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REVIEW ARTICLE

Cyanotoxins: producing organisms, occurrence, toxicity, mechanism of action and human health toxicological risk evaluation

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Abstract Cyanobacteria were present on the earth 3.5 billion years ago; since then they have colonized almost all terrestrial and aquatic ecosystems. They produce a high number of bioactive molecules, among which some are cyanotoxins. Cyanobacterial growth at high densities, forming blooms, is increasing in extension and frequency, following anthropogenic activities and climate changes, giving rise to some concern for human health and animal life exposed to cyanotoxins. Numerous cases of lethal poisonings have been associated with cyanotoxins ingestion in wild animal and livestock. In humans few episodes of lethal or severe human poisonings have been recorded after acute or short-term exposure, but the repeated/chronic exposure to low cyanotoxin levels remains a critical issue. The properties of the most frequently detected cyanotoxins (namely, microcystins, nodularins, cylindrospermopsin and neurotoxins) are here critically reviewed, describing for each toxin the available information on producing organisms, biosynthesis/genetic and occurrence, with a focus on the toxicological profile (including kinetics, acute systemic toxicity, mechanism and mode of action, local effects, repeated toxicity, genotoxicity, carcinogenicity, reproductive toxicity; human health effects and epidemiological studies; animal poisoning) with the derivation of health-based values and considerations on the risks for human health.

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Introduction

Cyanobacteria (CB), also known as 'blue-green algae,' are part of a primitive group of microorganisms which, according to fossil records, have existed for approximately 3.5 billion years (Tomitani et al. 2006; Schopf 2002; Fig. 1). They occupy many different niches and can be found in all terrestrial and aquatic ecosystems, ranging from deserts to tropical rain forests, to alpine and subsurface soils, and from the ultraoligotrophic open ocean to hypereutrophic lakes (Paerl 2014; Paerl and Huisman 2009; Rastogi et al. 2014). Most CB are an immense source of several secondary natural products with applications in the pharmaceutical, food, cosmetic, agriculture and energy sectors (Kim et al. 2014; Lau et al. 2015; Rastogi and Sinha 2009; Vijayakumar and Menakha 2015). Different CB species are also well-known cyanotoxin producers and are present in several areas around the world (Chorus and Bartram 1999; Funari and Testai 2008; Whitton 2012; Merel et al. 2013a, b; Rastogi et al. 2014; Corbel et al. 2014; Niamien-Ebrottie et al. 2015; Mowe et al. 2015; Fig. 2; Table 1).

Cyanobacterial blooms extension and frequency are increasing, following anthropogenic activities and climate changes (Funari et al. 2012; Paerl and Otten 2013). To give some examples, due to heavy rainfall and floods, the distribution of *Microcystis*-producing microcystins (MC) has now spread into several estuaries, in USA and in China, where many aquaculture plants are located, with possible consequences on fishery production (Funari et al. 2012; Manganelli et al. 2012; Preece et al. 2015a). The transport of CB cells and MC from inland seaward has been observed also in Italy

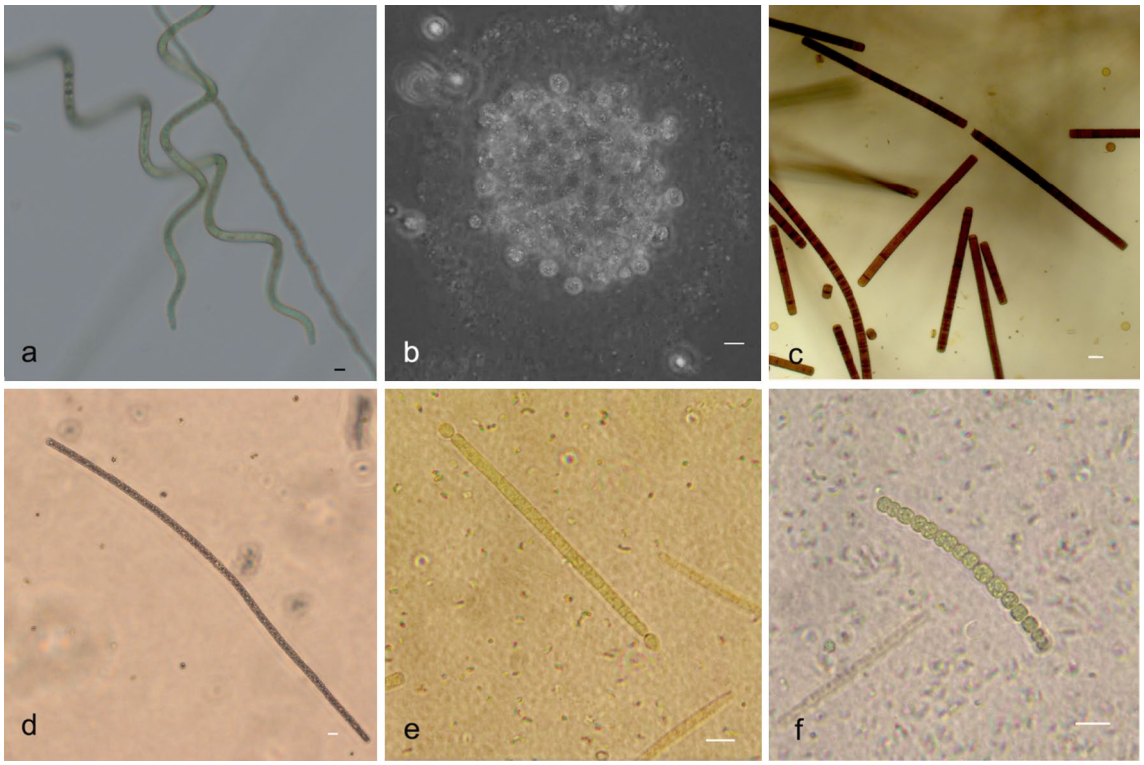


Fig. 1 Some examples of different cyanobacteria morphologies. **a** *Arthrospira* sp.; **b** *Microcystis botrys*; **c** *Lyngbya* sp.; **d** *Planktothrix rubescens*; **e** *Cylindrospermopsis raciborskii*; **f** *Nostoc* sp. *Scale bar* 10 μm except **c** *Lyngbya* sp. = 30 μm

Fig. 2 Cyanobacteria distribution around the world

for blooms of the filamentous *Planktothrix* (De Pace et al. 2014). Extended dry periods, with evaporation exceeding precipitation, favored the contribution of brackish groundwater to maintain lake water levels, increasing lakes salinity, thus favoring the diffusion of toxic CB like *Nodularia spumigena* in subtropical Australia (McGregor et al. 2012).

The recent colonization of many subalpine Italian lakes by *Dolichospermum lemmermannii* is another interesting

Table 1 Toxic cyanobacteria genera prevalence in the world

Country	Potential toxic genera
Argentina	Microcystis; Dolichospermum; Cylindrospermopsis; Raphidiopsis; Aphanizomenon; Planktothrix
Australia	Cylindrospermopsis; Anabaena; Nodularia; Aphanizomenon; Oscillatoria; Nostoc; Planktothrix; Scytonema
Bangladesh	Microcystis; Anabaena; Planktothrix
Belgium	Microcystis; Planktothrix
Brazil	Cylindrospermopsis; Microsystis; Planktothrix; Aphanizomenon; Oscillatoria; Anabaena; Dolichospermum; Raphidiopsis
Cambogia	Microcystis
Cameroon	Planktothrix; Oscillatoria; Microcystis
Canada	Microcystis; Anabaena; Cylindrospermopsis; Planktothrix; Aphanizomenon
Czech Republic	Microcystis; Planktothrix; Aphanizomenon; Anabaena
China	Raphidiopsis
Cuba	Microcystis; Oscillatoria; Anabaena; Planktothrix; Lyngbya; Synechococcus; Gomphosphaeria; Phormidium
Denmark	Microcystis; Anabaena; Aphanizomenon; Planktothrix; Nodularia
Egypt	Phormidium; Nostoc; Plectonema
England	Anabaena; Aphanizomenon; Microcystis; Oscillatoria
Ethiopia	Microcystis
Finland	Microcystis; Planktothrix; Anabaena; Nodularia
France	Microcystis; Aphanizomenon; Planktothrix; Anabaena; Phormidium; Woronichinia
Germany	Cylindrospermopsis; Aphanizomenon; Anabaena; Planktothrix; Microcystis
Ghana	Microcystis; Anabaena
Greece	Microcystis; Anabaena; Aphanizomenon; Cylindrospermopsis; Planktothrix
Guatemala	Lyngbya
Hungary	Cylindrospermopsis
Hong King	Microcystis; Anabaena
India	Microcystis; Trichodesmium
Indonesia	Microcystis; Cylindrospermopsis; Planktothrix
Israel	Aphanizomenon
Italy	Microcystis; Planktothrix; Anabaena; Aphanizomenon; Oscillatoria; Raphidiopsis; Dolichospermum
Japan	Microcystis; Umezakia
Kenya	Microcystis; Arthrospira; Anabaenopsis
Korea	Microcystis
Malaysia	Microcystis, Cylindrospermopsis; Planktothrix
Malawi	Anabaena
Mexico	Microcystis; Planktothrix; Cylindrospermopsis; Pseudanabaena; Anabaena; Nostoc; Oscillatoria
Morocco	Microcystis
Myanmar	Microcystis
Netherland	Anabaena; Aphanizomenon; Microcystis; Planktothrix; Woronichinia; Phormidium
New Zealand	Anabaena; Aphanizomenon; Cylindrospermopsis; Microcystis; Nodularia; Oscillatoria; Phormidium; Planktothrix; Scytonema; Nostoc
Nigeria	Cylindrospermopsis; Anabaena; Microcystis; Lyngbya; Aphanizomenon; Oscillatoria;
Peru	Microcystis; Nodularia
Philippines	Microcystis; Cylindrospermopsis
Poland	Microcystis; Anabaena; Aphanizomenon; Gloetrichia; Nodularia;
Portugal	Microcystis, Planktothrix, Anabaena, Aphanizomenon, Cylindrospermopsis
Russia	Microcystis, Anabaena, Aphanizomenon
Scotland	Aphanocapsa, Aphanothece, Microcystis, Woronichinia, Oscillatoria, Planktothrix, Anabaena, Aphanizomenon, Gloeotrichia
Saudi Arabia	Microcystis
Senegal	Microcystis; Cylindrospermopsis
Singapore	Anabaena, Anabaenopsis, Aphanizomenon, Cylindrospermopsis; Microcystis, Planktolyngbya, Planktothrix, Pseudanabaena, Raphidiopsis

Table 1 continued

Country	Potential toxic genera
Slovene	Microcystis
South Africa	Microcystis, Anabaena; Oscillatoria; Cylindrospermopsis
Spain	Microcystis; Cylindrospermopsis; Anabaena; Aphanizomenon
Sri Lanka	Microcystis; Anabaena; Planktothrix
Sweden	Nodularia
Switzerland	Oscillatoria, Phormidium; Planktothrix
Tanzania	Anabaena; Microcystis
Thailand	Microcystis; Cylindrospermopsis
Tunisia	Microcystis
Turkey	Planktothrix; Microcystis; Nodularia; Anabaenopsis; Cylindrospermopsis
Uganda	Microcystis; Anabaenopsis; Aphanizomenon; Anabaena; Cylindrospermopsis
USA	Microcystis; Anabaena; Cylindrospermopsis; Planktothrix; Anabaenopsis
Uruguay	Microcystis; Aphanizomenon; Dolichospermum; Cuspidothrix; Pseudanabaena; Anabaenopsis; Cylindrospermopsis; Nodularia; Raphidiopsis; Planktothrix; Planktolyngbya; Limnothrix
Venezuela	Microcystis; Anabaena; Anabaenopsis
Vietnam	Microcystis; Arthrospira; Oscillatoria; Planktothrix; Pseudanabaena
Zimbabwe	Microcystis; Cylindrospermopsis; Lyngbya; Anabaena; Aphanizomenon; Oscillatoria

example of the effects of complex relations between environmental factors affected by climate changes, namely increasing temperature and extreme events. The frequent lake-level fluctuations due to increased extreme events as dry periods followed by heavy rains, and consequent drying and rewetting of shoreline, would represent pulse inputs of phosphorus, which together with the increase of temperature would trigger the regular appearance of large blooms of *D. lemmermannii* also in oligotrophic lakes (Callieri et al. 2014). On the other hand, in specific areas combined effects of parameters related to climate changes can also be expected to go the opposite way, reducing CB bloom in the future, at least for some strains (Helbling et al. 2015).

Environmental factors are also responsible for the regulation of the ratio of toxic vs nontoxic genotypes, gene expression, free and bound toxins, probably acting in a very complex manner which is not fully elucidated yet (Pearson et al. 2016).

When blooms are formed, the risk of toxin contamination of surface freshwaters increases, posing in some cases serious health problems to animals and humans. The CB spreading in coastal waters and the identification of CB marine species represent an additional reason of concern since cyanotoxins may accumulate through ingestion of CB cells or contaminated zooplankton in coastal edible aquatic vertebrates and invertebrates such as fish, mussels, other crustaceans that are not routinely checked for their presence to provide a 'safe' product to consumers.

Numerous cases of lethal poisonings have been associated with cyanotoxins ingestion in wild animal and livestock exposed to toxic CB blooms, especially by drinking infested water. On the contrary, only few episodes of human poisonings have been recorded. The plausible reasons may lie in the general improved capabilities in managing the risk associated with cyanotoxins, especially in developed countries, but also to difficulties in finding a causal relationship when symptoms are subtoxic.

The most serious known episode associated with human exposure to MC occurred in Brazil, where 56 out of 130 hemodialyzed patients died after treatment with water accidentally contaminated with MC (Azevedo et al. 2002). Some episodes of acute/short-term human intoxications due to drinking water consumption have been reported in some countries as a consequence of failure or inefficiency of water treatment (Falconer 1994; Funari and Testai 2008) and recently in the USA after recreational activities (Hilborn et al. 2014).

Humans may be indeed orally exposed to cyanotoxins by drinking contaminated water, through consumption of cyanotoxin-containing freshwater fish, crops, vegetables and food supplements, or by ingesting water during recreational activities. During recreational, sport and professional activities (i.e., fishing) in contaminated waters dermal and inhalation exposure may also occur. The problem of toxic CB seems extremely relevant in arid and developing countries, where people are more dependent from stored (in some cases desalinated) drinking water in reservoir, and where toxin-producing cyanobacteria and related toxins have been found in recreational, drinking and irrigation water contained in dams and tanks, in groundwater wells, hot springs and rainwater pools (Costa et al. 2006; Mohamed and Al Shehri 2007, 2009; Mohamed 2008;

Chatziefthimiou et al. 2014; Mohamed et al. 2016-abstract; Chatziefthimiou et al. 2016).

The properties of the most frequently detected cyanotoxins (namely, microcystins, nodularins, cylindrospermopsin and neurotoxins) are here reviewed, although we are aware that known toxins very likely represent a limited fraction of the high number of bioactive molecules produced by CB. For other compounds already identified as microviridins, microginins, cyanopeptolins, other cyclic peptides and unusual fatty acids (Metcalf and Codd 2012) very few information is available at present.

Besides this introductory section, the review consists of chapters dedicated to single toxins that critically summarize the available information on the different producing CB, toxin occurrence in the environment and in biota; the genetic and biosynthesis of the toxin and its toxicological profile (including kinetics, acute systemic toxicity, mechanism and mode of action, local effects, repeated toxicity, genotoxicity, carcinogenicity, reproductive toxicity; human health effects and epidemiological studies; animal poisoning). The concluding part is devoted to the derivation of health-based values and consideration on the risks for human health.

Microcystins

Microcystins (MC) are among the most widespread cyanobacterial toxins detected in freshwaters. They were first isolated from the cyanobacterium *Microcystis aeruginosa* (Carmichael et al. 1990) but are produced by several other genera of planktonic and benthic CB (Table 2).

Microcystins are cyclic peptides consisting of seven amino acids, characterized by the presence of the amino acid Adda [(2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid], which is a peculiar characteristic of CB (Chorus and Bartram 1999).

The most common congeners are MC-LR, MC-RR and MC-YR, resulting from the presence of the L-form of leucine (L), arginine (R) or tyrosine (Y) in positions 2 and 4 (the letters are those used as abbreviation for identifying the amino acids found in proteins). More than 100 MC variants have been reported to date, with molecular weights of 900–1100 Da, originating by different amino acid combinations and various other changes (such as the methylation/desmethylation of several functional groups) (Puddick et al. 2015).

MC-LR is the most studied MC among the different congeners, due to its ubiquity, abundance and toxicity. It is characterized by the presence of leucine (L) and arginine (R) as L-amino acids in positions 2 and 4 (Sivonen and Jones 1999; Carmichael et al. 1990). The chemical structure of MC-LR and MC-RR is shown in Fig. 3.

Producing organisms

The most diffuse and abundant freshwater MC-producing pelagic genera are *Microcystis*, *Planktothrix* and *Dolichospermum* (ex *Anabaena*).

Blooms of the unicellular colonial *Microcystis* have been reported from every continent, except Antarctica (Zurawell et al. 2005), and their toxicity has been demonstrated in 79 countries (Harke et al. 2016 and references therein). This genus is highly diffuse in eutrophied water, both N and P enriched; it can also regulate its buoyancy, moving along the water column of shallow, turbid water systems to uptake P resuspended from sediments (Paerl 2014).

Planktothrix spp. are very efficient light harvester, thanks also to accessory pigment–protein complexes (blue-green phycocyanin, PC, and red phycoerythrin, PE); therefore they can reach high production also at low light intensities, like in turbid water, or at depth, at the thermocline. The two most abundant and diffuse species, *Planktothrix rubescens* and *P. agardhii* (sensu Suda et al. 2002), thrive in quite diverse habitats from all the continents (Kurmayer et al. 2016 and references therein), *P. rubescens* (higher PE/PC) being more typical of deep, stratified mesotrophic lakes of temperate areas, and *P. agardhii* (higher PC/PE) more adapted to eutrophic shallow lakes in temperate and, less frequently, subtropical areas (Kurmayer et al. 2016; Ostermaier et al. 2012).

Another widespread bloom-forming taxa are the filamentous diazotrophic heterocystous group of *Dolichospermum/Anabaena/Aphanizomenon*, whose expansion, duration and intensity of blooms are increasing (Li et al. 2016a and references therein). *Dolichospermum* species produce blooms under a wide range of nutrient and temperature conditions and can be adapted to cold environment. However, some strains have T optima between 19 and 26 °C and generally form huge blooms in summer, in stratified condition (Salmaso et al. 2012). Some species are very tolerant to salinities and are major contributors to blooms in brackish regions of the Baltic Sea, together with *Nodularia* (Ploug 2008).

Other planktonic genera producing MC are *Aphanizomenon*, *Gloeotrichia*, *Hapalosiphon* and *Radiocystis*.

Benthic taxa are much less well known, but the more they are investigated, the more they look relevant and spread also in extreme environments (Quiblier et al. 2013). In California it has been estimated that 90% of stream kilometers state-wide of a slow-flux river could support toxigenic genera and 14% MC-producing species; the genera more frequently associated with MC (average 46 µg MC/m^2, with a maximum of 4767 µg MC/m^2) are *Nostoc* and *Phormidium* (Fetscher et al. 2015). These two genera have also been found to produce MC in Nile River, Antarctica, New Zealand, Finland and Switzerland (Quiblier et al.

Table 2 MC cell quota in isolated strains, from experimental or laboratory conditions, and field samples

Gender/species/strain	Cell quota (µg/g DW)	Country of origin	Analytical method	References
Anabaena BIR246	2.1×10^3	Easternmost Gulf of Finland	HPLC–DAD	Halinen et al. (2007)
Anabaena BIR250A	3.9×10^3			
Anabaena BIR257	1.9×10^3			
Anabaena BIR258	1.3×10^3			
Anabaena BIR260	2.1×10^3			
Anabaena 315	1.8×10^3	Coastal waters Helsinki		
Anabaena 318	1.3×10^3			
Anabaena sp. 90	1.26×10^3 low P	Southern Finland	HPLC–UV	Rapala et al. (1997)
	5.1×10^3 high NP			
Anabaena sp. 202 A1	0.76×10^3 low P			
	6.91×10^3 high NP			
Dolichospermum sp. BIR257 (ex *Anabaena*)	Min value high CO_2; low pH: 3.6 mg/g C		ELISA	Brutemark et al. (2015a)
	Max value high T: 6.7 mg/g C			
Dolichospermum sp. BIR257 (ex *Anabaena*)	3 Salinities (0, 3, 6 psu)		ELISA confirmed by HPLC–DAD	Brutemark et al. (2015b)
	Max value: $24.66 \times 10^3 \pm 14.54$ 6 psu			
	Different experimental conditions		ELISA	Gantar et al. (2009)
Geitlerinema sp. W-1	Control ~0.40[a]			
	Min–max ~0.15–0.30			
Leptolyngbya sp. FLK BBD1	Control ~0.10[a]			
	Min–max ~0.06–0.20			
Different isolated strains	Min–max	Florida		
Geitlerinema	0.02–0.10			
Leptolyngbya	0–0.08			
Pseudanabaena	0.02–0.043			
Synechococcus	0.08–0.27			
Spirulina 96-4	0.12			
Phormidium 73-2	0.026			
Microcystis sp.	62 (bloom)	Algeria	LC–MS/MS-PP2A	Bouhaddada et al. (2016)
Microcystis sp.	Large colonies (>500 µm)[b]	Grangent Reservoir (France)	PP2A	Sabart et al. (2013)
	0.030–0.300 pg/cell			
	Small colonies (25–50 µm)[b]			
	0.100–4.000 pg/cell			

Table 2 continued

Gender/species/strain	Cell quota (μg/g DW)	Country of origin	Analytical method	References
Microcystis sp.	Tot. cell[c]	Lake Erhai (China)	HPLC-PDAD	Yu et al. (2014)
	0.001–1.326 pg/cell			
	mcyB^{+} cells[c]			
	0.012–1.876 pg/cell			
Microcystis sp.	0.019–0.644 pg/cell	New Zealand	ADDA-ELISA	Wood et al. (2011)
Microcystis sp.	0.1–1.38 pg/cell	New Zealand	ELISA	Wood et al. (2012a)
Microcystis sp. 2006-B	0.2×10^3–3.0×10^3	Lake Erie	ELISA	Horst et al. (2014)
Microcystis aeruginosa	0.01–3.40 pg/cell	6 sampling sites in the river Loire (France)	PP2A	(Sabart et al. 2010)
M. aeruginosa PCC 7806	9.48 fg/cell low N		HPLC–UV	Sevilla et al. (2010)
	9.55 fg/cell high NP			
M. aeruginosa PCC 7806	Mixed antibiotic-treated group		LC–MS/MS	Liu et al. (2014)
	Max value: 40.6 fg/cell			
M. aeruginosa PCC 7806 WT	Different nutrient treatments[a] ~0.5–1.5 fg/μm^3		HPLC–DAD	Huang et al. (2015)
	D. magna effect			
M. aeruginosa UTEX LB2385	40.3–62.4 fg/cell	Ontario, Canada	ELISA	Pineda-Mendoza et al. (2014)
M. aeruginosa Ch 10	34.5–136.3 fg/cell	New Mexico		
M. aeruginosa MK 10.10	283.55×10^3 lowest salinity	Baltic sea	HPLC	Mazur-Marzec et al. (2010)
Microcystis aeruginosa UTCC 300	Strain alone and mixed culture		PPI	LeBlanc et al. (2011)
	~700–1100 low light[a]			
	~500–1200 high light[a]			
M. aeruginosa AB2011/53	0.024 pg/cell	South Africa	LC–MS	Ballot et al. (2014)
M. aeruginosa FACHB 915	40.93 fg/cell no UV-B		HPLC–PDA	Yang and Kong (2015)
	32.22 fg/cell UV-B 0.52 W/m^2			
	30.64 fg/cell UV-B 1.02 W/m^2			
M. aeruginosa FACHB	Different [urea], min–max 1.01–1.93 pg/cell	China	HPLC–PDA	Huang et al. (2014)
M. aeruginosa AB2005/31	Different nitrate level	China	LC–MS/MS	Krueger et al. (2012)
	*t*0: 925.4–*t*fin: ~150–700[a]			
	Different phosphate level			
	*t*0: 1097.8–*t*fin: ~450–1700[a]			
	Different pH value			
	*t*0: ~950[c]–*t*fin: ~100–650[c]			
	Different exposure times to DMSO			
	*t*0: 1534.3–*t*3 min: 551.3.–*t*6 min: 153.4			

Table 2 continued

Gender/species/strain	Cell quota (µg/g DW)	Country of origin	Analytical method	References
M. aeruginosa 905	Under light and dark conditions after H^2O^2 treatment		HPLC-CD	Qian et al. (2012)
	min–max 0.05–0.114 pg/cells			
M. aeruginosa CYA140	0.52 µg/mm^3		HPLC–DAD	Van de Waal et al. (2011)
M. aeruginosa CPCC299	38 fg/cell high light and zinc		ELISA	Xu et al. (2013a, b)
Microcystis flos-aquae	16.86–484.48 (bloom sample)	South Africa	LC–MS	Eguzozie et al. (2016)
Microcystis novacekii T20-3	53.62–1.714	Botswana	LC–MS	Mbukwa et al. (2015)
Nostoc sp. 152	116 fg/cell low P		HPLC–DAD	Kurmayer (2011)
	47.2 fg/cell high NP			
	Different condition, min–max	Finland	HPLC–DAD	Sivonen (1999)
Oscillatoria agardhii CYA 128	0.61×10^3–5.65×10^3			
Oscillatoria agardhii 97	1.38×10^3–7.9×10^3			
Planktothrix agardhii 126-3	N limited–N saturated growth		HPLC–DAD	Tonk et al. (2008)
	MC-LR: 0.025–0.042 µg mm^3			
	MC-RR: 0.149–0.245 µg mm^3			
P. agardhii 126-3	0.06 mg/mm^3 low N			Van de Waal et al. (2010)
	0.15 mg/mm^3 high NP			
	Different light and temperature		PPA2	Briand et al. (2008)
P. agardhii PMC 75.02	Limiting growth condition 43 fg/cell			
	Optimal growth condition 69 fg/cell			
P. agardhii PMC 86.02	Limiting growth condition 33 fg/cell			
	Optimal growth condition 50 fg/cell			
P. agardhii PMC 212.03	Limiting growth condition 24 fg/cell			
	Optimal growth condition 34 fg/cell			
Planktothrix rubescens	0.680 pg/cell	Lago Pozzillo (Italy)	HPLC–DAD	Naselli-Flores et al. (2007)
P. rubescens	0.01–0.42 pg/cell	Lago Vico (Italy)	HPLC	Manganelli et al. (2016)
P. rubescens TCC 29	Exponential growth phase 0.7 pg/cell	Lac du Bourget	HPLC–DAD	Briand et al. (2005)

Table 2 continued

Gender/species/strain	Cell quota (µg/g DW)	Country of origin	Analytical method	References
	MC-RR/MC-YR	Brazil	HPLC–DAD	Pereira et al. (2012)
Radiocystis fernandoii 28	25 µmol photons/m^2/s 117/171 fg/cell			
	65 µmol photons/m^2/s 262/150 fg/cell			
	95 µmol photons/m^2/s 140/186 fg/cell			
R. fernandoii 86	25 µmol photons/m^2/s 200/0 fg/cell			
	65 µmol photons/m^2/s 62/0 fg/cell			
	95 µmol photons/m^2/s 49/0 fg/cell			
R. fernandoii 28	~0.15–0.18 pg/cell[a] 100% Fe	Brazil	LC–MS/MS	Pereira et al. (2015)
R. fernandoii 86	~0.16–0.04 pg/cell[a] 1% Fe			

For *Microcystis* strains, only data from 2010 onward have been reported. When variants are not specified, MC are expressed as MC-LReq (ELISA and PP) or sums of MCs

DW dry weight, *P* phosphate, *NP* nitrate and phosphate, *C* carbon, *T* temperature, *N* nitrate, *DMSO* dimethyl sulfoxide, *t* time, *Fe* iron

[a] Values taken from figures; [b] calculated on the fraction of *mcyB*$^+$ cells; [c] recalculated from monthly averages in the text

2013). Beyond these genera, a benthic MC producer strain of *Planktothrix* has been isolated in a river in New Zealand (Wood et al. 2010a).

MC have also been detected in few benthic marine environment. *Geitlerinema* and *Leptolyngbya* sp., isolated from some field samples from Black Band Disease, a migrating mat rich in sulfide growing on coral reefs, in the wider Caribbean (Florida Keys and Bahamas), produced MC (Richardson et al. 2007). A recent study reported a high diversity in toxin-producing CB on mats in three mangrove areas, very productive areas and important for local economies, located along the southern Red Sea coast of Saudi Arabia (salinity 38‰) (Mohamed and Al-Shehri 2015). The authors isolated many MC-producing species (*Aphanothece elabens*, *Leptolyngbya tenuis*, *Oscillatoria tenuis* and *Calothrix breviarticulata*, producing MC in the range 489–974 µg/g DW) and detected up to ~900 and ~1400 µg/g DW of MC in pneumatophore and sediments mats, respectively.

In the extreme environment of hot springs in Saudi Arabia, accessible to the public as recreational places and some also used as cooking resources by rural communities, *Oscillatoria limosa* and *Synechococcus lividus* produced MC at concentrations ranging from 468 to 512.5 µg/g (Mohamed 2008).

Finally, MC have also been detected in terrestrial habitats, in *Nostoc* symbionts of lichens (Oksanen et al. 2004; Gehringer et al. 2012).

Intracellular vs dissolved toxin

MC are mostly intracellular until cell lysis (Rohrlack and Hyenstrand 2007). Extracellular MC (either dissolved in water or bound to other materials) typically make up less than 30% of the total MC concentration in raw water (Graham et al. 2010). MC concentrations vary considerably both from one bloom to another and during the course of a single bloom. This variability is due in part to changes in the levels of MC production by the MC-producing cells (Wood et al. 2011), in part to changes in the relative proportions of potentially MC-producing and non-MC-producing cells (Manganelli et al. 2016; Sabart et al. 2010) and in genotypes with higher and lower cell quota (Kosol et al. 2009) in the bloom-forming populations. Intracellular MC content can vary up to 40-fold during the same bloom, from 10 to 420 fg/cell in *P. rubescens* and from 100 to 4000 fg/cell in *M. aeruginosa* (Manganelli et al. 2016; Sabart et al. 2013), and is usually higher at the beginning of the bloom. Smaller but still significant variations in cell quota in *Microcystis* on a very short timescale of a few hours, due to a change in *mcyE* expression rate following a sudden increase in cellular density (as it happens during surface foam formation, at densities of 4–7 × 10^9 cell/L), have

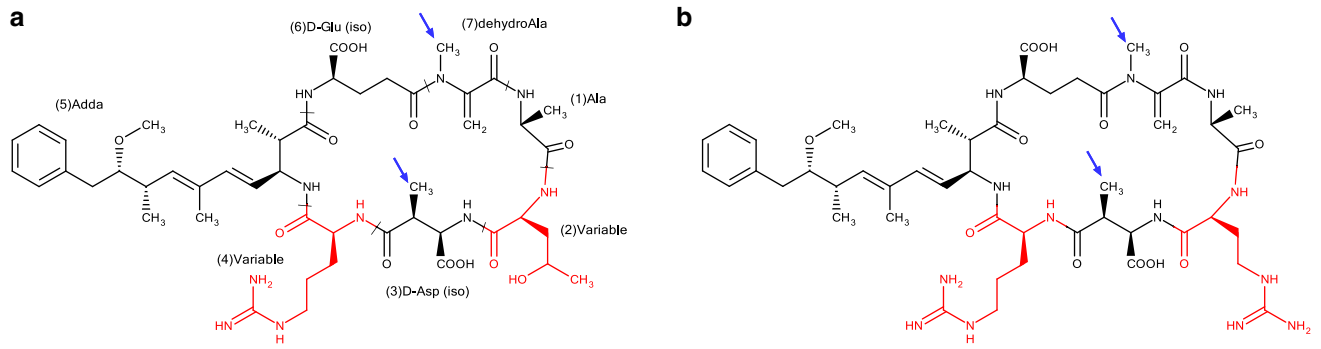


Fig. 3 Chemical structure of MC-LR (**a**) and RR (**b**), with leucine (L) and arginine (R) and two arginine, as L-amino acids in positions 2 and 4, respectively. Possible positions for demethylations are indicated by the *arrows*

been observed both in the field, from 0.019 to 0.644 pg/cell, and in a mesocosm experiment, from 0.1 to 1.38 pg/cell (Wood et al. 2011, 2012a). This implies that during the sudden formation of foam or scums, a phenomenon that can happen in few hours, cells can be induced to produce more cyanotoxins, and that animals and humans, in the presence of this material, can be exposed to a significantly higher dose of MC.

Very few data are available for other MC-producing species (Table 2). *Dolichospermum* can produce different toxins. The MC-producing strains, *D. flos-aquae* e *D. lemmermanni,* have been isolated from lakes in Canada (Krishnamurthy et al. 1986), Finland and Norway (Sivonen et al. 1992) and from less saline areas of Baltic Sea (Halinen et al. 2007). Even if the abundance of MC-producing strains of *Anabaena*/*Dolichospermum* is favored in less saline areas, MC intracellular concentrations increased in time, in the isolates exposed to higher salinities, between 0 and 6 ppm ranging from 1.70 ± 1.34 mg/g BM to 24.66 ± 14.54 mg/g BM at 15 days, salinity 6 (Brutemark et al. 2015b). However, the extracellular concentration was much higher in freshwater, where it reached the maximum values of 180.65 fg/cell vs a minimum of 0.50 fg/cell (Brutemark et al. 2015a, b).

Occurrence

Many papers have been published during the last decades reporting data of occurrence of cyanotoxins worldwide in different matrices. Among cyanotoxins, MC and particularly some specific variants (e.g., MC-LR, MC-RR and their demethylated forms) are for sure the most frequently investigated and detected. This can be partially due to limited availability of certified standards and also to the low sensitivity of methods for toxins other than MC (Testai et al. 2016a, b).

Tables 3, 4 and 5 report literature data of the last ten years on the presence of cyanotoxins in water and other matrices. The different detection methods are also reported, since the methodology used gives important information on the reliability of the results. Indeed, methods for detection in water and/or in CB such as HPLC-FD or UV, LC-MS or LC-MS/MS, MALDI-TOF/MS, are sufficiently reliable and some of them validated (see US-EPA 2015a); in addition immunological methods (i.e., ELISA) are used, although they provide only semiquantitative results and do not allow to distinguish among variants. Whenever matrices other than water are concerned, validated methods are not available so far for MC neither for any other cyanotoxin. All the above-mentioned methods are prone to matrix effects, responsible for inaccuracy due to ion enhancement or suppression, especially in electrospray ionization (ESI) mode, as well as interference in the ELISA optical density detection. Matrix effects as well as the recovery following the use of sample cleaning steps are generally not accounted or neither reported. The performance of the sample preparation and the analytical method is crucial information to evaluate the reliability of data on occurrence (Testai et al. 2016a).

In addition, some toxins including MC can occur either as free toxins or bound to proteins; it is therefore important to use an adequate extraction procedure to obtain reliable data on the total MC content, or at least provide a clear indication about the detection of free, bound or total toxin form, in the absence of which results cannot be properly interpreted and compared.

Regarding occurrence our group co-authored two recent reviews on alkaloid neurotoxins as well on the occurrence in food items for all cyanotoxins (Testai et al. 2016a, b). The readers are invited to consult them for further details; in this paper an update is given.

Surface and drinking water

As shown in Table 3, MC are the most widely and frequently found cyanotoxins, with levels up to 126.42 µg/L in

Table 3 Cyanotoxins occurrence in surface water, bloom and scum

	Country	Area	Producing organism	CTX	Surface water (µg/L)	Bloom and scum (µg/g DW)	Analytical method	References
Africa	South Africa	Swartspruit River	*Microcystis flos-aquae*	MC		16.86–484.48	LC–MS	Eguzozie et al. (2016)
	South Africa	Water impoundments in the Kruger National Park		MC		Up to 103000 µg/L	ELISA	Masango et al. (2010)
	Algeria	Lake Oubeira	*Microcystis* sp.	MC		1120	Ion trap LC/MS	Nasri et al. (2008)
	Algeria	Lake des Oiseaux	*Microcystis* sp.	MC		62	LC–MS/MS	Bouhaddada et al. (2016)
	Kenya	Lakes Nakuru, Bogoria	*Arthrospira fusiformis, Anabaenopsis abijatae, Spirulina subsalsa Phormidium terebriformis*	MC ATX		16–4593 0.3–223	HPLC–PDA	Ballot et al. (2004)
America	Greenland	18 Arctic lakes		MC	0.005–0.4		ELISA	Trout-Haney et al. (2016)
	Canada	6 different lakes in Quebec		BMAA	ND-0.3		HRMS	Roy-Lachapelle et al. (2015a)
				DAB	ND-0.04			
				AEG	ND-0.08			
				ATX	ND-0.1			
				CYN	ND-0.2			
				STX	ND			
	Canada	St. Lawrence River	*Lyngbya wollei*	LWTX		209–279	LC-QqQMS	Lajeunesse et al. (2012)
				ATX		ND		
				NOD		ND		
				MC		ND		
				STX		ND		
	USA	National survey in freshwater (1161 lakes)		MC	3 (230)		ELISA confirmed by LC–MS/MS	Loftin et al. (2016b)
				CYN	0.56 (4.4)			
				STX	0.061 (0.38)			
				Mean (max) levels				
	USA (Washington)	Puget Sound area lakes	*Anabaena* spp.; *Microcystis* spp.	MC			ELISA confirmed by	Preece et al. (2015a)
				Lake water	2700		LC–MS/MS	
				Marine water	Up to 0.34			
	USA (Washington)	Several lakes in the Washington state		MC	Up to 10000 ug/L		–	Trainer and Hardy (2015)
				ATX	Up to 1170 µg/L			
				STX	ND-193 µg/L			
				CYN	ND-1.12 µg/L			
	North America	Lake Erie	*Microcystis*	MC	ND-1.9		ELISA	Hu et al. (2016)

Table 3 continued

	Country	Area	Producing organism	CTX	Surface water (µg/L)	Bloom and scum (µg/g DW)	Analytical method	References
	Nebraska	Nebraska Lakes and Impoundments		ATX	ND-35.7		ELISA for MC	Al-Sammak et al. (2014)
				BMAA	ND-39.6		LC–MS/MS for BMAA, DABA and ATX	
				DABA	ND-37			
				MC	ND-44.5			
	USA (Kansas)	Lake Milford	*Microcystis* spp. *Anabaena* spp.	MC	Up to 1600		ELISA	Trevino-Garrison et al. (2015)
	USA (Piedmont region)	75 wadeable streams		MC (in headwater streams)	Up to 3.2		ELISA	Loftin et al. (2016a)
	Guatemala	lake Amatitlán	*Microcystis*	MC intracell extracell	1931 90		LC–MS/MS	Romero-Oliva et al. (2014)
	Argentina	Los Padres Lake		MC	0.22–14.96		LC–MS/MS	Amé et al. (2010)
	Argentina	San Roque reservoir	*Microcystis*	MC ATX	ND-119 ND-0.006		HPLC–UV and MS/MS	Ruiz et al. (2013)
			Pseudanabaena					
			Oscillatoria					
			Anabaena					
Asia	China	Lake Chaohu	*Microcystis Dolicospermum*	MC	0.28–8.86		HPLC–DAD	Shang et al. (2015)
	China	Lake Taihu	*Microcystis*	BMAA		2.03–7.14	LC–MS/MS	Jiao et al. (2014)
	Korea	Rivers and reservoirs in South Korea	*Microcystis* *Oscillatoria* *Anabaena*	MC ATX	0.057–19.1 0.01–0.08	133–2612 –	–	Srivastava et al. (2015)
	Japan	Artificial reservoir of Isahaya Bay	*M. aeruginosa* *Planktothrix rubescens*	MC	ND-18		ELISA	Takahashi et al. (2014)
	Russia	Lake Baikal	Synechococcales Chroococcales Oscillatoriales Nostocales	STXs		up to 0.293	ELISA confirmed by MALDI-TOF	Belykh et al. (2016)
	Taiwan	3 Reservoirs in Kinmen island	*Nodularia* spp. *Microcystis* spp.	MC ATX	0.08–3.38 –		LC–MS	Yen et al. (2011)
				NOD	0.3–3.7			
				CYN	ND-36			
	Bangladesh	Water sources in Mymensingh district	*M. aeruginosa* *M. flosaquae* *Anabaena crassa* *Aph. flosaquae*	MC	0.025–82.3		ELISA	Affan et al. (2015)
	India	Lakshmikund pond	*Microcystis* spp.	MC	7.5–126.42		QTRAP-LC–MS/MS	Singh and Asthana (2014)
	India	A pond in Varanasi	*Nostoc sp.*	MC		25.2	ELISA confirmed by MALDI-TOF	Bajpai et al. (2009)
	Turkey	Lake Eğirdir	*M. aeruginosa*	MC	2.9–13.5	320	HPLC–PDA	Gurbuz et al. (2016)

Table 3 continued

	Country	Area	Producing organism	CTX	Surface water (µg/L)	Bloom and scum (µg/g DW)	Analytical method	References
	Qatar	Water impoundments	Chroococcales, *Microcoleus*	MC BMAA DAB ATX-s	ND-0.8902 ND ND ND		LC–MS/MS	Chatziefthimiou et al. (2016)
	Saudi Arabia	Lake Tendaha	*Anabaenopsis arnoldi*	MC	1.6–8.3	Up to 364	ELISA confirmed by HPLC	Mohamed and Al Shehri (2009)
	Saudi Arabia	Lake Gazan Dam	*C. raciborskii*	CYN	0.03–23.3	568	ELISA confirmed by LC–MS	Mohamed and Al-Shehri (2013)
Australia	Queensland	Awoonga Dam and Yabba Creek	*Lyngbia wollei*	CYN Deoxy-CYN		ND-2.9 3–86	QTRAP-LC–MS/MS	McGregor and Sendall (2015)
	Queensland	Carbrook Cable Ski Lake	*N.spumigena*	NOD	Up to 220		HPLC–PDA	McGregor et al. (2012)
	New Zealand	Drinking water pre-treatment reservoir	*Scytonema* cf. *crispum*	STX ATXs CYNs MC		65.6 ND ND ND	HPLC-FD for STX LC–MS for other CTXs	Smith et al. (2011)
Europe	Sweden	Lake Finjasjön		BMAA		ND-0.006	UPLC–MS/MS	Lage et al. (2015)
	Finland	Gulf of Finland	*Anabaena*	MC NOD	ND-0,05 ND-0,48		LC–MS	Fewer et al. (2009)
	Russia-Lithuania	Curonian Lagoon	*M. aeruginosa* *P. rubescens* *D. flos-aquae*	MC ATX CYN STX		Up to 153.6 µg/L ND ND ND	QTRAP-LC-MS/MS	Sulcius et al. (2015)
	Poland	four oxbow lakes of the Vistula River	*M. ichthyoblabe* *M. wesenbergii* *O. tenuis* *D. planctonicum* *D. spiroides* ….	MC	ND-0.246		HPLC–DAD	Krzton et al. (2016)
	Poland	94 water reservoirs		MC (median)	Up to 37 (0.26)		ELISA	Bláhová et al. (2007)
	Poland	Gulf of Gdansk	*N. spumigena*	NOD (seawater)	ND-42300 (0.1–0.2)		HPLC–PDA	Mazur-Marzec et al. (2013)
	Poland	36 randomly selected lakes in western Poland	*Cylindrospermopsis raciborskii* *Aphanizomenon gracile*	CYN	ND-3 µg/L		LC–MS/MS	Kokociński et al. (2013)
	Germany	21 Lakes in North-easter Germany		CYN Dissolved Particulate Total	 0.08–11.75 0.002–0.484 Up to 12.1		LC–MS/MS	Rücker et al. (2007)
	France	River La Loue	*Phormidium favosum*	ATX		8000	HPLC–DAD confirmed by QTOF-LC–MS/MS	Gugger et al. (2005)
	Italy	Lake Garda	*Tychonema bourrelly*	ATX MC	ND-11.32 ND		QTRAP-LC–MS/MS	Shams et al. (2015)

Table 3 continued

	Country	Area	Producing organism	CTX	Surface water (µg/L)	Bloom and scum (µg/g DW)	Analytical method	References
	Italy	Lakes Garda, Iseo, Como and Maggiore	*Tychonema bourrellyi, Planktothrix rubescens*	MC ATX HomoATX	ND 1.42–154.23 ND-1.53		QTRAP-LC–MS/MS	Salmaso et al. (2016)
	Italy	Lake Vico	*P. rubescens*	MC	ND-5	Up to 56 µg/L	LC–MS/MS	Manganelli et al. (2016)
	Spain	Ebro River		ATX MC	ND-0.007 ND		LC–MS/MS	Rivetti et al. (2015)
	Spain	11 reservoirs and lakes in Castellon province		MC NOD	ND-0.14 ND		UHPLC–MS/MS	Beltran et al. (2012)
	Spain (Donana national park)	Los Ansares Lagoon	*Microcystis aeruginosa*	MC		Up to 11400 µg/L	ELISA	Lopez-Rodas et al. (2008)
	Greece	Lake Kastoria and Marathonas		MC CYN ATX	0.03–63 ND ND		LC–MS/MS	Zervou et al. (2016)
	Greece	Lakes and reservoir	*C. raciborskii* *A. flos-aquae*	MC CYN STX	3.9–108 0.3–2.8 0.4–1.2		ELISA	Gkelis and Zaoutsos (2014)

DW dry weight

Asia (Singh and Asthana 2014), 660 µg/L in Europe (Pekar et al. 2016) and 10,000 µg/L in the USA (Trainer and Hardy 2015). MC have been reported in streams in New Zealand and Egypt (Loftin et al. 2016a, b). Levels up to 103 mg/L, associated with the death of rhinos, zebras, blue wildebeest, have been reported in South Africa (Masango et al. 2010). The highest MC concentrations are detected in cyanobacterial accumulated materials, like blooms and scum (see above).

Few data are available for MC in drinking waters probably because most of them go in routine monitoring. Table 4 shows that MC concentrations in tap waters rarely exceed the WHO guideline value for drinking water of 1 µg/L, indicating that in general drinking water treatment system processes are efficient. Yet if raw water is affected by high MC concentrations (650 µg/L) these processes might lose efficiency (2.5 µg/L in treated water) (Svircev et al. 2009). Water storage reservoir in arid zone, when not properly managed, is an issue that deserves a special attention to prevent CB growth and the consequent MC occurrence in tap waters (Mohamed and Al Shehri 2007).

Pure MC are relatively stable in filtered deionized water as well as at high temperature; however, in natural water or reservoir in presence of bacteria and photosynthetic pigments extracellular toxin concentrations can rapidly decrease (Rastogi et al. 2014).

Fish and seafood

Very recently, in an extensive review of literature data, mean and median ranges of 0.19–0.20 and 0.01–0.07 µg/g wet weight (WW), respectively, have been reported in fish muscle (Testai et al. 2016a). MC concentrations in fish liver and viscera have been found up to 20 times those in fish muscle in some studies, although this relationship is not described in other ones (Testai et al. 2016a). In seafood (mollusks and crustaceans) max and median values of 74.7 µg/g and around 0.23 µg/g have been detected. As shown in Table 5, MC levels up to 2.8 µg/g WW have been reported in muscles of silver carp in China (Zhang et al. 2007). Preece et al. (2015b) reported MC contamination of 6.5 ng/g in sea mussels, where much higher levels, up to 63.4 µg/g, have been reported in previous investigations (Williams et al. 1997).

The high temperature typical of cooking procedures (e.g., boiling in water, microwave), can affect the level of contamination. Zhang et al. (2010) found that the mean concentrations determined in bighead carp-contaminated muscles were 300% higher in boiled muscles than unboiled, likely due to the release of phosphatase-bound MC. However, other authors obtained opposite results (Guzman-Guillen et al. 2011).

Food supplements

The market of these products, known as BGAS (blue-green algal supplements), seems to be increased during the last decades all over the world due to the claimed beneficial effects for consumers' health. They are harvested from natural environments and mainly obtained as extracts or lyophilized nontoxic species like *Aphanizomenon flos-aquae* and *Spirulina*. Yet toxic CB can be present as contaminating species. Indeed, MC have been reported at concentration up to 5.8 µg/g dry weight (DW) (Heussner et al. 2012; Vichi et al. 2012; Vinogradova et al. 2011).

Spirulina food supplements in the Chinese market have been found to contain MC in 94% of the 34 tested samples, with concentrations from 2 to 163 ng/g (mean 14 ± 27 ng/g) (Jiang et al. 2008).

Extracts from food supplements of *Aphanizomenon flos-aquae* with MC concentration ≤ 1 µg MC-LR equivalents/g DW and none of the other known toxins found, still showed cytotoxicity, implying the occurrence of some unknown toxin (Heussner et al. 2012).

Finally, it seems of interest that levels up to 0.166 and 0.96 µg/g DW of MC-LR and MC-LA, respectively, have been reported in a 100% *Aphanizomenon flos-aquae* food supplement for dogs in California, responsible for a case of dog intoxication (Bautista et al. 2015).

Crops

Few data are available on MC occurrence in crops, with levels up to 2.3 µg/g DW (Chen et al. 2012; Li et al. 2014; Xiao et al. 2009) in China.

Even though most of the available data refer to crops irrigated with intentionally contaminated water, depicting an unrealistic scenario, yet they show that MC can accumulate in crops (Bittencourt-Oliveira et al. 2016; Gutierrez-Praena et al. 2014; Hereman and Bittencourt-Oliveira 2012; Jarvenpaa et al. 2007; Kittler et al. 2012; Maejima et al. 2014). Therefore, it is not clear whether, after contamination, crops are still fit for consumption. Freitas et al. (2015), for example, found that lettuce plants in nonearly development stages are able to cope with low concentrations of MC-LR, cylindrospermopsin (CYN) and the MC-LR/CYN mixture; however, high concentrations (100 mg/L) can affect both lettuce yield and nutritional quality. Also Maejima et al. (2014) found that after seven-day exposure, MC-LR and MC-RR (0, 0.01–10 µg/mL) affected the growth of rice and broccoli, showing, for rice, a NOEL less than 0.1 µg/mL; for broccoli growth inhibition was confirmed in response to exposure to more than 1 µg/mL. Pflugmacher et al. (2007) showed that different variants of the same spinach species can react in different ways to the same toxin. In any

Table 4 Cyanotoxins occurrence in water used for drinking

	Country	Area	Producing organism	CTX	Raw water (µg/L)	Tap water (µg/L)	Analytical method	References
America	USA	Utilities located in five US states	*Microcystis* *Aphanizomenon* *Anabaena*	MC CYN ATX	0.05–0.25 0.41 ND	ND ND ND	LC–MS/MS and HPLC/PDA	Szlag et al. (2015)
	USA	33 water supplies		MC (mean)	ND-5.6 (0.307)	Plant effluent ND-0.36 (0.036)	ELISA	Haddix et al. (2007)
Asia	South China	25 urban reservoirs for drinking water	*C. raciborskii*	MC CYN	1.99 8.25		–	Lei et al. (2014)
	Taiwan	Reservoirs and WTPs in Kinmen Island		MC ATX NOD CYN	0.08–0.73 ND 0.64–1 0.71–36	ND-0.1 ND ND-0.05 0.69–2.2	LC–MS	Yen et al. (2011)
	Bangladesh	Tap water in Mymensingh district	*M. aeruginosa* *M. flosaquae* *A. crassa* *Aph. flosaquae*	MC	0.025–82.3	0.030–0.032	ELISA	Affan et al. (2015)
	Qatar	Water impoundments	Chroococcales *Microcoleus*	MC BMAA DAB ATX-s	0.6628–1.3309 ND ND ND		LC–MS/MS	Chatziefthimiou et al. (2016)
	Saudi Arabia	Open reservoirs without filters	*A. parietina* *C. minor* *G.alpina* *L. epiphytica* *L. lagerhimii* *P. inundatum* *P. tenue* *Xenochoccus sp.*	MC	ND-0.14	0.02–0.3	LC–MS	Mohamed and Al Shehri (2007)
		Open reservoirs with filters			0.02–0.1	0.04–0.2		
		Covered reservoirs without filters			ND	ND		
		Covered reservoirs with filters			ND	ND		
	Saudi Arabia	Lake Tendaha	*Anabaenopsis arnoldi*	MC	1.6–8.3	0.33–1.6	ELISA confirmed by HPLC	Mohamed and Al Shehri (2009)
Australia	New Zealand	A drinking water pre-treatment reservoir	*Scytonema agardh*	STX	65.6 µg/g		HPLC-FD	Smith et al. (2011)
Europe	Sweden	Source waters and infiltration ponds		ATX HomoATX CYN MC	ND-1.3 ND- 10.6 <LOQ ND-660.8	ND ND ND ND	UPLC–MS/MS	Pekar et al. (2016)
	Poland	26 Drinking water supplies	*Woronichinia* spp. *M. aeruginosa*	MC	ND-2,1		ELISA	Bláhová et al. (2007)
	Greece	Lake Marathonas (auxiliary drinking water source for the metropolitan area of Athens)		MC	ND-0.063		LC-MS/MS	Zervou et al. (2016)
	Serbia	Celije Reservoir		MC	Up to 650	Up to 2.5	–	Svircev et al. (2009)

Table 5 Cyanotoxins occurrence in fish and seafood

	Country	Area	Producing organism	CTX	Matrix	DW/WW	Level (ng/g)	Analytical method	References
Asia	China	Lake Taihu		MC	Shrimp fish	DW	6–12 4–46	LC-MS/MS	Jia et al. (2016)
	China	Tiesha River	*M. aeruginosa*	MC	H. molitrix	WW		LC–ESI–MS	Zhang et al. (2007)
					Muscle		2860		
					Liver		4520		
					Intestine		6490		
	China	Lake Taihu	*M. aeruginosa*	BMAA	Mollusks Crustacean Fish	DW	Up to 6720 Up to 8760 Up to 35910	LC–MS/MS	Jiao et al. (2014)
	Japan	Artificial reservoir	*M. aeruginosa* *P. rubescens*	MC	Fish muscle Prawn Mollusk	DW	1.3–46 5.1 280	ELISA	Takahashi et al. (2014)
	India	Lakshmikund pond	*Microcystis* spp.	MC	carp muscles catfish muscles	DW	5 70	QTRAP-LC–MS/MS	Singh and Asthana (2014)
America	Nebraska	Nebraska Lakes and Impoundments		ATX BMAA Bound (free)	Fish samples	DW	ND ND-2570 (ND-400)	HPLC/FD and LC–MS/MS	Al-Sammak et al. (2014)
	Canada	three different lakes in the province of Québec		MC	Fish muscle Whole fish	–	9–130 3–7	LDTD-APCI-HRMS	Roy-Lachapelle et al. (2015b)
	Washington	Puget Sound area lakes	*Anabaena* spp.; *Microcystis* spp.	MC	Sea mussels	–	ND-6.5	ELISA confirmed with LC–MS/MS	Preece et al. (2015b)
	USA	Lake Mascoma		BMAA DAB MC	Fish muscle	–	1270 ND ND	LC–MS/MS	Banack et al. (2015)
	Argentina	Los Padres Lake		MC	Odontesthes bonariensis muscle (liver)	WW	4 ± 2 (34 ± 37)	LC–MS/MS	Amé et al. (2010)
	Mexico	Lake Catemaco	*Cylindrospermopsis*	CYN PSP MC	Tegogolos (Pomacea patula catemacensis)	DW	3.35 ± 1.90 1.04 ± 0.42 ND	ELISA confirmed with HPLC–UV and LC–MS/MS	Berry and Lind (2010)
	Mexico	Lake Catemaco	*Cylindrospermopsis*	CYN PST	Finfish from local market	WW	Up to 1 Up to 0.7	ELISA	Berry et al. (2012)
	Brazil	Two hydro electric reservoirs		MC	Fish muscles	DW	9–12	PPI confirmed with HPLC–PDA	Deblois et al. (2008)
	Brazil	Artificial lake		MC ATX CYN STXs	Fish muscle Before depuration (After depuration)	–	ND ND ND 20 (<LOD)	HPLC–PDA for STXs LC–MS/MS Other CTX	Galvao et al. (2009)
	Brazil	Alagados Reservoir		STXs	Fish muscle		Up to 30.6 ± 14.5	HPLC-FD	Clemente et al. (2010)

Table 5 continued

	Country	Area	Producing organism	CTX	Matrix	DW/WW	Level (ng/g)	Analytical method	References
Australia	Queensland	Recreational artificial lake	*N. spumigena*		*Mugilidae*	WW		LC–MS/MS	Stewart et al. (2012)
				NOD	Muscle Liver		ND-18 ND-13,000		
				MC	Muscle and liver		ND		
Europe	Sweden	Swedish west coast		BMAA	Sea mussels	WW	<40	HILIC-UHPLC–MS/MS	Rosen et al. (2016)
	Sweden	Lake Finjasjön		BMAA	Fish muscles	DW	0.1–6	UPLC–MS/MS	Lage et al. (2015)
	Finland	Baltic sea	*N. spumigena*	NOD	Herring liver Stickleback viscera	DW	ND-5 ND-700	LC–MS	Sipia et al. (2007)
	Poland	Gulf of Gdansk (Baltic sea)	*N. spumigena*	NOD	Mussels Fish	DW	3–816 4–347	QTRAP-LC–MS/MS	Mazur-Marzec et al. (2013)
	France	French coasts		BMAA	mollusk	DW	190–6700	LC–MS/MS	Reveillon et al. (2016)
	France	Thau Lagoon	*Diatoms*	BMAA	Mussels Free (Total)	DW	ND-1200 (ND-9700)	LC-MS/MS	Reveillon et al. (2015)
				DAB			ND-6200 (ND-10,700)		
				AEG			ND-310 (ND-1200)		
	Italy	Two Sardinian ponds	*A. spiroides*	MC			Up to 8.5 ND	ELISA LC-MS/MS for MC-LR	Sedda et al. (2016)

DW dry weight, *WW* wet weight

case monitoring of crop products irrigated with lake water affected by cyanobacterial blooms needs further consideration as they can move MC into farm animal and food chains.

Biosynthesis and genetics

Microcystins are synthesized by a nonribosomal peptide synthetase (NRPS) encoded by the *mcy* (microcystin synthetase) cluster, which has been characterized in several cyanobacterial genera, including *Microcystis, Planktothrix* and *Anabaena* (Tillet et al. 2000; Christiansen et al. 2003; Rouhiainen et al. 2004).

The cluster contains nine to ten genes, coding for peptide synthetases, polyketide synthases, chimeric enzymes composed of peptide synthetase and polyketide synthase (PKS) modules, putative thioesterases, putative transporters and putative peptide-modifying enzymes. For more details, see Vichi et al. (2016).

Detection of *mcy* genes generally indicates the presence of potentially toxic CB. A high degree of DNA polymorphism has been observed to occur in toxin biosynthetic genes, which leads to the co-occurrence of toxic and nontoxic subpopulations, morphologically indistinguishable from each other, in a ratio extremely variable during blooms (Vichi et al. 2016).

The clarification of the genetic basis behind the production of microcystins has allowed an increased use of molecular techniques for the discrimination of toxic and nontoxic cyanobacterial cells, improving the water resource management. Recently, the use of assays based on real-time PCR technique as the Taq nuclease assay (TNA) has spread, allowing the accurate quantitative estimate of a cyanobacterial community composition and toxigenicity, according to the number of *mcy* copies present in a sample with respect to the general population (16SrDNA positive), as well as the possibility to predict MC concentration during a bloom (Kurmayer and Kutzenberger 2003; AL-Tebrineh et al. 2012; Savichtcheva et al. 2011; Ostermaier and Kurmayer 2010; Manganelli et al. 2016). However, while some studies have shown positive correlations between toxin gene abundances and MC concentrations, others seem to exclude any connection; therefore, the relationship between these variables is still controversial (Manganelli et al. 2016; Beversdorf et al. 2015).

About bloom toxicity, an effect of the environmental conditions on toxin production is undoubtedly plausible, as well as the regulation of *mcy* genes; however, the exact mechanisms at a molecular level involved in MC production are still not completely clear (Neilan et al. 2012).

A strong variability of the genotype ratio (toxic vs nontoxic strains) has been reported to occur, even at small temporal scale (such as on a weekly or biweekly scale) for *P. rubescens*, especially during the blooms, and this should be kept into account for the evaluation of human exposure (Kurmayer and Kutzenberger 2003; Manganelli et al. 2010, 2016).

In *Planktothrix*, it is known that the partial deletion of *mcy* genes or the insertion of transposable elements can affect the *mcy* gene cluster by both recombination and inactivation processes (Christiansen et al. 2006, 2008; Kurmayer et al. 2004, 2005). In a recent paper, Chen and coworkers discovered that insertion events within the *mcy* cluster in *Planktothrix* can be predicted since their location correlates with the presence of sequences similar to the repetitive extragenic palindromic DNA sequences (REPs), which in the past have been widely used for taxonomic purposes. Moreover, they observed that these REP regions within the *mcy* cluster in *Planktothrix* occur more frequently than in *Microcystis* and *Anabaena*, thus explaining the more frequent occurrence of mutations in *Planktothrix* with respect to other CB genera (Chen et al. 2016a, b). However, compared to the neutral mutations (7.5–20.6%), those mutations leading to the inhibition of MC production were considered more rare (0–6.9%).

Toxicological profile

Kinetics

As for other xenobiotics, toxicokinetic has a crucial role in the toxicity of MC. Because of hydrophilic chemical structure of most of the variants, passive transport is not possible; therefore, absorption at gastrointestinal (g.i.) level as well as cellular uptake is facilitated by the presence on the outer membrane of the organic anion transport system (OATP), particularly by the OATP1B1 and 1B3 isoforms, as demonstrated by several in vitro studies (Eriksson et al. 1990; Fisher et al. 2005, 2010). In vivo, OATP null mice, completely lacking the OATP-mediated transport system, showed complete resistance to MC-LR-induced hepatotoxicity, while wild-type mice, treated with the same dose, presented extensive hepatic hemorrhagic necrosis (Lu et al. 2008).

Recently this aspect has been further studied to search possible molecules which can prevent MC-induced toxic effects as the grapefruit flavanone glycoside naringin using human hepatocyte uptake transporter OATP1B3-expressing HEK293-OATP1B3 cells (Takumi et al. 2015) or captopril in A549 (human lung carcinoma), SK-Hep-1 (human liver adenocarcinoma), FL (human amniotic normal cells) (Teneva et al. 2016). Both compounds, inhibitors of the OATP transporters, were able to attenuate the cytotoxicity of MC-LR, in a dose-dependent manner (naringin), blocking the uptake of MC-LR into the tested cells line. In pancreatic cancer cell lines BxPC-3 and MIA PACA-2,

in which OATP1B1 and 1B3 were found to be expressed, the cytotoxic antiproliferative and proapoptotic effects of MC-LR were proportionally related to the expression of these transporters. These properties suggested that MC structure can be used as a model for the development of new anticancer compounds targeted to pancreatic cells, being more potent than present drugs (Kounnis et al. 2015).

The OATP active transport is not present only in the entero- and hepatocytes but also in other organs, as the kidney and the blood–brain barrier (Feurstein et al. 2009), explaining the toxicity in organs other than liver (thyroid, kidney brain) recently investigated by several studies (see below), as well as some effects reported in dialysis patients after the Brazil outbreak (Azevedo et al. 2002) and the demonstration that some congeners (e.g., MC-LF, MC-LW and MC-LR) can display neurotoxic effects (Feurstein et al. 2011; Li et al. 2012a, b). Furthermore, it has been recently evidenced MC-LR induced the production of cytotoxic and cytostatic products (nitric oxide (NO)) as well as proinflammatory mediators [e.g., tumor necrosis factor α, TNF-α, and interleukin 6 (IL-6) on the macrophage line RAW 264.7 which do not express OATPs 1a1, 1a5 and 1b2: the uptake occurred via the Toll-like receptors (TLRs)] (Adamovsky et al. 2015).

The cell uptake is therefore a complex mechanism, and therefore, the equilibrium between uptake, metabolism and extrusion is likely to be the relevant metrics, as demonstrated by a comparison between the MC-LR-induced effects on HepG2 cells and primary human hepatocytes. At similar nominal exposure concentrations, MC-LR did not show any morphological change or cytotoxicity in the cell line at variance with the hepatocytes. This was attributed to a lower intracellular toxin (Ikehara et al. 2015), despite HepG2 are characterized by a similar transporter expression. This evidenced that, due to kinetic features, not all cell lines are appropriate to evaluate MC-induced toxicity.

The uptake of radiolabeled dihydro-MC-LR has been demonstrated to be rapid in fresh hepatocytes in suspension and in perfused liver at 37 °C: hepatocyte necrosis was observed 45 min after initiation of perfusion (Hooser et al. 1991). About 65–77% of the radiolabel was in the cytosolic fraction (of which 60–80% was bound to protein); 13–18% was in the plasma membrane (Hooser et al. 1991). When isolated rat livers were perfused with 0.3 and 0.5 μg/mL of tritiated MC-LR for 60 min distribution of radiolabeled was: 1.7% to bile, 79% to perfusate (the great majority of which was associated with the parent toxin) and 16% to the liver, associated with the cytosolic fraction and corresponding to parent toxin (15%) and a more polar component (85%, likely the conjugation metabolites). The elimination half-life from perfusate was 130 ± 10 min (at 0.5 μg/mL) (Pace et al. 1991).

Organ distribution studies after oral and i.p. administration of the same external dose in mice, indicate a 80-fold difference in hepatic content of radiolabeled 3H-dihydro-MC-LR (Nishiwaki et al. 1994). *In vivo*, after i.p. injection of 70 μg/kg to mice, from 3 to 30 min, the plasma levels correspond to 5.5–8% of the administered dose with an elimination half-life of 29 min (Robinson et al. 1989). From 3 to 90 min the radiolabel was detected in the kidney (0.92%), small intestine (6.4%) and large intestine (2.1%); a linear accumulation was observed in the liver for 30 min (1.6%/min) with a corresponding decrease in the carcass. A similar distribution was described in another study 1 h after treatment, with approximately 72% of the administered radiolabel MC-LR dose detectable in the liver, 1.4% in the small intestine, 0.5% in the kidney, 0.4% in the lungs and 0.3% in the stomach (Nishiwaki et al. 1994). Similar results (67% of the dose of tritiated MC-LR in the liver) were obtained after i.v. injection of 35 μg MC-LR/kg bw, with a biphasic plasma clearance ($t1/2 = 0.8$ and 6.9 min, respectively) (Robinson et al. 1991).

These data suggest that the route of administration (parenteral vs oral) highly influences MC kinetics and therefore the internal dose in the target organ. Systemic distribution of MC in the organs depends on types and expression level of OATP/Oatp carriers, besides the degree of blood perfusion.

Glutathione conjugation, occurring either spontaneously or catalyzed by GST, is the accepted main step in the detoxification of MC: indeed, conjugates retain only a minimal residual inhibitory activity with respect to the parent compound (Botha et al. 2004; Dittmann and Wiegand 2006). The reaction, involving the methyl group of N-methyldihydroalanine (opposed to Adda), has been reported to occur in vivo and/or in vitro in plants, aquatic organisms, fish, mammals (Kondo et al. 1996; Takenaka 2001; Pflugmacher et al. 2005; Huang et al. 2008; Šetlíková and Wiegand 2009; Wu et al. 2010; He et al. 2012a, b), and recently, it has been characterized using recombinant human GST and human hepatic cytosol with MC-LR and MC-RR (Buratti et al. 2011, 2013, Buratti and Testai 2015). Due to the optimal temperature for fish cells physiology, the spontaneous reaction between MC-LR and GSH is limited, whereas in mammalian cells at physiological conditions it occurs at similar levels or predominates on the enzymatic one (depending on the variant) (Buratti et al. 2011, 2013, Buratti and Testai 2015). When GSH levels are depleted or at low MC-LR concentrations (≤10 μM), representative of repeated oral exposure at GSH concentration around 1 mM, the enzymatic reaction became predominant (Buratti and Testai 2015). The role of GSH and its concentration are crucial factors for MC detoxification; in fact besides the GSH depletion following MC-LR-dosing (Dittmann and Wiegand 2006), when SD (Sprague–Dawley)

rats were treated i.p. with MC-LR and buthionine-(S,R)-sulfoximine, an inhibitor of GSH synthesis, the hepatic tissue showed MC-LR accumulation and higher vulnerability to its toxicity (Li et al. 2015a).

In mice, approximately 24% of the i.p. MC-LR-administered dose was eliminated in the urine (9%) and feces (15%) over 6 days: parent compound accounts for 60% of the excreted MC-LR (Robinson et al. 1991), although this percentage is expected to be different depending on the lipophilicity of the variants. MC-GS conjugate can be further degraded to the cysteine conjugate (enzymatically and sequentially by γ-glutamyl-transpeptidase and cysteinylglycine-dipeptidase), mainly in the kidney, and excreted at least partially in this form: in rats i.p. treated with MC-LR, MC-LR-Cys was detected at higher-level respect to MC-LR-GSH being likely more stable (Li et al. 2015a, b, c). Furthermore, in vivo in bighead carp tissues besides the conjugates, the presence of free MC (MC-RR) has been also reported, suggesting that MC conjugation with the thiol of GSH/Cys can likely be a reversible process. On the other hand, the presence of free MC in the urine of mammals (Robinson et al. 1991) may derive also from the breakdown of conjugates due to the hydrolytic enzymes present in the kidney. Consequently, also the kidney can be a potential target for MC-LR toxicity, as evidenced from several studies (Li et al. 2013).

Toxicokinetics seems to be the base for the described congener-dependent toxicity, since the inhibitory capacity of single MC congeners on PP1 (protein phosphatase 1) and PP2A (protein phosphatase 2A) in vitro is comparable, with IC_{50} (inhibitory concentration 50%) values in the nanomolar range (Hoeger et al. 2007; Monks et al. 2007; Fischer et al. 2010; Vesterkvist et al. 2012). In addition a similar modulations of gene transcription levels with induction of overlapping transcriptomic profiles (suggesting a common mechanism of toxicity) were reported in Caco-2 cells treated with 10–100 μM MC-RR or MC-LR (Zeller et al. 2012).

In fact the uptake, tissue distribution and excretion can result in threefold to fourfold differences among some congeners (Meriluoto et al. 1990); MC-LR and MC-RR showed different affinities for OATP1B1 and 1B3 transporters responsible for the uptake into primary human hepatocytes and organic anion transporter-transfected HEK293 cells (Fischer et al. 2010). Also MC detoxification efficiency seems to be variant dependent: for example MC-RR is less toxic and more efficiently conjugated than MC-LR, especially at low concentrations (Buratti and Testai 2015). Differences in the metabolism of the two MC variants were more pronounced in rodent cytosol than in human samples (Buratti and Testai 2015).

Considering the differences between two variants comparatively hydrophilic in the range of physiological pH (Liang et al. 2011), extrapolation of toxicological information from MC-LR, the most studied congener, to the whole MC group family might be difficult: at the moment very few information is available on other variants, especially the more lipophilic ones for which kinetic parameters can be significantly different. Caution should be used also when extrapolating from animal data to human: indeed rodent cytosols showed higher catalytic efficiency than human samples for both MC-LR and MC-RR (Buratti and Testai 2015), supporting the default consideration that during the risk assessment process humans are considered more susceptible. For the two studied variants the differences between animal and the 'average' humans cytosol are limited to a factor of three and, therefore, are covered by the default factor of 10 used for interspecies differences, which should account also for other processes, such as active transport. The involvement of polymorphic enzymes in human only, in those conditions in which the enzymatic reaction is predominant in MC detoxification, may be of importance due to the high human interindividual variability of GSTs content.

The only data on the kinetics of MC in humans derive from the follow-up of dialyzed patients i.v. (intravenously) exposed to MC in two separate outbreaks in Brazil (one in Caruaru, one in Rio de Janeiro), when MC were still present in patients' serum more than 50 days after the exposure (Soares et al. 2006; Hilborn et al. 2007). Levels of 0.2–0.96 μg/L (0.2–1 nM) were found in asymptomatic individuals after the Caruaru episode; up to 28.8 μg/L (29 nM) was detected in serum from intoxicated but surviving patients: however, not knowing the initial exposure no further kinetic information can be derived.

Acute systemic toxicity

Acute toxicity in rodents of MC-LR is highly dependent on the route of exposure as expected due to the different kinetics as described above: after i.v., i.p. and oral administration to mice LD_{50}s value are, respectively: 28 μg/kg bw (Kondo et al. 1992), 30–150 μg/kg bw (Table 6) and ≥5000 μg/kg bw (similar values obtained in rats). An LD_{50} (lethal dose 50%) = 250 μg/kg bw was reported after intratracheal application of MC-LR (poorly representative of human exposure via inhalation) with necrosis starting in the high airways and progressing to alveoli and liver damage as well (Fitzgeorge et al. 1994).

Acute toxicity is highly variable among MC variants, likely dependent on the presence of different substituents; the range of i.p. LD_{50} values in rodents being from 50 to >1200 μg/kg bw in rodent (Funari and Testai 2008). The change in a single amino acid is enough to determine a tenfold difference in acute i.p. toxicity (e.g., MC-LR vs MC-RR).

Table 6 MC acute toxicity in mammals expressed as LD_{50} values

Variant	LD_{50}	Value	Unit	Species	References
MC-LR	LD_{50} oral (gavage)	>5	mg/kg bw	Rat	Fawell et al. (1999a)
	LD_{50} oral (gavage)	5–10.9 Overall range	mg/kg bw	Mouse	Fawell et al. (1999a), Yoshida et al. (1997), Rao et al. (2005)
	LD_{50} i.p.	72–122 Overall range	µg/kg bw	Rat	(Moreno et al. 2005; Miura et al. 1991; Li et al. 2015a)
	LD_{50} i.p.	32.5–158 Overall range	µg/kg bw	Mouse	Lovell et al. (1989); Stotts et al. (1993); Stoner et al. (1989); Robinson et al. (1989); Gupta et al. (2003); Fawell et al. (1999a); Chen et al. (2006); Yoshida et al. (1997); Rao et al. (2005); Wang et al. (2012); Chernoff et al. (2002)
	LD_{50} i.v.	80	µg MC-LR_{equiv}/kg bw	Rat	Wang et al. (2008)
	LD_{50} i.v.	28	µg/kg bw	Mouse	Kondo et al. (1992)
MC-RR	LD_{50} i.p.	111–650 Overall range	µg/kg bw	Mouse	Stotts et al. (1993), Stoner et al. (1989), Gupta et al. (2003), Chen et al. (2006)
	LD_{50} i.v.	80	µg MC-LR_{equiv}/kg bw	Rat	Wang et al. (2008)
MC-WR	LD_{50} i.p.	140, 171	µg/kg bw	Mouse	Chen et al. (2006), Stotts et al. (1993)
MC-FR	LD_{50} i.p.	100, 249	µg/kg bw	Mouse	Chen et al. (2006), Stotts et al. (1993)
MC-AR	LD_{50} i.p.	≈249	µg/kg bw	Mouse	Stotts et al. (1993)
MC-LA	LD_{50} i.p.	=39	µg/kg bw	Mouse	Stoner et al. (1989)
MC-LY	LD_{50} i.p.	=91	µg/kg bw	Mouse	Stoner et al. (1989)
MC-YR	LD_{50} i.v.	91	µg/kg bw	Mouse	Kondo et al. (1992)
[O-demethyl-ADDA^5]MC-LR	LD_{50} i.p.	≈ 97	µg/kg bw	Mouse	Stotts et al. (1993)
[desmethyl^7]MC-RR ([Dha^7]MC-RR)	LD_{50} i.p.	180, 420	µg/kg bw	Mouse	Kiviranta et al. (1992), Chen et al. (2006)
[desmethyl^3]MC-RR ([D-Asp^3]MC-RR	LD_{50} i.p.	250, 350	µg/kg bw	Mouse	Meriluoto et al. (1989), Chen et al. (2006)
MC-[methionine-S-oxide]R	LD_{50} i.p.	≈750	µg/kg bw	Mouse	Stotts et al. (1993)
[desmethyl^3]MC-FR ([D-Asp^3]MC-FR)	LD_{50} i.p.	=90	µg/kg bw	Mouse	Chen et al. (2006)
[desmethyl^3]MC-WR ([D-Asp^3]MC-WR)	LD_{50} i.p.	=95	µg/kg bw	Mouse	Chen et al. (2006)
[3H]dihydroMC-LR (epimer 1)	LD_{50} i.p.	=120	µg/kg bw	Mouse	Meriluoto et al. (1990)
[3H]dihydroMC-LR (epimer 2)	LD_{50} i.p.	=135	µg/kg bw	Mouse	Meriluoto et al. (1990)
GSH-MC-LR (conjugate)	LD_{50} i.v.	630	µg/kg bw	Mouse	Kondo et al. (1992)
Cys-MC-LR (conjugate)	LD_{50} i.v.	267	µg/kg bw	Mouse	Kondo et al. (1992)
GSH-MC-YR (conjugate)	LD_{50} i.v.	304	µg/kg bw	Mouse	Kondo et al. (1992)
Cys-MC-YR (conjugate)	LD_{50} i.v.	217	µg/kg bw	Mouse	Kondo et al. (1992)

i.p. intraperitoneal, *i.v.* intravenous

Acute studies are available, allowing the identification of a range of values as the acute threshold of toxicity after i.p. injection: 25–50 µg/kg bw produced no effects in the mouse liver (Fromme et al. 2000). Since the ratio between the no-effect concentration and the one inducing severe adverse effects, up to mortality, is very small (<2), the dose–response curves for MC-LR seem to be very steep. This is confirmed by the indication of MC-LR-induced inhibition of PP1 and PP2A at levels of i.p. dosage >32 µg/kg bw in vivo (Gehringer 2004), which is in the same range of no effects seen in various studies. After oral administration signs of hepatic toxicity were present also at the lowest dose tested (LOAEL, lowest observed adverse effect level = 500 µg/kg bw) (Funari and Testai 2008).

The main target of acute toxicity is the liver, but neurotoxic effects have been reported for some specific MC, such as MC-LF, MC-LW and MC-LR (Feurstein et al. 2011; Li et al. 2012a, b), and some neurological disorders were observed in humans during a fatal outbreak in Brazil (Azevedo et al. 2002). More recently it has been reported

that also other organs (thyroid, lung, etc.) seem to be affected.

Mechanism and mode of action

The inhibition of the soluble and highly conserved protein serine/threonine phosphatases (PP1 and to a larger extent PP2A) is the generally accepted cellular/molecular processes or mechanism of action (MeA) of MC toxicity at the base of the functional or physiological changes or mode of action (MoA) caused by the toxin in mammals and fish, as reported by studies using the omic technologies. PP2A accounts for approximately 1% of the total cellular proteins and 80% of total serine/threonine phosphatases in mammalian cells.

The inhibition occurs because of a covalent binding with a protein cysteine (Cys269, within the PP2A/C catalytic subunit) (Harada et al. 1990), blocking the access of any substrate in the catalytic site.

The interaction of MC-LR with PP1 and PP2A was described as a two-step mechanism, where the toxin first binds to the enzyme and subsequently forms covalent adducts (MacKintosh et al. 1990; Craig et al. 1996; Campos and Vasconcelos 2010). Although Adda is necessary for MC to inhibit PP1 and PP2A, it is not toxic when i.p. injected in mice even at very high doses (10 mg/kg bw) (Harada et al. 2004) suggesting the need for a steric hindrance, provided by the toxin molecule, to cause an efficient inhibition. And indeed it has been shown that the binding with Cys 269 occurs with terminal carbon atom of the Mdha side chain; on one end of the binding pocket, four amino acids in the catalytic subunit of PP2A form a hydrophobic cage, which accommodates the long hydrophobic Adda side chain of MC-LR (Xing et al. 2006).

Inhibition of PP2A, leading to downstream proteins hyperphosphorylation, reversing the action of protein kinases and regulating a diverse set of cellular process (e.g., apoptosis, metabolism, proliferation, DNA repair) is the key primary event of the adverse outcome pathway (AOP) of MC-induced toxicity. The role of PP2A-associated proteins and related signal pathways in MC-LR toxicity has been recently reviewed (Liu and Sun 2015): it induces abnormal signaling in multiple pathways mediated by PP2A, resulting in increased protein phosphorylation that triggers a cascade of events leading to series of cellular responses such as:

- Modification of cytoskeleton and disruption of actin filaments. The alteration of microtubule seems to be the early event associated with MC-LR-induced PP2A inhibition also in cells of human origin, with PP2A activity decreased starting from 1 μM MC-LR, with a concentration-dependent decline (Sun et al. 2014). Besides the alteration of protein phosphorylation related to PP2A inhibition, MC-LR also acts via protein kinase (MAPK) pathway activation, causing hyperphosphorylation of different types of microfilament-associated proteins, such as Tau, vasodilator-stimulated phosphoprotein (VASP) and HSP27 (Zeng et al. 2015; Sun et al. 2015). The phosphorylated status of these protein influences their ability to bind and stabilize cytoskeleton in all type of cells. MC-LR not only induced the phosphorylated expression of HSP27, VASP and Tau but also influenced their distribution and binding ability to the cytoskeleton (Sun et al. 2015).

The alpha4 protein is a regulator of PP2A through binding with catalytic subunit C, forming a complex with low phosphatase catalytic activity, which serves as a reserve of catalytic subunits that promote survival in times of stress. In normal cells MC-LR triggers alpha4/PP2A/C subunit dissociation, which can compensate for reduced PP2A activity mediated by the toxin (Liu and Sun 2015): it is therefore plausible that PP2A inhibition occurs when the reserve is overwhelmed. And indeed, HEK293 cells overexpressing alpha4 protein are less prone to cytoskeletal alterations mediated bt PP2A inhibition after MC-LR exposure (Huang et al. 2016). In addition alpha4 protein binding with PP2A is crucial for the phosphorylation of the cytoskeleton-related proteins such as HSP27 and VASP and then for the alteration of the assembly of actin filament and microtubules.

Dysfunction of cytoskeleton, crucial in maintaining cellular architecture, is strictly related also to alteration in cell division, migration and signal transduction. A recent review on the MC-LR-induced cytoskeletal disruption in animal cells is available (Zhou et al. 2015).

- Oxidative stress. The increased formation of reactive oxygen species (ROS) and/or GSH depletion, leading to oxidative stress, drives cells toward cell apoptotic death, rather than to necrosis due to release of apoptotic factors (see Campos and Vasconcelos 2010). In vivo changes in the mRNA and in the protein expression of ER (endoplasmic reticulum) stress signaling molecules were evidenced after seven-days repeated i.p. treatment (Qin et al. 2015). In primary hepatocytes ROS induction was observed in the nanomolar range (as for the PP2A inhibition) (Žegura et al. 2004). Although the mechanism underlying MC-mediated ROS production is not fully characterized, the involvement of mitochondrial dysfunction (loss of membrane potential and permeability transition) and activation of calpain and Ca^{2+}/calmodulin-dependent protein kinase II (Ding and Nam Ong 2003) have been suggested. Meng et al. (2015) evidenced in vitro that MC-LR had time-

and concentration-dependent effects on ROS generation, p38-MAPK activation and TAU phosphorylation. In vivo i.p injection of 0.25 and 0.5 μg MC-LR/kg bw produced time-dependent alterations of GSH levels in rat liver, significant changes of antioxidant enzymes, including GSH peroxidase (GPX) and GSH reductase (GR), activation of NF-kB and the expression of p53, Bax and Bcl-2 (Chen et al. 2016a, b). To support the role of oxidative stress in MC-induced toxicity, pretreatment with the antioxidants, N-acetylcysteine and vitamin C significantly decreased the MC-LR-induced apoptosis improving mitochondrial function and suppressing oxidative stress (Meng et al. 2015; Xue et al. 2015).

- Induction of apoptosis. It can be also due to the phosphorylation of some proteins such as p53, Bcl-2 and Bad, cytochrome c and caspase (Liu et al. 2016; Wang et al. 2013). In addition, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a member of the family of serine/threonine protein kinases, is also necessary for MC-LR-induced apoptosis: the use of CaMKII inhibitors limits the phosphorylation of apoptotic-related proteins (Liu and Sun 2015). Increase in p53 protein expression has been reported in vitro in apoptotic HepG2 cells, cultured hepatocytes and rat liver tissues after MC exposure (Campos and Vasconcelos 2010). Cellular proapoptotic Bax and Bid proteins increased, while antiapoptotic Bcl-2 decreased (Fu et al. 2005; Weng et al. 2007; Xing et al. 2008; Takumi et al. 2010; Huang et al. 2011; Li et al. 2011a).
- Reduced DNA repair. Loss of activity of DNA-dependent protein kinase (DNA-PK, regulated by phosphorylation mechanisms) induced by MC could potentially reduce capacity of cell to repair DNA double-strand break, related to oxidative DNA damage (Douglas et al. 2001; Kleppe et al. 2015).
- Cell proliferation leading to tumor promotion. The tumor suppressor role of PP2A is known, since it is active in regulating mitogenic signaling pathways in cancer pathogenesis in mammalian cells. The alteration of cell proliferation, division, signal transduction and gene expression with activation of Akt/S6K1 pathways, due to the hyperphosphorylation of transcription factors c-Myc and c-Jun, has been also reported (Liu et al. 2016). The in vitro activation of nuclear factor kappaB (NF-kB), the phosphatidylinositol 3-kinase (PI3-K)/AKT-mediated MMP-2/9 hyperexpression observed in vivo (Xu et al. 2013a, b) and the modulation of tumor necrosis factor alpha (TNF-α), support the tumor-promoting activity for MC, through PP2A inhibition (Zhang et al. 2013). Using transcriptomics and proteomics techniques, Zhao et al. (2012) evidenced the alteration of the expression of some 37 miRNAs and 42 proteins related to cancer or involved in the tumorigenesis pathways such as glutathione metabolism and MAPK signaling pathway (as already evidenced in targeted studies). Also the overexpression of the proto-oncogenes *c-myc, c-jun and c-fos* induced by MC has been reported (Wang et al. 2013; Li et al. 2009). The activation of endoplasmatic reticulum (ER) stress pathways, unfolded protein response (UPR) and endoplasmic reticulum-associated degradation seem also to be involved (Christen et al. 2013).

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

Overall, it can be concluded that MC toxicity is a multipathway process (Vichi et al. 2016; Fig. 4), and it has been proposed that it is the result of 'cross-talking' and cooperative effects between different pathways, responsible for cytoskeleton alterations, lipid peroxidation, oxidative stress and apoptosis, leading to centrilobular toxicity with intrahepatic hemorrhagic areas due to damage of sinusoidal capillaries as the results of acute relatively high doses; at low doses (below 20 μg/kg bw; Gehringer 2004) typical of repeated long-term exposure, phosphatase inhibition induces cellular proliferation and hepatic hypertrophy and tumor promotion activity. Since up to know PP2A inhibition has been shown in vitro in all organotypic cells and by all the studied congeners. It is plausible that the toxicity induced in organs other than the liver, including effects in neuronal cells shown by some variants, is associated with the same general AOP(s).

Local toxicity

Potential local toxic effects at the site of contact have been tested with freeze-dried algal aqueous suspensions from both *Microcystis* and *Anabaena* blooms: very low potential for skin irritation and contrasting results for eye irritation were obtained (Funari and Testai 2008). The possible toxic effects of MC-LR on the skin were investigated in vitro with human keratinocytes: short-term exposure to MC-LR does not affect cell proliferation, but after longer incubation times a dose-dependent disruption of the actin cytoskeleton and inhibition of keratinocyte migration were evident (Kozdeba et al. 2014).

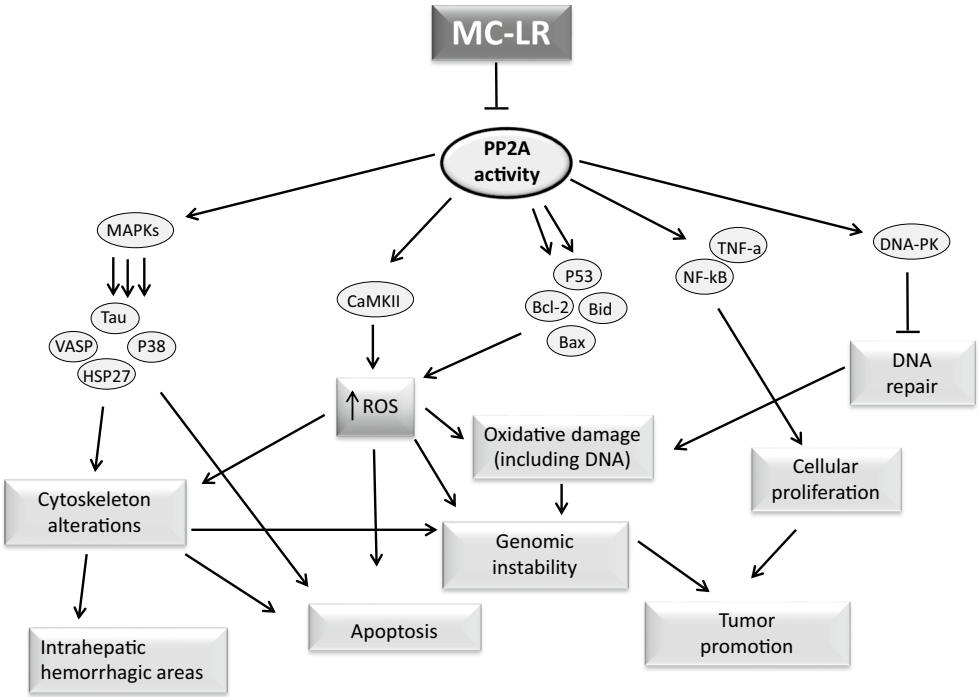


Fig. 4 Pathways of MC-LR mechanism of action

Although positive results were obtained in the skin sensitization test, components other than MC present in the algal extract are likely endowed with irritation and sensitizing potential. Indeed, no correlation was found in animal testing between the MC content in the extract and the allergic reaction, and pure MC-LR showed only slight skin sensitizing potential. This was confirmed in a human study with 259 chronic rhinitis patients tested with nontoxic CB extract, showing that cyanobacterial allergenicity (IgE response in a skin-prick test) resides in nontoxin-containing components of this organism (Bernstein et al. 2011). Further testing, with sera from patients and CB extracts from toxic and nontoxic strains, identified phycobiliprotein complexes in *M. aeruginosa* as the relevant sensitizing agent (Geh et al. 2015), whereas MC was shown to inhibit in a dose-dependent manner the sensitization of the extracts, although the interplay between immunogenicity and toxicity of CB in different conditions has to be further studied (Geh et al. 2015).

Repeated-dose systemic toxicity

Several repeated toxicity studies are available, but only a few are useful to derive a relevant reference values (such as NOAEL, no observed adverse effect level, or LOAEL) (Table 7). Indeed many of them have been carried out by using the i.p. route (Solter et al. 1998; Guzman and Solter 1999; Dong et al. 2008; Li et al. 2012c; Huang et al. 2013; Zhou et al. 2013; Zhao et al. 2015a, b), which has a limited relevance for the more frequent human exposure route for kinetic reason (Funari and Testai 2008). In addition some other studies used poorly characterized extracts (likely containing other toxic components) as the test item instead of pure toxins, so that the exposure could not be clearly established. Finally some others administered a single dose (e.g., (Ueno et al. 1999) or were focused on single endpoints ignoring any other effect (Table 7).

The short-term study by Heinze (1999) is the study chosen as the critical one by Health Canada (2016) and US-EPA (2015a, b) for determining the 10-day Health Advisory value for drinking water. MC-LR (0, 50 or 150 μg/kg bw) was administered via drinking water to 11-week-old male rats (n = 10/group) for 28 days. The LOAEL was determined to be 50 μg/kg/day: indeed, at that dose (the lowest tested) the relative liver weight was increased (17%); slight to moderate liver lesions with or without hemorrhage were observed in relation to dose. However, other histological effects, including Kupffer cell activation and PAS staining, showed no dose–response since all ten animals at the low and high doses displayed similar degree of damage. In addition, it is quite surprising that no changes were measured in the mean levels of AST (aspartate aminotransferase) and ALT (alanine aminotransferase) as early markers for hepatotoxicity, whereas other serum parameters (i.e., ALP and LDH) were altered.

Table 7 MC-LR subacute/subchronic toxicity, with identification of a reference value

Duration	Reference value	Value	Unit	Species/limitations/note	References
Oral					
10 days (gavage)	NOAEL	=600	µg/kg bw/day	**Mouse**. Three doses (200, 600, 2000 µg/kg bw/day). Reproductive toxicity study. No evidences of maternal toxicity (although the number of parameters checked for general toxicity is rather limited in this kind of studies)	Fawell et al. (1999a)
1 month (gavage)	LOAEL	=50	µg/kg bw/day	**Mouse.** Two doses (50, 100 µg/kg bw/day). Treatment every two days: alterations of SOD activity and glutathione content, slight hepatic steatosis. No alterations of hepatic toxicity biomarker (ALT, AST and ALP).	Sedan et al. (2015)
28 days (drinking water)	LOAEL	=50	µg/kg bw/day	**Rat.** Two doses (50, 150 µg/kg bw/day). Although histological hepatic damage indicated in the text (no picture nor detailed description) no alteration of enzymatic markers (ALT and AST) for hepatic damage observed	Heinze (1999)
13 weeks (gavage)	NOAEL	=40	µg/kg bw/day	**Mouse.** Three different doses tested (40, 200, 1000 µg/kg bw/day). Slight hepatic damages were observed at the lowest observed effect level (LOAEL) of 200 µg/kg bw/day in a limited number of treated animals, whereas at the highest dose tested (1 mg/kg bw/day) all the animals show hepatic lesions, consistent with the known action of MC-LR. Key Study	Fawell et al. (1999a)
6 months (drinking water)	NOAEL	=1	µg/L	**Mouse.** Three doses (1, 10, 40 µg/L), water consumption not reported. Hepatic effects not checked. Alveolar collapse, lung cell apoptosis and destruction of cell junction integrity were evidenced especially at the highest concentration (40 µg/L). Some tissue alterations at middle dose of 10 µg/L	Wang et al. (2016)
12 months (drinking water)	NOAEL	=1	µg/L	**Mouse.** Five doses (1, 5, 10, 20, 40 µg/L), water consumption not reported. Hepatic effects not checked. No pathological change at 1 µg/L. Thickening of the lung alveolar septa and an increase in collapsed areas started from 5 µg/L. SOD, MDA and stability of mitochondrial DNA unchanged or with spotted alterations (and high variability). Changes in the expression of mitochondrial genes in the lung cells starting from 1 µg/L, but the meaning of the alteration not clear with respect to adversity	Li et al. (2016b)
18 months (drinking water)	NOAEL	≥ 20	µg/L	**Mouse.** A single dose was tested, no effects* seen at 20 µg/L MC-LR in drinking water. Water consumption was monitored during the treatment. Histopathology of main organs and serum biochemistry was reported. Unbound NOAEL (*) sporadic alterations not associated with other treatment-related changes	Ueno et al. (1999)

Table 7 continued

Duration	Reference value	Value	Unit	Species/limitations/note	References
Intraperitoneal					
7 days	NOAEL	=3.125	µg/kg bw/day	**Mouse**. Four doses (3.125, 6.25, 12.5, 25 µg/kg bw/day). The paper measured few blood parameters, no histopathology was performed. The NOAEL is derived based on the alterations of ALP and LDH at the immediately higher dose (6.250 µ/kg) which on the other hand were the only alterations observed.	Huang et al. (2013)
7 days	NOAEL	=3	µg/kg bw/day	**Mouse.** Three doses (3, 6, 12 µg/kg bw/day). Limitation: no positive control. The NOAEL is derived considering DNA–protein cross-links and micronucleus test in mice testicle cells, the only checked parameter (uncertain reliability as a marker for adversity)	Dong et al. (2008)
20 days	LOAEL	=5	µg/kg bw/day	**Mouse.** Four doses (5, 10, 15 and 20 µg/kg bw/day). Treatment of 10 injections every other day. Hepatic necrosis and inflammation in all treated groups; at the same doses impairment of respiratory mechanics with increased central airways resistance and viscoelasticity/heterogeneities of peripheral lung was evidenced in all tested doses	Carvalho et al. (2016)
28 days	NOAEL	=16	µg/kg bw/day	**Rat.** Three doses (26, 32, 48 µg/kg bw/day). Dose-dependent hepatic alterations were reported. Limitation: low number of animals; use of an i.p. osmotic pump	Guzman and Solter (1999)
28 days	NOAEL	=3	µg/kg bw/day	**Rat.** Three doses (3, 6, 9 µg/kg bw/day) Limitations: focus only on some hepatic parameters, use of an i.p. osmotic pump, use of only 5 animal per group, use of ELISA to detect MC in the tissue (possible matrix effect not considered at all)	Solter et al. (1998)
30 days	LOAEL	=0.5	µg/kg bw/day	**Mouse.** Three doses (0.5, 2, 8 µg/kg bw/day). The paper only measured parameters related to normocytic anemia after i.p. injection (altered only at high doses): there are even no data on mice weight. The LOAEL is derived on the basis of altered MN frequency at the lowest dose tested. However, there is no positive control, high apoptosis level possibly affecting results of MN induction	Zhou et al. (2013)
50 days	LOAEL	=1	µg/kg bw/day	**Rat.** Two doses (1, 10 µg/kg bw/day). The paper focuses on the spleen impairment after i.p. injection; therefore the LOAEL only refers to limited spleen-related parameters. Spleen effects at the LOAEL very slight, although increased at the higher dose	Li et al. (2012c)
4 weeks	LOAEL	=5	µg/kg bw/day	**Mouse.** Two doses (5, 20 µg/kg bw/day). The paper focuses on thyroid dysfunction after i.p. injection; therefore the LOAEL only refers to limited thyroid-related parameters. More general alterations, linked to metabolic disorders seen only at 20 µg/kg. Some mechanistic data available (protein and mRNA expression related to thyroid hormone receptors also available)	Zhao et al. (2015a, b)

Table 7 continued

Duration	Reference value	Value	Unit	Species/limitations/note	References
8 months	LOAEL	= 10	µg/kg bw/day	**Rat.** A single dose was tested, treatment every second day with MC-LR or MC-YR. The paper focuses only on kidney damage. Collapsed glomeruli with thickened basement membranes were revealed. In the epithelial tubular cells cytoplasmic aggregation and accumulation of fibrillar actin filaments were evidenced. The changes due to MC-LR exposure appeared more severe than those induced by MC-RY	Milutinović et al. (2003)
Inhalation					
7 days (aerosol)	NOAEL	=3	µg/kg bw/day	**Mouse.** Three doses (3, 6, 12.5 µg/kg bw/day) No clinical signs of toxicity (hepatotoxicity, renal toxicity). NOAEL derived on the basis of nasal/respiratory tract lesions	Benson et al. (2005)
30 days (intranasal)	LOAEL	=6.7	ng/kg bw/day	**Mouse**. A single daily dose was tested. The paper focuses on pulmonary dysfunction; therefore, the LOAEL only refers to limited lung-related parameters such as increased amount of PMN cells and higher l static elastance. No changes in the nasal epithelium	Oliveira et al. (2015a, b)

The study of Fawell et al. (1999a), where mice were treated by gavage for 13 weeks with MC-LR (0, 40, 200, 1000 µg/kg bw), is yet to be considered the key subchronic study from which a NOAEL of 40 µg/kg bw has been derived (Fawell et al. 1999a), taken as the point of departure (PoD) also by WHO for the derivation of drinking water guideline value. Only slight hepatic damages were observed at the lowest observed effect level (LOAEL) of 200 µg/kg bw/day in a limited number of treated animals, whereas at the highest dose tested (1 mg/kg bw/day) all the animals showed hepatic lesions, consistent with the known action of MC-LR. When mice were subchronically administered with MC-LR-containing extracts through the diet, a regimen more similar to human exposure although possible interference could be present due to other cyanobacterial components, the NOAEL value was higher (333 µg/kg bw/day) (Schaeffer et al. 1999), due to toxicokinetic differences as discussed in Funari and Testai (2008). In rats treated orally by gavage for seven days at 40 µg MC-LR/kg bw alterations of hepatic metabolism, using omics tools, were evident also in the absence of other marker of toxicity or histological changes (He et al. 2012a, b), although the meaning of the findings with respect to adverse effect induction has to be further elucidated.

Recently Sedan et al. (2015) claimed that MC-LR-induced toxic effects are observed at concentration lower than 40 µg/kg bw: they administered by gavage 50 µg/kg bw every other day for a month and extrapolated the daily dose at 25 µg/kg bw/day. This extrapolation is not correct based on the available information on MC-LR kinetics. In addition the described effects such as alterations of SOD activity and glutathione content, slight hepatic steatosis with no necrosis and a decrease in intraepithelial lymphocytes, without any alterations of hepatic toxicity biomarker (ALT, AST, and ALP) (Sedan et al. 2015) were compatible with the already identified LOAEL in the Heinze study.

Only 1 study is available on inhalation (nose-only): up to 265 µg MC/m^3 MC for 7 days produced slight to moderate multifocal degeneration and necrosis in the respiratory epithelium and atrophy of the olfactory epithelium in male BALB/c mice; the authors identified a NOAEL for nasal lesions after inhalation corresponding to 3 µg/kg bw or 20 ng/cm^2 (Benson et al. 2005).

Other specific effects in the liver include: hepatic lipid metabolism abnormality, which caused steatosis, in mice treated i.p. 5–20 µg/kg bw/day for 14 days (Qin et al. 2015); inhibition of CYP1A1 and CYP3A11 activities in mice receiving a daily i.p. injection of 2, 4 and 8 µg MC-LR/kg bw for 7 days, paralleled by CYP2E1 induction, possibly contributing to enhance ROS generation (Zhang et al. 2015). Although these studies could be hardly compared to the oral ones, in terms of internal dose of exposure due to MC-LR kinetics, they can provide information on the hazard concerning other possible targets. And indeed, recently some studies have been focused also on effects on organs other than the liver.

The kidney is an additional target for MC toxicity, as shown in rats repeatedly treated (by i.p. injections) with

a single dose (10 µg/kg bw) of MC-LR and MC-YR; the mechanism of renal damages was described as similar to the one for hepatotoxicity (Milutinović et al. 2003). MC-LR (1–200 µM) induced cytotoxicity and apoptosis in human embryonic kidney and human kidney adenocarcinoma cell lines (Piyathilaka et al. 2015).

Effects on thyroid are reported in vivo in mice i.p. injected with doses 5 and 20 µg/kg bw MC-LR for 4 weeks. At the higher dose MC-LR altered glucose, triglyceride and cholesterol metabolism with sign of hyperphagia, polydipsia and weight loss due to the increased expression of TH receptor (Tra) and mTOR expression in the brain induced by the higher free triiodothyronine (FT3) level (Zhao et al. 2015a, b). The i.p. treatment, however, limits the relevance of these findings.

The lung as a possible target for MC-LR toxicity has been investigated in acute (Soares et al. 2007) and repeated studies using i.p. (10 injections every other day of MC-LR in the range 5–20 µg/kg bw/day; Carvalho et al. 2016) and intranasal administrations (6.7 ng MC-LR/kg bw for 30 consecutive days; Oliveira et al. 2015a, b), showing pulmonary mechanical impairment, alveolar collapse and recruitment of inflammatory cells. Besides i.p. studies, highlighting the potential hazard, alveolar collapse and lung cell apoptosis were observed also after treating mice with 0.1, 1, 4 µg MC-LR/kg bw daily for 6 months in drinking water, together with the breach of cell junction integrity (Wang et al. 2016). Analogously, when mice were treated orally with 1, 5, 10, 20 and 40 µg MC-LR/L for 12 months, in the lung cells mtDNA stability was influenced and the expression of mitochondrial genes was changed (Li et al. 2016b). In both studies, the authors did not report the water consumption and therefore the dose expressed as µg/kw bw and the actual meaning of these changes in inducing adverse effects has to be elucidated, before considering them as PoD for the risk assessment. In contrast to their findings no lung effects were described in the Ueno study (Ueno et al. 1999) in which 20 µg/L of MC-LR in drinking water was administered for 18 months to mice with no alterations in the major organs. For this reason this aspect deserves further investigation.

Some data show that chronic exposure to MC-LR has also the potential to impair immune responses, possibly leading to increased risk of various diseases; however, further investigation is definitely needed to elucidate the possible MC-LR-related immunotoxicity, useful to understand the relationship between development of (any) disease and MC-LR exposure (Lone et al. 2016).

Genotoxicity

In vitro MC-LR is not genotoxic in the Ames test (Ding et al. 1999; Sieroslawska 2013); as already discussed some positive results (Rao et al. 1998; Mankiewicz et al. 2002; Zhan et al. 2004; Žegura et al. 2008; Sieroslawska and Rymuszka 2010) could be attributed to secondary effects related to cytotoxicity or to the use of extracts which show a different genotoxic pattern compared with the pure toxin (Funari and Testai 2008). In some mammalian cell lines, the micronucleus and comet assay evidenced DNA damage associated with aneugenic effects (Dias et al. 2014), although MC-LR was not active in other assays (in many cases due to absence of active uptake by the cells).

After oral administration at a relatively high dose (2 and 4 mg/kg bw), a statistically significant dose-dependent but transient DNA damage in the blood cells was observed after 3 h (although not persistent at 24 h) (Gaudin et al. 2008). When as in this case in vivo data have been obtained after a single exposure at doses in the range of the LD_{50}, or even higher, the presence of DNA damage could very likely be associated with necrosis occurring in the liver as well as in other organs (e.g., testes) having therefore a limited relevance.

MC-LR (2 and 10 ng/mL) significantly enhanced 8-oxo-dG, a common biomarker of oxidative DNA damage in time- and dose-dependent manner in vitro in primary cultured hepatocytes and in vivo in rat liver cells: the effect is compatible with the oxidative stress induced by the MC-LR MoA, not implying a direct interaction with DNA. MC-LR significantly decreased the amount of endogenously formed DNA adducts, especially after prolonged exposure (Bouaïcha et al. 2005). Similar results (increase of 8-hydroxy-2′-deoxyguanosine levels of DNA in liver cells with damage to mtDNA and nuclear DNA) were obtained in mice treated orally in drinking water for 12 months at concentrations of 1, 5, 10, 20 and 40 µg/L (Li et al. 2015c), likely associated with the oxidative stress induced by MC-LR cytotoxicity or by the increase in nitric oxide (NO) production, as shown in human–hamster hybrid (AL) cells exposed to MC-LR (Wang et al. 2015).

In in vivo DNA damage (measured as % DNA tailin the comet assay) was detected in several organs: liver > kidney medulla > kidney cortex > lung after MC-LR (Filipič et al. 2007). However, it is accepted that positive results with the comet assay, although suggestive of a possible DNA damage, can still be repaired and therefore there is no biunivocal association with genotoxicity.

Significant increase in DNA damage of primary cultured rat hepatocytes treated with MC-RR (0.01, 0.1, 1 µg/mL) (compared to the control) was observed only after prolonged treatment (13 h) (Žegura et al. 2002).

The induction of DNA breaks seems to be associated with ROS formation, mutagenic oxidative DNA lesions, interference with DNA repair and clastogenic activity, related to the MeA of MC, suggesting a secondary genotoxicity rather than a direct interaction between MC-LR and DNA (Funari and Testai 2008; Žegura et al. 2011).

Carcinogenicity

No direct carcinogenic effects have been evidenced in in vivo studies after i.p. or oral treatment (Ito et al. 1997; Lian et al. 2006; Labine and Minuk 2014). Ito et al. (1997) administered 80 μg MC-LR/kg bw/day by gavage to mice for 80 or 100 days over 28 weeks (7 months). This single dose failed to induce neoplastic nodules of the liver. In the other available oral study, four groups of adult, male CD-1 mice ($n = 20$/group) were dosed for 28 weeks with water (control group), water containing 1.0 μg MC-LR/L, water containing thioacetamide and water containing both MC-LR and thioacetamide. No tumors were present in the control or MC-LR-alone groups, while in the other two groups four and five mice developed liver tumors, whose mean size, Ki-67 staining, number of atypical mitoses and liver cancer gene expression profiles were similar (Labine and Minuk 2014). The lack of hyperplastic nodules at 7 months suggests that MC-LR is not a mutagenic initiator of tumors; the fairly short duration may have been a limiting factor, but the same results obtained in two independent studies are supportive.

Although the recent study from Labine and Minuk (2014) seems to limit the ability for MC-LR to enhance existing tumor growth, the toxin tumor-promoting activity was described already early in cyanotoxin research (Nishiwaki-Matsushima et al. 1992) and then confirmed by cyanotoxin administration with known carcinogenic (initiator) compounds, such as aflatoxin B1 and diethyl-nitrosamine (Sekijima et al. 1999). Repeated MC-LR i.p. administration (10 μg/kg bw) induced the formation of GST-P(+) liver foci in the presence of an initiator (Sekijima et al. 1999). The biological plausibility for tumor promotion is associated with the inhibition of protein phosphatases involving TNF-α, as an endogenous tumor promoter and a central mediator of tumor promotion (Fujiki and Suganuma 2011).

On this basis and considering the tumor-promoting activity the International Agency for Research on Cancer (IARC 2010) classified MC-LR as possibly carcinogenic to humans (Group 2B) because still there are not adequate evidences in both humans and experimental animals to demonstrate the direct carcinogenicity of MC-LR.

Reproductive/developmental toxicity

Possible effects on reproductive and developmental toxicity have triggered some discussion, since controversial results are available: no teratogenic effects were seen in some studies, whereas some evidences of effects on the offsprings as well on male and female reproductive systems have been reported more recently. However, many of the papers originate from the same research laboratory and are associated with i.p. administration; they present some weaknesses in experimental design and reporting. For this reason US-EPA (2015a) and Health Canada (2016) considered them not adequate to identify PoD values for the derivation of guideline values for drinking water. Additional studies published more recently are discussed in the following.

Generally no teratogenic effect was observed after i.p. (128 μg/kg bw for 2 days) (Chernoff et al. 2002) or oral (up to 2000 μg/kg bw for 10 days) administration (Fawell et al. 1999a) also in the presence of maternal toxicity. A NOAEL for reproductive toxicity of 600 μg/kg bw has been derived (no evidences of either maternal or fetal toxicity) from the oral study (Fawell et al. 1999a).

Cognitive effect on offsprings has been observed on rat pups from dams treated orally with very high MC-LR doses (1000, 5000, 20,000 μg/kg bw for 8 weeks), without evident pathological alterations in the CA1 regions of rat brain (Li et al. 2015b). No indication was reported related to other organ toxicity, but it is reasonable to expect that at the tested dose the cognitive impairment was not the critical effect to be used in the risk assessment.

The mechanism for cognitive impairment has been recently studied in three-month-old male Wistar rats i.p. treated with 10 μg MC-LR/kg bw/day, for 14 days: spatial memory injury was observed in the animals, with alterations in endoplasmic reticulum (ER) ultrastructure and apoptotic cell in CA1 neurons, suggesting the involvement of stress pathway, supported by the upregulation of glucose-regulated protein (GRP78) and caspase 12 (Cai et al. 2015). A role for the oxidative stress was supported also by the increase in malondialdehyde (MDA) level and decrease in GHS brain content in SD rats pups, following the infusion of dams with 10 μg MC-LR/kg bw/day from gestational day 8 (GD8) to postnatal day 15 (PD15) of lactation. The rat offspring brains contained 3.75 ± 0.94 ng MC-LR/g DW. The proteomic analysis evidenced the up- and downregulation of 49 proteins related to neurodevelopment, protein folding and degradation (Zhao et al. 2015a, b). With a similar experimental design, effects in the offspring liver were studied: the levels of MC-LR reached 6.94 ± 0.833 ng/g DW, and presence of oxidative stress with ROS production and MDA formation, lipid peroxidation and reduction of GSH level were evidenced, together with alteration of serum markers of liver toxicity. The proteomics analysis also showed that MC-LR significantly altered 45 proteins related to cytoskeleton, metabolism and oxidative stress (Zhao et al. 2016), compatible with MC-LR toxicity in adults. Although useful to highlight potential 'hazards,' the use of one single dose and of the i.p. route of exposure limit the possibility to use these findings in the risk assessment.

Regarding the male reproductive system, some studies (mainly using i.p.) showed in vivo the possibility to affect the sperm quality and hormone levels at different doses (Li et al. 2008; Chen et al. 2011, 2013a, b; Wang et al. 2012).

In the only oral study devoted to male reproductive system, mice (strain and age not specified) were dosed for 3 and 6 months with MC-LR in drinking water (commercial product; purity not stated) at concentrations of 0, 1, 3.2 or 10 μg/L (approximately 0, 0.25, 0.79 or 2.5 μg/kg bw/day using default data; body weight and amount of water consumed were measured, but these data were not presented, and doses to the animals were not calculated by the authors). No clinical signs of toxicity were observed; body weight and testes weight were not affected by treatment. The authors described a dose- and time-dependent decline of sperm quality at doses ≥3.2 μg/L associated with testicular atrophy (at 6 months) together with a decrease of testosterone and increase of FSH and LH (Chen et al. 2011). However, methodological and reporting limitations affect this study (e.g., use of methanol as a vehicle for MC-LR not administered to control animals, effects present also in the control group, poorly described analysis on sperm, no purity of the test item reported, nor check for MC-LR concentration in the administered drinking water). Many of these criticisms were shared by US-EPA (2015a), concluding that the quantitative data on decreased sperm counts and sperm motility were not appropriate for determining the PoD for the derivation of the reference dose (RfD) for MC in drinking water. In addition the possibility for an uptake by human testis cells due to the presence of OATP transporters is not yet completely clarified although it has been reported that in animals MC-LR can be detected in the testes, mainly within seminiferous tubules, and in vitro the toxin has been shown to enter spermatogonia and Sertoli cells, but not Leydig cells (Zhou et al. 2012; Whang et al. 2013). Interestingly enough, although the striking difference in kinetics, similar effects at the same range of doses (5–15 μg/kg bw, purity not reported) were observed after 28-day i.p. treatment (Li et al. 2008), casting additional doubts about the relevance of the Chen et al. (2011) study.

Considering the female reproductive system, some indication of effects are suggested by an oral study in which female mice were treated for 3–6 months in drinking water containing 0, 1, 10 and 40 μg MC-LR/L (isolated from *Microcystis aeruginosa*, purity ≥95%; again body weight and amount of water consumed were not reported, so that it is not possible to calculate the exact dose of exposure). Slight reduction of gonadosomatic index (GSI), follicle atresia (a natural physiological process which occurs throughout female reproductive life during all stages of follicular development), decrease of serum estradiol and increase of progesterone, and disturbs of estrus cycles were statistically significant at 40 μg/L (3 months) and at 10 μg/L (6 months). Somatic granulosa cells (surrounding each oocyte within the follicle, responsible for secretion of steroid hormones) are indicated as the target of MC-LR toxicity and induction of oxidative stress as the MeA for the described effects. The MC-LR uptake by primary cultured mouse granulosa cells was qualitatively reported in the text, measured by immune-fluorescence methods using 1.25 μM MC-LR 48-h in vitro exposure, although as stated by the authors themselves, which kinds of OATPs (if any) are present in granulosa cells still remains unclear (Wu et al. 2015).

Inconsistently with this, serum progesterone decreased after 28-day administration of 5–20 μg/kg bw MC-LR by i.p. injection in the previous toxicity study from the same group, although the other effects were surprisingly similar (Wu et al. 2014).

Human health effects and epidemiological data

The toxicological effects in humans exposed orally or dermally or by inhalation to water infested by cyanobacterial blooms producing MC and to ingesting contaminated food items gave rise to some outbreaks in the late past century (Funari and Testai 2008). Symptoms range from gastroenteritis, abdominal pain, flu-like symptoms, ear and eye irritation and rashes to kidney and liver damage. Fatal outcomes were reported only in poisoning episodes occurred in Brazil, one due to consumption of drinking water (Teixera et al. 1993), the second one to the use of contaminated water for dialysis purposes, therefore referring to a parenteral route of exposure, when the toxin injected in the blood stream, with 100% bioavailability in 1996 in Caruaru (Jochimsen et al. 1998, Carmichael et al. 2001). In the Caruaru episode the concentrations of unbound MC were 19.5 μg/L and the average concentrations in human liver, namely for MC-YR, -LR and -AR, were estimated 223 ng/g (Hilborn et al. 2013; Pouria et al. 1998).

Despite the increased attention and control, a further episode occurred in Rio de Janeiro in 2001, when 44 patients were found to have been exposed to 0.32 μg MC/L, as detected in the activated carbon filter used in an intermediate step for treating drinking water to prepare dialysate (Soares et al. 2006). Concentrations of MC-LR in the serum ranged from 0.46 to 0.96 μg/L about 1 month after exposure with some alterations in serum markers of hepatic cellular injury cholestasis (AST, ALT bilirubin, ALP and GGT).

A case study of acute intoxication to MC was reported in which exposure occurred likely via multiple routes (direct contact, ingestion and inhalation). Indeed, a young boy in Argentina accidentally falling in a freshwater lake infested by an intense bloom of *Microcystis* spp. during a paragliding run, remained in water for 2 h (MC-LR level in water was 48.6 mg/L). Four hours after exposure signs as nausea, abdominal pain and fever were evident, followed by dyspnea and respiratory distress, leading to an atypical

pneumonia. Finally, the patient experienced alteration of hepatic damage biomarkers (ALT, AST and γ-GT) (Giannuzzi et al. 2011).

A case study was also related to the prolonged consumption of MC-contaminated *A. flos-aquae* products, used as food supplements (the so-called BGAS) which was suggested as the most likely cause responsible for liver failure and death of a 34-year-old woman: 2.62–4.06 μg MC-LR eq/g DW was found in BGAS, the patient liver was MC-positive at immunostaining and hepatic damage was compatible with MC-induced toxic effects (Dietrich et al. 2007). BGAS, found affected by MC-LR, MC-LA and other variants contamination (Vichi et al. 2012), are generally proposed as health-promoting natural products and can become a potential public health problem for the easy availability and the uncontrolled costumer's use.

Several epidemiological studies are available: unfortunately, most of them are affected by lack of quantitative information about exposure and/or by the failure to control for extremely relevant confounding factors, as the co-exposure with other potent carcinogens and other contaminants in drinking water (Ueno et al. 1996) or alcoholism and chronic kidney disease among the enrolled individuals (Falconer et al. 1983).

Some ecological or cohort studies tried to search for correlation between hepatic and colorectal cancer and the consumption of contaminated drinking water and/or food (Fleming et al. 2001, 2002; Zhou et al. 2002; Svirčev et al. 2009, 2013). When drinking water was the source of exposure in Florida (Fleming et al. 2001, 2002) and Serbia (Svirčev et al. 2009, 2013), even if the studies evidenced geographic 'hot spot' of exposure with possible eventual effects, they have some limitations as lack of information on exposure (e.g., duration of permanence/residence in the same place, actual contamination of consumed water vs simple visual information about blooming in the reservoir). When both drinking water (from untreated ponds) and food in China were indicated as source of MC-LR exposure responsible for liver cancer, the poor quality of water with presence of other contaminants or other confounding factors (such as the endemic presence of viruses and aflatoxin B1) was not considered, not allowing any conclusions about a possible correlation with colorectal carcinoma incidence (Ueno et al. 1996; Zhou et al. 2002). Hence, these studies cannot be used as determinant for a causal relationship between MC exposure and liver or colorectal tumors.

On the other hand, the proved tumor-promoting mechanism of MC in animals can help in the interpretation of data coming from those studies: MC exposure, acting as tumor promoter, might act synergistically with other risk factors to cause increased tumor incidence.

A correlation with hepatic or gastrointestinal effects has been evidenced in a cohort (on Chinese fishermen; Chen et al. 2009) and cross-sectional (on Chinese children: Li et al. 2011b) and in a Canadian prospective case–control study (Lavesque et al. 2014). In the fishermen study a positive correlation between the consumption of water and food (carp and duck) contaminated with MC (MC-RR, MC-YR and MC-LR, detected in Lake Chaohu water) and increase of serum markers for hepatotoxicity (ALT, AST, LDH and ALP) was evidenced (Chen et al. 2009). The proportions of MC-RR, -YR and -LR were 41.7, 13.3 and 45.0%, respectively, close to the proportions of MC-RR, -YR and -LR in the muscle of aquatic animals. The estimated daily intake of MC by a fisherman could be 2.2–3.9 μg MC-LReq (Chen et al. 2009). In the big cross-sectional study ($n > 1000$ enrolled individuals) the mean concentration in the high-exposed, low-exposed and unexposed children was 1.3, 0.4 and <LOD μg MC-LReq/L. AST and ALP were significantly increased in the high-exposed children when compared to the other groups. After adjustment for HBV infection and use of hepatotoxic medicines (considered as confounding factors) the OR (95% CI) for liver damage was 1.72 (1.05–2.76) (Li et al. 2011b). However, according to the authors hepatitis B infection was a greater risk of liver damage among these children than the MC exposure. In the Canadian study a correlation with several gastrointestinal symptoms (e.g., diarrhea or abdominal pain, nausea or vomiting) was found with the presence of cyanobacterial cells in a lake affected by MC-producing blooms (Lavesque et al. 2014).

Recently Labine et al. (2015) found no geographic association between liver cancer data in Canada and surrogate markers of cyanobacterial contamination in freshwater lakes, by using a negative binomial regression model. However, the lack of an actual quantification of cyanotoxin presence, replaced by the use of surrogate markers of potential cyanobacterial contamination (e.g., % of land devoted to agriculture, cattle and swine densities), strongly limited the value of the analysis.

An additional cross-sectional study ($n = 5493$ enrolled persons) was recently conducted in Southwest China to investigate the association of renal function indicators (RFIs, including blood urea nitrogen, serum creatinine and estimated glomerular filtration rate) with exposure to MC and aflatoxin B1 (AFB1). The MC-LR levels in aquatic products were measured by ELISA, with a concentration range of 0.02–1.74 μg/kg (although the reliability of MC detection in complex matrix as food is generally problematic and refers exclusively to free forms). The highest quartile of MC-LR exposure group showed significantly higher risks of abnormal RFIs (OR 1.80–4.58), not observed for the AFB1 exposure group and similar to the ones measured when MC-LR and AFB1 were in combination. The data were confirmed also after adjustment to consider age, alcohol use, HBV infection, etc., although other relevant

factors, possibly affecting renal function, such as Cd and As contamination, or liver damage were not considered (Lin et al. 2016).

Numerous reports of skin irritations and local effects associated with recreational contact with MC-producing CB in lakes or freshwater basins exist, although recent studies in humans indicate that components other than cyanotoxins are responsible for skin sensitization, namely phycobiliprotein complexes (Geh et al. 2015). This new finding allows to interpret the epidemiological studies (Pilotto et al. 1997; Stewart et al. 2006a, b) aimed to understand effects (mainly skin and eye irritation), exclusively due to direct contact with contaminated water in a different perspective: the relevant metrics for the local effects cannot be MC concentration but rather biovolume or cell surface area. However, during the recreational use of water, the dermal exposure is generally paralleled by involuntary ingestion of water and by inhalation. The Kansas Department of Health and Environment received 25 reports of human illnesses after recreational activity in the Milford Lake potentially associated with CB in 2011. Seven cases were confirmed, and 2 patients were hospitalized showing sore throat, cough, malaise, headache and fever. Both cases were in periods of high CB cell densities and microcystin toxin levels as confirmed by the water samples (110 and 1600 µg/L maximum concentrations by ELISA) (Trevino-Garrison et al. 2015). Recently the US Center for Disease Control (CDC) has published data of health surveillance about the algal bloom-associated disease outbreaks among users of freshwater lakes for 2009 and 2010. In 11 cases (46%) MC were identified as the suspected (MC < 20 µg/mL) or true etiological agent (MC > 20 µg/mL) (Hilborn et al. 2014). Whenever contact exposure was reported, affected persons developed rash or skin irritation; in each of the outbreaks for which ingestion exposure was reported, affected persons had gastrointestinal signs or symptoms, and in outbreaks for which inhalation exposure was reported, affected persons had respiratory signs or symptoms (Hilborn et al. 2014).

There is still a high degree of uncertainty on the relevance of the inhalation route, related to the concomitant presence of other cyanobacterial cell components with irritative and allergenic properties, as well as cell debris in the aerosol, as well as to the bioavailability of the toxin in the aerosol. In fact in 81 individuals exposed to aerosols of lakes contaminated by MC in California (15–350 µg/L, with MC-LA as the dominant variant in water; 0.052, 2.89 ng MC/m^3 in the aerosol), the toxin was detected only in the nasal swabs but not in plasma (l.o.d.:1 µg/L) (Backer et al. 2008, 2010) and it was not possible to conclude on symptoms because of the limited number of enrolled individuals also for the disparity in the size of the control (non exposed individuals = 7), the wide range of aerosol particle size and analytical difficulties in detecting MC by ELISA in complex matrix. This area deserves further investigations, also considering the presence of dried scums on the shore, or terrestrial CB in crust, which can be a concern in windy days.

Animal poisoning

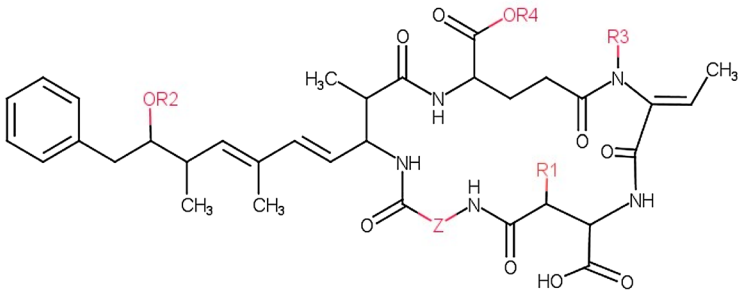
A wide variety of animals from wild to livestocks and domestic ones have been reported to be affected by toxicosis due to MC exposure. This phenomenon in the past was generally reported only based on temporal association between death of animals and blooms in the waterbodies used for watering and on the observation of typical 'symptoms' (Stewart et al. 2008). The awareness of the problem and the possibility to detect toxins in tissues and biological samples, such as stomach contents, tissues, hair (although with some analytical limitations), allow in recent times to conclude more firmly on a possible cause–effect relationship.

Several reports are about dogs poisoning with MC watering in lake affected by blooming during summer with levels up to 126,000 µg/L in water: MC presence was not always confirmed in animal tissues, vomitus or hair, sometimes due to the delay in the analysis (Wood et al. 2010a; van der Merwe et al. 2012; Lürling and Faassen 2013; Trevino-Garrison et al. 2015). A case report of MC intoxication in a dog was reported, assessed by liver dysfunction, after using a commercially available blue-green algae dietary supplement, containing 166 ng/g of MC-LR and 962 ng/g of MC-LA (Bautista et al. 2015).

Furthermore in 11 alpine sites of southeastern Switzerland in two decades the death of more than 100 cattle was reported with a range of concentrations in water of 0.01–17.5 nM MC-LR eq according to the protein phosphatase inhibition (Metz et al. 1997).

In 2004 in southwest Spain a blue-green surface scum was indicated as the cause of thousands of fish death followed by herbivorous waterfowl and piscivorous birds death (6000 birds belonging to 47 species) (Lopez-Rodas et al. 2008). Mass fish mortality due to toxic blooms of *Cylindrospermopsis raciborskii* and *Microcystis aeruginosa* was registered during 2003 in northeast Brazil. The MC levels varied from 0.07 to 8.73 µg/L in the seston samples and from 0.01 to 2.59 µg/L in the liver samples using the standard HPLC method (Chellappa et al. 2008).

Recently a possible turtles deaths in a zoo have been associated with recurring blooms of toxic *Microcystis aeruginosa* in the moats (MC range 0.0001–7.5 µg/L), since in the liver of dead animals up to 166 ng/g of MC were found (Doster et al. 2014). In Norway a case of hepatotoxicosis in a wild roe deer (*Capreolus capreolus*) due to MC was reported. The MC content in the liver was 1361 ng of



	R1	R2	R3	R4	Z
Linear NOD	CH_3	CH_3	CH_3	H	L-Arg
[L-Val[2]] NOD	CH_3	CH_3	CH_3	H	**L-Val**
[L-Har[2]] NOD	CH_3	CH_3	CH_3	H	**L-Har**
[D-Asp[1]] NOD	**H**	CH_3	CH_3	H	L-Arg
[DMAdda[3]] NOD	CH_3	**H**	CH_3	H	L-Arg
[dhb[5]] NOD	CH_3	CH_3	**H**	H	L-Arg
[Glu[4](Ome)] NOD	CH_3	CH_3	CH_3	**CH_3**	L-Arg
[(6Z)-Adda3] NOD	double bound of Adda is in *cis* configuration				

Fig. 5 General chemical structure of NODs: in the table the chemical nature of different R substituents; in *bold* the main differences from linear NOD are highlighted

MC-YR, -LR and -RR per gram (WW, wet weight) (Handeland and Østensvik 2010).

In Lake Oubeira (Algeria) in 2005 two terrapins died during a bloom of *Microcystis* spp.: the MC-LR, -RR and -YR were detected (1.12 mg MC-LR eq/g dried bloom material, detected as protein phosphatase inhibition). MC content was 1192.8 mg MC-LR equivalent/g DW in the liver and 37.19 mg MC-LR equivalent/g DW in the viscera (Nasri et al. 2008).

In South Africa (Nhlanganzwane Dam), a bloom of *M. aeruginosa* leading to MC level of 23,718 µg/L in water was associated with mortalities of mega herbivores; although no measurement was taken in the animal tissue, and MC-LR daily intake for an adult male white rhinoceros was estimated as 754.29 µg/kg bw (Oberholster et al. 2009). In the Kruger National Park, MC levels up to 103 mg/Lin water have been associated with the death of 15 white rhinos, 8 zebras, 5 blue wildebeest. According to Masango et al. (2010) wildlife mortality from CB poisoning has increased in southern Africa during the past few years, even though published data are limited, reflecting probably an underestimation of the problem.

Also the marine environment could be affected after blooming in freshwater habitats: 21 southern sea otters died after ocean discharge of freshwater lake, to within 1 km, where MC were present up to 2900 ppm. Hepatic MC concentrations varied from 1.36 to 348 ppb WW (MC-RR was present in 19/21 animals). The possible biomagnification by marine clams, mussels and oysters, often consumed by sea otters, was evaluated in the laboratory, showing invertebrate tissue concentrations of MC-LR up to 107 times higher than adjacent seawater (Miller et al. 2010).

Nodularins

Nodularins (NOD) are cyclic peptides of five (NOD) amino acids mainly produced by the CB *Nodularia spumigena*. They share a similar structure with MC, consisting of Adda, D-glutamic acid (D-Glu), *N*-methyldehydrobutyrine (MeDhb), D-erythro-β-methylaspartic acid (D-MeAsp) and L-arginine (L-Arg). Since the first description of NOD in 1988 (Charmichael et al. 1988), to date about eight NOD isoforms have been characterized (Mazur-Marzec et al. 2006a, b), whose chemical structure is shown in Fig. 5. Variations occurring within the Adda residue reduce or abolish the toxicity of the compound; amino acid substitutions in position 2 of NOD give rise to nodularin-Har (when the L-Arg is replaced by L-Har) or motuporin (when the L-Arg residue is replaced by L-Val). Esterification of free carboxyl of the D-Glu residue suppresses toxicity, while variation at position 1 has minor effects (Pearson et al. 2010).

Producing organisms

The filamentous diazotrophic *Nodularia spumigena* is the main NOD producer (Table 8). It is the most conspicuous constituents of the CB water blooms in the Baltic Sea, with the nontoxic *Aphanizomenon flos-aquae* and the MC-producing *Anabaena* sp. (Ploug 2008; Stal et al. 2003). *Nodularia* forms not densely packed colonies, and for this reason, turbulent conditions may have a great impact on aggregates of this cyanobacterium. It blooms during late summer, in nitrogen-depleted condition, when other phytoplanktonic species cannot grow. Necessary conditions for it to bloom seem a low N/P ratio, adequate concentrations of N and P and high temperature, which stabilizes the water column and allows organisms to float, thanks to the production of gas vesicles (Stal et al. 2003). *Nodularia* is an important food source for Baltic copepods and mysids; indeed, nodularin has been detected in zooplankton, fishes and mussels (Karjalainen et al. 2005; Mazur-Marzec et al. 2015; Sipiä et al. 2002); however, the exposure of Baltic fauna to NOD is not limited to the surface where the floating organisms accumulate, but is extended along the water column (Koskenniemi et al.

Table 8 NOD cell quota in isolated strains, from experimental or laboratory conditions, and field samples

Gender/species/strain	Cell quota (µg/g DW)	Country of origin	Analytical method	Reference
Nodularia spumigena NSPH-02 *N. spumigena* NSPH05	Min–max of averaged values (all strains) at 0, 12, 24 and 35 g/kg salinities	Western Australia	HPLC–UV	Blackburn et al. (1996)
N. spumigena NSOR11 *N. spumigena* NSOR13	≈60–160 fg/cell Logarithmic phase[a]	South Australia		
N. spumigena NSLA01 *N. spumigena* NSLA02	≈110–250 fg/cell Stationary phase[a]	Tasmania		
N. spumigena BY1	4.35×10^3 low P 15.4×10^3 high NP	Baltic Sea	HPLC	Lehtimäki et al. (1997)
N. spumigena BY1	2.3×10^3 low P 3.08×10^3 high NP		HPLC	Lehtimäki et al. (1994)
N. spumigena HEM	$0.66x\ 10^3$ low P 1.48×10^3 high NP			
N. spumigena AV1	4.2–5.8 mg/g C		ELISA	Engstrom-Ost et al. (2011)
N. spumigena AV1	t0: 0.011–0.042 No, low, high grazing pressure: ambient water tfin: 0.003–0.091 Sea water tfin: 0.009–0.022		ELISA	Gorokhova and Engström-Öst (2009)
N. spumigena 001E	3.58 µg/mg POC	South Australia	HPLC–UV	Hobson and Fallowfield (2001)
N. spumigena KAC 13	Intracellular: 0.13–0.22 pg/cell Direct grazer exposure 0.07–0.18 pg/cell Indirect grazer exposure Extracellular: 0.03–0.13 pg/cell Direct grazer exposure 0.06–0.16 pg/cell Indirect grazer exposure	Baltic Sea	HPLC–DAD	Lundgren et al. (2012)
N. spumigena KAC 66	Mono- and mixed cultures, at different pH ~2–2.5 pg/trichome[a]	Baltic Sea	HPLC	Møgelhøj et al. (2006)
N. spumigena KAC 66	1.66 µg/µg Chl-a low P 0.63 µg/µg Chl-a high NP	Baltic Sea	HPLC–PDA	Stolte et al. (2002)
N. spumigena NSGG-1	High value, 35psu, 30 g 0,68 NOD/Chl-a ratio	Baltic Sea	HPLC–DAD	Mazur-Marzec et al. (2005)
N. spumigena KAC71	4.24 pg/cell low P 0.41 pg/cell high NP	Baltic Sea	HPLC–DAD	Pattanaik et al. (2010)
N. spumigena	2001: 0.3–8.9 pg/filaments 2003: 60–150 pg/filaments	Baltic Sea	HPLC–DAD ELISA	Kanpaanka et al. (2009)
Nodularia GR8b	1.0×10^{-4}–4.4×10^{-4} ng/cell	Gulf of Finland	HPLC–DAD	Repka et al. (2001)
Nostoc sp. symbionts of '*Macrozamia serpentina* 73.1'	232 NOD per g WW	Australia	LC–MS/MS HESI-MS/MS	Gehringer et al. (2012)
'*M. riedlei* 65.1'	346 NOD per g WW			
Nodularia PCC 7804	4.2×10^3 [b]		HPLC–PDA and H-NMR	Beattie et al. (2000)

When there is no reference number close to the name of the strain, it refers to field population

DW dry weight, *P* phosphate, *NP* nitrate and phosphate, *C* carbon, *t* time, *POC* particulate organic carbon, *NOD* nodularin, *Chl-a* chlorophyll-a, *WW* wet weight

[a] Values taken from figures, [b] [L-Har^2] nodularin production

2007). A genome-transcriptomic analysis of a strain isolated from Baltic Sea revealed that *Nodularia* possesses complex systems for uptake and assimilation of Fe and P compounds, and for the formation of vesicles, which confer a competitive advantage in nutrient-limited brackish water ecosystems (Voß et al. 2013). And indeed, toxic strains

of *Nodularia* have been found worldwide in brackish or estuarine environments, in temperate brackish areas (Huber 1984; Jones et al. 1994) in Nevada (Horne and Galat 1985), New Zealand (Carmichael et al. 1988) along the German coast (Nehring 1993) and recently in a subtropical lake in Australia (McGregor et al. 2012). They have been also found in freshwater lakes, originally connected to the sea, in Australia (Heresztyn and Nicholson 1997), and in a freshwater lake in Turkey (Akcaalan et al. 2009).

Extracts of *Nodularia* PCC 7804, a benthic mat-forming originally isolated from a freshwater thermal source, produce the variant [L-Har^2]nodularin as the major component (4.2 µg/mg DW), in addition to lower quantities of NOD (Beattie et al. 2000).

Nodularin can be produced also by some strains of *Nostoc* symbiont of the gymnosperm Cycadaceae, at a concentration of 1.34 ng/mL extract from coralloid root, or 3 pmol/g plant, showing that NOD can be diffused on terrestrial habitats as well (Gehringer et al. 2012).

Intracellular versus dissolved toxin

In growing *N. spumigena* cultures, NOD is usually only intracellular, appearing in the dissolved phase only at the stationary phase, when filaments start to decay (Sivonen and Jones 1999; Møgelhøj et al. 2006). NOD does not seem to have an allelopathic effect, since in mono- and mixed cultures of a strain of *Nodularia* and the alga *Rodomonas salina*, it was always intracellular in the exponential growth phase (~2–2.5 pg/trichome), and the extracellular components in the stationary phase were 9–12% of the total NOD with no differences between systems (Møgelhøj et al. 2006). In the presence of grazers, the intracellular content of NOD decreased, from 0.042 to 0.003 µg/µg DW vs an increase in the control from 0.042 to 0.091 µg/µg DW, thus suggesting that the toxin is not even a defense against predation (Gorokhova et al. 2009; Table 8). In a second experiment the share of extracellular NOD was measured, and in the grazed system it increased from undetectable at time 0 to 45% of the total in the end vs no increase in ungrazed control, probably due to sloppy feeding of the grazers (Gorokhova et al. 2009). Cell quota of NOD from other strains in other regions in the world are lower, varying from a maximum of one strain tested at 12 g/Kg salinity of 1.6 mg/g DW in Australian strains (and a range, in several strains, of 60–500 fg/cell) (Blackburn et al. 1996) to 0.578 mg/g DW in the freshwater strain from Turkey (Akcaalan et al. 2009).

Also in the field NOD is mainly intracellular, even if in dense blooms of *N. spumigena* around 10–20% (Mazur and Plinski 2003) and 57–100% (in senescent blooms) (Kankaanpää et al. 2009) of the toxin concentration can be in the dissolved phase. Intracellular concentration in Baltic Sea was highly variable between years, since it ranged from 0.3 to 8.9 pg/*N. spumigena* filament in 2001 and 60–150 pg/filament in 2003 (Kankaanpää et al. 2009), even if the former data have been obtained by HPLC/DAD, while the latter by ELISA, which is less specific.

Occurrence

Surface and drinking water

As NODs are produced almost exclusively from the genus *Nodularia*, they occur mainly in slightly saline or brackish waters and in saline and coastal lakes (Table 3). NODs have been reported mainly in Australia, New Zealand and the Baltic Sea (Merel et al. 2013a, b). Recent investigations in the Baltic Sea water have reported NOD levels up to 42,300 µg/L (Mazur-Marzec et al. 2013).

NOD degradation, in lake water that was supporting a bloom, was found to be faster than in water without blooms for several months. Probably in a water that was supporting a bloom, microorganisms capable of degrading the toxin were present (Heresztyn and Nicholson1997). Mazur-Marzec et al. (2006a, b) investigating the NOD UV decomposition, found that NOD was fairly stable under white fluorescent light (VIS). UV-B had the most pronounced degradation rate, and it was higher in *Nodularia* extract than in pure water.

In 2009 Mazur-Marzec et al. found that incubation of NOD with bacterial isolates from seawater and sediment of Baltic Sea did not reveal any NOD-degrading activity, while natural microbial communities from the Baltic sediments were very effective in NOD removal (complete degradation within 7 days), showing that brackish water microorganisms play an important role in cyanotoxin removal.

Few data have been published in the last 10 years on NODs in drinking, in any case concentrations within 1 µg/L are generally reported in raw water (Yen et al. 2011).

Fish and seafood

As reported in Table 5, the highest NOD concentrations have been found in aquatic organisms of the Baltic Sea, with values of 0.91 µg/g DW in fish muscle and 2.5 µg/g in mussels.

Food supplements

Heussner et al. (2012) tested thirteen products consisting of *Aph. flos-aquae*, *Spirulina* and *Chlorella* or mixtures; thereof, none of them was positive for NODs.

Crops

No field data are available on NOD occurrence in crops. A laboratory study reported evidence for NOD uptake by the terrestrial plant, *Spinachia oleracea* (up to 66 ± 5.1 pmol/g WW in old leaves), and showed that the exposure of spinach to cyanobacterial crude water extract from nodularin-producing strain AV1 results in inhibition of growth and bleaching of the leaves (Lehtimäki et al. 2011).

Biosynthesis and Genetics

Nodularins are synthesized nonribosomally by multienzyme complexes encoded by the *ndaS* gene cluster which was full sequenced and characterized in 2004 (Moffitt and Neilan 2004) in the filamentous planktonic cyanobacterium *Nodularia spumigena* NSOR10.

As reviewed by Pearson et al. (2010), the gene cluster spans 48 kb (encoding nine ORFs, namely *ndaA-I*) and it is transcribed from a bidirectional regulatory promoter region. The proposed biosynthetic pathway for NOD is similar to that for MC, due to its high sequence homology with the microcystin synthetase (*mcy*) gene cluster, encoding nonribosomal peptide synthetase modules, polyketide synthase modules and tailoring enzymes.

An hybrid NRPS/PKS pathway produces the Adda side chain starting from a phenylacetate starter unit and several malonyl-CoA extensions (NdaC, D and F). Then, the NRPS module NdaF adds D-Glu to the raising chain. Finally, the cyclic pentapeptide is completed by the addition of the final amino acid residues L-Thr, D-MeAsp and L-Arg, through the NRPS enzymes NdaA and B.

The two NRPS modules responsible for the activation of the amino acids D-Ala and L-Leu in the *mcy* gene cluster are not present in the structure of *ndaS* (Moffit and Neilan 2004). A posttranslational modification of NRPS and PKS proteins is required for activity, and the gene encoding this phosphopantetheinyl transferase is not clustered or adjacent to the biosynthetic genes (Copp et al. 2007).

The *ndaS* cluster also encodes several tailoring enzymes that modify nodularin during and after chain elongation, such as the *O*-methyltransferase *ndaE*, the L-Asp/L-Glu racemases *ndaG*, the ABC transporter ndaI and also a D-3-PGDH homolog, *NdaH*, putatively involved in the production of D-MeAsp (Pearson et al. 2010).

While NOD expression appears to be constitutive, a transcriptional regulation of the biosynthetic pathway played by environmental factors was suggested in Nodularia spumigena strain AV1, since phosphate depletion was found to increase NOD expression, while ammonia supplementation was reported to inhibit it. However, despite the changes observed in nda expression, intracellular and extracellular nodularin concentration was shown not to vary significantly (Jonasson et al. 2008).

Toxicological profile

Kinetics

Specific studies related to ADME on NOD are not available. However, due to its chemical similarity with MC it is likely that its uptake depends on OATP transporter system. In fact, a similar uptake of NOD and MC-LR was recently evidenced studying the role of Oatp1d in zebrafish (Faltermann et al. 2016). Furthermore, some authors investigating the expression of OATP1A2, OATP2B1, OATP1B1, OATP1B3 and OCT-1 in different human liver cell lines observed that the sensitivity to NOD-induced toxic effect correlated with the presence of OATP transporters in the cells (Meili et al. 2016). The conjugation with GSH is the hypothesized NOD pathway of detoxification, based on the identification of NOD-GSH adduct in fish tissue (Chen et al. 2013a, b).

Acute systemic toxicity

In the literature several studies are present where an i.p. LD_{50} in mice has been identified: unfortunately, the studies often reported only the value, without further explanation (Lankoff and Kolataj 2000), or with very poor description of the experimental conditions used (Namikoshi et al. 1993), so that their quality/reliability is not easy to check. Some other studies are available although affected by uncertainties in reporting (e.g., low number of animals) (Carmichael et al. 1988; Eriksson et al. 1988; Runnegar et al. 1988) on the basis of which a range of LD_{50} = 50–70 μg/kg bw, similar to the one for MC-LR, can be derived.

Mechanism and mode of action

NOD is a hepatotoxic compound causing liver damages very similar to the typical ones following MC intoxication; for this reason and based on the similar chemical structure their MoA and MeA are considered overlapping with MC. There are few specific studies on NOD molecular mechanism of toxicity: they allow to confirm the similarity, but also to evidence some differences. The major one is related to the protein phosphatases inhibition: although it mainly involves PP2A (IC_{50} = 0.17 nM in human red cell) (Ufelmann et al. 2012), and it is the base of MeA also for NOD, at variance with MC, phosphatase inhibition is characterized by a noncovalent binding (Bagu et al. 1997).

In vitro PP1 and inhibition PP2A stimulate cell response through a cascade of events related to oxidative stress, induction of apoptosis and cell proliferation with increase of caspase activities (at concentrations ≥ 100 nM), in phosphorylated ERK1/2, p90RSK, p85S6K, p70S6K as well as

p38 and a late induction of the antiapoptotic Bcl-xL (Ufelmann and Schrenk 2015). Apoptosis was actually reported in HepG2 with induction of Fas receptor and Fas ligand expression, at both mRNA and protein level, in a time- and dose-dependent manner. Nuclear translocation and activation of p65 subunit of NF-κB seemed to be involved in this process as evidenced by the reduction of effects using p65 knockdown cells (Feng et al. 2011). Furthermore, during the early phase of NOD-induced apoptosis, with ROS formation, the cytosolic antioxidant Cu, Zn superoxide dismutase (SOD1) was phosphorylated with disruption of its co-localization with actin in hepatocytes and consequent cytoskeletal rearrangements (Hjørnevik et al. 2012). The involvement of the phosphorylation of acyl-CoA-binding protein (ACBP) in the apoptotic process was also reported (Solstad et al. 2008).

NOD induces inflammation, ER stress and associated UPR in liver cells acting via TNF-α with influence on the expression of IL8, DR5 and Caspase 8 and involvement of MAPK pathway, as evidenced by induction of C*jun* and C*fos* in human hepatoma cells (Huh7) (Meili et al. 2016). Using MC-LR, in the same cell line, the inflammatory process was activated via NFκB with induction of INF-α and TNF-α (Christen et al. 2013), a further difference between the two groups of toxins.

Finally, in B6C3F1 mouse splenocyte cultures NOD affected T lymphocyte functions with a concentration-dependent inhibition of the lymphoproliferative activity via transcription factor NF-AT, which leads to a diminution in IL-2 and IL-4 gene transcription (Yea et al. 2000). In vivo the i.p. treatment with 8 μg/kg bw of NOD induced several lysosomal enzymes in mouse liver (Lankoff and Kolataj 2000, 2001).

Local toxicity

No specific data on NOD data are available.

Repeated-dose systemic toxicity

No data on repeated toxicity relevant for risk assessment are available. However, it is reasonable to consider that, as a worst case, NOD repeated toxicity can be compared with MC-LR and therefore to adopt the NOAEL value identified for MC-LR, expressing NOD concentrations as MC-LR equivalents.

Genotoxicity

NOD genotoxicity has not been tested on bacteria. In an in vitro study with HepG2 cells, NOD (1–10 μg/mL) increased in a dose-dependent manner the basal DNA-strand breaks and the relative frequencies of micronuclei containing centromeres (MNC+), likely due to aneugenic activity (Lankoff et al. 2006). Furthermore, NOD seemed to interfere with the NER DNA repair via the ERCC1/XPF inactivation, inducing oxidative DNA damage in HepG2 and CHO cells (Lankoff et al. 2008). Consistently, the 8-oxo-dG, a common biomarker of oxidative DNA damage, was significantly enhanced in primary cultured hepatocytes and in vivo in rat liver cells (NOD 2 and 10 ng/mL and 50 μg/kg bw, in vitro and in vivo, respectively). It has however to be noted that the in vivo treatment dose was similar to the LD_{50}, meaning that toxicity in the liver with hepatocyte necrosis was present possibly interfering with the obtained results. When NOD and MC-LR were compared, difference in ROS production kinetics and oxidative DNA damage induction was observed: in primary cultured rat hepatocytes NOD seemed to induce 8-oxo-dG formation more rapidly than MC-LR, but at lower level, in vivo the rate of adduct formation induced by NOD was delayed (Maatouk et al. 2004; Bouaïcha et al. 2005).

Carcinogenicity

NODs are considered tumor promoters without initiation capability (Ohta et al. 1994; Song et al. 1999). In an in vivo study NOD did not induce hepatic tumors in transgenic (HBVgene^{+}) and wild-type mice i.p. treated once a week for 15 weeks with 10 μg NOD/kg bw (Lian et al. 2006). In the two-stage carcinogenesis models in rat, NOD (25 μg/kg bw i.p.) induced the formation of GST-P($^{+}$) liver foci in initiated animals with higher volume and area of foci/liver, but lower number, than MC-LR. NOD alone induced hepatocyte proliferation until week 18 (increased PCNA index) (Song et al. 1999).

However, according to the IARC evaluation, NODs are not classifiable in relation to their carcinogenicity to humans (Group 3), due to the scant amount of available data (IARC 2010).

Reproductive/developmental toxicity

In a study testing the promoting activity of NOD in rats, testosterone levels decreased after NOD i.p. injections (25 μg/kg bw for 2 days, no effect when initiator alone was administered) (Park et al. 2002). Also 17α-hydroxylase and pregnenolone decreased levels as well as effects on spermatogenesis and spermatogonia were reported with respect to control animals after NOD treatment, but since the initiator alone showed similar or higher effects, no conclusion can be drawn. Lysosomes proliferation in Sertoli cells and increase of peroxisomal granules in Leydig cells with signs of apoptosis were observed in initiated and noninitiated rats. Inhibition of expression of mRNA expression of stAR gene up to 80, 60 and 30% in the initiator alone, plus NOD,

and NOD alone rats, respectively, was evidenced (Park et al. 2002).

In in vitro an estrogenic activity of NOD with a dose-dependent increase in the luciferase activity (EC_{50} = 66.4 nM) was evidenced (Oziol and Bouaïcha 2010).

Human health effects and epidemiological data

No data are available.

Animal poisoning

There are several papers reporting wild and domestic animal poisoning in the past decades (1970–1990) attributed to NOD exposure (see Chen et al. 2013a, b). However, the association was often based on indirect observations (e.g., presence of NOD in water bodies), rather than by detecting NOD in the tissues with a clear cause–effect relationship. Two recent studies evidenced centrilobular hepatocellular and renal tubular necrosis (Simola et al. 2012) and also lymphoid depletion from different organs (Algermissen et al. 2011) in dogs dead after NOD intoxication. In the first study NOD concentrations in liver, kidney and intestine were 490, 500 and 5 mg/kg DW, respectively, and no metabolic products were detected (Simola et al. 2012).

Cylindrospermopsin

Cylindrospermopsin (CYN) was first reported in 1979 after an hepatoenteritis outbreak occurred in Palm Island (Northern Queensland, Australia) (Bourke et al. 1983), ascribed to a *Cylindrospermopsis raciborskii* bloom in the local drinking water supply.

The chemical structure of CYN was firstly characterized in 1992 by Ohtani et al. (1992) according to which CYN is a tricyclic alkaloid consisting of a tricyclic guanidine group combined with a hydroxymethyl uracil (Fig. 6a). Due to its zwitterionic nature, CYN is a highly water-soluble compound (Chiswell et al. 1999). It is believed to derive from a polyketide that uses an amino acid starter unit such as glycocyamine or 4-guanidino-3-oxybutyric acid (Duy et al. 2000). Structural variants have been identified as naturally occurring compounds, such as the 7-epi-CYN and the 7-deoxy-CYN (Norris et al. 1999; Banker et al. 2000) (Fig. 6b, c). Other 2 variants were identified in the Thai strain of *C. raciborskii,* 7-deoxy-desulfo-CYN and 7-deoxy-desulfo-12-acetyl-CYN, but the authors themselves could not conclude on their nature: actual congeners, precursor of degradation products (Wimmer et al. 2014).

Producing organisms

Besides *Cylindrospermopsis raciborskii*, many other CB have been identified as CYN producers, including *Aphanizomenon* (now *Chrisosporum*) *ovalisporum* (Banker et al. 1997), *Anabaena lapponica* (Spoof et al. 2006), *Aphanizomenon flos-aquae* (Preußel et al. 2006) and *Raphidiopsis curvata* (Li et al. 2001), for which also molecular information is available. For a recent review on the new systematic assignation within the *Aphanizomenon* group see Cires and Ballot (2016). Other species producing CYN are *Umezakia natans* (Harada et al. 1994) and *Lyngbya wollei* (Seifert et al. 2007).

Cylindrospermopsis raciborskii is a N_2-fixing filamentous species, found in many tropical/subtropical regions of the world and more recently in temperate regions, thanks to its physiological flexibility and probably due to the increasing surface temperature (Burford et al. 2016; Funari et al. 2012). A high intraspecific variability allows the population to deal with fluctuating habitat conditions, in terms of nutrient concentration and other parameters like light (Burford et al. 2016). While it is probable that the increasing temperature due to climate change will favor the diffusion and the success of *C. raciborskii*, it is not clear whether the toxic or nontoxic strains will be favored (Funari et al. 2012). Only the Australian strains of *Cylindrospermopsis* produce cylindrospermopsin (CYN) (Saker and Griffiths 2000), whereas the Brazilian or European strains produce saxitoxin and a still unknown toxin, respectively (Fastner et al. 2003; Lagos et al. 1999).

Among the other CYN-producing organisms, *Chrisosporum ovalisporum* has a cosmopolitan distribution; *Aphanizomenon gracile* and *Aph. flos-aquae* are the most important CYN producer in Europe (Cires and Ballot 2016).

Intracellular versus dissolved toxin

CYN production in *Cylindrospermopsis* is probably constitutive (Willis et al. 2015), even if it is highly variable among many strains isolated throughout Australia, with a CYN cell quota ranging from 0 to 55.8 fg/cell (Orr et al. 2010; Willis et al. 2016). Also the ratio of CYN/deoxy-CYN varies between strains and during the different growth phases, even if CYN seems to be the most important product, ranging from 46 to 99% in two isolates of *C. raciborskii* (Davis et al. 2014).

At variance with MC, the dissolved CYN concentrations are generally higher than the intracellular ones; when present, they ranged between 20 and 99%, both in field in *Aphanizomenon* dominated lakes and in laboratory samples

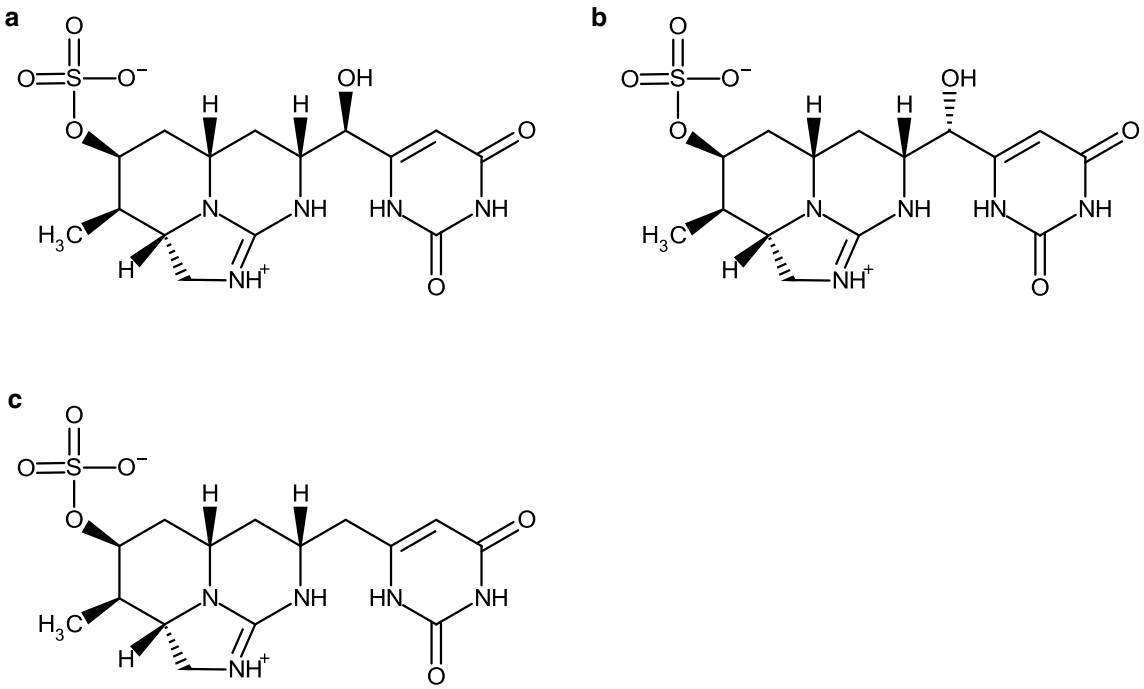


Fig. 6 Chemical structure of CYN (**a**), 7-epi-CYN (**b**) and deoxy-CYN (**c**)

of *C. raciborskii* (Davis et al. 2014; Rücker et al. 2007). Although the dissolved fraction level can be reduced by dilution in the water body, wind mixing, adsorption to the sediment and (bio)degradation, the persistence in water can be longer than one month (Wormer et al. 2008).

Other freshwater cyanobacterial species CYN producing have a more variable cell quota (Table 9) differing over the growth cycle, suggesting that CYN may have a different role in these species. The extracellular share of CYN in European *Aphanizomenon* strains seems related to stress conditions caused by environmental factors, like light, temperature and nutrients (N and P) concentrations, which promote the active export of CYN from the cells, with slight different outcome in diverse strains (Preußel et al. 2009, 2014). In three *Aphanizomenon* strains, with intracellular CYN + deoxy-CYN concentration ranging between 290 and 801 ng/mm^3, N limitation determined a twofold to sixfold increase in intracellular content, while P limitation caused an overall significant increase in CYN production and in the extracellular fraction between 15 and 90%; the share of deoxy-CYN ranged between 5 and 40% of the toxin production (Preußel et al. 2014). In an Israelian strain of *Aph. ovalisporum*, with an initial CYN content of 5.21 µg/mg (or 2.17 ng/cell), sulfur and P limitation reduced the CYN content to <20% (Bácsi et al. 2006).

The isolate PCC 6506 of a benthic *Oscillatoria* had a quite high share of extracellular CYN, >80% (intra-CYN between 1.9 and 2.6 fg/cell and extra-CYN between 9.3 and 12.4 fg/cell) (Bormans et al. 2014). Two populations of the freshwater mat-forming *L. wollei* from southeast Queensland, Australia, produced CYN and deoxy-CYN in a ratio opposite to planktonic species, with field concentrations of CYN ranging from <0.1 to 33 and deoxy-CYN from 0.5 to 546.8 µg/g DW (Seifert et al. 2007). The overall concentration was lower than that observed in planktonic *Aphanizomenon flos-aquae* of 2300–6600 µg/g DW (Preußel et al. 2006). Still, the authors estimated that concentrations up to 50 mg/m^2 CYN and 820 mg/m^2 deoxy-CYN can be expected if the Australian *L. wolley* populations reached a biomass comparable to those in other states, like USA (Seifert et al. 2007).

Occurrence

Surface and drinking water

As shown in Table 3, the highest levels of CYN (173 µg/L) in the environment have been reported in an arid lake in Saudi Arabia (Mohamed and Al-Shehri 2013). In natural conditions, photodegradation occurs and it is inversely proportional on the water depth (Chiswell et al. 1999; Wormer et al. 2010), whereas CYN biodegradation by the microbial community seems to be not relevant (Wormer et al. 2010), although Dziga et al. (2016) found that *Aeromonas* sp. was capable of CYN removal depending on pH and temperature.

CYN is generally reported at low concentrations in drinking water; however, in a study in Kinmen Island

Table 9 CYN and deoxy-CYN cell quota in isolated strains, in laboratory and experimental conditions, and field samples

Toxin	Gender/species/strain	Cell quota (µg/g DW)	Country of origin	Analytical method	References
Cylindrospermopsin	*Aphanizomenon flos-aquae*	2300–6600	Germany	LC–MS/MS	Preußel et al. (2006)
	Aphanizomenon ovalisporum ILC-164	5.21×10^3		capillary electrophoresis	Bácsi et al. (2006)
	A. ovalisporum UAM 289	max value: 190.6 fg/cell		LC–MS/MS	Cirés et al. (2011)
	A. ovalisporum UAM 290	gradient of T (10–40 °C); irradiance (15–340 µE/m^{2}s)			
	Cylindrospermopsis raciborskii	568 (bloom)	Saudi Arabia	ELISA-LC–MS/MS	Mohamed and Al-Shehri (2013)
	C. raciborskii SDS *C. raciborskii* SDC	different light intensities (18–140 µmol photons/m^2/s) min–max: 0–1.39 µg/µg Chl-a	Queensland	HPLC–UV	Dyble et al. (2006)
	C. raciborskii CR-CR7	≈1–4 µg/mm^3 cell[a]	Australia	LC–MS/MS	Saker and Griffiths (2000)
	Lyngbya wolley	0–2.9 (bloom)	Queensland	QTRAP-LC–MS/MS	Mc Gregor and Sendall (2015)
	Oscillatoria PCC 6506	Intracellular min–max: 1.9–2.6 fg/cell extracellular min–max: 9.3–12.4 fg/cell		ELISA	Bormans et al. (2014)
Cylindrospemopsis +deoxy-cylindrospermopsis	*Aphanizomenon* sp. 10E9 *Aphanizomenon* sp. 22D11 *Aphanizomenon* sp. 30D11	Intracellular min–max: 290–801 ng/mm^3 Extracellular min–max: 22–80 ng/mm^3	Germany	LC–MS/MS	Preußel et al. (2014)
	C. raciborskii	24 strains min–max: 95–232 fg/cell	Queensland	HPLC	Willis et al. (2016)
	C. raciborskii CS-505	~ 2.5×10^3–3.5×10^3[a]		LC–MS/MS	Stucken et al. (2014)
	Lyngbya wolley	CYN = 0–2.9 Deoxy-CYN = 3–86	Queensland	QTRAP-LC–MS/MS	Mc Gregor and Sendall (2015)
	L. wolley[b]	CYN = <0.1–33 Deoxy-CYN = 0.5–546.8	Queensland	LC–MS/MS	Seifert et al. (2007)
	Raphidiopsis curvata HB1	CYN: 0.56 Deoxy-CYN: 1.3 x10^3	China	HPLC	Li et al. (2001)

DW dry weight, *Chl-a* chlorophyll-a

[a] Values taken from figures, [b] field sample isolated and cultured for 18 months

(Taiwan), CYN was found in all the 15 tap water investigated, in a range of 0.69–2.2 µg/L. In the same samples *Cylindrospermopsis* spp. cells were efficiently removed from raw waters during the treatment with slow sand filters. Therefore, the presence of CYN in tap waters may be very likely due to its occurrence in raw water mainly in the dissolved form (Rücker et al. 2007). This is true especially in old blooms (Chiswell et al. 1999), where it can persist, since dissolved CYN along the water column is slowly degraded (Chiswell et al. 1999).

In a stability test in chlorinated water (Senogles et al. 2000) when the dissolved organic carbon content is low, relatively low chlorine doses (<1 mg/L) are sufficient to degrade CYN.

Fish and seafood

As shown in Table 5, the few available data show that generally CYN occurs at low levels in fish muscles, with values up to 1.3 ng/g. No data are available in seafood, except a value of 3.35 ± 1.90 ng/g in a freshwater snail in Mexico (Berry and Lind 2010). It is not degraded at boiling temperature (100 °C for 5 min); therefore, cooking procedures do not alter its concentration in food.

Food supplements

In two published studies, CYN was not detected in food supplements (Heussner et al. 2012; Liu and Scott 2011).

Crops

No field data are available on CYN occurrence in crops. Laboratory studies indicate that exposure of Brassica roots to the toxin may result in CYN levels in leaves ranging from 10 to 21% the concentration applied to the root. In seedlings, CYN concentrations up to 49 µg/g FW were observed (Kittler et al. 2012).

Biosynthesis and genetics

Characterization of the gene cluster for CYN biosynthesis (*cyr*) was firstly described in *Cylindrospermopsis raciborskii* AWT205 (Mihali et al. 2008). It encompasses 15 open reading frames, spanning a total of 43 kb, encoding an amidinotransferase (*cyrA*), an hybrid NRPS/PKS (*cyrB*), PKS (*cyrC*, *cyrD*, *cyrE* and *cyrF*), amidohydrolases (*cyrG* and *cyrH*), tailoring (*cyrI*, *cyrJ* and *cyrN*), putative transport (*cyrK*) and regulation (*cyrO*) enzymes, that assemble the various constituents into the CYN structure, as well as two transposases (*cyrM* and *cyrL*), plausibly responsible for the horizontal transfer of the whole cluster, and therefore the toxicity potential. The *cyr* gene cluster was later described for other CYN-producing genera, as *Anabaena*, *Aphanizomenon* (Stuken et al. 2009) and *Raphidiopsis curvata* (Jiang et al. 2012), showing a high similarity of the sequences, while the main difference was seen in their organization and location, suggesting the ability of the cluster of jumping along the genome as a mobile genetic element, which has already been observed in other CB.

Number of *cyr* cluster in samples positively correlates with densities of potentially toxic CB, while the absence of toxicity in *C. raciborskii* has been observed to be associated with the loss of the gene cluster, rather than to point mutations or partial deletions (Stuken et al. 2009). This hypothesis was further supported by a recent study (Sinha et al. 2014) in which the genomes of three closely related *C. raciborskii* strains, the toxic CS-505 (CYN+) and CS-506 (CYN+) and the nontoxic CS-509 (CYN−), were compared, showing that the CS-509 (CYN−) strain lacked *in toto* the complete set of *cyr* genes, possessed, on the contrary, by the two CYN producer strains CS-505 and CS-506.

In addition, two further genetic clusters were identified in *C. raciborskii*, named NRPS1 and NRPS2, involving polyketide synthetase (PKS) and nonribosomal peptide synthetase (NRPS), whose function has not clearly characterized so far, but plausibly responsible for the synthesis of additional bioactive secondary metabolites (Sinha et al. 2014).

A posttranscriptional regulation of the CYN biosynthesis was proposed to occur in *C. raciborskii* when exposed to different light and CO_2 regimens, due to the observation that transcriptional levels of *cyr* genes such as *cyrA* (amidinotransferase) and *cyrK* (putative multidrug exporter) did not showed any correlation with total CYN cell quotas, thus suggesting that the cellular CYN pool size is probably regulated at the protein level (Stuken et al. 2014; Pierangelini et al. 2015).

Toxicological profile

Kinetics

Only few studies are available, aimed to understand CYN kinetic behavior. The molecular structure of CYN confers it hydrophilic features; for that reason its intestinal absorption and the uptake into hepatocytes need to be mediated by an active transport systems, such as the bile acid transport system, e.g., cholate and taurocholate (Chong et al. 2002). When [^{14}C]-CYN (0.2 mg/kg bw) was i.p. injected to mice, radioactivity was recovered mainly in the liver and, to a lesser extent, in the kidney (20.6 and 4.3% of the administered dose 6 h after the treatment, respectively). A limited passive diffusion through biological membranes occurs, as evidenced in cell line not expressing bile acid transport system, where cytotoxic effects were observed (Chong et al. 2002), likely due to the small size of the molecule. In the primary mouse hepatocytes, a rapid uptake can be hypothesized since CYN-induced effects were already evidenced 4 h after treatment (Froscio et al. 2003), while in Vero cell line the entry was slow, even if progressive (Froscio et al. 2009a, b) and the permeation across the Caco-2 cell monolayer were limited but time and concentration dependent (Fernandez et al. 2014): integrity of the monolayer was not affected, and no cytotoxicity was evidenced.

There are several papers suggesting on the basis of indirect observations the possible CYP450-mediated bioactivation of CYN (see Funari and Testai 2008). This hypothesis is supported by the co-localization of hepatic toxicity mainly in the periacinar region, where substantial

CYP450-mediated xenobiotic metabolism occurs (Shaw et al. 2000) and by the in vivo and in vitro effects of inducers and inhibitors of P450 on CYN toxicity (Norris et al. 2002; Froscio et al. 2003; Humpage et al. 2005; Bazin et al. 2010b). Despite these observations, direct proof of CYN biotransformation was not provided, except for a recent paper on HepaRG cells, a cell line of human origin known to be metabolically competent, rat and human liver tissue, showing that after 24 h incubation of 72 nM CYN no phase I biotransformation can be detected in vitro (Kittler et al. 2016). Phase II metabolism was not investigated. The decrease in parent toxin concentration at the end of the incubation period with liver HepaRG cell line or mammalian liver tissue fractions was minimal, suggesting that other phenomena (e.g., irreversible protein binding) were not significantly masking metabolite formation (Kittler et al. 2016). However, since 9% of the initial CYN concentration was lost, it is not possible to exclude that some reactive metabolites are formed. And indeed treating the same cells with ketoconazole, a known CYP3A4 inhibitor, the cytotoxicity is significantly decreased: to explain it, the authors hypothesized that ketoconazole can affect the cellular uptake/efflux (indicating that further research is needed also in this respect) or be protective against the oxidative stress (Kittler et al. 2016), but again it cannot be ruled out that inhibition of active metabolite(s) formation could be one of the cause for the reduced toxicity. When HepG2 was treated with CYN (10 µg/L) with or without cytochrome P450 inducers, no effect was observed (Liebel et al. 2015), but the metabolic competence of that cell line is so poor that actual differences were not expected. The substantial GSH depletion, observed after CYN oral administration to rats, leads to the hypothesis that CYN can be further metabolized by GSH conjugation or alternatively that GSH synthesis is inhibited by the toxin (Runnegar et al. 1995).

Urinary and fecal excretions were quite rapid: indeed, the great majority occurred during the first 0–12 h (25 and 26.9% of the administered dose in urine and feces, respectively) with negligible excretion after 24 h. Most of the excreted radiolabel (about 70%, corresponding to around 50% of the administered dose) was associated with the parent compound (Norris et al. 2001). At 48 h after exposure the average hepatic content was 7.7% (range 4.7–23.2%), whereas less than 1% was found in kidneys or spleen. Considering the tissues content, 48 h after treatment the recovery was 85–90% (Norris et al. 2001).

Acute systemic toxicity

Liver and kidney are the main target of CYN acute toxicity, but effects in other organs are also reported (Terao et al. 1994; Falconer et al. 1999; Seawright et al. 1999). Acute hepatic damage is localized in the centrilobular areas with hepatocyte vacuolization and increased pigmentation of nuclei and cytoplasm. Renal toxicity is characterized by necrosis and increased lumen of proximal tubules and alterations in the glomerulus. Data are available on crude extracts or freeze-dried cells after oral or i.p. administration (Banker et al. 1997; Falconer et al. 1999; Seawright et al. 1999), but the sometimes poor toxin quantification and the co-presence of other potentially toxic components made them of limited value to derive an LD_{50} value. Regarding the pure toxin, only an i.p. study in mice is available showing that LD_{50} is dependent on the duration of the observation time, being 2.1 mg/kg bw after 24 h, 0.2 mg/kg bw at 120–144 h (Ohtani et al. 1992). Although the value is cited in most of the papers as the relevant LD_{50}, it is simply indicated without any further experimental detail in a 'note' to the cited paper, dealing with a purification method for CYN. However, data are in line with results obtained with freeze-dried cyanobacterial extracts (i.p. LD_{50} at 7 days: 0.18 mg CYNeq/kg bw); when they were administered via the oral route, the LD_{50} was 4.4–6.9 mg CYN equivalent/kg bw after 2–6 days (Seawright et al. 1999), likely due to kinetics differences.

After intratracheal injection of 70 µg/kg bw semipurified extracts to mice, CYN impaired lung mechanics with parenchyma inflammation and production of inflammatory mediators IL-1b, IL-6 and KC, induced alveolar collapse, polymorphonuclear cells, fiber deposition and oxidative stress (Oliveira et al. 2012, 2015a, b).

Several in vitro studies from the same group reported on the possible immunotoxic effect of CYN. In human peripheral blood lymphocytes proliferation was stimulated by 1 µg CYN/mL (Poniedziałek et al. 2012), likely related to CYN-induced cytopathic response at lower concentration CYN (0.01–1 µg/mL). Cytotoxic effects are associated with oxidative stress due to concentration-dependent increase in H_2O_2 content, decrease of SOD and CAT activity, elevated level of glutathione peroxidase and apoptosis with potential reduced capacity to fight pathogenic microorganisms (Poniedziałek et al. 2014b, 2015). Human peripheral blood neutrophils reacted to CYN (0.01–1 µg/mL) decreasing ROS production (necessary for pathogen elimination), although their phagocytic activity remained unchanged (Poniedziałek et al. 2014a). However, no in vivo indication of immunotoxic effect has been reported so far.

Mechanism and mode of action

The mechanisms involved in CYN toxicity in different organs are not fully elucidated, although some pathways have been described and schematically illustrated in Fig. 7.

It is known and proved both in vitro (in primary mouse hepatocyte cultures; Froscio et al. 2003) and in vivo (Terao et al. 1994) that CYN induced a concentration-dependent

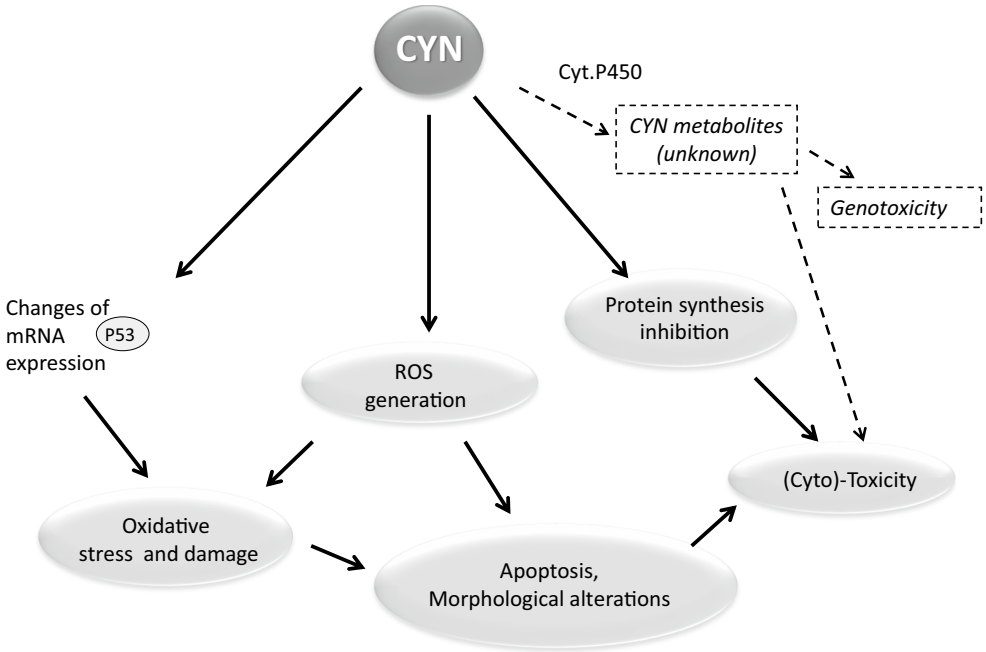


Fig. 7 Pathways of the CYN mechanism of action. *Dotted lines* indicate the hypothesized but not yet clarified pathways

protein synthesis irreversible inhibition leading to (cyto)-toxicity in the liver and the kidney. By using cytochrome P450 inhibitors, CYN toxicity decrease but no protection against the impairment of protein synthesis was observed (Froscio et al. 2003) suggesting that the parent compound and the possibly formed metabolites could exert toxicity with a different mechanisms, also depending on CYN concentrations. To support this, treatment of poorly metabolically competent cells (HepG2, Caco2 and human dermal fibroblasts) with up to 5 µg CYN/mL, results in inhibition of cell proliferation within 48 h, but no LDH activity release (as marker of cytotoxicity) was measured (Bain et al. 2007). These aspects are still unclear, since—as discussed above—CYN metabolites have not been identified so far, and indication of metabolism still remains indirect, including the evidence that in HepG2 cells CYN induced the upregulation of genes coding for phase I enzymes (CYP1A1, CYP1B1, ALDH1A2 and CES2) and phase II enzymes (UGT1A6, UGT1A1, NAT1 and GSTM3) (Straser et al. 2013c). Some in vitro studies suggested some possible key steps in CYN MeA, such as induction of stress responses that result in the activation of the p53 transcription factor as described by the concentration-dependent increase in mRNA levels of p53 target genes CDKN1A, GADD45a, BAX and MDM2 in cultured human dermal fibroblasts and HepG2. CYN induced an early activation of p53 (Bain et al. 2007), presumably due to the native toxin, since the used cells do not show a significant metabolic activity. Involvement of oxidative stress was confirmed by other studies: in human umbilical vein endothelial cell line (HUVEC) the intracellular ROS production was increased with ≥0.3 µg CYN/mL, c-glutamylcysteine synthetase and GSH content showed a concentration-dependent increase starting from 0.75 µg CYN/mL (Gutierrez-Prena et al. 2012a); intracellular formation of ROS was observed also in HepG2 (0.05, 0.1 and 0.5 µg CYN/mL) just after 30 min of exposure, steadily increasing with incubation time (Straser et al. 2013c). Consistently with oxidative stress induction, apoptosis was observed in (i) HUVEC cells, together with morphological alterations as nucleolar segregation with altered nuclei, degenerated Golgi apparatus, increases in the presence of granules (Gutierrez-Prena et al. 2012a) and (ii) in primary rat hepatocytes (treatment range 90–360 nM CYN) with induction of the transcription of the antioxidant response element (ARE)—binding factor, Nrf2 (Lopez-Alonso et al. 2013). Also in human intestinal Caco-2 cells CYN was able to induce oxidative stress and ultrastructural alterations, mitochondrial damage and nucleolar segregation with altered nuclei at the lowest concentrations tested (0.3 µg/mL) (Gutierrez-Praena et al. 2012b).

Despite these many evidences for an involvement of oxidative stress in the CYN MeA, a toxicogenomic study on HepG2 showed that only among the oxidative response genes only catalase (CAT) and thioredoxin reductase (TXNRD1) were upregulated, whereas other genes as NOS2 and SOD1 or the glutathione detoxification and antioxidant pathway (GSTs, GSS, GSR, GPXs and GGT1)

seemed not to be affected (Straser et al. 2013c). When the transcriptomic profile of Caco-2 cells exposed to a subtoxic concentration of CYN (1.6 µM for 24 h) was studied, proteins involved in DNA repair were overexpressed (upregulation of *aptx* and *pms2* genes) and nucleosomal histones were modified (Huguet et al. 2014). In HepG2, at noncytotoxic concentrations, in the absence of apoptosis, some DNA damage was observed: the authors considered it not as the consequence of oxidative stress (Straser et al. 2013a, b), but no alternative explanation was given.

There was also some evidence of effects after 21-day exposure to purified CYN (66 µg/kg bw/day) on morphology of red blood cells (RBCs) showing an abnormal spiked surface tentatively associated with an increased cholesterol content in the RBC membrane due to inhibition of plasma lecithin cholesterol acyl transferase (Reisner et al. 2004). However, the study evidenced that the kidney was the most sensitive target of toxicity (Reisner et al. 2004).

Local toxicity

Lyophilized extracts from CYN-producing CB have been associated with moderate skin irritation and skin sensitization potential (Torokne et al. 2001), but the positive response was likely due to other components as supported by results obtained with the mouse ear swelling test (MEST) according to which the purified CYN produced a response in 22% of animals versus the 80% elicited by a *C. raciborskii* suspension (Stewart et al. 2006d) and by results in humans (Bernstein et al. 2011).

Repeated-dose systemic toxicity

Several repeated toxicity studies are available, but only one has been conducted with the pure toxin: CYN was orally administered to mice for 11 weeks by gavage (0, 30, 60, 120 and 240 µg/kg bw/day) (Humpage and Falconer 2003). A dose-dependent increase in liver and kidney weight (relative at ≥60 µg/kg bw/day 12–23% greater than controls), alteration in plasma enzymes (used as markers for hepatic and renal toxicity) and consistent histopathological changes at the high doses were evident. The renal effects occurred at lower doses, and a NOAEL of 30 µg/kg bw/day was identified on the basis of increased kidney weight observed at the immediately higher dose tested (dose at which also the urinary protein content was altered) (Humpage and Falconer 2003). When *C.raciborskii* extracts dissolved in water were administered orally for 10 weeks effects on kidney and liver were evidenced at the lowest dose tested (216 µg toxin equivalents/kg bw/day) (Humpage and Falconer 2003). Results are qualitatively supported by an additional oral subchronic study with CYN administered in drinking water for 42 weeks at gradually increasing daily doses, ranging from 10 to 55 µg/kg bw (Sukenik et al. 2006). The experimental design does not allow to derive a robust enough reference value (see Funari and Testai 2008).

Genotoxicity

No genotoxicity response in the Ames test was reported. Neither an increase in the number of revertants nor an inhibition of the growth of bacteria with or without metabolic activation was observed (Sieroslawska 2013).

However, when mammalian cells have been used, a dose-dependent increase in DNA damage was evidenced, prevented by chemical CYP450 inhibitors (Humpage et al. 2005). No chromosome aberrations were detected in CHO-K1 cells after the exposure to CYN with or without metabolic activation (Fessard and Bernard 2003; Lankoff et al. 2007). In contrast CYN induced MNi and MNBNC formation in two human cell lines HepaRG (hepatic origin, high metabolically competent) and Caco-2 (from colon carcinoma) (Bazin et al. 2010b). In Caco2 the presence of ketoconazole (P450 inhibitor) reduced cytotoxicity and genotoxicity to 50%; with HepaRG the presence of ketoconazole leads to no micronucleus induction and significant protection from CYN cytotoxicity at all tested concentrations (Bazin et al. 2010b). DNA double-strand breaks in HepG2 (human hepatoma cell line) were also reported (Straser et al. 2013a, b).

In vivo CYN induces DNA-strand breakage in the colon of i.p. injected mice (100 and 200 µg/kg bw) and in the bone marrow (1 and 2 mg/kg bw) and colon (4 mg/kg bw) after oral administration (Bazin et al. 2010a). It has however to be noted that the treatment doses were quite high (inducing high levels of cytotoxicity, which is always associated with DNA damage), thus limiting the validity of the study. The genotoxicity of CYN (and/or its metabolites) is therefore still controversial.

Carcinogenicity

No in vivo reliable data with pure CYN are available. Indeed some preliminary data on CYN carcinogenicity (Falconer and Humpage 2001), questionable due to flawed study design (see Funari and Testai 2008), were never confirmed.

In the Syrian hamster embryo (SHE) cells the CYN cell-transforming potential was reported at very low, but not cytotoxic, doses (10^{-7}–10^{-2} ng/mL), while at higher concentrations cell transformation was not observed (Maire et al. 2010).

Reproductive/developmental toxicity

After oral treatment of rats (0.03–3 μg/kg bw for 20 days), parameters used as marker for reproductive performance and malformations in the fetus were not altered (de Almeida et al. 2013). This is in line with previous findings (Rogers et al. 2007) showing that i.p. administration in the range 8–128 μg/kg bw to pregnant mice (GD 8–12), reduced litter size in treated groups only in the presence of maternal toxicity at doses ≥32 μg/kg bw (16 μg/kg bw was considered as the NOAEL for maternal toxicity); however, pups at PND 6 showed normal development (Rogers et al. 2007). Changing the exposure window to GD 13–17, a significant number of pups were found dead and incidences of blood in the gastrointestinal tract and hematomas in the tips of the tails were reported in survivors, despite similar maternal toxicity (Chernoff et al. 2011, 2014).

In vitro CYN up to 3 μM (6 h treatment) was not cytotoxic and did not alter the balance of progesterone/estrogen ratio in primary human granulosa cells from women with normal reproductive physiology (Young et al. 2008, 2012), although protein synthesis was inhibited at the high concentration of the tested range (0.15–3 μM). The only effect was the hCG-stimulated progesterone production inhibition observed when cells were obtained from a cohort of females including infertile subjects at 0.15 μM CYN (Young et al. 2008).

When undifferentiated mouse embryonic stem cell (mES) was treated in vitro with CYN (0–1 μg/mL for 24–168 h), the inhibition of protein synthesis (as marker for viability), the expression of differentiation marker genes, and the morphology of differentiating neural-like cells were not affected up to 1 μg CYN/mL for 72 h; proliferation only decreased at the highest concentration after 168 h. The NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) were therefore 0.5 and 1 μg/mL (Reid et al. 2015).

Human health effects and epidemiological data

Data on outbreaks associated with the presence of *C. raciborskii* were reported in Australia, as for the 'Barcoo fever' in outback Australia (Hayman 1992), or the hepatoenteritis outbreak at Palm Island (Byth 1980). In the latter case, a dam was treated with copper sulfate to control a dense algal bloom of *C. raciborskii* causing cellular lysis: 139 children and 10 adults were hospitalized with reversible liver and renal damages (Byth 1980; Hawkins et al. 1985). In famous poisoning episode of dialyzed patients in Brazil, the presence of CYN in contaminated water was also postulated. However, since CYN quantification was not reported, the possible contribution to the observed effects cannot be evaluated (Carmichael et al. 2001). The report published by the US CDC about the algal bloom-associated disease outbreaks among users of freshwater lakes for 2009 and 2010, indicated that CYN was detected in water in 2 outbreaks. However, the co-occurrence of other cyanotoxins (MC and anatoxin-a) does not allow to understand the relative contribution to the symptoms (Hilborn et al. 2014). Dermal exposure to CYN using skin-patch testing in humans shows mild irritation, but no statistically significant dose–response relationship (Pilotto et al. 2004; Stewart et al. 2006c).

Animal poisoning

The death of cows and calves which drank from a dam infested by *C. raciborskii* was reported, with CYN detected in water and in a cyanobacterial culture isolated from the bloom by means of the mouse bioassay (Thomas et al. 1998; Saker et al. 1999).

Neurotoxins

The most studied cyanobacterial neurotoxins are alkaloid compounds, namely anatoxins and saxitoxins (also known as paralytic shellfish poisoning toxins—PSPs, mainly produced by marine dinoflagellates) (Kellmann et al. 2013). The available literature on environmental occurrence, biosynthesis/genetics, toxicology (including a description of what is known so far about their MeA) and epidemiology of alkaloid neurotoxins has been extensively addressed in a very recent review by our group to identify potential risk to human health: the reader is invited to consult it (Testai et al. 2016b). Here only some crucial issues are summarized, together with some brief considerations on other neurotoxins of concern.

The cyanobacterial alkaloid neurotoxins act on cholinergic synapses or voltage-gated ion channels blocking skeletal and/or respiratory muscles; in mammals death can occur at lethal doses due to respiratory failure (Funari and Testai 2008; Kellmann et al. 2013).

Anatoxins

Anatoxin-a (ATX) is a secondary amine bicyclic alkaloid (Devlin et al. 1977) first synthesized as a mixture of stereoisomers (Campbell et al. 1979). It is structurally related to homoanatoxin-a (HTX): the two molecules differ for the presence of a propionyl instead of the acetyl group at C-2 (Fig. 8a, b). Anatoxin-a(S) (ATX-s) is an N-hydroxyguanidine methyl phosphate ester and is the only natural organophosphate known (Fig. 8c). No structural variants of ATX-s have been detected so far.

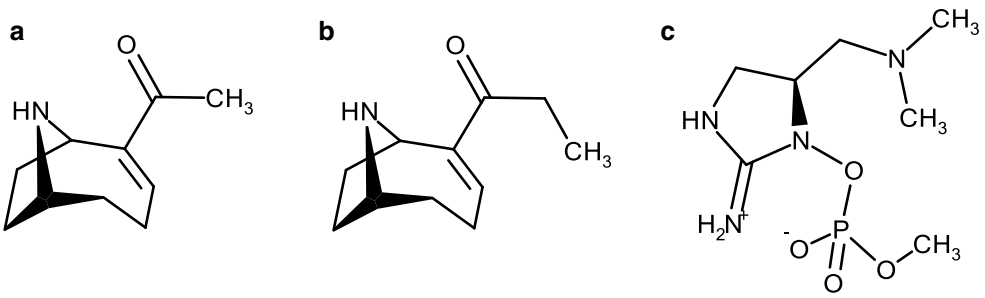


Fig. 8 Chemical structure of ATX (**a**), HTX (**b**), ATX(s) (**c**)

Producing organisms

ATX was first reported from the planktonic *Anabaena flos-aquae* (now *Dolichospermum flos-aquae*) from Canada (Carmichael et al. 1975) and later from blooms of *Anabaena* and *Aphanizomenon* spp. (*Dolichospermum* spp.) from Finland (Sivonen et al. 1989). Now several genera are known to produce ATX/HTX, the most recent discovered is the planktonic Oscillatoria *Tychonema bourrellyi* in Italy (Shams et al. 2015), but most of them are benthic ones.

Mats of Oscillatoriales *Phormidium* and *Oscillatoria* in several European countries, in Ontario (USA) and New Zealand, produce ATX/HTX at concentrations highly variable, between <1 mg/kg and 8 × 10^3 mg/kg DW (Cadel-Six et al. 2007; Faassen et al. 2012a, b; Gugger et al. 2005; Heath et al. 2010; Puschner et al. 2008; Wood et al. 2007).

In New Zealand *Phormidium*-dominated mats are very diffuse: blooms of such mats can be favored by low concentration of dissolved reactive phosphorus (<0.01 mg/L) and concentration of dissolved inorganic nitrogen >0.20 mg/L (Wood et al. 2014). The most important toxins identified in these mats are ATX, HTX, dhATX and dhHTX (Wood et al. 2007, 2014), with a high spatial and temporal variability (McAllister et al. 2016). The highest concentration was detected in the Oreti River, at 712 mg/kg DW, with a median <1 mg/kg DW. In these mats the concentration of toxins can be due to a variable ratio between toxic and nontoxic strains (Heath et al. 2010) but also to a variable concentration in toxic strains (100-fold) (Wood et al. 2012b). In one of the very few experimental studies on factors affecting toxin production in benthic species, Heath et al. (2016) showed that high nutrient concentration (P and N) reduced the content of ATX per cell in cultures; limiting P concentrations increase ATX cell quota, which is higher during the lag phase.

The production of HTXa has been shown from mats of another Oscillatoriale *Hydrocoelum lyngbyaceum* in New Caledonia, where giant clams intoxication has been reported, suggesting a possible relationship between the episodes and the presence of the neurotoxic cyanobacterium, even if it was not possible to quantify the toxin concentration (Méjean et al. 2010). Recently ATX-a has been detected for the first time in Argentina, San Roque reservoir, at concentration of 6.6 ng/L; the toxin correlated with *Oscillatoria* and *Anabaena* densities (Ruiz et al. 2013).

ATX-s is an organophosphate isolated from *Anabaena flos-aquae* in North America, *A. lemmermannii* in Denmark and *Anabaena crassa* in South America (see references in Testai et al. 2016b). The toxin has also been possibly detected by acetylcholinesterase assay in the desert crust in Qatar, where the dominant mat-forming species were *Microcoleus* and *Phormidium* (Metcalf et al. 2012). The maximum concentration detected was 3300 µg/g DW in three Danish lakes samples dominated by *A. lemmermannii* (Henriksen et al. 1997). The isolates from the same blooms showed very different toxicities, suggesting that the coexistence in different ratio of many genotypes was partly responsible for the variability of the levels detected in the bloom (Henriksen et al. 1997).

Occurrence

The update of data compared to what reported in Testai et al. (2016a) confirms that ATX is unfrequently detected in surface water. Yet, ATX can reach very high concentrations in bloom samples, up to 1 mg/L (Trainer and Hardy 2015). In a recent study in Northern Italian lakes largely used for recreational activities, ATX was found up to levels of 154 µg/L (Salmaso et al. 2016). No new data on drinking water have been published after the publication of Testai et al. (2016a).

ATX is rapidly degraded in water due to photolysis and chemical instability; therefore, its bioaccumulation into aquatic organism tissues is likely to be low (Funari and Testai 2008). The rather few data available on the occurrence of ATX in seafood generally indicated low concentrations (Testai et al. 2016a, b).

In food supplements Draisci et al. (2001) identified dihydrohomoATX (10 µg/g) and epoxyATX (19 µg/g) two nontoxic ATX metabolites in *Spirulina*-based tablets. When *Aphanizomenon*-based food supplements were analyzed, three out of 39 (7.7%) contained a range of 2.5–33 µg ATX/g (Rellàn et al. 2009), whereas other studies found the analyzed products free of ATX (Heussner et al. 2012; Rawn et al. 2007).

Regarding the occurrence of ATX-s in the environment and in food, also due to the relevant methodological difficulties given by the absence of analytical standards, little updates can be made to what reported in Testai et al. (2016a). Chatziefthimiou et al. (2016) examined drinking and irrigation water of urban and rural environments in desert areas, and none of the analyzed samples was positive for ATX-s.

Biosynthesis and genetics

Compared to the most characterized producers of MC, only a small number of anatoxin-a (ATX)-producing species have been identified and elucidated. The use of a genetic molecular approach based on the identification of the ATX-encoding genes was not feasible until the genes involved in the biosynthetic pathway leading to the production of the toxin were firstly characterized for *Oscillatoria* sp. strain PCC 6506 by Méjean et al. (2009, 2010a).

Since then, other ATX biosynthesis genes clusters have been identified in other CB species as *Oscillatoria*, *Phormidium*, *Aphanizomenon* and *Anabaena* strains by comparison with the nucleotide sequences of the *ana* gene cluster (Cadel-Six et al. 2009; Ballot et al. 2010; Wood et al. 2010b; Rantala-Ylinen et al. 2011; Shams et al. 2015), which showed a high percentage identity both in the sequence and in the relative protein function, while the main differences were seen in the organization of the genes (Rantala-Ylinen et al. 2011; Shih et al. 2013), suggesting the hypothesis of a common ancestor. Moreover, since transposase genes were shown to flank the *ana* clusters, it is highly likely that these elements had played a role in the horizontal *ana* gene transfer within the genome of anatoxin producers CB (Mejean et al. 2014).

The biosynthetic pathway of both anatoxin-a and homoanatoxin-a has been shown to be catalyzed by multifunctional enzymes belonging to a polyketide synthases (PKS) family with a modular structural organization. Details of the biosynthetic route are given in the paper by Testai et al. (2016b). Experiments performed with purified enzymes from recombinant overproducers *Escherichia coli* which fully reproduced in vitro the first steps of the proposed ATX/HTX biosynthetic pathway had demonstrated to fully agree with the predicted function of the enzymes (Mann et al. 2011; Mejean et al. 2009, 2010a, b; Dittmann et al. 2013).

The identification of *ana* cluster in several producer strains has greatly improved the monitoring of anatoxin producers in the environment and the management of water bodies in particular. Indeed, the use of specifically designed primers directed to selected genes within the *ana* gene cluster, i.e., the *anaC* and *anaF* genes, has allowed to identify the toxic populations in environmental samples (Ballot et al. 2010; Cadel-Six et al. 2009; Rantala-Ylinen et al. 2011; Shams et al. 2015), although the factors affecting the regulation of the anatoxin biosynthesis remain still unknown.

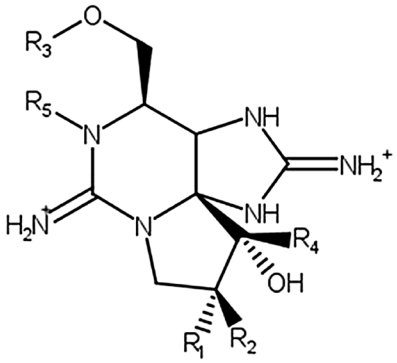
In a recent paper by Shams et al. (2015) a polyphasic approach based on microscopic identification, genetic characterization and phylogenetic analyses was applied to detect the cyanobacterial species responsible for anatoxin-a (ATX) production in Lake Garda (Italy). The identification of the toxic cells was carried out by using primers specifically designed for the *anaC* of the anatoxina synthetase (*ana*) gene cluster in the *Oscillatoria* genus (Rantala-Ylinen et al. 2011), proving to be capable also in the amplification of the anaC gene in *Tychonema*, the species producing ATX in Lake Garda, suggesting a plausible potential for a wider use also to other Oscillatoriales.

To date, the genetic cluster responsible for the ATX-s biosynthesis has not been identified. However, using feeding studies with radiolabeled amino acids, a putative biosynthesis pathway was proposed, starting from L-arginine and involving (2S,4S)-4-hydroxyarginine (or erythro-4-hydroxyarginine) as intermediate steps to yield ATX-s (Moore et al. 1992; Hemscheidt et al. 1995).

Toxicological profile

ATX is a potent pre- and postsynaptic depolarizing agent, acting by efficiently competing with acetylcholine for nicotinic receptors in neuromuscular junctions and in the central nervous system (Aronstam and Witkop 1981; Campos et al. 2006). The acute toxicity is highly dependent on the route of exposure: the i.p. LD_{50} of the >95% pure toxin in mice is 260–315 µg/kg bw (Stevens and Krieger 1991; Valentine et al. 1991) with death occurring within 2–6 min, whereas after oral administration the LD_{50} is >5000 µg/kg bw (Stevens and Krieger 1991; Fitzgeorge et al. 1994). The two studies available on subchronic toxicity of ATX (28-day treatment of mice by gavage (Fawell et al. 1999b) and 54-day treatment of rats via drinking water (Astrachan et al. 1980), indicate that a NOAEL could not be derived, since no toxic effects were evidenced even at the highest dose tested (2460 and 510 µg pure ATX/kg bw/day). For this reason WHO (2004) and US-EPA (2015c) consider the available toxicological database not adequate to derive any TDI (tolerable daily intake) for ATX, due to the high level of uncertainty for long-term exposure. Nevertheless, some countries have established legal values or given recommendations in order to prevent and/or manage the possible health effects induced by ATX exposure (Testai et al. 2016b).

Anatoxin-a(s) (ATX-s) is structurally related to carbamate and organophosphorus insecticides (Carmichael and Falconer 1993) with consequent similar mechanism of



	R1	R2	R3	R4	R5
STX	H	H	$CONH_2$	OH	H
neoSTX	H	H	$CONH_2$	OH	OH
dcSTX	H	H	H	OH	H
GTX1	H	OSO_3^-	$CONH_2$	OH	OH
GTX2	H	OSO_3^-	$CONH_2$	OH	H
GTX3	OSO_3^-	H	$CONH_2$	OH	H
GTX4	OSO_3^-	H	$CONH_2$	OH	OH
B1	H	H	$CONHOSO_3^-$	OH	H
B2	H	H	$CONHOSO_3^-$	OH	OH
C1	H	OSO_3^-	$CONHOSO_3^-$	OH	H
C2	OSO_3^-	H	$CONHOSO_3^-$	OH	H
C3	H	OSO_3^-	$CONHOSO_3^-$	OH	OH
C4	OSO_3^-	H	$CONHOSO_3^-$	OH	OH

Fig. 9 Chemical structure of STX; in the table the chemical nature of different R substituents

toxicity as noncompetitive irreversible inhibitor of acetylcholinesterase (AChE). However, while the insecticides act on AChE independently on the tissue, the action of ATX-s is limited to the peripheral nervous system. The LD_{50} in mice after i.p. injection is in a range spacing from 40 to 60 µg/kg bw (surviving time 5–30 min) (Mahmood and Carmichael 1987) to 228 µg/kg bw (Cook et al. 1988). In rats a lower i.p. LD_{50} of 5.3 µg/kg bw is reported (Cook et al. 1989). Data on oral administration as well as on subchronic and/or chronic toxicity are not available.

Saxitoxins (STX)

Saxitoxins (STX) are a group of more than 30 natural alkaloids produced by some marine dinoflagellate genera but also by freshwater CB. They are generally grouped into carbamate (STX, neoSTX and GTX1-4), sulfamate (GTX 5-6, C1-4) and decarbamoyl toxins (dcSTX dcneoSTX, dcGTX1-4) on the basis of the substituent in position R4 (Fig. 9).

Producing organisms

In America STX are produced by *Cylindrospermopsis raciborskii*, *Geitlerinema amphibium*, *Geitlerinema lemmermannii*, *Cylindrospermum stagnale*, *Phormidium uncinatum* (Borges et al. 2015) and *Lyngbya wollei* (Carmichael et al. 1997; Foss et al. 2012). In Australia the production of STX in freshwater is mainly due to *Anabaena circinalis* (Beltran and Neilan 2000). The production and allocation (intra-/extracellular) of STX in *Anabaena* and *Cylindrospermopsis* is related to environmental stress, mainly ionic stress (pH and Na^+ concentration), and is species specific. In two isolates of *A. circinalis* and *C. raciborskii,* the production of intra-/extracellular STX was 5/3 and 1/1.5 mg/L, respectively (Ongley et al. 2016); a Na^+ increase in the medium produced a decrease in STX production in *A. circinalis* and the opposite in *C. raciborskii*, although the ratio between the intra- and extracellular content was not altered in both species (Ongley et al. 2016). When pH was increased up to 9, in *A. circinalis*, STX production was reduced, and the intra-/extraratio was reversed; in *C. raciborskii* total STX was increased mainly associated with a significant increase of extracellular toxin (Ongley et al. 2016). The results on *A. circinalis* have been confirmed by a proteogenomic study, which showed that high extracellular NaCl led to a decrease in total STX and dcGTX2/3 after 48-h exposure, in addition to a decrease in the expression of SxtC at the proteomic level (D'Agostino et al. 2016).

Among the marine CB species, *Trichodesmium erythraeum* produced 2–10 µg/g STX equivalent in a bloom off the Brazilian coast (Proença et al. 2009). Along the coast of South America, in South Western Southern Ocean several blooms of *Trichodesmium* have been sampled, containing STX 0.45–3.9 µg/L STXeq, corresponding to intracellular contents of 0.2–5.7 fg/cell (Sacilotto Detoni et al. 2016).

Occurrence

STX are commonly controlled in marine edible organisms; nevertheless, in recent years their relevance in freshwater environment has increased. Loftin et al. (2016) found STX in 7.7% of the 1161 inland lakes surveyed in the USA. In a three-year study of Finnish lakes and brackish coastal waters the genes involved in STX biosynthesis were identified in 31% of the analyzed samples (Savela et al. 2015). A widespread STX distribution has been reported (Testai et al. 2016a, b), with levels up to 4466 µg STX eq/g DW in South Australia after a persistent bloom of *Anabaena circinalis* and 193 µg/L in a lake in Washington State (Trainer and Hardy 2015).

Few data are available in freshwater fish and seafood. The highest levels of 30.6 ± 14.5 and 1 ng/g were reported in Brazil in fish muscles (Clemente et al. 2010) and in freshwater snail in Mexico (Berry and Lind 2010), respectively. Regarding the occurrence of STX in food supplements, Heussner et al. (2012) tested 13 products consisting of *Aph. flos-aquae*, *Spirulina* and *Chlorella* or mixtures; thereof, none of them was positive for STX.

Biosynthesis and genetics

The potential correlation between STX producers and their genetic profile is of crucial importance owing to the increased risks posed to human health by STX toxins worldwide. The biosynthetic pathway and genes responsible for STX synthesis have been identified in CB, among which *Cylindrospermopsis raciborskii, Anabaena circinalis and Aphanizomenon* sp. (Kellmann et al. 2008; Mihali et al. 2009; Stuken and Jakobsen 2010) and also in multiple species of neurotoxic dinoflagellates (*Gymnodinium catenatum* and several *Alexandrium* species) (Stuken et al. 2011; Hackett et al. 2013).

The complete sequence determined in the STX-producing strain *Cylindrospermopsis raciborskii T3* encodes 26 genes, namely sxtA-sxtZ (Kellmann et al. 2008). Since then, the STX gene cluster has been characterized in several other CB species. A large number of the stx genes (sxtA, sxtG, sxtB, sxtD, sxtS, sxtU, sxtH/T and sxtI) appear to code for proteins which have diverse catalytic functions directly related to the biosynthesis of the toxin, while others (sxtL, sxtN and sxtX) seem to be involved in the modification of STX into respective congeners and to act as STX potential transporters and binding proteins (Pearson et al. 2010). In both CB and dinoflagellates, STX is synthesized by a similar biosynthetic route (for details, see Testai et al. 2016b).

A recent study by Tsuchiya et al. (2016), conducted on the cyanobacterium *A. circinalis* by incorporating the [$^{15}N_2$]-labeled intermediates Int-A' (3) and Int-C'2 (4)′ into the major STX analogues, C1 (8) and C2 (9), confirmed that the genes responsible for STX production proposed by Kellmann et al. (2008) are consistent with the results of feeding experiments reported by Shimizu et al. (1984) and Shimizu (1986).

Recently, a large-scale study by Penna et al. (2015) was carried out on the STX gene and toxin content using both quantitative PCR (qPCR) and HILIC-HRMS (hydrophilic interaction liquid chromatography with high-resolution mass spectrometry) techniques, to investigate the STX-related risk in the Mediterranean Sea due to the presence of the toxic dinoflagellate *A. minutum,* co-occurring with *A. pacificum* in Syracuse Bay (Italy). As known for many other CB species, toxic (with regard to STX synthesis) *Alexandrium* strains may coexist with other morphologically indistinguishable nontoxic strains within the same species.

In the study, both the two seawater *Alexandrium* species could be analyzed for sxtA1 and sxtG presence by qPCR assay, proposing a reproducible and effective molecular tool to estimate the abundance of multiple *Alexandrium* sp. harmful blooms.

Toxicological profile

They can cause in humans a syndrome known as paralytic shellfish poisoning (PSP): symptoms vary from a slight tingling or numbness to complete respiratory paralysis, depending on the involved toxin within the group. Indeed, the degree of toxicity has been demonstrated to be different for the different groups, although the MeA is assumed common to all of them, on the basis of results obtained with around 50% of the natural analogues. They act by blocking Na^+-channels in neuronal cells (Kao 1993) and Ca^{++} and K^+ channels in cardiac cells: this action prevents the propagation of electrical transmission within the peripheral nerves and skeletal or cardiac muscles (Wang et al. 2003; Su et al. 2004). More recently a large variation of the interaction of STX variants has been reported in relation to sodium channel subtype (Alonso et al. 2016) possibly explaining differences in toxicity.

In mice, i.p LD_{50} of STX is in the order of 10 μg/kg bw, whereas the oral LD_{50} is 260–263 μg/kg bw (Mons et al. 1998); similar values have been reported in rats (Llewellyn 2006). Only recently the acute toxicity of variants other than STX was evaluated in mice after oral (gavage and diet) administration as well as after i.p. injection for comparison (Munday et al. 2013). The ranking of LD_{50} values for any STX variant was dietary exposure > gavage > i.p. injection. An acute NOAEL could be identified after gavage, according to which neoSTX was the most toxic (87 μg/kg bw), followed by STX (163 μg/kg bw), dcSTX; GTX1&4 have a similar toxicity potential (228 and 337 μg/kg bw, respectively), while the less toxic of the group is GTX2&3 (486 μg/kg bw) (Munday et al. 2013). The ranking of the toxicological potential can change significantly depending on the route and type of exposure (Testai et al. 2016b).

The neoSTX formation by N-oxidation of STX could then be considered as a bioactivation process. This reaction might occur in vivo after ingestion: neoSTX was detected in the tissues and urine (but not in the gastric content) of individuals dying after consumption of shellfish contaminated only by STX (Garcia et al. 2004). Another bioactivation may involve the loss of the SO_3^- group of the N-sulfocarbamoyl toxin under acidic conditions, such as those in the gastric environment, converting the toxins in the carbamoyl analogue (Aune 2001) having a much higher toxicity. These interconversions may therefore have relevant health consequences, resulting in severe poisoning episodes. Data related to human intoxication by PSP through seafood are available on more than 500 individuals; a broad range of symptoms (tingling or burning of the lips, tongue and throat, increasing to total numbness of the face, respiratory paralysis and death) have been reported with a high variability in susceptibility attributed to uncertainties in

estimating the exposure levels as well as to interindividual characteristics of intoxicated individuals (Llewellyn 2006). Available data have been used to derive a LOAEL around 1.5 µg STXeq/kg bw.

Other emerging neurotoxins

In the last years among the marine and terrestrial species the occurrence of more than 65 cyanobacterial neurotoxins has been reported (e.g., jamaicamide, antillatoxin and kalkitoxin), for which the available data are still scant (Testai et al. 2016b). Besides these relatively new compounds, known toxins such as ciguatoxin and palytoxin, generally not associated with CB so far, are becoming an emerging issue. In fact, some benthic and pelagic species of marine cyanobacteria such as *Trichodesmium, Lyngbia majuscula* and *Hydrocoleum* spp. have been suspected to produce ciguatoxin (Laurent et al. 2008; Kerbrat et al. 2010), which has been mainly related to benthic dinoflagellates of the genus *Gambierdiscus* (Yasumoto et al. 1977). The *Trichodesmium* genus has been demonstrated to produce palytoxin and 42-hydroxy-palytoxins (Kerbrat et al. 2011), generally detected in soft corals of the genus *Palythoa* and also produced by algae of the genus *Ostreopsis*. In a recent External scientific EFSA Report on an extensive literature search on cyanotoxins toxicological features of ciguatoxin and palytoxin are reviewed (Testai et al. 2016a).

Among the neurotoxins an intense scientific debate is going on related to the effects induced by *β-N*-methylamino-L-alanine (BMAA), on which still a high degree of uncertainty is present. The issue will be briefly summarized here.

BMAA is a nonprotein amino acid with molecular weight of 118 Da (Fig. 10). It has been assumed that BMAA may be produced by all known groups of CB (Cox et al. 2005), but to date this has not been clearly verified due to unclear reporting and unsupported conclusions in key studies on BMAA research (Faassen 2014). It seems to be produced also by diatoms (Jiang et al. 2014a) and dinoflagellates (Lage et al. 2014). It was first isolated from cycad trees on Guam in 1967, but very little is known regarding the biosynthesis of this nonprotein amino acid. A putative two-step pathway has been proposed by Brenner et al. (Brenner et al. 2003a, b), in cycad plants, starting with the transfer of an ammonia group at the beta-carbon of the substituted alanine followed by the addition of a methyl group to produce BMAA. A similar pathway was proposed initially by Araoz et al. (2010) for BMAA biosynthesis in CB, assuming the existence of putative cyanobacterial genes orthologous to the cycad ones, but this hypothesis was not confirmed by subsequent studies (Downing et al. 2011).

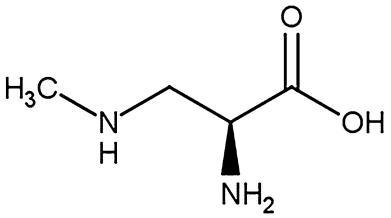


Fig. 10 Chemical structure of BMAA

BMAA can occur in the environment and in tissues as a free molecule but mainly bound to protein, and in this form can be transferred and accumulated along the trophic chain (Jiang et al. 2014b; Jiao et al. 2014). Its function and impact on human health are still scarcely known; however, it is in the spotlight of the scientific community because of its neurotoxic potential on the basis of which BMAA has been hypothesized as a possible etiological agent of widely diffused, severe human neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS). The hypothesis started from the observation in the 1950s of high incidence and prevalence of neurodegenerative diseases in three islands of Western Pacific, up to 100 times higher than that observed in comparable populations in the continental USA (Kurland and Mulder 1954).

BMAA was isolated from cycas seeds: according to Banack and Cox (2003), it is synthesized by symbiotic *Nostoc* CB in the roots of cycads of the Western Pacific and released into the plant, although it has been also shown that BMAA occurs in *Cycas micronesica* seedlings grown without endophytic CB symbiosis (Marler et al. 2010). BMAA has been detected in flying foxes, feral deer and pigs that feed on cycad seeds in Guam (Spencer et al. 1987; Banack et al. 2006). Flour from Cycas seeds and the above-mentioned animal bioaccumulating it eaten by the local population were considered the specific sources of exposure for BMAA. These exposures ceased or were strongly reduced after the Second World War, with important lifestyle modifications and the introduction of westernized diet, coherently with the observation of strong decrease of neurodegenerative diseases (Spencer et al. 2016). The presence of BMAA in the brains of patients deceased from neurodegenerative diseases was considered a further support for this hypothesis (Murch et al. 2004; Pablo et al. 2009), although a number of other studies failed to confirm these data (Montine et al. 2005; Kushnir and Bergquist 2009; Snyder et al. 2009, 2010; Combes et al. 2014) and a number of criticisms were associated with the methodology used for BMAA detection (Faassen et al. 2012a, b; Faassen 2014).

As other nonprotein amino acids, BMAA might be incorporated into proteins (Holtcamp 2012) leading to protein misfolding, a hallmark of many neurodegenerative

disease (Moreno-Gonzalez and Soto 2011; Glover et al. 2014).

Although the administration of BMAA has not been associated with in vivo neurotoxic effects in mice (Cruz-Aguado et al. 2006), some neurotoxic and behavioral effects (Lobner et al. 2007; Karlsson et al. 2009a, b; Liu et al. 2010) have been associated with BMAA exposure, affecting motor neurons with different mechanisms including direct agonist action on glutamate NMDA (*N*-methyl-D-aspartic acid) and AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors or induction of oxidative stress and depletion of glutathione (Lobner et al. 2007; Banack et al. 2010). However, none of the studies giving positive results provides a clear reproduction of the behavioral and neurodegenerative alterations observed in humans suffering with ALS–PDC and changes observed in postmortem tissues from ALS patients. This was attributed to the fact that the majority of in vivo studies have been carried out with rodents, an animal model that could have a limited capability in mimicking the upper motor neuron element of human ALS (Karamyan and Speth 2008).

On the other hand, quite high doses have to be administered to primates in vivo to induce some degenerative effects or in vitro (1–3 mM) to induce in vitro neuronal death (Ross et al. 1987) and this is one of the major criticisms to the hypothesis attributing a role to BMAA in ALS/PDC (amyotrophic lateral sclerosis/parkinsonism dementia complex) and other neurodegenerative disease etiology.

On the basis of this summary is evident why the available data have raised scientific disputes on their reliability and validity: the overall picture is not completely clear, and the issue deserves more in depth investigations.

Derivation of reference values and guidance values for cyanotoxins

Most of the data available are on MC-LR for which it is possible to derive a health-based reference value, based on animal studies, since the available data on humans do not fit for the purpose. This was the WHO approach (2004), using the subchronic NOAEL of 40 μg/kg bw/day (Fawell et al. 1999a) obtained with mice by oral dosing as point of departure (PoD). A provisional TDI for life-time exposure of 0.04 μg/kg bw/day was calculated, applying an assessment factor (AF) of 1000 accounting for: (1) interspecies variability; (2) intraspecies variability; (3) lack of lifetime toxicity data and gaps in the database. The procedure seems to be conservative and still valid (Funari and Testai 2008), even in the light of more recent studies showing potential toxicity for organs other than the liver. Indeed, these new studies show unfortunately some weaknesses in experimental design and reporting and are not robust enough to provide a new PoD for any health-based reference value and need to be confirmed. Therefore, so far the WHO provisional TDI value for MC represents a pragmatic, quantitative approach to the management of risks associated with exposure to this class of cyanotoxins, although as said, some limitations exist: first of all the toxicological profile is still not fully elucidated and it is necessary to collect more data to fill the gaps on either kinetic or dynamic aspects. Secondly, it can be discussed whether or not an additional safety factor should be included to account for the tumor-promoting activity of MC: since this process has a threshold, related to the inhibition of hepatic protein phosphatases, it is clear that by preventing this effect also the tumor promotion activity is prevented, with no need for any additional safety factor. Last but not least, data are available mainly on one single congener (MC-LR) and still they are not exhaustive; very few data are available on the other variants and generally obtained via i.p. injection, although it is clear that striking differences occur in their kinetics. Therefore, the validity of the acute i.p. toxicity ranking according to which MC-LR is the most toxic remains to be demonstrated. However, the WHO GV (Guidance Value) for drinking water in relation to the sum of MC variants (expressed as MC-LReq) derived as described in Funari and Testai (2008), is used as a reference by many countries around the world to establish legal values or give recommendations, as reported in Table 10, to prevent and/or manage the possible health effects induced by MC exposure through drinking water.

In deriving their Guidelines for Drinking Water US-EPA (2015a) and Health Canada (2016) considered results from the Heinze study (1999), in which MC-LR was administered to male rats in drinking water for 28 days and the related LOAEL = 50 MC-LR/kg bw/day was used as PoD. For shorter exposure periods (which are the most frequent ones considering the seasonality of some blooms), the value was divided for 300 as assessment factor (besides the default factor of 100 for inter- and intraspecies variation, an additional 3 was introduced for the use of a LOAEL), to reach a reference value for subacute/subchronic toxicity (or HA, Health Advisory) = 0.16 μg MC-LR/kg bw/day (Table 10). Since for short-term advisories EPA assumes 100% exposure is derived from drinking water, no allocation factor was applied. A 10-day HA was also derived for (1) bottle-fed infants and young children of pre-school age and (2) children of school age by using the appropriate water consumption and allometric scaling. They were, respectively, equal to 0.3 and 1.6 μg/L, which are considered protective of noncarcinogenic adverse health effects for the target population over a ten-day exposure to MC in drinking water.

Alternatively, by using the same approach as for the TDI derivation by WHO, but omitting the AF of 10 related to

Table 10 Recommendations by International Organizations and limit values set in different countries in order to prevent/manage the possible human health effects induced by MC exposure

Organization/country			Limit value	Note	References
WHO		Drinking water	1 µg/L	GV provisional	WHO (2003a, 2004)
		Recreational water	<10 µg/L (Low) 10–20 µg/L (Moderate) 20–2000 µg/L (High) >2000 µg/L (Very high)	Relative Probability of Acute Health Effects	WHO (2003b)
Europe		Recreational water	Bathing water directive (EU), national implementation: France: 25 µg/L Germany: >10 µg/L or more Hungary: <4 µg/L <10 µg/L <20 µg/L >20 µg/L Italy: >25 µg/L	Bathing is banned and recreational activity restricted Discourage bathing, temporary closure is considered or recommended Excellent Good Acceptable Unacceptable Bathing is prohibited	Ibelings et al. (2015)
US-EPA		Drinking water	0.7 µg/L (infants and pre-school children) 3 µg/L (school-aged children and adults)	HAL for periods up to 10 days	US-EPA (2015a)
		Recreational water	EPA is currently developing Ambient Water Quality Criteria for cyanotoxins for the protection of recreational activities in freshwater systems. EPA is expecting to release a draft document for public comment in Fall 2016.		US-EPA web site (*)
	California	Food	10 ng/kg ww human fish consumption 0.5 (0.01) mg/Kg dw acute (subchronic) crust and mat intake for dog 5 (0.1) mg/Kg dw acute (subchronic) crust and mat intake for dairy cow 6 (0. 1) mg/Kg dw acute (subchronic) crust and mat intake for beef cattle	Action level	Butler et al. (2012)
		Drinking water	100 (2) µg/L acute (subchronic) water intake for dog 50 (0.9) µg/L acute (subchronic) water intake for dairy cow 200 (3) µg/L acute (subchronic) water intake for beef cattle	Action level	Butler et al. (2012)
		Recreational water	0.8 µg/L	Advisory	US-EPA web site (*)
	Florida	Drinking water	1 µg/L chronic 10 µg/L 90 day	HAL	Chorus (2012)
	Illinois	Food	20 µg/l	GV: water concentration at which to recommend no consumption of fish	Illinois EPA (2012)
		Recreational water	≥10 µg/L	Advisory	US-EPA web site (*)

Table 10 continued

Organization/country			Limit value	Note	References
	Indiana	Recreational water	Level 1: <4 µg/L Level 2: 4 to 20 µg/L Level 3: >20 µg/L	Level 1: very low/no risk, use common sense practices Level 2: low to moderate risk, reduce recreational contact with water Level 3: serious risk, consider avoiding contact with water until levels of toxin decrease	US-EPA web site (*)
	Iowa	Recreational water	≥ 20 µg/L	Caution—bloom present no toxin data available Warning—when toxin levels exceed 20 µg/L	US-EPA web site (*)
	Kansas	Recreational water	>4 µg/L to < 20 µg/L >20 µg/L	PHA PHW: all contact with water is restricted	Trevino-Garrison et al. (2015); US-EPA web site (*)
	Massachusetts	Recreational water	14 µg/L	Advisory—avoid contact with water	US-EPA web site (*)
	Minnesota	Drinking water	0.1 µg/L	Short-term Noncancer Health-Based Value (nHBVShort-term)	MDH (2015)
	Nebraska	Food	≥20 µg/L	Not eat whole fish	Testai et al. (2016a)
		Recreational water	≥20 µg/L	Health alert	US-EPA web site (*)
	Ohio	Food (fish filet)	<3 µg/kg 7 µg/kg 28 µg/kg >28 µg/kg	GV: unrestricted 1/week 1/month Do not eat	Ohio (2010)
		Drinking water	0.3 µg/L	Do not drink—children under 6 and sensitive populations (pregnant women, nursing mothers, those receiving dialysis treatment, the elderly and immune-compromised individuals)	Ohio (2015a), US-EPA web site (*)
			1.6 µg/L	Do not drink—children 6 and older and adults	
			20 µg/L	Do not use (based on the Recreational No Contact Advisory thresholds)	
		Recreational water	6 µg/L 20 µg/L	PHA—swimming and wading are not recommended, water should not be swallowed and surface scum should be avoided. NCA recommends the public avoid all contact with the water	Ohio (2015b); US-EPA web site(*)
	Oregon	Food	1 µg/kg DW(food supplement BGAS)	S	Testai et al. (2016a)
		Drinking water	1 µg/L	GV	Farrer et al. (2015),
		Recreational water	10 µg/L	PHA	US-EPA web site (*)
	Oklaoma	Recreational water	>20 µg/L	Blue-Green Algae Awareness Level Advisory	US-EPA web site*

Table 10 continued

Organization/country			Limit value	Note	References
	Rhode Island	Recreational water	≥14 µg/L	HAL	US-EPA web site (*)
	Texas	Recreational water	>20 µg/L	Blue-Green Algae Awareness Level Advisory	US-EPA web site (*)
	Vermont	Recreational water	≥6 µg/L	Beach closure	US-EPA web site (*)
	Virginia	Recreational water	>6 µg/L	Immediate public notification to avoid all recreational water contact where bloom is present; continue weekly sampling	US-EPA web site (*)
	Washington	Recreational water	>6 µg/L	Tier 2. Warning: Toxic algae present Tier 3. Danger: Lake closed (in presence of unusual high concentration, Very dense blooms covering an entire lake, Confirmed pet illnesses or death, Reported human illness	Trainer and Hardy (2015), US-EPA web site (*)
Canada		Food	0.02 µg/kg bw/d	Tolerance Limit	Testai et al. (2016a)
		Drinking water	1.5 µg/L	MAC	Chorus (2012)
		Recreational water	≤20 µg/L		Ibelings et al. (2015)
Australia		Food	24 µg/kg (fish) 32 µg/kg (prawns) 51 µg/kg (mussels or molluscs)	GV used in Victoria and New South Wales States based on a TDI of 0.2 µg/kg bw/d	Mulvenna et al. (2012)
		Drinking water	1.3 µg/L 2.3 µg/L livestock		Chorus (2012)
New Zealand		Drinking water	1 µg/L	Provisional MAV	Chorus (2012)
		Recreational water	≥12 µg/L	Notify the public of a potential risk to health	Ibelings et al. (2015)
Turkey		Recreational water	<10 µg/L >25 µg/L	Recreational activities are permitted. Monitoring Discourage users from swimming and other water activities	Ibelings et al. (2015)
Brazil		Drinking water	1 µg/L	S	Chorus (2012)
		Recreational water			Chorus (2012)
Argentina		Drinking water	1 µg/L	WHO's GV provisional	Chorus (2012)
Singapore		Drinking water	1 µg/L	S	Chorus (2012)
South Africa		Drinking water	1 µg/L	S	Chorus (2012)
Uruguay		Drinking water	1 µg/L	GV	Chorus (2012)

(*) https://www.epa.gov/nutrient-policy-data/guidelines-and-recommendations

DW: dry weight, HAL: health advisory level, GV: Guidance Value, PHA: public health advisory, PHW: public health warning, NCA: No Contact Advisory, S: standard, MAV: maximum acceptable value, MAC: maximum acceptable concentration

study duration, a reference value for subchronic toxicity could be derived equal to 0.4 μg MC-LR/kg bw/day. Since the duration of the study in the Heinze study is shorter, the use of a LOAEL increases the level of uncertainty, and considering that in the short exposure period it is extremely unlikely that all the uncertainties related to chronic exposure may play a role, it can be assumed that the use of the NOAEL from the Fawell study (1999a) is a more appropriate approach for the reference values related to the chronic and subchronic exposure scenario.

The California EPA also used the Heinze study as the key study for the derivation of a health-based reference dose. The incidence of microscopic liver lesions was input into the EPA benchmark dose (BMD) software to estimate the $BMDL_{10}$ (that is the 95 percent lower confidence limit of the dose associated with a 10 % response rate), resulting equal to 6.4 μg/kg bw/day (Butler et al. 2012). It is accepted that the BMDL is a preferable PoD with respect to the use of the NOAEL (being less dependent on the experimental design and dose spacing and taking into account the slope of whole dose–response curve). However, since only two dose levels were used in the Heinze study and the estimated value is well outside of the dose range tested, this approach has strong limitations. The US-EPA highlighted also that the lack of dose–response in all the histological effects is not suitable to BMD modeling, and, as a consequence, the LOAEL of 50 μg/kg bw/day described by Heinze (1999) was used as the PoD.

As previously discussed in Funari and Testai (2008) from MC-LR toxicological data it is also possible to estimate no-effect concentrations with regard to the acute risk starting from the lowest acute NOAEL of 25 μg/kg bw, derived from i.p. injection studies in mice, and converting it to the oral route, by the application of a correction factor (CF) of 10 (to account for the kinetic differences) still representing a conservative approach, although with a high degree of uncertainty. An acute no-effect dose of 2.5 μg/kg bw, corresponding to 150 μg/kg bw for an adult of 60 kg bw, can be derived, although due to the steepness of the dose–response curve, special attention should be given when the exposure to MC-LR is close to the acute adverse no-effect dose. This route-to-route extrapolation would be substantially impossible to be applied to repeated exposure due to the kinetic differences for which insufficient data are available.

The WHO has also examined the possible human health implications associated with exposure to cyanobacterial cells and cyanotoxins via bathing activities and has provided provisional guidelines for avoiding the risk from skin irritation and more severe effects. However, the WHO guidelines for bathing waters (2003a, b) do not take into account more recent investigations and present several limitations, including the focus on MC-LR only. The approach and the limitations are described in details in Funari and Testai (2008). As a consequence, the values adopted by many countries are quite different, depending on the approach followed, although in a number of cases the WHO guidelines are still a reference (Table 10). In many cases the GVs were calculated to protect children as the most prone to risky exposure, during playing especially along the shore.

Interestingly the EPA of the State of California (USA) has defined action levels to protect not only human health (only applicable to incidental exposure through recreational use and should not be used to judge the drinking water quality), but also pets and livestock health, particularly dairy cows, beef cattle and dog from acute and subchronic exposures by drinking natural or impounded waters or by eating cyanobacterial crusts or mats and—for dog only—also swimming (Butler et al. 2012). The action levels for livestock and pets were calculated to identify the concentration of cyanotoxins in water that represents little or no risk of toxic exposures to cattle. They have been derived starting from information on lethal doses in 15 sheeps exposed to lyophilized *Microcystis aeruginosa* collected from a natural bloom in amounts of dry material equivalent to approximately 2.7–6.7 mg MC/kg bw (Jackson et al. 1984). The highest nonlethal dose was approximately 3.7 mg MC/kg bw; animals treated with higher doses died and exhibited marked liver histopathological changes consistent with MC poisoning. The nonlethal dose was divided by 100 (10 for interspecies variation and 10 for severity of the endpoint) yielding an acute RfD of 3.7×10^{-2} mg/kg bw.

The subchronic reference value was derived considering the Heinze study and the above-mentioned $BMDL_{10}$, divided by 10 to cover the uncertainty in extrapolating from mice to cattle and dogs, and the uncertainty due to incomplete toxicology profiles; the result is a reference value of 6.4×10^{-4} mg/kg bw/day (Butler et al. 2012). The reference doses by the exposure related to the different scenarios give the chemical concentration in water (mg/L) that would result in exposure at the RfD level or below, defined as the action level.

The water consumption rate for small breed dairy cows and beef cattle of 0.23 and 0.07 L/kg bw/day, respectively, was divided for an uncertainty factor of 3 to account for the reported preferential consumption of water contaminated by CB with respect to clean water shown by different animal species (Codd et al. 1992; Lopez and Costas 1999), to obtain a final exposure level of 0.69 and 0.21 L/kg bw/day.

Since high concentrations of MC are generally found in dried scum and mats, also the scenario foreseeing animals eating that kind of material on the edge of natural or impounded water bodies is worth of consideration. It was estimated that dairy cows and beef cattle have an average

intake of 1.2 kg/day, which divided by the average weight for small breed dairy cows (454 kg) or for beef cattle (635 kg) gives an estimated intake rate of 0.0026 or 0.0019-kg crusts or mats/kg bw/day, respectively. The action levels are calculated by dividing the reference values for the intake.

Exposure of dogs can be associated with drinking and grooming; indeed, after swimming in contaminated water they use to lick their coats. The California State EPA calculated that a 20-kg dog exercising in water for 1 h can drink up to 0.01 L/kg bw and that up to 1.5 L of bloom waters may cling to the coat, for an amount of water ingested during drinking and grooming is 0.085 L/kg bw. This value is further divided for a factor of 3, in analogy to the approach used for livestock, for a final exposure of 0.255 L water/kg bw/day. Based on the calories requirements and the corresponding amount of dry food that a 20-kg dog can eat in few minutes, the potential ingestion of crust or mat material was assumed to be 0.5 kg (equivalent to 0.025 kg/kg bw/day). By applying the uncertainty factor of 3 the final exposure level resulted in 0.075 kg/kg bw/day.

The levels of uncertainty associated with derivation of reference value for CYN are also high, especially when its potential but still controversial genotoxicity is concerned; this toxicological property has been associated with its metabolites, which on the other hand have not been identified yet. In principle a genotoxic activity should not allow to derive any reference value, assuming that there is no threshold. These aspects need an adequate clarification. In order to estimate the risk associated with CYN oral repeated exposure for noncarcinogenic adverse health effects, the most appropriate PoD is the subchronic NOAEL = 30 μg/kg bw/day (Humpage and Falconer 2003). Similarly to the approach used for MC, dividing the NOEL for a UF (uncertainty factor) of 1000 (100 for inter- and intraspecies variability and 10 for the limited toxicological database), a provisional TDI value of 0.03 μg/kg bw/day can be derived. Recently US-EPA (2015b) used the same PoD for the derivation of the Drinking Water Health Advisory. Indeed, the similarity in the type of effects observed and the LOAELs from the Humpage and Falconer (2003) and Reisner et al. (2004) studies, justified the choice of the NOAEL from the Humpage and Falconer study as the most appropriate PoD for 10-day exposures in infants, children and adults despite its longer exposure duration. An uncertainty factor of 300 was used to account for inter- and intraspecies differences (100) and for deficiencies in the database for CYN (3).

The ten-day HA derived for bottle-fed infants (0.7 μg/L) and for children of school age through adults (3 μg/L) obtained by using the appropriate water consumption and body weight is considered by US-EPA protective of noncarcinogenic adverse health effects over a ten-day exposure to CYN in drinking water (US-EPA 2015b).

The values for CYN used as legal limits or as recommendation over the world are reported in Table 11. It can be seen that values are reported to protect human health from different exposure scenarios (e.g., drinking and recreational water); in addition the California State EPA also defined action levels for CYN, by using the same approach described above for MC (Butler et al. 2012).

Regarding neurotoxins, the available toxicological data for ATX are considered not adequate to derive a TDI or any other reference value by WHO (2004) and US-EPA or Health Canada. The two subchronic studies carried out so far did not show any significant treatment-related effect even at the highest doses tested. However, a worst-case approach can be applied to derive a conservative reference value to be used in a pragmatic way to manage the risk of exposure to ATX (Testai et al. 2016b). The approach considers as PoD the NOAEL of 98 μg ATX/kg bw/day, that is the low dose in the 28-day study (Fawell et al. 1999b) in which two deaths in the high- and mid-dose group were reported, although they could not be clearly attributed to the treatment. It is possible to obtain a GV = 1 μg/L to protect a 10-kg bw child consuming 1 L drinking water per day, providing an adequate margin of safety (10^3). On this basis, some countries have established legal values or given recommendations in order to prevent and/or manage the possible health effects induced by ATX exposure (Testai et al. 2016b).

Also for STX, the toxicity database is not suitable for the derivation of any reference value for repeated exposure. However, both data on acute toxicity on animal models and on a number of human intoxication episodes following contaminated seafood allow to derive limit values for the acute toxicity of STX-group toxins.

An ARfD of 0.5 μg STX equivalents/kg bw was adopted in 2009 by EFSA (2009) derived from human data on more than 500 individuals, starting from a LOAEL of 1.5 μg STXeq/kg bw, divided by an assessment factor of 3 (due to the use of a LOAEL instead of a NOAEL as PoD). The value is supported by more recent experimental animal data (Munday et al. 2013), on the basis of which ARfD in the same order of magnitude can be derived. The dose–response curve for acute effects both in human and in animals is quite steep, indicating the need for caution at exposures levels close to the limit (Testai et al. 2016b).

For regulatory purposes all STX variants can be expressed as STX concentration equivalents, assuming a common mode of action, although it is known that toxicity is variant-specific. In its opinion EFSA applied a toxicity equivalent factor (TEF) approach, based on the mouse bioassay (MBA) data or the in vitro blocking of Na^+ channel

Table 11 Recommendations by International Organizations and limit values set in different countries in order to prevent/manage the possible human health effects induced by CYN exposure

Organization/Country			Limit value	Note	References
US-EPA		Drinking water	0.7 µg/L (Infants and Pre-school children) 3 µg/L (School-aged children and Adults)	HAL for periods up to 10 days	US-EPA (2015b)
		Recreational water	EPA is currently developing Ambient Water Quality Criteria for cyanotoxins for the protection of recreational activities in freshwater systems		US-EPA web site (*)
	California	Food	70 ng/kg ww Human fish consumption	Action level	Butler et al. (2012)
		Animal Safety	Dog 0.5 (0.04) mg/Kg dw acute (subchronic) crust and mat intake; 200 (10) µg/L acute (subchronic) water intake	Action level	Butler et al. (2012)
			Dairy cow: 5 (0.4) mg/Kg dw acute (subchronic) crust and mat intake; 60 (5) µg/L acute (subchronic) water intake		
			Beef cattle 7 (0.5) mg/Kg dw acute (subchronic) crust and mat intake; 200 (20) µg/L acute (subchronic) water intake		
		Recreational water	4 µg/L	Advisory	US-EPA web site (*)
	Indiana	Recreational water	5 µg/L	Warning level	US-EPA web site (*)
	Ohio	Drinking water	0.7 µg/L	Do Not Drink—children under 6 and sensitive populations (pregnant women, nursing mothers, dialysis patients, elderly and immune-compromised individuals)	Ohio (2015a), US-EPA web site (*)
			3.0 µg/L	Do Not Drink—children 6 and older and adults	
			20 µg/L	Do Not Use (based on the Recreational No Contact Advisory thresholds)	
		Recreational water	5 µg/L	PHA—swimming and wading are not recommended, water should not be swallowed and surface scum should be avoided	Ohio (2015b), US-EPA web site (*)
			20 µg/L	NCA-recommend the public avoid all contact with the water	
	Oregon	Drinking water	1 µg/L		Farrer et al. (2015), US-EPA web site (*)
		Recreational water	6 µg/L	PHA	US-EPA web site (*)
	Washington	Recreational water	4.5 µg/L	Tier 2. Warning: toxic algae present	Trainer and Hardy (2015), US-EPA web site (*)
				Tier 3. Danger: lake closed (in presence of unusual high concentration, very dense blooms covering an entire lake, confirmed pet illnesses or death, reported human illness	

Table 11 continued

Organization/Country		Limit value	Note	References
Australia	Food	18 µg/kg (fish) 24 µg/kg (prawns) 39 µg/kg (mussels or molluscs)	GV used in Victoria and New South Wales States based on a TDI of 0.2 µg/kg bw/d	Mulvenna et al. (2012)
	Drinking water	1 µg/L		Chorus (2012)
New Zealand	Drinking water	1 µg/L	Provisional MAV	Chorus (2012)
Brazil	Drinking water	15 µg/L	GV	Chorus (2012)

HAL health advisory level, *GV* Guidance Value, *PHA* public health advisory, *PHW* public health warning, *NCA* No Contact Advisory

(*) http://www2.epa.gov/nutrient-policy-data/guidelines-and-recommendations

in cultured neuronal cells (EFSA 2009). However, more recent data evidenced differences between the TEF values based on MBA and those calculated using the oral LD_{50} (Munday et al. 2013). It seems therefore appropriate to revise the relative potency of the different STX variants (Testai et al. 2016b). Some countries have proposed specific limits to protect people from dangerous exposures to STX through drinking or recreational water, as well as through consumption if contaminated fish and shell fish (Testai et al. 2016b).

Exposure media

Water for hemodialysis

The parenteral route of exposure can occur when surface waters infested by cyanobacteria are used for hemodialysis and not properly purified. This exposure scenario represents a remarkable risk for the patients also in view or their pre-existing diseases; indeed since cyanotoxins directly enter into the bloodstream, their bioavailability is 100%. Likely enough this is a quite low-frequency event, and up to now only described twice in Brazil (Jochimsen et al. 1998; Azevedo et al. 2002; Soares et al. 2006), where acute effects up to death were recorded in the first episode, whereas subchronic effects could be associated with the second episode. In particular, regarding repeated exposure to low concentrations, the impact of this potential route of exposure is probably underestimated, more likely in countries where there are no alternatives to the use of surface waters (potentially infested by cyanobacteria) in hemodialysis therapy.

As it was shown by the contamination event at a dialysis center in Rio de Janeiro, Brazil, in 2001, the 44 dialysis 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). Without detection of MC in the activated carbon filters for water purification and then in the patients serum, it can be expected that the event would not have been identified and possible symptoms likely attributed to the pre-existing diseases of dialyzed patients. Although intermittent, exposure to cyanotoxins can represent a high risk for dialysis patients. Indeed, also in those countries where quality of water used for dialysis is regulated, contamination by cyanotoxins is not among the parameters to be routinary checked, with the exception of Brazil.

The experience of the Brazilian outbreaks indicates that cyanotoxins pass easily through dialyzer membranes used to prepare dialysate. Filtration through granular-activated carbon and reverse osmosis are the usual dialysis water treatment systems: they have been reported to reduce the

MC concentration in water by >90% (although for a limited period of time after which they are no more efficient and in a specific range of working temperatures). Yet, data from the second event in Brazil suggest that such a level of performance is not always achieved (Soares et al. 2006).

According to the current US standards for dialysis water, the maximum allowable levels of potentially toxic contaminants are set at 10% of the levels allowed in drinking water, when specific toxicity data are not available. On that basis, it has been proposed to use as the maximum concentration for MC in dialysis water 10% of the WHO provisional drinking water guideline that is 0.1 µg/L (Hilborn and Ward 2016). However, the confidence on such a proposed limit is low due to the high degree of uncertainty associated with the WHO GV, to the additional susceptibility of dialyzed patients, plus the lack of information on the differences in bioavailability between the oral and the parenteral route and effects on humans. The available data on levels in human serum are not robust enough, due to the late sampling time (with respect to the actual exposure) and the limitation of the detection methodologies.

Regarding possible controls, since cyanotoxin concentrations can dramatically change over time, as well as the efficiency of the depuration system, intermittent testing can result not sufficiently protective with variable cyanotoxin levels in the dialysate. Consequently, it is extremely important to guarantee the maximum effectiveness in the preparation of water and to plan adequate quality controls, when surface water has to be used.

Drinking water

The episodes of human intoxications described in the literature indicate that depending on cyanotoxin levels in drinking water, acute and short-term effects may occur in humans and in animals (Funari and Testai 2008); although more difficult to identify and demonstrate the chronic effects are the most probable especially in developed countries.

The awareness of the potential risk associated with cyanotoxins triggers the implementation of adequate treatments of raw water and monitoring plans in many countries worldwide, which prevents the use of contaminated water, and therefore limits the availability of published data reporting cyanotoxin levels in drinking water. However, the probability for its contamination is likely, where the effectiveness of treatment methods for removing cyanobacteria and cyanotoxins is low, or in less developed countries, where many people need to use unfiltered/untreated surface waters. In this latter case recommendations can be made not to use surface waters infested by cyanobacterial blooms without filtering to remove cells (i.e., simple sand filters) and to avoid the use of water when the bloom is senescent and extracellular cyanotoxin concentration is expected to be higher. The efficiency of treatment methodologies is also related to high concentration in raw water, and cases were recently reported in Serbia, following a *P. rubescens* heavy bloom in the reservoir providing drinking water to the city of Uzice, and in the USA (Toledo, Ohio), where the use of tap water coming from Lake Erie infested by cyanobacteria and contaminated by MC was not allowed for drinking and cooking.

As reported above in the section dedicated to the derivation of reference values, many countries adopted the recommendations and guidelines values coming from WHO, or derived their own limit values for the quality of drinking water, which, despite the high degree of uncertainties for all the different cyanotoxins, represent a pragmatic and up to know effective way to manage the risk, limiting exposure to cyanotoxins.

A special consideration should be given to wild animals, livestock and pet dogs for which the probability to drink raw water is high. Levels of MC, CYN and ATX reported in surface water (see above) are much higher than the action levels set for each toxins by the State of California (USA) to protect dairy cows, beef cattle and dog health from both acute and subchronic exposures by drinking natural or impounded waters. The number of case reports of animal poisoning witnesses the need to give increasing attention to this phenomenon, which applies also to cyanobacterial crusts from dried scums and mats related to benthic cyanobacteria eaten by animals, since they contain high levels of cyanotoxins.

Food items

Human exposure from food can be due to consumption of fish, crops, food supplements based on algae, or items of animal origins, following the use of contaminated water for irrigation or in farming activities. The scientific literature suggests that cyanotoxins can be accumulated in food at concentrations higher than provisional limits set for MC-LR in drinking water to protect consumers from repeated chronic exposure. However, no episode on human outbreaks related to contaminated food items has been reported so far. This could be due to difficulties in recognizing cyanotoxins as the cause of possible symptoms and to the nature of effects likely associated with long-term exposure, rather than to acute intoxications. In this line, biochemical alterations as markers of hepatic damage have been associated in Chinese fishermen and children with reiterated consumption of fish and ducks coming from a contaminated lake (Chen et al. 2009; Li et al. 2011b), apparently not affected by any specific symptoms.

Among the potentially contaminated food items, many different aquatic organisms (e.g., fish, bivalves shellfish

and crustacean) may accumulate cyanotoxins via ingestion of CB cells or via the transdermal route as dissolved toxins (a particularly relevant route for CYN) (Rücker et al. 2007). Although some data indicate negligible residues in meat, milk and dairy products for hydrophilic MC variants (Orr et al. 2003), it cannot be ruled out that the uptake of more lipophilic MC congeners or other cyanotoxins occur in animal husbandry, through consumption of contaminated waters. Cyanotoxins have been detected in some edible crops, translocated to plants from surface irrigation waters. Finally BGAS products contamination has been repeatedly evidenced in different world area, and a preliminary risk assessment indicates a potential high risk for consumers (Vichi et al. 2012). These considerations lead EFSA to list cyanotoxins among the emerging issues and to fund a tender for an extensive literature search to identify the levels of human exposure and understand whether or not cyanotoxins can be considered an actual emerging risk. The results of the survey indicated that even for MC, accounting for the great majority of the available information, data are limited. Indeed, due to the poor reliability of the analytical methods used, many papers were not considered, evidencing the need for collecting more good quality data and the availability of validated analytical method for complex matrix as one of the major data gaps (Testai et al. 2016a). However, the available data suggest the possibility of risky exposure for fish and shell fish as well for BGAS consumers in relation to MC contamination; for other food items as well as for other cyanotoxins the database of occurrence is extremely scant, although a possible risk can be anticipated (Testai et al. 2016a).

The concern linked to the diet as a source of exposure derives also from the increasing evidence of cyanotoxins produced by marine CB, as well as from the more and more frequent occurrence of CB in brackish water and estuaries, where aquaculture plants are often located, based on the higher seafood consumption by the general public when compared to freshwater organisms. However, in order to carry out a sound evaluation, data should be produced to characterize i) the kinetics of bioaccumulation/biomagnification in different aquatic organisms and fish species and ii) the cyanotoxins bioavailability for humans (e.g., stability following cooking processes, bound vs unbound fraction).

Water for recreational activities

When recreational and/or professional activities (e.g., swimming, water-skiing, fishing, boating, sail boarding, canoeing, playing along the shore) are carried out in or around water bodies infested by CB, the oral, dermal and inhalation routes of exposure are concurrent, and a cumulative risk should be taken into account. The analysis of anecdotal evidence, case reports and epidemiological studies show that a range of symptoms is associated with recreational exposure to CB, the most frequent includes dermatological signs or symptoms (e.g., rash, skin irritation, swelling, sores) and eye irritation likely related to direct contact with contaminated water and with cyanobacterial cells; gastrointestinal and respiratory signs or symptoms, likely associated with ingestion of inhalation; neurological signs or symptoms and headache generally present when neurotoxins are detected in water (Stewart et al. 2006a, b; Hilborn et al. 2014). More severe symptoms, including throat, cough, malaise, headache and fever atypical pneumonia, myalgia, vertigo, blistering in and around the mouth, and liver damage have been more rarely reported to be associated with prolonged exposure to high levels of cyanotoxins ($\gg$100 µg/L) (Turner et al. 1990; Giannuzzi et al. 2011; Trevino-Garrison et al. 2015).

Besides the effects due to direct contact, people exercising in contaminated water may experience also systemic effects since they may inadvertently swallow contaminated water (around 100 mL per swimming exercise according to WHO), being the body immersed into the water cyanotoxin may be absorbed by penetrating the skin, and by inhalation after vaporization, favored by water movements. Although on the basis of the physical–chemical properties of MC-LR and CYN it can be anticipated a negligible vaporizing or penetrating the skin, it is not possible to exclude these routes for alkaloids neurotoxins as well as for other MC variants. Inhalation could be a relevant exposure for lakeside populations or water users, when dried scums accumulated during windy days. However, the oral route gives generally a higher contribution than the other two routes. Regarding risk assessment following exposure to cyanotoxins during recreational activities, the most relevant effects are related to acute and short-term exposure; indeed, in temperate areas, the seasonality of blooms makes chronic exposure unlikely and at least where species blooming during cold seasons (e.g., *Planktothrix* sp.) are present the exposure period due exclusively to bathing activities is limited. Only in areas with persistent/continuous blooms where sports/recreational activities are frequently performed, the typical exposure scenarios can give rise to some subchronic health risks. Therefore, in order to prevent systemic effects following ingestion the provisional TDI (applying a conservative approach) or the subchronic health-based reference value related to each toxin, when available, can be used to calculate the maximum concentration that could be present in water to protect the potentially exposed population, using the appropriate exposure scenario.

Since the presence and density of CB are easy parameters to be checked, and due to the indication given by the WHO guidelines for bathing water (2003), most recommendation or limit value to manage the risk associated with the recreational use of water is based on cell counts

(Tables 10, 11). If this approach or even better the cell biomass can be useful to protect toward local effects (likely associated with component other than cyanotoxins), it provides no adequate information on the concentration of toxins responsible for severe toxicity after ingestion. Yet, it is not possible to estimate toxin concentrations from CB cell density, due to the complex relationship between cell counts and concentrations of cyanotoxins in the water, variable even within the same species.

Considering exclusively the cell count can cause an overestimate of systemic effects risk, when the ratio toxic versus nontoxic individuals is low and when strains with low cell quota are the majority; on the other hand, the risk can be underestimated during bloom senescence, when cyanotoxins may persist in the water after the bloom has subsided and is no longer visible. Therefore, the detection of specific toxin in water is the optimal metrics to protect water users from systemic effects.

Overall conclusions

The potential risk arising from exposure to cyanotoxins for both animal and human health is assuming an increasing relevance due to the higher demands upon water resources for drinking, recreation, aquaculture and irrigation and for wastewater discharge due to population rise. This is paralleled by an enhanced spread out of CB and the potential increase in extension and frequency of blooms related to climate changes. In addition, some aspects are still little explored, including the occurrence of marine CB and terrestrial cyanobacterial communities, especially symbionts of edible plants, and the importance of freshwater CB communities in providing potential inocula of biomass in costal water bodies after flooding periods or heavy rains. For risk management procedures to be effective, advances in knowledge are crucial in order to adopt adequate regulations or guidelines appropriate for human and animal health protection. Last but not least, it is necessary to improve the awareness of the hazard related to CB and cyanotoxin exposure and to implement an effective communication, between scientists, professionals of water usage sectors and end users.

In this paper, the available literature on the most frequently found cyanotoxins has been critically reviewed, although being aware that many other bioactive compounds can be of relevance. This consideration further pointed out the need to improve knowledge about CB and cyanotoxins, and the possibility of complex interactions between different toxins/variants/bioactive compounds that can be concurrently present in water, soil, air as well as in edible items.

The number of producing organisms in the freshwater, marine and terrestrial environment as well as toxin detection is possibly underestimated due to the lack of validated analytical methods and standards, especially for matrices other than water (e.g., food items and/or biological fluids). As a consequence, data on human exposure are still limited. The toxicological database also needs to be expanded, in order to reduce the degree of uncertainty related to derivation of health-based values. To achieve these goals, more appropriate studies are recommended.

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