green and red phenotypes can be genetically and functionally closer than strains of the same color (Tooming-Klunderud et al., 2013; Rohrlak et al., 2008); indeed Pancrace et al.(2017) allocate all the planktonic strains to the same species. Thanks to the accessory pigment–protein complexes (bluegreen phycocyanin, PC, and red phycoerythrin, PE, each conferring the respective color to the respective phenotype) *Planktothrix* is a very efficient light harvester, and can thus attain high growth rates even at low light intensities, as in turbid water or in deep layers such as the thermocline of oligotrophic deep lakes (Manganelli, 2016). For the red phenotype of *Planktothrix*, the *mcy* gene cluster (responsible for MC synthesis) has been reported to be stable, present in a high percentage of the population and persistent (Kurmayer et al., 2004; Ostermaier et al., 2012). This contrasts with findngs for the green phenotype (Ostermayer et al., 2012) and other species, in which the toxic/non toxic genotype ratio is usually highly variable, such as in *M. aeruginosa* (Kurmayer and Kutzenberger, 2003; Okello et al., 2010). However, a high variability of the toxic/non toxic genotype ratio has also been observed in red *Planktothrix* during the declining phase of the winter bloom (Manganelli et al., 2016).

The other widespread bloom-forming taxa are the filamentous diazotrophic heterocystous *Anabaena*/*Dolichospermum*/*Aphanizomenon* (ADA) from the order of Nostocales, whose expansion, duration and intensity of blooms appear to be increasing (Li et al., 2016a and references therein). Recent sequencing of complete genomes from this clade (Osterholm et al.,

2020; Dreher et al., 2021) highlighted a wide range of physiological strategies explaining their success under variable nutrient concentrations and speciation, since most of them are able to use various forms of N, P and S (such as nitrate, nitrite and urea, phosphonates, sulfonates) (Dreher et al., 2021). Species from the clade can also tolerate and even compete successfuly under a wide range of temperature, from temperate to cold environments, even if some strains have T optima between 19 and 26 °C and generally form huge blooms in summer, under stratified condition (Salmaso et al., 2012). Finally, some species of these 3 genera are very tolerant to salinities, therefore they are the main taxa that bloom in the Baltic Sea (Ploug 2008), besides *Nodularia* that do not produce microcystins. Several strains of this clade are among the few planktonic cyanobacteria for which it has been demonstrated that they can simultaneously produce T&O substances and at least one major cyanotoxin (anatoxin-a, cylindrospermopsin, microcystins and saxitoxins) (Dreher et al., 2021 Manganelli et al., 2023).

While toxic benthic cyanobacteria have been studied much less than planktonic blooms, the increasing number of studies now becoming available show their ubiquitous distribution (Quiblier et al 2013; Wood et al., 2020; Ibelings et al., 2021b). They can grow on many different substrates, forming complex communities organized in mats, spreading laterally or vertically, producing thick mats (>70 cm thick), and they can proliferate under a wide range of nutrients concentrations, from oligo to eutrophic waters (Wood et al., 2020). They are found on the bottom of a wide range of different water bodies, drinking water reservoirs, lakes, rivers, meltwater, geothermal ponds, and coastal seawater (Fisher et al., 2015; Mohamed, 2008; Mohamed et al., 2015; Izaguirre et al., 2008; Gaget et al., 2017). The genera containing toxin-producing species include *Anabaena*, *Nostoc*, *Oscillatoria*, *Phormidium* (now also known as *Kamptonema*/*Microcoleus*), *Microcoleus*, and *Microseira* (previously *Lyngbya*). As for the planktonic species, mats can contain toxic and non toxic strain of the same species, even if the information on the conditions that lead to the dominance of one or the other is still very scant (Wood et al., 2020). The number of reports on toxic benthic cyanobacteria is increasing, partly due to a higher awareness and partly because in some ecosystems conditions are changing, favoring their occurrence. One such change includes water becoming clearer where eutorphication is declining, increasing light penetration and thus growth of photosynthetic organisms on sediments (Ibelings et al., 2021b).

Quantifying human health risks caused by exposure to benthic cyanobacteria is challenging. For most drinking water supplies risks seem unlikely because of their growth on the waterbody sediment and the primarily intracellular occurrence of microcystins. However, where parts of mats detach, they could occur in raw water, and assessing their occurrence in drinking water reservoirs or rivers used to abstract raw water is therefore recommended, most effecti-

vely in the context of hazard analysis and risk assessment when developing a water safety plan (WHO, 2022). Exposure risks via recreational water use are different: patches of mats may detach from the bottom, possibly due to buoyancy from photosynthetically produced oxygen trapped in the matrix or due to disturbance of the substrate, and these can accumulate along the shorelines or banks of rivers and lakes. Through this mechanism of concentration such mat material can be quite toxic and cause risks if ingested. While ingestion of such material seems unlikely for adults, risks cannot be excluded for children playing in shallow water along the shorelines. Moreover, some animals, chiefly dogs, are particularly attracted by decaying mats and many events of dogs deaths after ingestion of such mats have been reported, even if mostly for the presence of cyanotoxins other then microcystins (Backer et al., 2013).

The inclusion of benthic cyanobacteria in regular monitoring programs is challenging: standardized monitoring protocols are lacking and difficult to establish because of the heterogeneity of mat's substrates. A large number of samples would be required to cover this complexity (Wood et al., 2020). Furthermore results obtained pertain to toxins per unit area (cm^2) or per unit biomass (mg fresh or dry weight) rather than to a volumetric concentration (Welker and Raymond, 2021). A dose people could potentially ingest could thus at best be estimated as mass of mat material, but not as volume of water.

Microcystins occurrence have been extensively studied, and they have been identified worldwide, (Figure 1), including in Antarctica (Usman et al., 2022). Their concentration in water depends, as mentioned above, primarily on the presence of toxic strains and on the ratio between them and non toxic ones, which in turn is shaped by environmental conditions like light, temperature, CO_2, N and P speciation and concentration. Environmental conditions, as well as the stage of the bloom, can also influence the rate of microcystins production (Chorus and Welker, 2021). As a consequence, a wide range of MC concentrations has been detected during the blooms (Table 1). While pelagic concentrations are rarely above a few µg/L or tens of µg/L, very high concentrations may occur in surface scums: particularly where wind accumulates them in bays and along shorelines, concentrations may be in the range of mg/L or even tens of mg/L (Table 1). Besides the high cell density in these formations containing a high amount of toxin, high density may also promote a very rapid increase in *mcy* transcription and thus in MC cell quota (Wood et al., 2010; Wood et al., 2021). An increase in trascription of *mcy* has been observed also in condition of co-occurrence of *Microcystis* with specific organisms. Feng et al. (2020) showed that some strains of *Microcystis* exposed to negatively charged nanoplastic (<200nm) increased both intra and extracellular MC quota, showing a new "environmental" factor affecting MC production.

An extensive study conducted in temperate boreal areas in Canada by MacKeigan et al. (2023) used a standardized sampling plan for 440 lakes along 12 ecoregions (regions defined by unique climate, geology and vegetation), measuring total MC concentrations as well as those of MC variants, cyanobacterial abundance and species together with a wide range of environmental conditons (such as temperature, nutrients, hydrology,) and further biological variables (occurrence of Daphnia and other grazers in the zooplakton). The results showed the best predictors for MC concentrations to be *Microcystis* abundance and nutrient concentrations (total and soluble reactive P): these explained about 60% of the variability. Due to the complexity of the interacting conditions, it is still difficult to provide mechanistic or provisional model to predict the toxicity of a bloom (Bte Sukarji et al., 2022; Buley et al., 2022), so that the most important parameter for risk assessment during a bloom is the concentration of toxins, that has to be measured.

An important aspect of the problem is related to the increasing diffusion of microcystins, tipical of freshwater environments, along coastal areas, where they can possibly enter the food web via contamination of seafood in aquaculture plants and for accumulation in small animals, preyed upon by larger mammals (Miller et al., 2010; De Pace et al., 2014).

Preece et al. (2017) showed the occurrence of freshwater cyanoHABs (cyanobacteria harmful algal bloom) and microcystins in coastal waters of all continents. In some cases MC were discharged from freshwater environments after heavy rains in bloom seasons, but there are cases of occurrence of typical freshwater taxa such as *Microcystis* and *Anabaena* causing MC-producing blooms also in brackish environments. Other episodes of freshwater cyanobacteria and microcystins in coastal waters have been reported from the coast of Nigeria (Kadiri et al., 2020) and from the Atlantic coast in South America. Here, Kruk et al. (2021) documented a bloom that covered the whole strip of the Rio de la Plata estuary and the Uruguayan Atlantic coasts (around 500 km) for approximately 4 months, caused by the *Microcystis aeruginosa* complex (MAC). Microcystins concentration in water ranged from undetectable to 3 mg/L and the total average microcystin concentration for the period of the bloom was higher in samples from the Atlantic beaches than in estuarine sites.

Furthermore, in the anthropized areas is becoming usual the co-occurrence of microcystins with other natural toxins or chemical compounds, or also with pathogenic bacteria, raising the need to evaluate the risk for human health considering the exposure to a mixture of compounds (Metcalf and Codd, 2020).

On the Pacific coast of North America, microcystins have often been found co-occurring with marine toxins. Shartau et al. (2023) studied the occurrence of Net Pen Liver Disease

in salmons in the coastal areas of British Columbia and Vancouver Island (Canada), where many aquaculture plants occur; for the 2 years of sampling these authors found the concurrent presence of microcystins and 4 groups of marine toxins, okadaic and domoic acid, dinophysistoxin-1, pectenotoxin-2 and yessotoxin (Table 1). In San Francisco Bay (SFB), an area used for recreation and for the collection of mussels, water is routinely monitored for the presence of PSP and DST toxins. After years of recording temperature and intense drought that increased the intensity and duration of *Microcystis* blooms in the SFB/Delta system (Lehman et al., 2017), Peacock et al. (2018) analyzed water also for the presence of domoic acid and microcystins. They found all four classes of toxins in water and in 37% of the mussels sampled between 2012 and 2015. Coastal zone episodes of freshwater cyanobacteria and microcystins detection have also been documented in Southern California: in 2017 an extreme tide event caused the discharge of cyanotoxins from a coastal lagoon (Santa Clara River Estuary) into coastal water, where microcystins have been found all the year long together with anatoxin and domoic acid (Tatters et al., 2021). Howard et al. (2021) detected the occurrence of multiple toxins for the first time in Canyon Lake and Lake Elsinore, in Southern California, with the highest concentration of microcystins, anatoxin-a and cylindrospermopsin ever found in Southern California lakes (Table 1).

On the Atlantic side, examples include North Carolina, where Anderson et al. (2023) found persistent microcystins and domoic acid in the Bogue Sound from 2015 to 2020, and Florida. Here, the coast affected by the discharge of water from Lake Okeechobee harbours frequent blooms of *Microcystis* with high concentrations of MC (up to 2.8 mg/L), together with brevetoxins produced by a concomitant *Karenia brevis* bloom in seawater (Metcalf et al., 2021).

Cyanotoxins can co-occur with metabolites of cyanobacteria or of other aquatic organisms (particularly Actinomyces) that cause objectionable taste and odor (T&O). Such substances, particularly Geosmin with its low threshold of detection by the human nose (5 ng/L) can render drinking water unpleasant (Ackaalan et al., 2022) and thus easily rejected by consumers. A recent review analyzed the studies reporting their co-occurrence with cyanotoxins and concluded that there are not enough studies and not enough evidence of co-occurrence to consider T&O, detectable also at very low concentrations (ng/L), as a valid warning signal for blooms of toxic cyanobacteria (Manganelli et al., 2023). In their conclusion the authors also stressed that for a proper risk assessment of water, in case of co-occurrence of both the classes of substances, more toxicological studies are needed, considering the combined exposure to multiple chemicals.

3. Toxicity Profile

Microcystins are a family of more than 300 congeners, but the number of identified congeners is expected to increase further over time (Jones et al., 2021). The general structure of MCs is: cyclo-(D-Ala1-X2-D-Masp3-Z4-Adda5-D-γ-Glu6-Mdha7), D-Masp is D-erythro-β-methyl-isoaspartic acid, Mdha is N-methyl-dehydro-alanine, and Adda, an amino acid, (2S,3S,4E,6E,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) that has been identified exclusively in MCs and in the structurally similar pentapeptidic cyanotoxins, known as nodularins (NODs). Variants are due to substitutions with other aminoacids in position 2 and 4 (positions X and Z in Figure 2, marked red) and to other modifications (e.g. de-methylations) also occurring in other positions. As a result of leucine (L), tyrosine (Y), or arginine (R) in positions 2 and 4, the most prevalent congeners are MC-LR, MC-YR, and MC-RR (Figure 2).

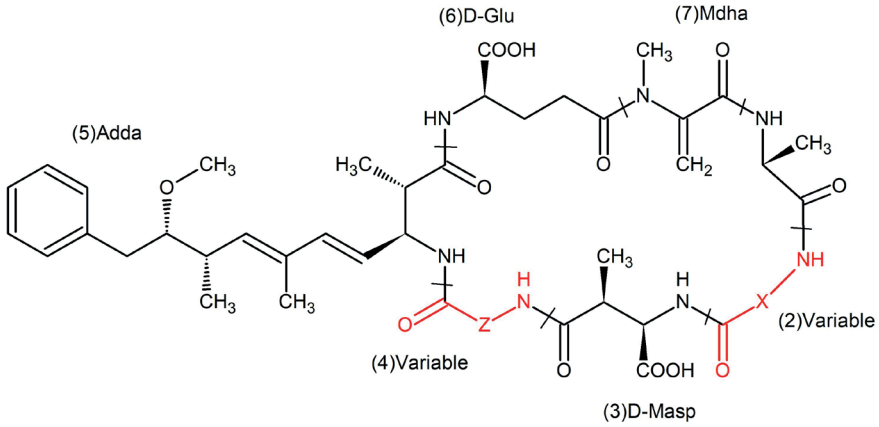

Figure 2. Microcystins chemical structure. Z and X mark positions at which amino acids may vary (see text).

Microcystins are produced by a hybrid polyketide synthase/non-ribosomal peptide synthetase (PKS/NRPS) enzyme complex, encoded by the genes (mcyA-J) in a 60 kbp-long cluster. Numerous strains and taxa, such as *Microcystis*, *Planktothrix*, *Dolichospermum*, Nostocales, and *Fischerella* posess PKS/NRPS gene cluster sequences. This makes it possible to profile cyanobacterial strains or wild populations to determine whether the MC gene cluster is present and, thus, whether there is a chance that MC could be produced (WHO, 2020). Many toxigenic strains simultaneously produce a variety of MC variants, although the number of variants produced by a given strain is typically limited. The strains (or genotypes) present in a bloom determine which variants occur and thus the quantity of MCs present (Testai et al., 2016), although it is yet poorly understood how environmental conditions influence strain dominance.

MCs toxicity to wild and domestic animals and humans can occur following exposure via the oral route, i. e. consuming contaminated drinking water or food items. The possibility of ingesting a dose higher than that recommended by the World Health Organisation for safe lifetime daily oral uptake has been suggested particularly for fish and shell-fish as well blue-green algal supplements (BGAS). MCs have also been found in or on vegetables irrigated with water containing cyanobacteria, although the levels found so far seems not to represent a concern for human health. Cyanotoxins presence has been reported also in dust containing dried planktonic biomass and aerosol formed e.g., through motorboats or strong wind. Therefore MCs uptake from such sources is possible through inhalation of dust or aerosol drops during showering, watersports or professional/recreational activities in waterbodies (Codd et al., 2005, 2020; Funari and Testai, 2008; Carmichael and Boyer, 2016; Chorus and Welker, 2021). Whether or not this constitutes a health risk depends on the dose and frequency of exposure discussed below.

The parenteral exposure can also occur when surface waters contaminated with cyanobacteria and not properly treated are used for haemodialysis. This exposure scenario represents a great threat for human health: beside the pre-existing diseases of patients, the toxins by-pass any absorption step and directly enter the blood circulation, determining a higher internal dose, when compared with the one attained with the other routes, at the same level of water contamination (Funari and Testai, 2008; Buratti et al., 2017). The most known outbreak was caused by the parenteral route in the Brazilian town of Caruaru in 1996, when patients undergoing regular renal dialysis started to experience headache, eye pain, blurred vision, nausea, and vomiting (Jochimsen et al., 1998; Azevedo et al., 2002). Due to using water contaminated with cyanotoxins, 76 patients ultimately passed away from liver failure. The water filtration system at the clinic used activated carbon, which was found to contain MCs and CYN through analysis. In a subsequent event again in Brazil in 2001, patients exposed to MC were almost asymptomatic, with biochemical outcomes (e.g., elevation of markers of hepatic cellular injury and cholestasis) varying among the patients (Soares et al., 2006). The event could be easily masked by the pathologic conditions of the dialyzed patients, but the awareness associated to the previous outbreak, lead to analyse the carbon filter and the blood of patients, where MCs were detected (Soares et al., 2006; Zanchett and Oliviera-Filho, 2013). Indeed, so far, Brazil then implemented a legal requirement to include cyanotoxins among the parameters to be routinely checked for the quality of water used for dialysis (Buratti et al., 2017).

3.1. Kinetics of MCs

Kinetics has a crucial role in MC toxicity, starting from the absorption, which can occur via gastrointestinal tract (oral exposure), respiratory tract (inhalation) or skin (dermal contact).

Indeed, absorption is almost exclusively mediated by transport proteins: in the intestinal mucosa it has been recently reported that in a 3D human reconstructed intestinal epithelium, the absorption of 5 MC variants is characterized by the equilibrium between uptake and extrusion (Turco et al., 2022). The intestinal uptake is only partially attributable to OATP 1A2 and 2B1, suggesting the involvement of additional transporters: MCs resulted not to be substrates of the efflux protein Pgp, but MRP2 and to a lesser extent BCRP are active in extruding MCs (Turco et al., 2022; Kaur et al., 2019). After oral exposure, the percentage of the administered dose detected in the liver is less than that observed after intraperitoneal (i.p.) or intravenous (i.v.) administration, which suggests that uptake from the intestine is slow and limits systemic exposure (WHO, 2020). Indeed, it has been recently reported that at concentrations in the range of 10-40 μM, plausible levels attained in the intestinal lumen after contaminated sea-food ingestion, MC-RR, LR, YR shows absorption values <5% of the administered dose (Turco et al., 2022).

The uptake of MCs after inhalation has been postulated as potentially relevant, considering the high blood perfusion and large surface area in lungs (Fitzgeorge et al., 1994), although the environmental levels measured so far are in the pg MC/m^3 range (Backer et al., 2008; Murby and Haney, 2016). Considering the chemical structure of MCs and the virtual absence of transporters in the skin, the dermal absorption of MCs is expected to be very limited (Funari et al., 2017).

MCs distribution to tissues is mainly governed by OATP transporter expression mediating the cellular uptake and level of blood perfusion, in addition to the type of congener (WHO, 2020).

Accumulation of MC in the liver has been reported in vivo in mice with the use of various techniques (e.g. radiolabeled MCs and immunostaining) (Nishiwaki et al., 1994; Ito et al., 2000). Uptake of MCs by hepatocytes is facilitated by the OATP1B1 and 1B3 isoforms, as demonstrated in several in vitro studies (Fischer et al., 2005, 2010; Niedermeyer et al., 2014; Rozman et al., 2017). The transport is congener-dependent: less water-soluble MC congeners (e.g. MC-LF, -LW, -LY) have higher affinity for OATPs (Fischer et al., 2010; McCord et al., 2018). Following oral exposure, a portion of MCs are subjected to the first pass effect, while the unchanged MCs can be systemically available and distributed to organs beyond the liver (WHO, 2020).

Biotransformation of MCs starts with conjugation to reduced glutathione (GSH, γ-glutamyl-cysteinyl-glycine, γ-Glu-Cys-Gly), to the methylene group of Mdha (Kondo et al.,

1992), either spontaneously or catalyzed by glutathione S-transferase (GST, Buratti et al., 2011, 2013), which has been demonstrated in aquatic plants, crustacean, mollusks, fish (Pflugmacher et al., 1998, 2016), rodents (Kondo et al., 1996; Takenaka, 2001), fungi (Esterhuizen-Londt et al., 2018) and humans (Buratti et al., 2011, 2013). The conjugates are much weaker in terms of toxicity compared to the parent molecules determined both in vivo, when mice were exposed via i.v. injection (Kondo et al., 1992), and in vitro in the PP inhibition assay (Metcalf et al., 2000).

As for the transport activity, also the conjugation reaction efficiency differs i) among variants and seems to be correlated to MC hydrophilicity, with MC-RR and MC-LF being highly and poorly conjugated, respectively (Santori et al., 2020) and ii) among species, with rodent GST having a greater catalytic efficiency for MCs than human GST (Buratti and Testai, 2015).

Although in human samples in the presence of physiological GSH concentrations the spontaneous reaction predominates, being up to 10-fold greater than the enzymatic reaction for some variants (Santori et al., 2020), when GSH is depleted, the enzymatic conjugation gives the major contribution to conjugation of low concentrations of MC-LR and -RR, likely representative of repeated exposure through contaminated food and/or water (Buratti et al., 2013; Buratti and Testai, 2015).

MCs metabolites are transported via the bloodstream from the liver to the kidneys to be excreted via urine or via the bile fluid to the intestines before excretion in faeces (WHO, 2020). However, at present, the cellular export of MCs and their metabolites has not been well studied and described.

3.2. Toxicity of MCs

The route and dose of exposure play crucial roles in kinetics and toxicity of MCs (Buratti et al., 2017; Chen et al., 2018; WHO, 2020). Regarding acute toxicity, i.p. LD_{50} of 50 μg MC-LR/kg bw in mice is 100-fold less than the oral LD_{50} = 5,000 μg/kg bw (Fawell et al., 1994, 1999), a difference attributed to kinetics reasons (Funari and Testai, 2008). For few MC variants (a small % with respect to the known ones) the acute toxicity potency after i.p. injection could be compared, showing that a single amino-acid difference (e.g. MC-LR vs MC-RR) can result in a 10 fold difference in the i.p. LD_{50} (Table 2).

Table 2. MC acute toxicity in mammals expressed as LD_{50} values (Buratti et al., 2017)

Variant	LD_{50}	value	Unit	Species
MC-LR	LD_{50} oral (gavage)	>5	mg/kg bw	Rat
	LD_{50} oral (gavage)	**5-10.9** Overall range	mg/kg bw	Mouse
	LD_{50} i.p.	**72-122** Overall range	µg/kg bw	Rat
	LD_{50} i.p.	**32.5-158** Overall range	µg/kg bw	Mouse
	LD_{50} i.v.	80	µg MC-LR_{equiv} / kg bw	Rat
	LD_{50} i.v.	28	µg/kg bw	Mouse
MC-RR	LD_{50} i.p.	**111-650** Overall range	µg/kg bw	Mouse
	LD_{50} i.v.	80	µg MC-LR_{equiv} / kg bw	Rat
MC-WR	LD_{50} i.p.	140, 171	µg/kg bw	Mouse
MC-FR	LD_{50} i.p.	100, 249	µg/kg bw	Mouse
MC-AR	LD_{50} i.p.	≈ 249	µg/kg bw	Mouse
MC-LA	LD_{50} i.p.	= 39	µg/kg bw	Mouse
MC-LY	LD_{50} i.p.	= 91	µg/kg bw	Mouse
MC-YR	LD_{50} i.v.	91	µg/kg bw	Mouse
[O-demethyl-ADDA^{5}]MC-LR	LD_{50} i.p.	≈ 97	µg/kg bw	Mouse
[desmethyl^{7}]MC-RR ([Dha^{7}] MC-RR)	LD_{50} i.p.	180, 420	µg/kg bw	Mouse
[desmethyl^{3}]MC-RR ([D-Asp^{3}] MC-RR	LD_{50} i.p.	250, 350	µg/kg bw	Mouse
MC-[methionine-S-oxide]R	LD_{50} i.p.	≈ 750	µg/kg bw	Mouse
[desmethyl^{3}]MC-FR ([D-Asp^{3}] MC-FR)	LD_{50} i.p.	= 90	µg/kg bw	Mouse
[desmethyl^{3}]MC-WR ([D-Asp^{3}] MC-WR)	LD_{50} i.p.	= 95	µg/kg bw	Mouse
[3H]dihydroMC-LR (epimer 1)	LD_{50} i.p.	= 120	µg/kg bw	Mouse
[3H]dihydroMC-LR (epimer 2)	LD_{50} i.p.	= 135	µg/kg bw	Mouse
GSH-MC-LR (coniugate)	LD_{50} i.v.	630	µg/kg bw	Mouse
Cys-MC-LR (coniugate)	LD_{50} i.v.	267	µg/kg bw	Mouse
GSH-MC-YR (coniugate)	LD_{50} i.v.	304	µg/kg bw	Mouse
Cys-MC-YR (coniugate)	LD_{50} i.v.	217	µg/kg bw	Mouse
i.p.= intraperitoneal, i.v.= intravenous				

The liver is considered as the main target organ of MC toxicity (WHO, 2020; Arman and Clarke, 2021): acute effects of MC-LR on liver are characterised by centrolobular toxicity

with intrahepatic hemorrhagic areas, due to damage of sinusoidal capillaries from cytoskeletal disruption within liver cells, allowing blood to flow between cells. Long-term exposure to small doses results in uncontrolled cellular proliferation and hepatic hypertrophy (Gehringer, 2004; WHO, 2020).

The generally accepted molecular initiating event of MC toxicity is the inhibition of the soluble and highly conserved serine/threonine protein phosphatases (PPs), including PP1, PP2A (Mackintosh et al., 1990; Yoshizawa et al., 1990), PP3 (Honkanen et al., 1994), PP4 and PP5 (Hastie et al., 2005). Various PPs are not equally sensitive to effects of MC congeners: hydrophilic congeners such as MC-RR and -LR have similar median inhibitive concentrations (IC50) for PP1 and PP2A, whereas PP2A is ≥2-fold more sensitive to the more hydrophobic congeners MC-LW, -LA and -LF (Altaner et al., 2019). However, this is not the major determinant for the variant-specific toxicity, which is due mainly to kinetic factors (Ito et al., 2002; Chen et al., 2006; Altaner et al., 2019; Santori et al., 2020).

Interaction with PP1 and PP2A involves ADDA, through which MCs covalently binds with a protein cysteine, residue, blocking the access of any substrate in the catalytic site, thus inhibiting enzymatic activity (Funari and Testai, 2008). However, ADDA *per se* is not able to inhibit PP1 and PP2A, and it is not toxic when i.p. injected in mice even at very high doses (10 mg kg^{-1} body wt (Harada et al., 2004)), suggesting the need for some steric hindrance, provided by the toxin molecule, to cause efficient inhibition.

Inhibition of phosphatases leads to hyperphosphorylation of downstream proteins, reverses the action of protein kinases, and activates a diverse set of signaling pathways and cellular processes, triggering a cascade of events including oxidative stress, cytoscheleton disruption, induction of apoptosis via mithocondrial dysfunction and endoplasmic reticulum stress, stimulation of possible inflammatory responses by disrupting the influx of neutrophils into affected organs, cell proliferation and decreased repair of damaged DNA associated to tumor promotion (Kujbida et al., 2009; Liu and Sun, 2015; Buratti et al., 2017; WHO, 2020). Overall, it can be concluded that MCs elicit toxicity via multiple pathways (Chen and Xie, 2016; Vichi et al., 2016; Buratti et al., 2017), as the result of 'cross-talking' and cooperative effects between different pathways.

Although the hepatotoxicity mainly characterizes MC toxicity, other systemic effects have been reported, which are indeed consistent with the expression of OATP transporters in various tissues, e.g. in the kidney and in the blood-brain barrier. These extrahepatic effects have been investigated in more recent studies, focusing mainly on reproductive toxicity, or organ

specific toxicity in thyroid and lung. Unfortunately, most studies used i.p. administration, which is not representative of animal and human exposure to MCs and has different kinetics. Also, these studies have not followed guideline test methods, and study designs as well as data reporting are questionable. Many of these studies only applied a single dose and thus could not establish a clear dose-response, which is necessary to identify dose without an effect as basis for an adequate risk assessment (Testai et al., 2016). Furthermore, studies in which MCs have been administered orally reported only MC concentrations in drinking water, without measuring the actual uptake of MC (e.g. Li et al., 2015, Zhao et al., 2020). The latter is generally estimated on the basis of an assumption for the average water uptake (with different studies based on different assumptions), without taking into account the increased body mass of rodents which is expected in long term studies (e.g. 12 months).

Some recent studies reported the use of OMICs technologies for Mode of Action (MoA) studies, many using fish species which do not readily allow extrapolation of the results to mammals; others conducted in rodents used an insufficient regime for administering the MCs, e.g. administration every other day, which due to kinetic effects cannot be directly extrapolated to daily doses (He et al., 2012, 2017; Sedan et al., 2015). While these results can be used to support the understanding of toxic mechanisms, they have been considered not robust enough for regulatory purposes (WHO, 2020).

One reason for the lack of 'good quality' toxicological studies, adequate for deriving guideline values or standards, could be the limited availability of the toxins; for this reason studies are often carried out with poorly characterised cyanobacterial extracts (with a number of confounding factors) (e.g. Schaeffer et al., 1999), also providing results that are insufficient for risk assessment. Indeed, the available data indicate that cyanobacterial components other than cyanotoxins are responsible for some toxicity and contribute to increased toxicity of the extracts when compared to 'pure' toxins (Testai et al., 2016).

The Weight of Evidence (WoE) indicates that MC-LR can cause DNA oxidative damage, likely associated with apoptotic/necrotic events, but is not a DNA-reactive genotoxin (Žegura et al., 2011; US EPA, 2015; Buratti et al., 2017; WHO, 2020). The damage to mitochondrial DNA (mtDNA) reported (Li et al., 2015; Li et al., 2016; Wang et al., 2018) in mice exposed to MC-LR (1-40 μg MC-LR/L) via drinking water was not statistically significant when compared to mice in the control group.

There are no long-term studies of MC carcinogenicity in animal models. Reports of chronic exposure to MCs via unspecified sources including drinking water and diets have claimed

an association with hepatocellular and colorectal carcinoma (Ueno et al., 1996; Fleming et al., 2002; Zhou et al., 2002; Svirčev et al., 2009; Zheng et al., 2017). However, because exposure was generally poorly characterized and results affected by many confounders, the causal association between exposure to MCs and cancer could not be determined (IARC, 2010; Buratti et al., 2017). The International Agency for Research on Cancer (IARC) concluded that there is inadequate evidence for the carcinogenicity of MC-LR (IARC, 2010). However, the tumor-promoting activity of MC-LR was recognized, leading to classification as potential carcinogen (Group 2B), with a known threshold effect. More than a decade has passed since this assessment, and although some additional data on animals have been produced, these did not lead to a change in the overall conclusions on MC carcinogenicity (WHO, 2020; Chorus and Welker, 2021).

A number of studies show that both male and female reproductive tissues in rodents may be a possible target for MCs. However, as detailed in the WHO Drinking Water Guidance (WHO, 2022) those studies are affected by a number of relevant limitations in the study design and in data interpretation (e.g. the use of i.p. dosing causes the reproductive tissues to be exposed to extremely high doses as compared to the oral route) (Chen et al., 2011; Wu et al., 2015; Pan et al., 2018; Zhou et al., 2020). Due to these methodological and reporting deficiencies they are not supported by mechanistic understanding of the claimed effects, limiting their use for human health risk assessment. Overall, the availability of robust data on developmental toxicity to overcome the uncertainties related to this end-point is limited, and well designed studies are necessary to assess whether microcystins damage reproductive tissues or performance.

The presence of OATP on the blood-brain-barrier and detection of MCs in the brain of several experimental models makes neurotoxic effects biologically plausible. Neurotoxicity caused by MCs has been reviewed (Hu et al., 2016; Hinojosa et al., 2019). Some variants, such as MC-LF or MC-LW, appeared to be more toxic in vitro in neural cells than MC-LR (Fischer et al., 2010). However, the available in vivo data are not robust enough to draw conclusions on the type of effects and the dose-response relationships. Also, for potential immunotoxicity of MCs, in vitro and in vivo studies data (reviewed by Diez-Quijada et al., 2021) remain too limited to draw any firm conclusions.

So, despite the increasing number of publications on MC-LR toxicity, when WHO updated the providional guideline value (GV) for MC-LR in drinking water (WHO, 2020), after thorough review of the available literature on repeated toxicity, used the repeated dose toxicity study by Fawell et al., (1999) as key basis for deriving the GV. This study administered MC-

LR at doses of 0, 40, 200 and 1,000 μg MC-LR/kg bw/day by gavage for 13 weeks to CD-1 mice. The lowest dose, 40 μg/kg bw/day, was identified as that not causing any detectable symptoms, i.e. the NOAEL, on the basis of slight hepatic histopathological damage and serum enzyme changes observed at 200 μg/kg bw/day in a limited number of treated animals. More severe hepatic damage was observed at 1,000 μg/kg bw/day, which indicated a clear dose-response relationship (Fawell et al., 1999). Another study on male rats (Heinze, 1999) was considered by USEPA for the derivation of the short-term Health Advisory levels. This study administered MC-LR (0, 50 or 150 μg/kg bw/day) for 28 days via drinking water to male rats and evidenced liver effects as the critical ones, identifying a lowest-observed adverse effect level (LOAEL) of 50 μg/kg bw/day. Although the administration via daily gavage may have limited MC absorption to the period of small intestinal transit in the Fawell study, the WHO experts have chosen it as the key one for a number of reasons: the availability not only of a LOAEL but also of a NOAEL avoids the need to use of an additional uncertainty factor for extrapolation from a LOAEL to a NOAEL, which would increase the total uncertainty and reduce confidence in the derivation of the GV; also, more doses were tested, covering an appreciably wider dose range; the duration of the study was longer; in the Heinze study some parameters show no dose-dependence and the lack of alteration in known markers of hepatotoxicity such as ALT and ASP was not consistent with the reported liver damage; the strain of rats used by Heinze was unusual (F1 generation of female WELS/Fohm × male BDIX) and is less well characterized; regarding the species differences, 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). In addition, the NOAEL of 40 μg/kg bw/day identified by Fawell et al. (1999) is supported by results of Ito et al. (1997) who describe 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.

For deriving guideline values for substances with a toxicity threshold such as Microcystins, it is standard practice to divide the NOAEL by a factor of 100, i.e. 10 to account for differences in sensitivity between species and 10 to account for differences in sensitivity between individuals. In many cases, including for Microcystins, division by a further factor of 10 is applied to account for limitations in the data base used – here for the NOAEL being based not on a life cycle but only on a part of it, i.e. 13 weeks, with daily administration of MCs. This leads to a tolerable daily intake (TDI) which is 1000-fold below the dose that did not cause any recognized effect in the test animals. For deriving guideline concentrations that reflect safe exposure to water, this

TDI is then multiplied with values for body weight, amount of water ingested and the fraction of the daily dose likely to be caused by water (relative to other pathways such as food). For Microcystin-LR the result is a provisional lifetime WHO guideline value for Microcystins in drinking water of 1 µg/L (see WHO (2020) and below for details).

4. Effects in Humans

For assessing cyanobacteria- and MC-associated human health effects (reviewed in Svirčev et al., 2019; Chorus and Welker, 2021) it would be important to differentiate between those caused by microcystins or other cyanotoxins, those caused by other constituents of cyanobacteria (possibly including organisms inhabiting their mucilage) and those happening to be in water harbouring blooms. Where blooms occur, waterbodies are typically not only affected by excess nutrients but also by various sources of pollution such as sewage (which, even after treatment still contains considerable amounts of pathogens), runoff from roads and runoff from agricultural land which can carry pathogens from animals as well as many other contaminants. Studies reporting human health effects after exposure to water containing cyanobacteria can scarcely differentiate between this range of further hazards. Typically, the information available is limited to the type of symptoms observed and whether or not they are likely to be caused by microcystins. Effect type and severity also depends on the exposure scenario, meaning the route, level and duration of exposure. The non carcinogenic symptoms reported in connection with exposure to cyanobacteria include:

- gastrointestinal effects such as diarrhea, vomiting and abdominal pain (Teixeira et al., 1993; Annadotter et al., 2001; Hilborn et al., 2014; Backer et al., 2015; Roberts et al., 2020). These symptoms are typical of gastrointestinal infections, and while some of these reports explicitly mention that these were excluded, this is routinely only done by checking for *E. coli* as indicator for bacterial pathogens; analysing virus or protozoa requires different, more challenging methods, and if these had been applied, they would likely have been reported. The primary concern from microcystins, depending on the dose and length of exposure, are systemic effects in the liver and kidney, and reports of plausible evidence for these exist worldwide (see e.g. Falconer et al., 1983; Vidal et al., 2017; Giannuzzi et al., 2011);

- respiratory effects ranging from respiratory irritation with rhinitis and dry cough to severe wheezing, shortness of breath, fever and pneumonia (Turner et al., 1990; Giannuzzi et al., 2011; Trevino-Garrison et al., 2015; Lin et al., 2016; Wu et al., 2021);

- dermatitis ranging in severity from skin irritation, pruritis, and mild rash to vesiculation and ulceration (Cohen and Reif, 1953; Turner et al., 1990; Lévesque et al., 2014);

• neurologic illnesses such as confusion, visual and cognitive changes and muscle pain have been reported after recreational water exposures, and for some cases the concomitant exposure to neurotoxins was reported, (Hilborn et al., 2014; Backer et al., 2015) and after intravenous exposure of haemodialysed patients (Pouria et al., 1998, Jochimsen et al., 1998, Azevedo et al., 2002);

The only known incidence of fatal cyanotoxin poisoning occurred in the 90ies of the last century in Brazil, where hemodialysis patients were exposed by parenteral route: indeed, insufficiently treated water highly contaminated by MCs (and, as later found, presumably also by cylindrospermopsins) was used for the treatment and more than 70 patients experienced liver failure and death. Further episodes, due to dysfunction of the treatment plant with a lower MC dose compared to the first outbreak was associated with sub-lethal liver injury (Jochimsen et al., 1998; Azevedo et al., 2002; Hilborn et al., 2013; Soares et al., 2006; Hilborn and Ward, 2015).

Some cases of illness attributed to cyanobacteria have been reported when drinking water was inadequately treated, resulting in contamination with MCs (Miller and Tisdale, 1931; Bourke et al., 1983; Falconer et al., 1983; Teixeira et al., 1993; McCarty et al., 2016), typically resulting in sub-lethal effects and rarely in more severe gastrointestinal symptoms (Teixeira et al., 1993).

Since MCs have been detected in dietary cyanobacterial supplements, also known as blue-green algal supplements (BGAS), or in contaminated terrestrial or aquatic foods, food has been considered as a relevant source of exposure: but robust data on occurrence are still limited (Gilroy et al., 2000; Magalhães et al., 2001; Testai et al., 2016; Ibelings et al., 2021a), due to analytical limitations of many studies. However, a general trend in literature indicates higher MC content in fish and seafood with respect to other food items, with levels in muscle tissue typically low and the highest levels recovered from liver and viscera. Therefore, molluscs and crustaceans, which are eaten with the viscera, seems to be related to higher risks (Testai et al., 2016; Chorus and Welker, 2021- Chapter 5). However, no clinical episodes of human health effects have been reported so far, although alterations in biomarker for hepatic toxicity have been described (Chen et al., 2009; Li et al., 2011).

In general, despite the widespread occurrence of of cyanobacteria and cyanotoxins across the globe the number of adverse human health effects with strong evidence for cyanotoxins as cause is small (discussed in Chorus and Welker, 2021 - Chapter 5). While detection of cyanotoxin concentrations in patient tissues would provide clear evidence of exposure, such

data are largely missing. One of the reasons for this lack of information can be that unspecific symptoms co-occurring with exposure to cyanobacteria are typical for other common illnesses, mostly mild and self-limiting so that those affected resort to self-medication rather than seeking medical care (Funari et al., 2017). In addition, physicians and health care providers may not be sufficiently aware of the possibility of illness being associated to cyanobacterial exposure. Targeted education and active case-finding (surveillance) in collaboration with health care providers can improve detection as well as reporting of patient visits attributed to cyanobacteria-associated illnesses (Funari et al., 2017; Chorus and Welker, 2021).

5. Recommendations by International Organizations and Limit Values

As discussed above, since human data are not sufficiently robust to be used for the derivation of a health-based value for life-time exposure, WHO used the NOAEL of 40 µg /kg bw per day as Point of Departure (PoD) divided by an uncertainty factor (UF) of 1000 for derivation of a provisional tolerable daily intake (TDI) value of 0.04 µg/kg bw per day. This is extrapolated to indicate that an adult with a body weight of 60 kg could be orally exposed to 2.4 µg MC-LR per day throughout life, without experiencing any toxicological effect. On this basis, the WHO has derived a provisional Guideline Value (pGV) of 1µg/L for the total concentration of MC-LR in drinking water, considering a daily consumption of 2 L of drinking water and assuming that potential daily exposure via drinking water would account for 80% of the total ingestion of MC. The value is considered provisional, since due to the significant area of uncertainty it could be revised as soon as more robust data will be available.

WHO also recognized a pronounced need for guideline values for short-term exposure, both via recreational waterbody use and for situations when short-lived blooms occur in waterbodies used for abstracting drinking water. These give risk managers an option to respond to seasonal occurrence of short duration, including to invest in sustainable mitigation measures rather than in costly “bandaid-type quick fixes” that may be costly without providing a health benefit. Their derivation followed the same PoD and procedure as for the derivation of the lifetime TDI and provisional lifetime drinking water guideline value, with the following differences:

1) the omission of the uncertainty factor for database deficiencies to calculate the provisional short-term, since the PoD is based on a sufficiently relevant period of exposure, thus reducing the total uncertainty factor from 1000 to 100; this is justified as the available data seem to exclude evidence for developmental toxicity from short-term exposure (WHO, 2022) and the tumour promoting activity mediated via protein phosphatase inhibition (a threshold effect) is not relevant to short-term exposures,.

2) the WHO default allocation factor reflecting the fraction of exposure allocated to drinking-water was set to 100% for short-term exposure, as during a bloom drinking-water is expected to be the far most significant source of exposure for most of the population.

These assumptions result in a short term pGV in drinking water of 12 μg/L, suggested to be used for not more than 2 weeks and not more than once per season.

For the recreational GV, the differences with respect to the short term pGV in drinking water are:

1) body weight for a child (default for WHO=15 kg) and

2) consumption of 250 mL, to account for the possible incidental water consumption for a child playing along the shoreline in the presence of a dense bloom. This derivation procedure gives a pGV for recreational exposure of 24 μg/L.

Although the TDI is derived for MC-LR, WHO suggests to use the pGVs for the sum of concentratons of all MC congeners in a sample, assuming this approach to be sufficiently protective, because MC-LR is among the most acutely toxic MCs. However, there is a caveat to this assumption because for most of the congeners toxicity has only been characterized as acute toxicity following intraperitoneal (i.p.) injection (Buratti et al., 2017; Santori et al., 2020) and the validity of extrapolation from this to other routes of exposure is questionable. Also, most importantly, the validity of extrapolation of relative acute toxicity of MC congeners to their chronic toxicity remains to be demonstrated.

WHO has no executive power and thus cannot set legally binding standards but only give recommendations. WHO strongly emphasizes that when setting national, legally binding limit values it may be appropriate to adapt WHO guideline values to national or even local public health priorities, so as to focus first on those hazards that cause the highest risks to publich health. Indeed WHO GVs are used as a reference by many countries around the world to establish legal values or give recommendations, to prevent and/or manage the possible health risks from MC exposure through drinking water and/or recreation (Buratti et al., 2017). However, other Organizastions, such as US EPA (2015) and Health Canada (2016), have derived limit values following different procedures and/or based on other studies, such as Health Canada's reference value for subacute/subchronic toxicity (or HA, Health Advisory) equal to 0.16 μg MC-LR/kg bw per day (similar to the WHO TDI, although the PoD was different, based on the LOAEL from the study by Heinze (1999)). A selection of values adopted by different countries around the world are summarized in Table 3.

Table 3. Recommendations by International Organizations and limit values set in different countries in order to prevent/manage possible human health risks from exposure to microcystins (MCs).

Organization	Country	Limit Value		Note	Reference
WHO[a]		General	0.04 µg/kg/day	**provisional tolerable daily intake (TDI chronic) for MCLR**	WHO, 2020, 2022
		Drinking water	1 µg/L	**provisional lifetime GV for MCLR**	WHO, 2020, 2022
			12 µg/L	**provisional short-term drinking-water GV for MC-LR[b]**	
		Recreational water	24 µg/L	**provisional recreational water GV for MCLR**	WHO, 2021
Europe	France	Food	5.6 µg/kg wm fish (edible part)	adult (60 kg), consumption of fish of 86 g/d, 20% MCs from fish and 80% from water	Arnich, 2012
			1.4 µg/kg wm fish (edible part)	children (10kg), consumption of fish of 57 g/d, 20% MCs from fish and 80% from water	
			28 µg/kg wm fish (edible part)	adult, consumption of fish of 86 g/d, 100% MCs from fish	
			7 µg/kg wm fish (edible part)	children, consumption of fish of 57 g/d, 100% MCs from fish	
		Drinking water	1 µg/L (sum of MCs)	Standard	Humpage and Cunliffe, 2021
	Finland	Drinking water	> 1 µg/L	Restriction on water use	
			>10 µg/L	Ban on water use	
European Union (EU) Bathing water directive (BWD)	national implementation	Recreational water			Ibelings et al., 2014
	France	Recreational water	25 µg/L	Bathing is banned and recreational activity restricted	
	Germany	Recreational water	> 30 µg/L	Discourage bathing, considering temporary closure is recommended	
	Hungary	Recreational water	< 4 µg/L	Excellent	
			< 10 µg/L	Good	
			< 20 µg/L	Acceptable	
			>20 µg/L	Unacceptable	
	Italy	Recreational water	>25 µg/L	Bathing is prohibited	
	Czech Republic	Recreational water	1 µg/L	Standard	

European Union (EU) Drinking Water Directive (DWD) national implementation	 Italy	Drinking water Drinking water	1 µg/L (for MC-LR) 1 µg/L (for MC-LR)	Parameter measured only in case of increasing density of cyanobacterial cells or bloom forming potential.	DIRECTIVE (EU) 2020/2184 and S.I. No. 99 of 2023 D.Lgs. 23.02. 2023 n. 18
China		Drinking water	1 µg/L		GB, 2006
US-EPA[e]		Drinking water	0.3 µg/L	Infants and Pre-school children, HAL for periods up to 10 days	US EPA, 2015
			1.6 µg/L	School-aged children and Adults	
		Recreational Water Quality Criteria	8 µg/L	Duration: 1 in 10-day assessment period across a recreational season; Frequency: More than 3 excursions in a recreational season, not to be exceeded in more than one yearc	US EPA, 2019; 2021
		Swimming Advisory	8 µg/L	Duration: One day; Frequency: Not to be exceeded	
	Canada	Food	0.02 µg/kg bm/d	Tolerance Limit in finished product	Testai et al., 2016
		Drinking water	1.5 µg/L[d]	MAC for total MCs (intra- and extra-cellular)	Health Canada, 2020
		Recreational water	10 µg/L	Maximum concentration	Health Canada, 2022
	Australia	Food	39 µg/kg wm (fish); 39 µg/kg wm (prawns); 83 µg/kg wm (mussels)	≥ 17 years (74 kg), TDI of 0.2 µg/kg bm/d, consumption of 377 g fish g/d, 377 g prawns/d, 178 g mussels/d	Mulvenna et al., 2012; Mulvenna and Orr, 2012
			24 µg/kg wm (fish); 32 µg/kg wm (prawns); 51 µg/kg wm (mollusks)	2-16 years (38 kg), TDI of 0.2 µg/kg bm/d, consumption of 319 g fish g/d, 236 g prawns/d, 148 g mollusks/d	
		Drinking water	1.3 µg/L		NRMMC. 2011
		Recreational water	≥10 µg/L	Level 1 probability of adverse health effects GV for short term exposure	NRMMC. 2008

	New Zealand	Drinking water	1 µg/L	Provisional MAV	Humpage and Cunliffe, 2021
		Recreational water	≥ 12 µg/L	Notify the public of a potential risk to health	Ibelings et al., 2014
	Türkiye	Drinking water	1 µg/L(sum of MCs)	Standard	Humpage and Cunliffe, 2021
		Recreational water	< 10 µg/L	Recreational activities are permitted. Monitoring	Ibelings et al., 2014
			> 25 µg/L	Discourage users from swimming and other water activities	
	Brazil	Drinking water	1 µg/L	Standard	Humpage and Cunliffe, 2021
	Argentina	Drinking water	1 µg/L	WHO's GV provisional	Chorus, 2012
	Singapore	Drinking water	1 µg/L	Standard	Humpage and Cunliffe, 2021
	South Africa	Drinking water	1 µg/L	GV	Humpage and Cunliffe, 2021
	Uruguay	Drinking water	1 µg/L	Standard	Humpage and Cunliffe, 2021

Bm: body mass, dm: dry mass, wm: wet mass; HAL: Health Advisory Level, GV: Guidance Value, PHA: Public Health Advisory, PHW: Public Health Warning, NCA: No Contact Advisory, MAV: Maximum Acceptable Value, MAC: Maximum Acceptable Concentration, RUV: Recreational Use Value, WQS: Water Quality Standards.
a: Insufficient data are available to derive a GV for MC variants except MC-LR. GV derivation, allocation to water (for human consumption): 80% of TDI, for short-term exposure, 100% of TDI; weight : 60 kg adult; consumption: 2 litres/day
b: 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 mass (e.g. up to 5 times more drinking-water/kg bm 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.
c: An excursion is defined as a 10-day assessment period with any toxin concentration higher than the criteria magnitude. When more than three excursions occur within a recreational season and that pattern reoccurs in more than one year, it is an indication the water quality has been or is becoming degraded and is not supporting its recreational use. As a risk management decision, states should include in their WQS an upper-bound frequency stating the number of years that pattern can reoccur and still support its recreational use
d: when the Total MC concentration exceeds 0.4 µg/L, an alternative source of drinking water (e.g. bottled water) should be used for mixing infant formula.
e: It is to be noted that the single states can have own GVs due to their use of different starting values for dosing (RfD, NOAEL or LOAEL) (Buratti et al., 2017). For example, in Minnesota the GV for drinking water is 0.1 µg/L (MHD, 2015). Highest variability can be seen for food where the acceptable MCLR content can be from 1 to 28 µg/ kg fish (Testai et al., 2016).
Regarding the recreational water the public health advisory in Virginia and Oregon, start with MCs ≥8 µg/L (VDH, 2021; OHA, 2021) but in New Jersey with 3 µg/L of MCs (NJDEP, 2021).
Specific advisories for pets (Buratti et al., 2017), such as the Oregon RUV specific for dog (0.2 µg/L) (OHA, 2021), sometimes are available.

6. Concluding Remarks

Important developments in MC research have produced an increasing number of publications in the last decades, although still many gaps and questions remain, starting from the understanding of the actual biological function of MC (e.g. whether or not their production gives any advantage to MCs producing cyanobacteria in their competition against other phytoplankton organisms) to finish up with the collection of data on occurrence in different matrices and the elucidation of the toxicological profile of variants, for which there is a clear need for high quality studies. Cyanobacteria and cyanotoxins can represent a good example for the One Health approach (Hilborn and Beasley, 2015): their presence in the environment can affect the ecosystem, including animals and humans in both a direct and indirect way. In addition, animal poisoning, beside having direct impacts on animal welfare and productivity, can also serve as sentinel for potential risks to humans.

A major remaining challenge is to establish a causal relationship between human/animal exposure to toxic cyanobacteria and the development of adverse effects, due to non-specific symptomatology and the lag time between exposure and water sampling for cyanobacteria/cyanotoxins analysis (dissipation of the bloom, changes in toxin profiles and/or concentrations). Developing good and reliable analytical methods for complex matrices as well as specific biomarkers reflecting cyanotoxin exposure will be pivotal for clarifying the cause of symptoms co-occurring with cyanobacterial blooms and for improving toxicological derivations.

Furthermore, the effect of cyanobacterial mixtures, including other bioactive and taste and odor compounds produced by cyanobacteria (Manganelli et al., 2023) as well as other natural and man-made chemical pollutants and organisms, including bacteria, viruses and fungi, which often co-occur in cyanobacterial blooms and waterbodies, is poorly studied. These will be the research frontiers for tomorrow.

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