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Review

Occurrence and toxicity of microcystin congeners other than MC-LR and MC-RR: A review

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ABSTRACT

The occurrence of cyanobacterial toxins is being increasingly reported. This is a reason for concern as they can induce toxic effects both in humans and in the environment. Among them, microcystins (MCs) are the best described and most diverse group of cyanobacterial toxins, and MC-LR and MC-RR are the congeners most widely investigated. However, the number of MC variants has also increased in recent years. Some of these minority variants have been shown to have a different toxicokinetic and toxicodynamic profile, but research focused on them is still limited. Moreover, in some water bodies these minority variants can be the predominant toxins. Nonetheless, MC-LR is the only one used for risk evaluation purposes at present. In order to contribute to more realistic risk assessments in the future, the aim of this review was to compile the available information in the scientific literature regarding the occurrence and concentration of minority MCs in water and food samples, and their toxic effects. The data retrieved demonstrate the congener-specific toxicity of MCs, as well as many data gaps in relation to analytical or mechanistic aspects, among others. Therefore, further research is needed to improve the toxicological characterization of these toxins and the exposure scenarios.

1. Introduction

Cyanobacteria toxins are a diverse group of secondary metabolites that includes hepatotoxins (microcystins, nodularin), cytotoxins (cylindrospermopsin), neurotoxins (anatoxins, saxitoxins, β-methylamino-L-alanine), and dermatotoxins (lipopolysaccharide, lyngbyatoxins, aplysiatoxin) (Sanseverino et al., 2017). Among them, microcystins (MCs) are the best described and most diverse group of cyanobacterial toxins (Neilan et al., 2013; Preece et al., 2017). Moreover, they are the toxins most frequently produced by freshwater cyanobacteria genera, such as *Microcystis*, *Aphanizomenon*, *Planktothrix, Dolichospermum*, etc. (Buratti et al., 2017). Twenty-three cyanobacterial genera and at least 47 species are known or suspected to produce MCs (Catherine et al., 2017). Eutrophication is consistently recognized as the main driver of cyanobacterial blooms, which is the massive proliferation of these cyanobacteria species. Currently, MC-producing cyanobacteria blooms have been reported in 80 countries (Catherine et al., 2017), cyanobacterial abundance has increased disproportionately relative to other phytoplankton, and this trend is likely to continue in the coming decades (Huisman et al., 2018). This is a reason for concern, as bloom occurrence and cyanotoxin production are closely linked.

With regard to their chemical structure, MCs are cyclic heptapeptides with the general formula cyclo(D-Ala-L-X-D-*erythro*-β-methylAsp-L-Z-Adda-D-Glu-*N*-methyldehydro-Ala), where Adda refers to the β-amino acid (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, unique for cyanobacteria (Botes et al., 1984) (Fig. 1). The L-amino acid residues at positions 2 (X) and 4 (Z) result in structural variations of the molecule. Thus, for example, the most common MC congener, MC-LR, contains leucine (L) and arginine (R) at positions 2 and 4, respectively. However, all other MC residues are also variable to some extent. Thus, so far at least 246 variants have been identified (Spoof and Catherine, 2017). Among all these variants, as mentioned earlier, MC-LR is the most common and most studied congener, followed by MC-RR (with two arginines at positions 2 and 4). Currently, MC-LR is considered as the reference compound among MCs because of its toxicity and high occurrence. However, blooms frequently contain different MC variants at the same time. Graham et al. (2010) found that MCs were the most abundant cyanotoxins in blooms from the USA, being present in all of them. Moreover, different cyanotoxin types were found in 48% of cyanobacterial blooms and 95% had multiple MC variants. With regard to all these other MCs apart from MC-LR, the data available in the scientific literature about their occurrence and toxicity are more limited (Testai et al., 2016; Buratti et al., 2017).

The overall structure of MCs is relatively hydrophilic (polar), which comes from the carboxylic acids at positions 3 and 6 and the frequent occurrence of arginine at positions 2 and 4 (Catherine et al., 2017).

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Abbreviations list

A549 Human alveolar basal epithelial cells
ACHN Human Kidney Adenocarcinoma
ALP Alkaline phosphatase
ALT Alanine aminotransferase
AST Aspartate aminotransferase
ATP Adenosine triphosphate
BGAS Blue-green algae food supplements
b.w. body weight
Caco-2 Human colorectal adenocarcinoma cell line
CAT Catalase
CGNs Cerebellar granule neurons
CyA Cyclosporine A
Cys Cysteine
Didm Didemethyl
Dm Demethylated
Dmdm double demethylated
DOC Dissolved organic carbon
d.w. dry weight
EC_{50} Mean effective concentration
EFSA European Food Safety Authority
ELISA Enzyme-linked immunosorbent assays
EROD cytochrome P-450-dependent 7- ethoxyresorufin O deethylase
f.w. fresh weight
G Gills
Gb Gall bladder
G-GT Gamma-glutamyl transferase
GLU glucose
GPx Glutathione peroxidase
GR Glutathione reductase
GSH Glutathione
GST Glutathione S-transferase
(s)GST (soluble) Glutathione S-transferase
HEK293 Human embryonic kidney cell line
K Kidney
HeLa Human cervix epithelioid carcinoma cell line
HepG2 Human hepatocellular carcinoma
HPLC High performance liquid chromatography
HPLC-ESI-MS/MS High-performance liquid chromatography/electrospray ionization tandem mass spectrometry
HPLC-PDA High performance liquid chromatography with photodiode Array Detector
HPLC-UV High pressure Liquid chromatography-ultraviolet detector
I Intestine
i.p. Intraperitoneal
L Liver
LC-ESI-MS Liquid chromatography-electrospray ionization mass spectrometry
LC-ESI-MS/MS Liquid chromatography-electrospray ionization source-tandem mass spectrometry
LC-MS Liquid chromatography–mass spectrometry
LC-MS/MS Liquid chromatography-tandem mass spectrometry
LC-HRMS High-resolution LC-MS
LC_{50} Lethal Concentration 50
LD lactate dehydrogenase enzyme activity
LD_{50} Median lethal dose
LDH Lactate dehydrogenase
LPO Lipid peroxidation
M Muscle
MALDI-TOF Matrix assisted laser desorption ionization-time of light spectroscopy
MCs Microcystins
MDA Malondialdehyde
MN Micronucleus
mOatps Mouse organic anion-transporting polypeptide
MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt)
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide
mWBC Primary murine whole brain cells
NOM Natural organic matter
Nrf2 Nuclear erythroid 2-related factor 2
NMR Nuclear magnetic resonance
NRU Neutral red uptake
OATPs Organic-anion-transporting polypeptides
P19/A15 Murine embryonic carcinoma cells derived from P19 cell line
PC Protein content
PLHC-1 *Poeciliopsis lucida* hepatocellular carcinoma cell line
PMA Phorbol myristate acetate
POD Peroxidase
PP Protein phosphatase
PP2A Protein phosphatase 2
PPIA Protein phosphatase inhibition assay
RCA Rainbow trout gill-w1 cytotoxicity assay
Resazurin 7-Hydroxy- 3H-phenoxazin-3-one 10-oxide
ROS Reactive oxygen species
RTG2 *Oncorhynchus mykiss* trout Rainbow fibroblast gonad tissue cell line
RTgill-W1 *Oncorhynchus mykiss* rainbow trout gill cell line
SOD Superoxide dismutase
SRB Sulphorhodamine B assay
TBARS total thiobarbituric acid reactive species
TDI Tolerable Daily Intake
T&O four taste and-odor
UHPLC-MS Ultra-high performance liquid chromatography-mass spectrometry
WHO World Health Organization
WST-1 [2-(4-Iodophenyl)-3- (4-nitrophenyl)-5- (2,4-disulfophenyl)-2H-tetrazolium]
zfOatps Zebrafish Oatps

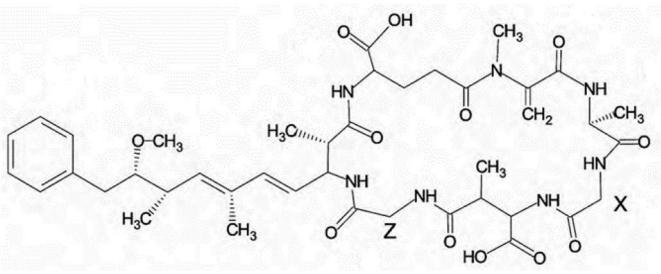

Fig. 1. General chemical structure of MCs.

However, among the wide range of MC variants there are also more lipophilic compounds than MC-LR. This is the case, for example, with MC-LW and MC-LF, which have tryptophan or phenylalanine, respectively, at position 4. This implies that the toxicity of congeners will differ, and some of them can be even more toxic than MC-LR. In this regard, Lürling and Faassen (2013) suggested that risk assessment can best be based on the toxicity contributions of all MC variants rather than only on MC-LR concentrations.

MCs are mostly intracellular (Rohrlack and Hyenstrand, 2007), although bloom lysis can lead to high extracellular concentrations. Owing to their predominant occurrence in freshwater ecosystems (although

they are also expanding in estuarine, marine, and even terrestrial ecosystems), they can be accumulated in aquatic organisms and transferred to higher trophic levels, with the risk of wildlife, livestock, pet, and human poisoning. In order to avoid toxic effects on humans, the World Health Organization (WHO) released a provisional drinking water guideline of 1 μg/L for MC-LR, excluding other known cyanotoxins because there was insufficient data to derive guideline values for them (Testai et al., 2016; WHO, 2003). Therefore, there is a need to generate new data and compile the existing information on minority MCs, this is, the MC variants that up to date are less studied than most abundant MCs, MC-LR or MC-RR.

Thus, the aims of this work are 1) to gather the available scientific information regarding MCs other than MC-LR and MC-RR (the latter was recently reviewed by Díez-Quijada et al., 2018) with respect to their occurrence in water and food, and toxicological studies in a wide range of experimental models (*in vitro*, *in vivo*, insects, aquatic organisms, birds, mammals, vegetables, and field studies), in the last few years (2010–2018); and 2) to derive conclusions about data gaps and future needs in this field in order to support more realistic risk evaluations.

2. Presence and concentrations of MC congeners other than MC-LR and MC-RR in water and food samples

2.1. Water reservoirs

In mixed field populations of cyanobacteria, MC variant occurrence depends on the interactions between the variable composition and predominance of cyanobacterial strains in freshwater, which are related to its trophic status, and environmental factors such as light, nutrients, temperature, hydrological parameters, etc., although these relationships are complex (Graham et al., 2010; Amé et al., 2010; Singh et al., 2015; Testai et al., 2016; Zhang et al., 2018; Turner et al., 2018). A study of the scientific literature published in the last decade demonstrates the presence of minority MC congeners other than MC-LR and MC-RR in water samples around the world, usually together in the same bloom with these predominant MCs, and the reports worthy of consideration are contained in Table 1. From the analysis of Table 1 a clear observation can be derived, that the occurrence of individual MC variants is very scarce, whereas a mixture of distinct MC variants is present in nearly all water reservoirs. Globally, among minority MC congeners, MC-YR and MC-LA are the variants most frequently detected in water samples, and in some cases they are predominant (Okello et al., 2010; Mankiewicz-Boczek et al., 2011; Jakubowska et al., 2013; Fetscher et al., 2015; Greer et al., 2016; Gurbuz et al., 2016; Simiyu et al., 2018). A more detailed description of MC congener distribution in each continent is provided below.

The occurrence of minority MC congeners in Africa could be a consequence of the particular vulnerability of this continent to cyanobacterial blooms owing to the eutrophication of many water bodies (Preece et al., 2017). The predominance of some variants, such as MC-RY, has scarcely been reported (Okello et al., 2009, 2010), and in some lakes the variant [*N*MeSer^7]-MC-YR was predominant. The authors concluded that *Microcystis* populations differ genetically with regard to the proportion of *mcy*B, which could explain the divergences in MC content of *Microcystis* cells among these lakes (qualitatively and quantitatively). They considered that these differences have a greater impact on MC production than the influence of several environmental factors (such as irradiance, etc.). In terms of toxicity, the fact that MC-LR was only rarely detected in Ugandan lakes (in contrast to its predominance in Europe), and its ten-fold higher toxicity to vertebrates when compared to MC-RR and other variants, suggest that these water samples should also be less toxic to livestock and humans (Okello et al., 2010). The presence of the MC-YR congener has also been reported in Kenya and Tanzania (Miles et al., 2012, 2013). MC-FR, a minor and relatively more hydrophobic MC variant, was detected in Moroccan and Tunisian freshwaters (Douma et al., 2010; Fathalli et al., 2011), together with 7 other MC variants in the latter case.

MC-YR and MC-LR have recently been reported as the major MCs present in some reservoirs (Major et al., 2018; Simiyu et al., 2018). In other African lakes MC-LR was the most abundant congener, with MC-YR, MC-RR, and MC-LA being less abundant (Douma et al., 2010; Sitoki et al., 2012; Krienitz et al., 2013; Willén, 2011). The occurrence of MC-WR at minor concentrations, and other MC variants (MC-(H4)YR), has also been reported and has been related to *M. aeruginosa* dominance (Mbukwa et al., 2012). However, in Moroccan water systems MC-WR and MC-RR were usually abundant in *Microcystis* strains, in comparison with other countries, and could constitute more than 70% of total MCs (Douma et al., 2017). The predominance in all cases of two MCs, in this case MC-WR and MC-RR, is in agreement with the fact that although many cyanobacterial strains can produce several MC variants, one or two are usually dominant in the same strain (Sivonen and Jones, 1999; Douma et al., 2017).

In many cases, a high diversity of MC analogs was reported, as in the case of 21 MC congeners identified in Algeria, in Lake des Oiseaux (Bouhaddada et al., 2016): 10 of them were well characterized, MC-RR being the major one (43.4%), followed by MC-LR, MC-FR, MC-WR, and MC-YR, and two new minor ones, [Asp^3]MC-HarAba and [Glu(OCH$_3$)^6] MC-FR, were identified. The last new minor MC congener is derived from the major congener MC-FR (9.4%), by the introduction of O-methylated D-*iso*-Glu instead of D-*iso*-Glu. Some studies have related the presence of D-Glu(OCH$_3$) in the structure with a lower toxicity (Namikoshi et al., 1992). The minor MC congener characterized as [Asp^3]MC-HarAba consists in the presence of the unusual residue of homoarginine (Har) and L-aminobutanoic acid (Aba) in positions 2 and 4, respectively. The presence of the Aba residue is very scarce in the scientific literature, and so far only 6 MC congeners have been characterized with this specific amino acid, with a worldwide distribution (Bouhaddada et al., 2016).

In the Americas, MC-LA has also been reported, which is of growing concern (Amé et al., 2010; Lawton et al., 2010; Fetscher et al., 2015); the latter authors detected MC-LA (and other variants) in liver and muscle of fish (see next section). MC-LA was also the most common MC congener detected in Pinto Lake (USA), among the following variants detected: MC-YR, MC-LF, MC-desmethyl-LR, MC-RR, and MC-LR; and it was implicated in the deaths of sea otters (Miller et al., 2010). In a wide survey of cyanotoxins in the USA, five MC congeners were detected, including MC-LA, MC-LY, and MC-YR (Loftin et al., 2016). Zastepa et al. (2014) have suggested that MC-LA has a longer half-life and greater persistence than the main MC congeners (MC-LR, MC-RR, MC-YR). In contrast to MC-RR, MC-LA was not observed to deposit onto sediments, and remained preferentially in the water phase, maybe owing to its net −2 charge, demonstrating congener-specific deposition (Zastepa et al., 2014). The same authors investigated the distribution and flux of 9 MC congeners in lake sediments (Zastepa et al., 2017). MC-LA again had a tendency to distribute more into the pore water phase in comparison to MC-LR. The low affinity of MC-LA for sediment was explained by electrostatic repulsion and/or high solvation resulting from the ionization of MC-LA in sediment pore waters. In contrast, MC-YR and MC-RR had a tendency to adsorb to sediment particles. The results suggested a higher remobilization of MC-LA compared to MC-LR, and, globally, the diffusion of MCs from sediments represents a potential risk for human exposure in the absence of visible surface blooms (Zastepa et al., 2017).

In Asia, most of the studies indicated the detection of MC-YR, although this toxin was present at lower concentrations in comparison to MC-LR or MC-RR (Li et al., 2010; Liu et al., 2011; Yen et al., 2011; Srivastava et al., 2012; Zhang et al., 2015, 2018; Jia et al., 2016). China was the country where more different types of MC congeners were detected (Krüger et al., 2010; Yen et al., 2011; Shang et al., 2018; Zhang et al., 2018) and where most of the studies were carried out. Other congeners were MC-LA, MC-LF, and MC-LW, and desmethylated

Table 1
Presence of MC variants in the different continents.

Country	Location	Congeners identified	Analytical method	Sample date	Reference
Africa	Two Moroccan lake reservoirs (Morocco)	**MC-YR, MC-FR, MC-WR,** MC-LR, MC-RR	HPLC-PDA LC-MS	2004	Douma et al. (2010)
	Uganda (five freshwater lakes)	**MC-RY** (40.5 ± 3.2 μg/L), **[NMeSer^7]-MC-YR** (15.5 ± 2.8 μg/L), **MC-YR** (6.6 ± 0.9 μg/L), **[Asp^3]MC-RY** (4.8 ± 1.2 μg/L), **[Asp^3]MC-YR** (0.3 ± 0.1 μg/L), MC-LR (3.3 ± 0.6 μg/L), MC-RR (23.0 ± 2.1 μg/L), [Asp^3]MC-RR	HPLC-PDA	May 2007 to April 2008	Okello et al. (2010)
	Tunisia (seven tunisian reservoirs)	**MC-FR, MC-YR, MC-WR, dmMC-YR,** MC-LR, MC-RR, dmMC-LR, dmMC-RR	MALDI-TOF	–	Fathalli et al. (2011)
	Rift Valley	**MC-YR** (0.4–9.7 μg/L), MC-LR (0.6–28.6 μg/L), MC-RR (0.2–2.9 μg/L), MC-dmLR	HPLC	December 2006	Willén, 2011
	South Africa's North-West (Hartbeespoort Dam)	**MC-YR** (0.14–71.22 μg/g DW), **MC-WR, MC-(H$_4$) YR,** MC-LR (0.38–140.68 μg/g DW), MC-RR (5.94–268.16 μg/g DW), (D-Asp^3, Dha^7) MC-RR	LC-ESI-MS	December 2010 to March 2011	Mbukwa et al. (2012)
	Lake Naivasha (African Rift Valley)	**MC-YR** (0.013 μg/L), **MC-LA** (0.003 μg/L), MC-LR (0.006–0.41 μg/L), MC-RR (0.001–0.011 μg/L)	LC-MS/MS	2008–2013	Krienitz et al., 2013
	Lake Victoria (Tanzania)	**[Mser^7]MC-YR**, [Mser^7]MC-LR, **MC-YR, MC-RA, MC-RY, [Asp^3]MC-YR, [Dha^7]MC-YR,** [Asp^3]MC-LR, **[Dha^7]MC-RY, MC-HilR, MC-FR, [Asp^3]MC-RY, [Mser^7]MC-RY,** [Dha^7]MC-LR, **MC-RAba, MC-RApa, MC-RL, MC-RF, MC-YA, MC-LA, MC-YAba, MC-LAba,** MC-LR, MC-RR	LC-MS^2, LC-MS/MS, LC-HRMS, NMR spectroscopy	2010	Miles et al. (2013)
	Lake des Oiseaux (North-eastern Algeria)	**MC-FR** (9.4%), **MC-WR** (7.4%), **MC-YR** (5.4%), **MC -LA** (2.2%), **[Asp^3]MC-Raba** (2.0%), **MC-HilR** (1.5%), **MC-(H4)YR** (1.2%), **[Glu(OCH$_3$)MC-LR** (0.9%), **[Glu(OCH$_3$)MC-FR** (0.9%), **[Asp^3]MC-HarAba** (0.6%), MC-LR (13.1%), MC-RR (43.4%)	LC-MS/MS PPIA	September 2013	Bouhaddada et al. (2016)
	Morocco (Natural Lakes)	**MC-WR** (42.58–47.86%), **MC-DM-WR** (14.53–18.06%), **MC-YR** (4.99–6.3%), MC-RR (28.59–37.9%)	HPLC-PDA	October 2005	Douma et al. (2017)
	Koka reservoir (Ethiopia)	**MC-YR, MC-LA,** MC-LR, MC-RR, MC-dmRR, MC-dmLR	HPLC-PDA LC-MS/MS	Ma, 2013 to April 2014	Major et al. (2018)
	Kisumu Bay (Lake Victoria)	**MC-YR** (0.5–343 μg/L), MC-LR (0.4–210 μg/L)	HPLC-PDA	October 2011 to January 2012	Simiyu et al. (2018)
America	Los Padres Lake (Argentina)	**MC-LA** (cellular: 0.29 ± 0.04 μg/L; dissolved: 0.13 ± 0.16 μg/L), **MC-YR** (cellular: 0.01 ± 0.01 μg/L; dissolved: 0.04 ± 0.07 μg/L), MC-RR (cellular: 1.30 ± 2.92 μg/L; dissolved: 1.50 ± 2.15 μg/L), MC-LR (cellular: 0.05 ± 0.09 μg/L; dissolved: 0.04 ± 0.05 μg/L)	LC-ESI-MS/MS	2007	Amé et al. (2010)
	Midwestern United States (23 lakes)	**MC-LA** (0.02–54 μg/L), **MC-LF** (0.02–51 μg/L), **MC-LW** (0.02–56 μg/L), **MC-LY** (0.02–200 μg/L), **MC-YR** (0.01–240 μg/L), MC-RR (0.03–16000 μg/L), MC-LR (0.06–2100 μg/L)	LC-MS/MS ELISA	August 2006	Graham et al. (2010)
	Ponds (Alabama)	**MC-LA** (13.7–33.6 μg/L)	HPLC	2007–2008	Lawton et al. (2010)
	Lake Pinto (EEUU)	**MC-LA** (2100 mg/L), **MC-YR, MC-LF,** MC-RR, MC-LR, MC-dLR	LC-MS/MS	2007	Miller et al. (2010)
	Lago de Patzcuaro (Mexico)	**MC-LA, MC-LY,** MC-LR, Didm-MC-LR, Didm-MC-RR	LC-MS/MS	July 2008 and June 2009	Berry et al. (2011)
	Lake Amatitlán (Guatemala)	**MC-YR** (3.31–6.48 μg/L), MC-LR (37.20–42.23 μg/L), MC-RR (19.95–41.29 μg/L)	LC-MS/MS	–	Romero-Oliva et al., 2014
	Ottawa (Canada)	Bloom 2009: **MC-LA** (intracellular 2472 ± 1336 μg/L; dissolved: 203 ± 14 μg/L). Bloom 2010: **MC-LA** (intracellular: 49.4 ± 13.1 μg/L; dissolved: 1.3 ± 0.3 μg/L), **MC-YR,** MC-LR (intracellular 0.9 μg/L), MC-RR (intracellular 0.2 μg/L)	Reverse phase HPLC-PDA	2009–2010	Zastepa et al. (2014)
	Monterey Bay (California)	**MC-LA** (43%), **MC-YR**, MC-LR (37%), MC-RR (6%)	LC-MS	2007–2013	Fetscher et al. (2015)
	Puget Sound (Washington)	**MC-LA,** MC-RR	ELISA UPLC-MS/MS	September to December 2013	Preece et al. (2015b)
	United States (1161 natural lakes and reservoirs)	**MC-LA, MC-LY, MC-YR,** MC-LR, MC-RR	LC-MS/MS	May and October 2007	Loftin et al. (2016)
	Lake of the Woods (Canada)	**MC-LA** (41%), MC-LR (59%)	LC-MS/MS	–	Zastepa et al. (2017)
Asia	Lake Chao (China)	**MC-YR, MC-LA, MC-LF, MC-LW,** MC-LR, MC-RR also dm-MCs and dmdm-MCs	LC-ESI-MS/MS	July 2007	Krüger et al. (2010)
	Yanghe Resevoir (China)	**MC-YR** (dissolved: 0.066 μg/L-intracellular: 3.76 μg/L), MC-RR (dissolved: 1.56 μg/L-intracellular: 70.1 μg/L), MC-LR (dissolved: 0.544 μg/L-intracellular: 24.6 μg/L)	LC-MS/MS	2007	Li et al. (2010)
	Lake Taihu (China)	**MC-YR,** MC-LR, MC-RR	HPLC-UV	2004	Liu et al. (2011)
	Taiwan (China): 3 eutropic lakes	**MC-YR** (40–240 ng/L), **MC-LW** (50–370 ng/L), **MC-LF** (90–1700 ng/L), **MC-LA** (30–100 ng/L), MC-LR (40–550 ng/L), MC-RR (50–790 ng/L)	Solid phase extraction (SPE) and LC-MS	2007	Yen et al., 2011a
	Moo-Tan Reservoir (Taiwan)	**MC-YR, MC-LW, MC-LF,** MC-LR, MC-RR	LC-MS	2003–2007	Yen et al., 2011
	Varanasi (India)	**MC-YR** (7.26–56.00 μg/L), MC-LR (17.15–237.09 μg/L), MC-RR (41.33–291.27 μg/L)	LC-MS	May 2010 to April 2011	Srivastava et al. (2012)
	Huai River Basin (China)	**MC-YR** (21–71%), MC-LR (24–60%), MC-RR (4–19%)	LC-PDA	2008–2009	Tian et al. (2013)
	Lakshmikund pond (India)	**MC-YR,** MC-RR, MC-LR	UHPLC-MS	2011–2012	Singh and Asthana, 2014

(*continued on next page*)

Table 1 (*continued*)

Country	Location	Congeners identified	Analytical method	Sample date	Reference
	Two freshwater ponds (India)	**MC-YR** (14–18%), MC-LR (23–24%), MC-RR (60–64%)	LC-MS	2010–2011	Singh et al. (2015)
	Poyand Lake (China)	**MC-YR** (Intracellular: 0.8%; extracellular: 2.63%), **MC-LA** (Intracellular: 0.02%), MC-LR (Intracellular: 4.65%; extracellular: 13.17%), MC-RR (Intracellular: 94.70%; extracellular: 84.73%)	UPLC-MS/MS	2012	Zhang et al. (2015)
	Lake Taihu (China)	**MC-YR** (13.9–50.6 ng/L), MC-LR (27.8–113.9 ng/L), MC-RR (35.1–111.5 ng/L)	LC-MS/MS	2013–2014	Jia et al. (2016)
	Lake Chaohu (China)	**MC-YR** (extracellular: up to 1.34 μg/L; intracellular: up to 0.82 μg/L), MC-LR (extracellular: up to 0.98 μg/L; intracellular: up to 1.15 μg/L), MC-RR (extracellular: up to 1.42 μg/L; intracellular: up to 0.56 μg/L)	HPLC	August 2011 to July 2012	Shang et al. (2018)
	Poyand Lake (China)	**MC-YR** (extracellular: up to 2.71 ng/L; intracellular: up to 30.61 ng/L), MC-RR (extracellular: up to 372.19 ng/L; intracellular: up to 8067.09 ng/L), MC-LR (extracellular: up to 70.27 ng/L; intracellular: up to 1975.07 ng/L)	UPLC-MS/MS	2013	Zhang et al. (2018)
Europe	North of Ireland (14 sites)	**MC-YR** (1.2–1388 ngMC/μgChla), **MC-LY** (1.8–150 ngMC/μgChla), **MC-LF** (2.4–290 ngMC/μgChla), MC-LR (3–3590 ngMC/μgChla), MC-RR (2.6–1510 ngMC/μgChla)	LC-MS/MS	September 2008	Mooney et al., 2011
	Greece (Lakes)	**MC-YR** (0–0.004 μg/L), MC-LR (0.004–0.014 μg/L), MC-RR (0.005–0.060 μg/L)	LC/PDA LC-MS/MS	–	Triantis et al. (2010)
	Jeziorsko Reservoir (Central Poland)	**MC-YR** (0.43 μg/L), MC-LR (0.38 μg/L), MC-RR (0.23 μg/L)	HPLC	2008	Mankiewicz-Boczek et al. (2011)
	Amvrakikos Gulf (NW Greece)	**MC-YR**, MC-LR	LC-MS	2006–2007	Vareli et al. (2012)
	The Netherlands (water samples and scum samples)	Water samples: **MC-YR** (0.01–41 μg/L), **MC-LY** (0.001–110 μg/L), **MC-LW** (0.05–260 μg/L), **MC-LF** (0.05–33 μg/L), MC-LR (0.01–2100 μg/L), MC-RR (0.02–66 μg/L), dm-7-MC-RR, dm-7-MC-LR Scum samples: **MC-YR** (1.2–1200 μg/L), **MC-LY** (24–2300 μg/L), **MC-LW** (10–990 μg/L), **MC-LF** (8.0–1800 μg/L),MC-LR (3–7900 μg/L), MC-RR (3.8–4600 μg/L), dm-7-MC-RR, dm-7-MC-LR	LC-MS/MS	–	Faassen and Lürling. (2013)
	Lake Marathonas, Athens (Greece)	**MC-YR** (2–717 ng/L), **MC-LA** (5–8 ng/L), MC-LR (2–451 ng/L), MC-RR (2–174 ng/L)	LC-ESI.MS/MS	July 2007–December 2010	Kaloudis et al. (2013)
	Great Mazurin Lakes (Poland)	**MC-YR** (0.006–0.074 μg/L), MC-LR (0.001–0.037 μg/L), MC-RR (0.003–0.048 μg/L)	LC-MS/MS	2007	Jakubowska et al. (2013)
	Alqueva and Boliche water reservoirs (Portugal)	**MC-YR** (33–346 ng/L), MC-LR (15–344 ng/L), MC-RR (14–212 ng/L)	LC- MS/MS (negative ionization mode)	2011	Rodrigues et al. (2013)
	Aland Islands (Finland)	**MC-YR** (17%), **dmMC-YR** (14%), **MC-LF** (1%), **MC-LW** (1%), **MC-LY** (1%), MC-LR (34%), MC-RR (44%), dmMC-RR, dmMC-LR, didmMC-RR, didmMC-LR	LC-MS	June to November 2009	Savela et al. (2014)
	Six countries throughout Europe (France, Italy, Ireland, Germany, Bulgaria, Turkey)	**MC-YR, MC-LY, MC-LF,MC-RL, MC-RY,** MC-LR, MC-RR	UPLC-MS/MS	2012–2014	Greer et al., 2016
	Bulgaria (6 water bodies)	**MC-YR** (0.3–71.8 μg/L), MC-LR (0.1–132.8 μg/L), MC-RR (0.07–103.2 μg/L)	LC-PDA	2012–2014 (July–October)	Pavlova et al. (2015)
	The Curonian Lagoon (Lithuania)	**MC-LW** (up to 8.80 μg/L), **MC-LF** (up to 7.60 μg/L), **MC-LY** (up to 4.80 μg/L), **MC-LA** (up to 0.80 μg/L), **MC-YR** (up to 0.10 μg/L), **MC-VR**, MC-LR (up to 62.40 μg/L), MC-RR (up to 7.20 μg/L)	LC-MS/MS	2014	Šulčius et al., 2015
	Lake Egidir (Turkey)	**MC-YR** (2.9–13.5 μg/L), **MC-LY** (4–6.6 μg/L), **MC-LF, MC-LW**	ELISA LC-PDA	2013	Gurbuz et al. (2016)
	Lakes and ponds (Sweden)	**MC-YR** (0.70–11.70 μg/L), **MC-LF, MC-LW, MC-LY**, **MC-HtyR- [D-Asp3- *(E)*-Dhb7]**, **MC- [D-Asp3- *(E)*-Dhb7]-Hphr**, MC-LR (0.68–16.80 μg/L), MC-RR (0.52–40.35 μg/L)	UPLC-MS/MS	–	Pekar et al. (2016)
	Occhito Lake (Italy)	**MC-HtyR,** [D-Asp^3]-MC-RR	LC-MS/MS	2010–2011	Nigro Di Gregorio et al., 2017
	Greece (lake water samples)	**MC-YR** (0.05–3.6 μg/L), **MC-HilR** (0.10–0.42 μg/L), **MC-WR** (0.11–0.51 μg/L), **MC-LA** (0.54 μg/L), **MC-LY** (0.16 μg/L),MC-LR (0.063–18 μg/L), MC-RR (0.062–63 μg/L), [D-Asp3]MC-RL, [D-Asp3]MC-RR	LC-MS/MS	October 2014 and 2015	Zervou et al. (2017)
	France	**MC-YR** (0.05 ± 0.09 μg/L), **MC-LF** (0.01 ± 0.02 μg/L), **MC-LW** (lower than 0.03 μg/L), **MC-LA** (lower than 0.03 μg/L), MC-LR (0.9 ± 1.3 μg/L), [Asp3]MC-LR, MC-RR (0.4 ± 1.1 μg/L), [Asp3]MC-RR	LC-MS/MS	2007, 2008, 2010	Pitois et al. (2018)
	England (70 water bodies)	**MC-YR** (≈25%), **MC-LA** (≈10%), **MC- LF** (≈5%), **MC-LW, MC-WR** (≈5%), **MC-LY, MC-HilR, MC-HtyR** (≈5%), MC-LR (≈35%), MC-RR (≈35%), D-Asp3-MC-LR, D-Asp3-MC-RR	LC-MS/MS	February to December 2016	Turner et al. (2018)
Oceania	Queensland (Australia)	**MC-LA** (9%), **MC-LF** (2%), **MC-FR** (1%), **MC-YR**, MC-LR (86%), dm-MC-LR	LC-MS	–	Cirés et al. (2014)

Abbreviations: dm: demethylated toxins; Didm: Didemethyl; dmdm: double demethylated toxins; DW: Dry weight; ELISA: Enzyme-linked immunosorbent assays; HPLC: High performance liquid chromatography; LC-PDA: Liquid chromatography- Photodiode Array detector; HPLC-UV: High pressure Liquid chromatography-ultraviolet detector; LC/ESI/Ion trap-MS/MS: LC-MS: Liquid chromatography-mass spectrometry; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; LC-HRMS: High-resolution LC-MS; MALDI-TOF: Matrix assisted laser desorption ionization-time of light spectroscopy; NMR: Nuclear magnetic resonance; PPIA: Protein phosphatase inhibition assay; UPLC-MS/MS: Ultra-light liquid chromatography-electrospray ionization tandem triple quadrupole/mass spectrometry; UHPLC-MS: Ultra-high performance liquid chromatography-tandem mass spectrometry.

variants and even double desmethylated variants were also found (Krüger et al., 2010). . Tian et al. (2013) investigated dissolved MCs in surface and ground waters in regions of China with high cancer incidence and found that the composition of MCs changed from surface to ground waters. Thus, in pond water MC-YR was dominant with 71%, but in river water MC-LR was dominant (60%). The results indicated that MCs are not transported equally through the aquifer, and suggested that MC pollution in groundwater might be due to the presence of these toxins in rivers, depending on the distance from them. The co-occurrence of MC-LR, MC-RR, and MC-YR, and four taste-and-odor (T&O) compounds is a growing concern and was recently investigated by Shang et al. (2018). The mutable relativities among the three MC congeners and the T&O compounds found were explained by the authors by the community dynamics of cyanobacterial strains, different metabolic pathways, and the different physicochemical properties such as volatility and degradability. In Indian water bodies with a predominance of *Microcystis* spp., the presence of MC-YR was reported, in a smaller proportion than MC-RR (usually dominant) and MC-LR, and it was the least prevalent variant (Srivastava et al., 2012; Singh and Asthana, 2014; Singh et al., 2015).

Europe was the continent where the occurrence of several MC variants was most frequently reported. Apart from MC-LR and MC-RR, MC-YR was one of the main MC congeners identified (Mooney et al., 2011; Savela et al., 2014; Pavlova et al., 2015; Greer et al., 2016), particularly in Mediterranean countries (Vareli et al., 2012; Kaloudis et al., 2013), usually associated with the dominance of *Microcystis* spp. (Savela et al., 2014). Actually, it was the most prevalent or dominant MC congener in water reservoirs in various European countries (Mooney et al., 2011; Mankiewicz-Boczek et al., 2011; Jakubowska et al., 2013; Greer et al., 2016; Gurbuz et al., 2016). In Irish lakes, MC-YR occurred at all the sites (Mooney et al., 2011). Moreover, MC-YR was the most frequently identified MC variant in Turkey (Gurbuz et al., 2016). Variations in the concentrations of three MC congeners (MC-LR, MC-RR and MC-YR) were time-dependent, and a seasonal trend was observed in Greece. In some periods MC-YR (and MC-LA) were found at trace levels, whereas in others MC-YR was the dominant MC congener (Kaloudis et al., 2013). Differences in the MC variant ratios during this study were associated with differences in cyanobacterial community composition, and environmental factors (light, nutrients, etc.). This important seasonal variation between the concentrations of MC congeners in different types of water samples collected from the same reservoir, indicating spatial and temporal variability, has also been reported in Portuguese reservoirs (Rodrigues et al., 2013). In addition, other MC variants, such as MC-LF, MC-LW, or MC-LY, were found in some water resources (Šulčius et al., 2015; Pekar et al., 2016). These variants tend to accumulate more efficiently in the exposed organisms, suggesting that, despite their relatively lower abundance, the toxicological effects of these hydrophobic congeners could be significantly higher than those of MC-LR or MC-RR (Lürling and Faassen, 2013).

Moreover, new and unusual MC variants have been detected and characterized in European waters in recent years. For example, Nigro Di Gregorio et al. (2017) reported the presence of MC-HtyR in Lake Occhito (Italy) for the first time, a MC variant unusually associated with a *P. rubescens* bloom in surface waters. This congener contains homotyrosine (Hty) in position 2 and arginine (R) in position 4, and the production of this peculiar toxin profile could be explained by genetic and environmental factors. Zervou et al. (2017) detected several minority MC congeners, such as MC-HilR. Turner et al. (2018) recently monitored cyanobacterial blooms from seventy water bodies throughout England, and a wide range of toxin analogs were detected. MC-LA, MC-LF, MC-WR, D-Asp^3-MC-LR, and MC-HtyR were each present at an average level of 5% of the total MCs, with MC-LY, MC-LW, MC-HilR, and D-Asp^3-MC-RR present only at trace levels. Another multi-toxin study, carried out in ten French water resource reservoirs in 2018 (Pitois et al., 2018), confirmed that multiple toxin-producing species are commonly encountered in cyanobacterial blooms, and toxin co-occurrence could be partially correlated with species composition and water temperature. The authors hypothesized that MC variant distribution is a direct consequence of species successional patterns, so, for example, MC-YR and MC-LF correlated with *Microcystis* biomass. The results also show that it is not appropriate to consider MC-LR as an indicator of MC occurrence, as other MC variants (MC-YR, -LW, -LF, etc.) did not correlate simultaneously with MC-LR, or were anti-correlated with [Asp^3]MC-RR/MC-RR.

In Australian freshwaters the presence of MCs has been widely reported during the last two decades, usually produced by the planktonic genus *Microcystis*. But the production of several MC variants by benthic bacteria, by *Stigonematales*, was reported for the first time by Cirés et al. (2014). In this case, MC-LR was the most abundant variant, followed by others such as MC-LA > MC-LF > MC-FR > dimethyl-MC-LR. Finally, the presence of cyanotoxins in extreme conditions has recently been reviewed (Cirés et al., 2017). Some minority MC congeners and MC-LR have been reported in polar regions, such as MC-FR, and variants characterized by rather uncommon amino acid substitutions. The variants MC-YR, MC-LA, and MC-LF were detected in phytoplankton populations in alkaline lakes, and the variants MC-YR and MC-LF in hot springs, and in tissues from dead flamingos. The authors pointed out the lack of geographically extensive studies in these extreme ecosystems and identified the main knowledge gaps to be overcome in the future.

Globally, although it is recognized that the variability of MC profiles produced by cyanobacteria is highly strain-dependent, further studies are needed to clarify the role of climatic and environmental factors on the variability in the production of MC variants, particularly those other than MC-LR, in natural waters. Moreover, although several multi-toxin methods have been developed to determine numerous MC variants (Greer et al., 2016; Pekar et al., 2016; Zervou et al., 2017), further robust, validated studies should be carried out in this direction, in order to improve the analytical characteristics that permit accurate determination of minority MC variants, because they are likely to contribute significantly to the total MC toxicity. In addition, there is a need for reference materials for correct identification of these minority MC congeners.

2.2. Food samples

The transference of MC through the food chain is well known (Gutiérrez-Praena et al., 2013), especially in the case of fish, in comparison to other aquatic organisms used as food (bivalves, etc.), and vegetables. The data collected show a broad prevalence of MCs in aquatic edible species around the world, confirming that accumulation of these toxins is a global concern (Testai et al., 2016). However, as in the case of the MC-RR congener (Díez-Quijada et al., 2018), in the last decade (2010–2018) only a few works reported the concentrations found for specific minority MC variants (Table 2). As can be seen in this table, more studies have been carried out in fish than in bivalves, MC-YR being the minority MC variant most frequently detected, or even the only one detected (Simiyu et al., 2018). The congener MC-LA was detected in bivalves and fish, and the concomitant presence of other MC congeners has scarcely been reported (Pawlik-Skowrońska et al., 2013; Gurbuz et al., 2016).

The European Food Safety Authority (EFSA) reported that the great majority of the studies have focused on the major congener, MC-LR, and only a limited percentage include the sum of the other toxins (Testai et al., 2016). Among the reasons given to justify the absence of these results were: 1) the need for chemical standards for variants of MCs and metabolites other than MC-LR; 2) the need for validated methodologies for matrices other than water (food), including detection techniques and adequate extraction procedures, depending on the objective (free toxin or total toxin); 3) available data of MC variant occurrence in field studies expressed as fresh weight are very scarce, etc. A brief description of the most important findings concerning the presence of minority MC variants in food follows.

Table 2
Presence of MCs congeners different to MC-LR and MC-RR in food samples.

Species (Common name)	MCs concentration (ng/g)	Location (Country, site)	Analytical method	Reference
BIVALVES				
Mytilus trossulus	**MC-LA** (1.5 ± 0.0–4.7 ± 0.3), **MC-LW,** MC-LR	Puget Sound (Washington)	HPLC	Preece et al. (2015a)
Mytilus trossulus	**MC-LA** (approx. up to 6.5)	Puget Sound (Washington)	ELISA	Preece et al. (2015b)
Sinanodonta woodiana	**MC-YR**	South Korea	HPLC-UV	Kim et al. (2017)
Sinanodonta arcaeformis	Muscle (M): approx. up to 2000			
Unio duoglasiae	M: approx. up to 2000			
	M: approx. up to 20000			
FISH				
Odontesthes bonariensis	Liver (L): **MC-LA:** ND-13.7 ± 1.6; **MC-YR:** ND-1.9 ± 0.7	Los Padres lake (Argentina)	LC-ESI-MS/MS	Amé et al. (2010)
	M: **MC-LA:** 0.5 ± 0.3–0.8 ± 0.8			
Oreochromis niloticus	**MC-YR**	Two freshwater bodies (Uganda)	LC-MS/MS	Nyakairu et al. (2010)
Lates niloticus	Intestine (I) > M			
	I			
Oreochromis niloticus (Nile tilapia)	**MC-YR**	Lakes (Uganda)	LC-MS	Semyalo et al. (2010)
	I > L > M			
Abramis brama	G: **MC-LF, MC-LY, MC-LA**	Zemborzycki dam reservoir (Poland)	HPLC-PDA	Pawlik-Skowrońska et al., 2013
	L: **MC-YR, MC-LY, MC-LA**, MC- LR, MC-RR			
	M: **MC-LY**, MC-RR			
Hypophthalmichthys molitrix (Silver carp)	**MC-YR**	Lake Taihu (China)	HPLC	Jia et al. (2014)
	I: 22.9 ± 43.6; L: 6.60 ± 4.01; M:5.66 ± 3.93; K: 5.57 ± 3.35;			
Aristicthys nobilis (Bighead carp)	I: 6.26 ± 4.40; K: 4.77 ± 1.31; L: 4.76 ± 2.84; M: 2.72 ± 2.54			
Carassius auratus (Crucian carp)	K: 14.2 ± 11.7; I: 12.4 ± 19.3; L: 7.40 ± 9.18; M: 3.15 ± 2.63			
Cyprinus carpio (Common carp)	I: 25.2 ± 59.0; M: 8.40 ± 12.1; K: 4.68 ± 1.50; L: 3.83 ± 1.99			
Cyprinus carpio (Common carp)	**MC-YR**	Lakshmikund pond (India)	UHPLC-MS	Singh and Asthana (2014)
Clarias batrachus (Catfish group 1)	L: 59.62 ± 5.41; K: 25.26 ± 2.38; I: 17.34 ± 2.18; Gb: 6.79 ± 0.46			
Clarias batrachus (Catfish group 2)	L: 94.66 ± 3.05; I: 74.06 ± 4.61; K: 64.66 ± 6.42; Gb: 33.61 ± 2.51; G: 3.60 ± 0.47; M: 1.39 ± 0.21			
	I: 134.11 ± 7.61; L: 115.33 ± 5.75; K: 75.31 ± 5.11; Gb: 38.28 ± 4.31; G: 4.16 ± 1.62; M: 1.71 ± 0.19			
Largemouth bass	**MC-LA**	Washington lakes	LC-MS/MS	Hardy et al. (2015)
Yellow perch	L: 2.5–12			
Rainbow trout	L: up to 14			
Cutthroat trout	L: < 1.0			
	L: < 1.0			
Cyprinus carpio (Common carp)	M: **MC-YR:** 180	Lake Egirdir (Turkey)	HPLC-PDA	Gurbuz et al. (2016)
Carassius gibelo	L: **MC-LA:** 240–25000; **MC-YR:** 850–1720; **MC-LF:** 2-300			
Atherina boyeri	M: **MC-YR**: 110–500; **MC-LY:** 205–345; **MC-LA:** 200-250			
	L: **MC-YR**: 550–750; **MC-LY**: 150			
	M: **MC-YR**: 950–1050; **MC-LA**: 570–630; **MC-LY** 450-940			
Hyporhamphus intermedius	MC-LR > MC-RR > **MC-YR**	Lake Taihu	LC-MS/MS	Jia et al. (2016)
Coilia ectenes				
Cyprinus carpio				
Neosalanx tangkahkeii taihuensis				
Silver carp				
Barbus sp	**MC-YR**	Kisumu Bay (Lake Victoria)	LC-MS/MS	Simiyu et al. (2018)
Haplochromis sp	8–20 ng MC/g DW			
Lates niloticus				
R. argentea				
Crustaceans				
Exopalaemon modestus	MC-LR > MC-RR > **MC-YR**	Lake Taihu	LC-MS/MS	Jia et al. (2016)
Macrobrachium nipponense				
VEGETABLES AND CROPS				
Plant species/Common name	**MCs concentration (ng/g)**	**Toxin source**	**Analytical method**	**References**
Lettuce	**MC-YR**: 22.8 (d.w.)/1 (f.w.)	Lettuce samples irrigated with Dianchi Lake water (China)	LC-MS/MS	Li et al. (2014)
Food Supplements	**MCs concentration (μg/g)**	**Toxin source**	**Analytical method**	**References**
BGAS	**MC-LA**: < LOD - 2.02; MC-LR: < LOD - 4.02	*Aphanizomenon flos aquae*	LC-MS/MS	Vichi et al. (2012)

Abbreviations: BGAS: Blue-green algae food supplements; d.w: dry weight; ELISA: Enzyme-linked immunosorbent assays; f.w: fresh weight; G: Gills; Gb: Gall bladder; HPLC: High performance liquid chromatography; HPLC-PDA High performance liquid chromatography with photodiode array detection; HPLC-UV: High pressure Liquid chromatography-ultraviolet detector; I: Intestine; K:Kidney; L: Liver; LC-MS: Liquid chromatography-mass spectrometry; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; LC-ESI-MS/MS: Liquid chromatography/electrospray ionization tandem mass spectrometry; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; M: Muscle; UHPLC-MS: Ultra-high performance liquid chromatography-tandem mass spectrometry.

Noteworthy in bivalves is the detection of three MC variants, MC-LA, MC-LR, and MC-LW, in mussels from Puget Sound Bay (USA). MC-LA was present during the entire sampling period, whereas MC-LR was detected earlier in the season, and MC-LW in samples harvested later (Preece et al., 2015a). The concentrations of MCs in mussels were below the Tolerable Daily Intake (TDI) guideline of 0.04 μg/kg body weight/day based on a consumption of 100 g of seafood daily. But the authors highlight two reasons why they may have significantly underestimated the risks: 1) they only measured 8 of the numerous known MC variants (because of limitations in analytical capabilities and available standards); 2) the values found were freely available MCs, and the covalently bound toxin content is variable and could account for > 75% in tissue (Williams et al., 1997; Catherine et al., 2017; Pham et al., 2017), because the bonded MC could be released during digestion processes (Díez-Quijada et al., 2018). Preece et al. (2015b) were also able to establish a direct relationship outside the laboratory (in the USA) between MC in inland lakes and marine seafood intoxication, reporting a high variability of MC values in mussels, marine receiving waters, and source lakes. Different accumulation of MCs may be explained in part because variants are assimilated unequally by filter organisms and eliminated as feces or pseudofeces; in addition, MC accumulation can vary with life stage and species type, etc. In this study, the highest measured MC concentration in mussels was obtained when no toxins were detected in water, in agreement with other studies (De Pace et al., 2014). Although the authors indicated that consumption of these mussels was safe on the basis of the established TDI (WHO, 2003), they pointed out the lack of standardized guidelines for accurate assessment of the risks of MCs to human health, and the need for further studies on the transfer of MC variants from freshwaters to marine environments (Preece et al., 2015b).

In fish, little research has been done on the distribution of minority MC variants, and variability of tissue bioaccumulation has been reported. In some cases the MC tissue concentrations decreased in the following order: gills > liver > muscles (Pawlik-Skowrońska et al., 2013); and not all congeners were detected. For example, MC-LY reached the highest amounts in gills, together with the isoforms MC-LF and MC-LA; whereas MC-LR was found only in the liver and MC-RR in liver and muscle. Differences in cellular uptake and organ distribution in fish could be partly explained by differences in cell membrane permeability (Vesterkvist and Meriluoto, 2003) and susceptibility to digestion (Smith et al., 2010). For example, the hydrophobic and proteolytic digestion-resistant MC-LY isoform that was detected in fish muscles is believed to be more cell-permeable than the hydrophilic variants (Pawlik-Skowrońska et al., 2013). In other studies, the highest levels of MC-YR (and MC-LR, and MC-RR) were detected in intestines, followed by liver and muscle (Nyakairu et al., 2010; Semyalo et al., 2010; Jia et al., 2014; Singh and Asthana, 2014). The fact that the gut contained MC, and that there was greater accumulation in this organ, has been reported previously and explained as being because the major route of fish exposure is through ingestion (Vasconcelos, 1995; Magalhaes et al., 2001). Once absorbed, MCs tend to accumulate in liver and muscle tissue, and in some cases the levels in muscle were not high (Table 2), in agreement with previous works which indicated that muscle accumulated toxins to a lesser extent (Magalhães et al., 2003; Vasconcelos, 1999; Xie et al., 2005). Jia et al. (2014) examined MC-YR (and MC-LR, and MC-RR) in phytoplanktivorous and in omnivorous fish species, and found no significant relationships between MC-YR in the intestinal wall and MCs in the liver and the kidney, suggesting that the uptake route of this congener may be different from that of MC-LR and MC-RR. Furthermore, the MC-YR levels in the intestinal wall were not significantly higher than in liver and kidney, and the toxin was not detected in the heart. In the case of *C. auratus* (omnivorous), the highest concentrations of MC-YR were found in the kidney, and the authors indicated that the main uptake route was probably direct uptake of dissolved MCs via the gills and not via the gastrointestinal tract (Zhang et al., 2009; Jia et al., 2014). As in the case of other major congeners (MC-LR and MC-RR), bioaccumulation of MC-YR was species-specific in fish (Singh and Asthana, 2014). Thus, levels of MC-YR were lower in carp than in catfish, and this could reflect the decreased grazing rate of carp. As stated earlier, the distribution of MC-YR in different organs was also variable, even within the same species (Table 2): liver was the organ with the highest values detected in some species (carp and catfish), whereas gut contained the maximum in others (catfish). In this work, no MCs were detected in brain or spleen (Singh and Asthana, 2014).

Surprisingly, MC-LA was the only variant detected in some fish liver when up to nine MC variants were determined by LC-MS/MS (Hardy et al., 2015), and no accumulation in muscle was detected. This variant was also found, together with MC-YR, in the liver and muscle of fish collected in Los Padres Lake (Argentina), and was the second congener found in water samples (Amé et al., 2010). The relative percentages of MC-LA in muscle during the dry and wet seasons were similar, and the results in this work indicated that the uptake of MC-LA and MC-LR were different, adding complexity to the association between the presence of MC in water and seston and the concentrations found in biota. The MC-LA levels in liver were higher than those found previously in tilapia (Deblois et al., 2008), and MC-YR liver contents in this work were lower than the levels reported in tilapia and carp (Deblois et al., 2008; Chen et al., 2007).

In crustaceans (Jia et al., 2016), vegetables, and crops (Li et al., 2014), knowledge about the presence of minority MC variants is even worse. Only Li et al. (2014) reported a frequency of 7.1% for MC-YR in vegetable samples collected from lakes in China, in the period of time reviewed. In blue-green algae food supplements (BGAS), the presence of various congeners, such as MC-LA and MC-LR, was demonstrated in products derived from *A. flos-aquae* (Vichi et al., 2012). The levels within the same brand varied significantly in different batches, indicating the need to monitor products of this kind, as they are proposed as health-promoting natural products.

Finally, the effects of several cooking procedures on MC concentration in food has been poorly studied. The few studies that have been conducted focused on MC-LR (Zhang et al., 2010; Guzmán-Guillén et al., 2011; Freitas et al., 2014), and the results were variable. In the case of MC-YR in fish muscle, experiments in the laboratory demonstrated considerable decreases, 24.6% or 56.4%, when samples were cooked in the microwave or by continuous boiling, respectively (Guzmán-Guillén et al., 2011).

From this review of the scientific literature it can be concluded that further studies focused on the seasonal variation of minority MC variant accumulation in fish and seafood, vegetables, and food in general are needed. As some authors indicated that bound MCs may become bioavailable in the digestive system of consumers, further *in vitro* and *in vivo* studies are required to investigate the bioaccessibility and bioavailability of some MC variants in different foods (fish, bivalves, vegetables), including the effect of various cooking procedures, following the recommendations of EFSA (Testai et al., 2016), in order to determine their contribution in risk assessment processes and their importance for the health of consumers.

3. Toxicokinetics

To date, there are very few studies on the toxicokinetic aspects of MCs, particularly with regard to minority MC congeners. Most studies have been performed with the MC-LR variant, which is highly hydrophilic and cannot penetrate cell membranes by passive transport, so its kinetic parameters may be significantly different from those of more lipophilic MC congeners (Testai et al., 2016). Indeed, uptake, tissue distribution, and excretion processes result in three-to four-fold differences among some congeners (Meriluoto et al., 1990; Testai et al., 2016). Knowledge of MC analog bioavailability is essential to evaluate the risk from oral ingestion of MCs (Roegner et al., 2014).

In general, absorption of MCs occurs through multispecific organic-

anion-transporting polypeptides (OATPs), and they are rapidly distributed to various organs (Ito et al., 2000; Roegner et al., 2014; Testai et al., 2016). Over 300 OATPs/Oatps have been annotated in different species (Hu et al., 2016), but only four out of eleven OATPs reported in humans (OATP1A2, -1B1, -1B3, and -2B1) have been shown to be involved in the uptake of MCs (Fischer et al., 2010). Most of the studies on transport of minority MC congeners have been carried out with *in vitro* models, in most cases using cells of the nervous system (Feurstein et al., 2009, 2010; Bulc Rozman et al., 2017). Feurstein et al. (2009) demonstrated the presence of various mOatps and uptake of various minority MC congeners in a mOatp-associated manner in primary murine whole brain cells (mWBC). Firstly, they observed positive bands at mRNA level for five tested mOatps (OATP1C1, -1A5, -3A1, -1A1, and -1B2) in mWBC. In order to show mOatp-associated MC transport, they carried out co-incubation studies of various MC congeners, such as MC-LF, MC-LW, and MC-LR, with OATP substrates (taurocholate and bromosulfophthalein) in this cell line, showing a significant reduction of MC-LW transport by Western blots. Moreover, an increase in cell viability was demonstrated in mWBC exposed to MC-LF, MC-LW, and MC-LR with OATP substrates by decreasing the transport of these cyanotoxins through OATPs. In agreement with these results, another study confirmed mOatp-mediated MC congener-specific uptake in mouse primary neurons (Feurstein et al., 2010). The authors investigated the identity of mOatps expressed in this cell line, demonstrating the expression of 12 mOatps (1a1, 1a4, 1a5, 1a6, 1b2, 1c1, 2a1, 2b1, 3a1, 4a1, 4c1, and 5a1). Moreover, they observed that MC-LF produced a greater reduction in protein phosphatase (PP) activity than other MC congeners such as MC-LR and MC-LW in exposed neuronal cells, which suggests that MC-LF was transported more efficiently into the cell and thus reached PP-inhibitive intracellular concentrations more quickly than MC-LR and MC-LW did (Feurstein et al., 2010). Similar results have been reported recently in cultured rat astrocytes exposed to MC variants such as MC-LF, MC-LW, and MC-LR. More hydrophobic MCs (MC-LF and MC-LW) have been reported to be distinctly more toxic than their hydrophilic congener MC-LR in this cell line, and intracellular localization of MC-LF and MC-LW has been documented in cultured rat astrocytes which expressed Oatp1a4, Oatp1c1, and Oatp1a5, but not Oatp1b2 (Bulc Rozman et al., 2017). The demonstration that various MC congeners (MC-LF, MC-LW, and MC-LR) can be transported into neuronal cells agrees with some neurotoxic effects reported in dialysis patients after the Brazil outbreak (Azevedo et al., 2002).

With regard to the absorption process, Fischer et al. (2010) confirmed the assumed OATP transport-dependent uptake of MC-LF, MC-LW, MC-LR, and MC-RR in OATP1B1- and OATP1B3-expressing HEK293 cells. In this study, MC-LW and MC-LF elicited higher cytotoxic effects than MC-LR and MC-RR in OATP-expressing HEK293 cells and primary human hepatocytes, showing congener-dependent effects in both *in vitro* models. Steiner et al. (2016), in zfOatp-transfected HEK293 cells, demonstrated that various zfOatps are capable of transporting minority MC congeners in zebra fish. Specifically, MC-LF can be transported by zfOatp1d1, zfOatp1f2-1, zfOatp1f4-1, and zfOatp1f4-2, and MC-LW by zfOatp1d1 and zfOatp1f2-1. Also, faster transport was observed of MC-LF and MC-LW than of MC-LR and MC-RR in zfOatp1d1-expressing HEK293 cells; this corroborates MC congener-dependent transport in zebra fish. In general, differences in the affinity and capacity of OATPs in transporting different congeners of MCs across the membrane could explain differences in the toxicity of these variants (Bulc Rozman et al., 2017; Feurstein et al., 2010; Fischer et al., 2010; Monks et al., 2007). Although OATPs are the main uptake mechanism recognized for MCs, other mechanisms of penetration into cells, such as pinocytosis processes, could be relevant in the case of more hydrophobic congeners. In this context, Vesterkvist and Meriluoto (2003) demonstrated differences in how hydrophobic (MC-LW and MC-LF) and hydrophilic (MC-LR and MC-RR) MCs interact with lipid membranes, showing that the toxin MC-LW was able to interact directly with bilayer membranes. *In vivo* studies focused on investigating the transport mechanisms of minority MC congeners have not been found.

In relation to minority MC congener distribution dynamics, only data from studies conducted on fish were found (Li et al., 2007a; Amé et al., 2010). In bighead carp i.p. exposed to algal cell extracts containing MC-YR, MC-LR, and MC-RR (200 or 500 μg MCs/kg), a significant decrease in MC-YR, similar to that presented by MC-RR and MC-LR congeners, was observed in intestines 1 h post-exposure, suggesting entry of MC-YR into the blood system and its rapid transport to various organs of the fish. The highest concentration of MC-YR was detected in liver at the highest exposure-dose, followed by kidney > gallbladder > intestine > muscle > spleen (Li et al., 2007a). However, these authors observed a different distribution pattern of this congener at the lowest exposure dose, detecting it mainly in gallbladder but not in the liver. In comparison with other major congeners, MC-YR follows distribution dynamics similar to those of MC-RR at high doses, but more similar to MC-LR at lower ones. A field study (Amé et al., 2010) investigated the relationship between levels of various MC congeners (MC-YR, MC-LA, MC-LR, and MC-RR) present in water and fish tissues collected from Los Padres Lake (Argentina). In the samples collected in dry seasons, a greater increase in MC-LR content was observed compared to MC-LA congener in muscle of *O. bonariensis* when the presence of these toxins increased in water samples, showing that some variants of MC presented different distribution dynamics. The authors also demonstrated the persistence of various MC minority variants (MC-LA, MC-YR) in liver and muscle of fish, even when these variants were absent in water samples.

In general, MC metabolism and excretion pathways include glutathione (GSH) conjugation, the kidney being an important organ for MC excretion. GSH conjugate formation reaction is normally catalyzed by the GST enzyme, although it can also occur spontaneously (Kondo et al., 1992; Roegner et al., 2014; Testai et al., 2016). Nevertheless, this reaction involving minority MC congeners is poorly documented. Only three *in vivo* studies have focused on investigating the role of GSH in the biotransformation of MC-YR (Kondo et al., 1992; Vasconcelos et al., 2007; Pasková et al., 2008). The analysis of GSH and cysteine (Cys) synthetic conjugates of MC-YR by frit-fast atom bombardment liquid chromatography/mass spectrometry showed that the thiol group of GSH and Cys is added to the *α,β*-unsaturated carbonyl of the *N*-methyldehydroalanine (Mdha) of the MC-YR congener moiety (Kondo et al., 1992). These authors corroborated the importance of the conjugation reaction as a detoxification route of this congener, as the MC-YR toxicity value (LD_{50}, up-and-down method) was greater than the LD_{50} values of GSH and Cys conjugates of MC-YR in mice (injected via the tail vein). Moreover, since the Mdha residue is unchanged in MC-LW and MC-LF congeners, conjugation products are probably formed with these other toxins (Vesterkvist and Meriluoto, 2003). Later, Vasconcelos et al. (2007) reported that exposure of a mixture of pure MC-YR and MC-LR led to an increase in GST activity in various organs of *Mytilus galloprovincialis*, highlighting the role of gut, gills, and mantle in the MC detoxification process. Similarly, in a different experimental model, Pasková et al. (2008) observed an increase in GST activity and GSH content in liver, heart, and brain of birds sub-chronically exposed to natural cyanobacterial biomass containing MC-YR (7%), unidentified MCs (13%), MC-LR (40%), and MC-RR (40%). Results of these parameters support the role of GSH in the MC detoxification pathway, but they cannot demonstrate whether GSH is involved in the process of removing minority congeners, main congeners, or both.

With regard to the MC excretion process, these toxins are mostly excreted by urine as cysteine conjugates that are previously oxidized to mercapturic acid metabolites. However, an elimination process as GSH-conjugates through biliary excretion is also possible (Soares et al., 2004; Schmidt et al., 2014). There are no studies focused mainly on the excretion of minority MC congeners, although they have been performed with MC-LR. Only two depuration studies carried out in tilapia fish exposed to a *Microcystis aeruginosa* bloom documented the elimination

of various MC congeners, evidencing toxin excretion through urine and feces (Soares et al., 2004; Mohamed and Hussein, 2006).

Globally, the results emphasize the importance of toxicokinetic aspects in order to understand the toxicity, organotropism, and bioaccumulation of minority MC congeners. A contribution of additional OATPs or other unknown transporters to the uptake of MCs could be possible. Further studies are certainly needed to fully determine the expression of individual OATPs and other possible transport mechanisms involved in the uptake, distribution, metabolism, and excretion pathways of minority MC congeners in various organs and experimental models. Also, the molecular characteristics involved in the various MC analog kinetics have yet to be explored. Moreover, most of the results published to date are derived from *in vitro* studies or intraperitoneal *in vivo* studies. In this context, the design of experimental studies, including the selection of adequate administration routes, is very important as it could influence kinetic parameters.

4. Toxicity

4.1. General aspects

MCs mediate their toxicity by uptake into hepatocytes, via a carrier-mediated transport system (OATP), followed by inhibition of serine/threonine protein phosphatases (PP). MCs are known to inhibit PPs of types 1, 2A, 3, 4, and 5, and, to a lesser extent, 2B (Catherine et al., 2017). The Adda moiety, present in all variants, is critical to MC activity (Campos and Vasconcelos, 2010). But the variations in the structure have an impact on the PP-inhibitory potency. Ikehara et al. (2009) investigated the effect of 21 MC analogs on PP2A activity and found that MC-LR was the strongest inhibitor. The substitution of leucine (L) by other L-amino acids at position 2 reduced the inhibitory potency, and the half maximal inhibitory concentration (IC_{50}) varied as follows: MC-LR < MC-RR < MC-FR < MC-YR < MC-WR (W: tryptophan, Y: tyrosine, F: phenylalanine, R: arginine). The substitution of R by other L-amino acids at position 4 also reduced the inhibitory potency as follows: MC-LR < MC-LF < MC-LW < MC-LA (A: alanine). Moreover, demethylation of the amino acid at position 7 also reduced the inhibitory potency on PP2A activity. However, these authors pointed out that there was a weak correlation between cytotoxicity data on normal human hepatocytes and PP2A inhibition activity of MCs. They explained this finding by assuming that the variations in the structure of MCs alter their membrane permeability. Other authors have also reported that MC analogs with more hydrophobic amino acids such as MC-LF and MC-LW show more pronounced cytotoxic effects than those of MC-LR on human intestinal Caco-2 cells (Vesterkvist et al., 2012), human hepatocytes, OATP-transfected embryonic kidney cells (Fischer et al., 2010), and also primary murine whole brain cells (Feurstein et al., 2009). In this context, Monks et al. (2007) and Fischer et al. (2010) also suggested that the structural variations in MC analogs conferred transporter selectivity.

These differences in toxicity have also been observed *in vivo*. Stoner et al. (1989) found that MC-LR and MC-LA were more toxic than MC-LY and MC-RR in adult mice. Substitution of a single L-amino acid for another markedly affected the dosimetric potency, but not the pathophysiology of its toxicity. The same finding was reported by Gupta et al. (2003), who observed that MC-LR was the most potent toxin in mice, followed by MC-YR and MC-RR. Stotts et al. (1993) also observed the different toxicity of MC variants in mice, with one of them non-lethal at 1 mg/kg ($C_3H_7O_2$ mono-ester of the α-carboxyl on the Glu unit of MC-LR) and others with median lethal doses ranging from 97 μg/kg to 750 μg/kg. All these reports support the congener-specific toxicity of MCs.

The inhibition of PP already mentioned results in an increase in protein phosphorylation that affects several processes, leading to various cellular responses such as: modification of cytoskeleton and disruption of actin filaments, oxidative stress induction, apoptosis, reduced DNA repair, tumor promotion, induction of neutrophil-derived chemokines, etc. (Campos and Vasconcelos, 2010; Buratti et al., 2017; Catherine et al., 2017). These responses have mainly been investigated with MC-LR, but there are also studies that report the same effects after exposure to minority MCs. For example, MC-LF induces oxidative stress (Cazenave et al., 2006; Peuthert et al., 2007), MC-LA and MC-YR induced the expression of interleukin-8 (IL-8) and cytokine-induced neutrophil chemoattractant-2αβ (Kujbida et al., 2008, 2009), etc.

Moreover, OATP is a multispecific transport system expressed in various cell types such as enterocytes, hepatocytes, and renal epithelial cells, and organs such as the heart, lung, spleen, pancreas, brain, and blood–brain barrier (BBB) (Feurstein et al., 2009). Therefore, it is not strange that MC-induced toxic effects have been reported in other organs apart from the liver. Systemic distribution of MC in the organs is therefore dependent on the degree of blood perfusion and types and expression level of OATP carriers (Campos and Vasconcelos, 2010). Thus, MCs have also been shown to induce nephrotoxicity (MC-LR, MC-YR, Milutinovic et al., 2003), cardiotoxicity (MC-YR, Suput et al., 2010), neurotoxicity (MC-LR, MC-LW, and MC-LF, Bulc Rozman et al., 2017), etc. Buratti et al. (2017) suggested 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 adverse outcome pathways.

On the other hand, human illnesses attributed to cyanobacterial toxins come into three categories: gastroenteritis and related diseases, allergic and irritation reactions, and liver diseases (Bell and Codd, 1994; Dawson, 1998). Moreover, they have also been involved in mortal episodes, such as the use of contaminated water for dialysis purposes in Caruaru (Brazil). In this case, the concentrations of unbound MCs were 19.5 μg/L and the average concentrations in human liver, for MC-YR, -LR, and -AR, were estimated to be 223 ng/g (Hilborn et al., 2013; Pouria et al., 1998). As in the case of human poisonings, a mixture of various MC variants has been responsible for intoxications in a wide range of animals, from wildlife to cattle and pets (Buratti et al., 2017). In this context, some authors have suggested that the contribution of minority MCs to the total toxicity should be taken into account (Lürling and Faassen, 2013).

As shown by the scientific literature, the increasing knowledge about the mechanistic pathways of MCs seems not to run in parallel with MC congener-specific effects. Accordingly, in order to establish the state of the art of minority MC toxicity, a review of the main toxicological studies on the topic is provided below.

4.2. In vitro toxicity studies

4.2.1. Cytotoxicity studies

Table 3 shows the *in vitro* assays that have been carried out with MC congeners other than MC-LR and MC-RR between the years 2000 and 2018. Most of the studies were performed in human and mammalian cell lines. However, there are also reports with an environmental point of view that investigated minority MCs in cell lines of fish origin (Pichardo et al., 2007; Sorichetti et al., 2014). There is also a single study conducted on plants (Laughinghouse et al., 2012).

Among the 246 structural variants already identified (Spoof and Catherine, 2017), MC-YR, MC-LF, and MC-LW were the most frequently evaluated in *in vitro* studies. This could be connected with the high occurrence of these variants. Currently, MC-YR is the third most common MC, after MC-LR and MC-RR (Buratti et al., 2017). It could also be because of the interest in knowing the toxicity of variants that are more hydrophobic than MC-LR.

Because MCs are potent hepatotoxins, most studies have been done in hepatic cells such as HepG2, human and rat hepatocytes, PLHC-1, etc. (Pichardo et al., 2007; Kujbida et al., 2008; Ikehara et al., 2009; Fischer et al., 2010; Ufelmann et al., 2012; Lundqvist et al., 2017). Furthermore, humans can come into contact with these toxins through oral exposure, so studies have also been carried out in intestinal cell

Table 3
In vitro studies performed with minority MC congeners.

Toxin	Experimental model	Assays Performed	Concentration ranges and exposure conditions	Main results	References
TOXICITY					
Commercial pure MC-LF, -LW and -YR standard (also MC-LR and −RR)	OATP- transfected HeLa cells	Growth inhibition by sulforhodamine B protein dye assay	Concentrations not revealed	After 72 h exposure, a greater cytotoxicity was observed to MC-LF and MC–LW than MC-YR in OATP-transfected cells. IC_{50} values were 0.4 nM and 0.9 nM MC-LF, 0.3 nM and 0.5 nM MC-LW and 90 nM and 45 nM MC-YR, in OATP1B1 and OATP1B3 transfected cells, respectively.	Monks et al. (2007)
Commercial pure MC-YR standard	RTG-2 and PLHC-1 cells	PC, NRU and MTS assays Morphological changes	0,10,50,75 and 100 μM for 48 h	Cytotoxic effects were observed in both cell lines after exposure. PC was the most sensitive endpoint for both cell lines with EC_{50} values of 35 μM and 67 μM for PLHC-1 and RTG-2 respectively. MTS was the least sensitive endpoint. 100 μM MC-YR produced apoptotic bodies in PLHC-1 cells and 75 μM in RTG-2 cells produced loss of cells, hydropic degeneration and necrotic cells. Thus, PLHC-1 cells were much more sensitive to MC-YR than RTG-2 cells.	Pichardo et al. (2007)
Commercial pure MC-YR and –LA standard (also MC-LR)	Rat and human neutrophils	Cell viability by flow cytometry	1 and 1000 nM for 24 h	MC-YR not affected human neutrophils after 24 h of exposure. An increase in cell viability with MC-LA was observed. No cytotoxicity was observed in rat neutrophils.	Kujbida et al. (2008)
Commercial pure MC-LW and –LF standard (also MC-LR)	mWBC cells	MTT assay	0.1–5 μM for 48 h	After exposure time, a total loss of cell viability was observed when mWBC was exposed to 5 μM MC-LF. At the same concentration, MC-LW and MC-LR produced a decreased in cell viability, 54% and 33%, respectively.	Feurstein et al. (2009)
Commercial pure MC-LW and –LF standards and purified extract containing MC-FR, -YR, -WR, -LA, and [DHA^7]MC-YR (among others)	Human normal hepatocytes	MTS assay	0–100 nM for 3 days	LC_{50} values ranged from 3.8 (MC-LA) to 540.5 nM ([Dha^7]MC-RR). The following cytotoxicity (EC_{50}) rank was obtained: MC-LA < MC-LR < [Dha^7]MC-LR < [Dha^7]MC-YR < MC-WR < MC-YR < [Dha^7]MC-RR < other MC-RR variants. The results showed a weak relation between cytotoxicity and PP2A inhibition assays.	Ikehara et al. (2009)
Commercial pure MC-YR standard (also MC-RR)	Undifferentiated and differentiated Caco-2 cells	PC, NRU and MTS assays Morphological changes	50, 100,150 and 200 μM for 24 and 48 h	Cytotoxic effects were observed. NRU was the most sensitive endpoint with an EC_{50} of 57.3 μM after 48 h in undifferentiated cells exposed to MC-YR. Reductions greater than 80% were observed at 100 μM MC-YR. Hydropic degeneration and detachment of cells were observed at the highest concentrations after 48 h.	Puerto et al. (2009)
Commercial pure MC -LF and -LW standard (also MC-LR and −RR)	Primary human hepatocytes and OATP- transfected HEK293 cells	MTT assay	0.09–200 nM for 48 h	Primary human hepatocytes were more sensitive than HEK293-OATP transfectants to MC-LF and MC-LW. The EC_{50} values were different between MCs congeners. The EC_{50} in donors of primary human hepatocytes were 0.5 nM and 0.4 nM MC-LW in donor 1 and donor 2 respectively and 0.5 nM and 0.6 nM MC-LF in donor 1 and donor 2 respectively. In OATP-transfected HEK293 cells the EC_{50} were 10.4 nM in HEK293-OATP1B1 cells and 4 nM in HEK293-OATP1B3 cells after exposure MC-LW and 11.1 nM and 3.7 nM MC-LF in HEK293-OATP1B1 and HEK293-OATP1B3 cells respectively.	Fischer et al. (2010)
Commercial pure MC-LW and-LF standard (also MC-LR)	Primary murine CGNs	MTT assay, caspase-3/7 activity and morphological changes	0.1, 0.2, 0.4, 0.8, 1, 3 and 5 μM for 48 h	Cell viability decreased in a concentration-dependent manner. After 48 h of exposure, cell viability was reduced to 8% of the control when CGNs cells were exposed to 5 μM MC-LF and 50% of control when were exposed to 5 μM MC-LW. 60% of apoptotic cells with highly condensed chromatin were observed when cells were exposed to 5 μM MC-LF. Only 5 μM of MC-LW resulted in a significant increase of apoptotic nuclei (32%). In contrast, MC-LR did not provide for a significant increase in apoptotic nuclei.	Feurstein et al. (2011)

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Table 3 (*continued*)

Toxin	Experimental model	Assays Performed	Concentration ranges and exposure conditions	Main results	References
Purified extracts containing MC-XR from *M. aeruginosa*	*Allium* cepa	Apoptosis and necrosis by *Allium* cepa test system	0.0658, 0.658 and 6.58 mg/L for 48 h	MC-XR was not cytotoxic in *Allium* cepa.	Laughinghouse et al. (2012)
Algal products containing MC-LA and MC-LR	A549 cells	MTT reduction assay	1.5, 4.6, 13.9 and 41.7 g dw algal product/L for 26 h	High cytotoxic effects were observed when A549 cells were exposed to 13.9 and 41.7 mg dw/L of algal products.	Heussner et al. (2012)
Pure MC-YR and dmMC-YR (and MC-LR, −RR and NOD and their demethylated variants) and/or purified from *M. aeruginosa* extract	Rat hepatocytes	Resazurin reduction assay and LDH leakage	0–200 nM for 24 h.	Resazurin reduction assay was the most sensitive endpoint in rat hepatocytes with an EC_{50} of 10.1 nM MC-YR and 10.2 nM dmMC-YR. LDH leakage showed cytotoxic effects with an EC_{50} value of 9.7 nM MC-YR and 21.4 nM dmMC-YR. The rank order of toxicity was MC-YR > NOD > MC-LR when both assays are taking into account.	Ufelmann et al. (2012)
Purified extract containing MC-LF, -LW and –LR from *Microcystis* PCC7820	Caco-2 cells	LDH leakage Cell proliferation by WST-1 Morphological changes	1, 10 and 50 μM for 22, 44 and 48 h	MC-LF was the most toxic MC congener. After 48 h of exposure, a 51% and 36% of LDH leakage was observed when Caco-2 cells were exposed to 50 μM MC-LF and MC-LW respectively. When cell proliferation and viability were measured, MC-LF was the most toxic, reducing mitochondrial dehydrogenases activity to 30% after 48 h of exposure to 50 μM MC-LF. Exposure to 50 μM MC-LF and –LW for 22 and 44 h showed apoptotic cells with shrinkage, loss of cells and cell-cell adhesion.	Vesterkvist et al. (2012)
Commercial pure MC-LA, -LF, -LW, -LY, -YR standard (also MC-LR, −RR and NOD)	RTgill-W1 cells	RCA assay	0.1, 1, 10, 100 and 1000 nM for 24, 48 and 72 h	No cytotoxicity effects were described. Reductions in cell viability higher than 50% were not detected.	Sorichetti et al. (2014)
Purified extract from *M. aeruginosa* containing MC-YR (also MC-LR and MC-*RR)*	P19/A15 cells	NRU assay	0.25, 0.5, 1, 2 g dm/L for 24 h	No cytotoxity was observed in P19/A15 cells after 24 h of exposure to the extract.	Javůrek et al., 2015
Commercial pure MC-LF and MC-LW (also MC-LR, MC-RR and Nodularin)	Hek293 and ACHN cells	SRB assay	1–200 μM for 24 h	Cytotoxic effects were observed in both cell lines. HEK293 was more sensitive than ACHN cells with IC_{50} values between 16.57 ± 0.035 to 1158.16 ± 9.025 μM and 62.36 ± 0.037 to 1589.78 ± 3.206 μM, respectively. MC-LR was the most toxic and MC-LW the least.	Piyathilaka et al. (2016)
Commercial pure MC-LW and MC-LF standard (also MC-LR)	Primary cultures of neonatal rats cortical astrocytes cells	MTT assay Apoptosis by annexin V labelling	0.5, 2 or 10 μM for 24 h	Dose-dependent toxicity was observed in rat cortical astrocytes. 38.2% and 74.2% reduction in cell viability was observed when cells were exposed to 10 μM MC-LF and MC-LW, respectively. An increase in apoptotic cells was also observed after exposure to MC-LF and –LW from 2 to 0.5 μM, respectively.	Bulc Rozman et al., 2017
Commercial pure MC-LY standard (also MC-LR and −RR)	HepG2 cells	MTS assay	0.1–3 μM for 24 h	No cytotoxicity was observed in HepG2 cells after 24 h of exposure to MC-LY and other MC variants. Data shown as cell viability was higher than 80% respect to the control in all cases.	Lundqvist et al. (2017)
OXIDATIVE STRESS					
Commercial pure MC-YR and –LA standard (also MC-LR)	Rat and human neutrophils	ROS release by lucigenin -enhanced chemiluminescence assay and ROS by flow cytometry	1 and 1000 nM for 24 h	The flow-cytometric measurement of RO metabolites did not cause significant changes in intracellular ROS levels. A significant increase in extracellular ROS level was observed in human neutrophils when were exposed to 1000 nM MC-YR and MC-LA. Rat neutrophils showed an increase in extracellular ROS level after exposure to 1 and 1000 nM MC-LA and MC-YR with PMA.	Kujbida et al. (2008)
Commercial pure MC-LY standard (and MC-LR and −RR)	HepG2 cells	Nrf2 activation	0.1–3 μM for 24 h	Nrf2 activity was significantly increased after exposure to 10 μM MC-LR and −RR, 3 μM MC-LY, and 1 μM [D-Asp3]-LR and MC-LR.	Lundqvist et al. (2017)
GENOTOXICITY					
Commercial pure MC-YR and –LA standard (also MC-LR)	Rat and human neutrophils	DNA fragmentation by flow cytometry	1 and 1000 nM for 24 h	No DNA fragmentation was observed in human neutrophils after exposure to MC-YR and MC-LA. MC-LR showed positive results. A significant difference was shown when rat neutrophils were exposed to 1 and 1000 nM MC-LA and to 1000 nM MC-YR.	Kujbida et al. (2008)

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Table 3 (*continued*)

Toxin	Experimental model	Assays Performed	Concentration ranges and exposure conditions	Main results	References
Purified extracts containing MC-YR (among others)	Human lymphocytes	MN test	0, 0.25, 0.5, 1 and 2 mg mL^{-1} for 20 h	No genotoxicity was observed in human lymphocytes after exposure	Abramsson-Zetterberg et al. (2010)
Purified extracts containing MC-XR from *M. aeruginosa*	*Allium* cepa	MN test and chromosomal aberration assay	0.0658, 0.658 and 6.58 mg/L for 48 h	MC-XR had genotoxic effects in *Allium* cepa bulbs. A 1.73% of chromosome aberrations and MN were detected when bulbs were exposed to 0.0658 mg/L of the extract containing MC-XR.	Laughinghouse et al. (2012)

Abbreviations: A549: Human alveolar basal ephitelial cells; ACHN: Human Kidney Adenocarcinoma; Caco-2: Human colorectal adenocarcinoma cell line; CGNs: Cerebellar granule neurons; HeLa: Human cervix epithelioid carcinoma cell line; HEK293: Human embryonic kidney cell line; Hep-G2: Human liver hepatocellular carcinoma cell line; LDH: Lactate Dehydrogenase; MN test: Micronucleus test; MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt); MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; mWBC: Primary murine whole brain cells; Nrf2: Erythroid 2-related factor 2; NRU: Neutral red uptake; OATP: Organic anion transporting polypeptides; PC: Protein content; PMA: Phorbol myristate acetate; P19/A15: Murine embryonic carcinoma cells derived from P19 cell line; PLHC-1: *Poeciliopsis lucida* hepatocellular carcinoma cell line; RCA: Rainbow trout gill-w1 cytotoxicity assay; Resazurin: 7-Hydroxy- 3H-phenoxazin-3-one 10-oxide; ROS: reactive oxygen species; RTG-2: *Oncorhynchus mykiss* trout Rainbow fibroblast gonad tissue cell line; RTgill-W1: *Oncorhynchus mykiss* rainbow trout gill cell line; SRB: Sulphorhodamine B assay; WST-1: [2-(4-Iodophenyl)-3- (4-nitrophenyl)-5- (2,4-disulfophenyl)-2H-tetrazolium].

lines (Puerto et al., 2009; Vesterkvist et al., 2012). Moreover, other organs may be affected by these MCs, and therefore studies have been performed in other cell lines, such as HeLa from cervix, RTG-2 from gonads, mWBC from primary murine brain cells, etc. (Monks et al., 2007; Feurstein et al., 2009; etc.).

With respect to hepatic cells, Pichardo et al. (2007) reported that PLHC-1 cells exposed to MC-YR showed a reduction in cell viability, with a mean effective concentration (EC_{50}) of 35 μM, as well as various morphological changes such as the induction of hydropic degeneration and cellular death mainly by necrosis but also by apoptosis. In normal human hepatocytes, Ikehara et al. (2009) evaluated the cytotoxicity of various MC variants and found that MC-LA was the most cytotoxic, and that there was no correlation between cytotoxicity and PP2A inhibition as MC-LR was the strongest PP inhibitor. This finding was attributed to the different permeability of the MC congeners. Also, Fischer et al. (2010) showed cytotoxic effects in primary human hepatocytes after 48 h of exposure to MC-LW and MC-LF. These authors reported that despite comparable PP-inhibiting capabilities, MC-LW and -LF elicited cytotoxic effects at lower equimolar concentrations than MC-LR and MC-RR, hence suggesting a congener-selective transport. Ufelmann et al. (2012) found the following ranking of toxicity in rat hepatocytes: MC-YR > nodularin > MC-LR. Moreover, desmethylation in ^{3}Asp or ^{7}Dha resulted in a higher toxicity for MC-LR, whereas it did not have an important influence on MC-YR. In this study, the resazurin reduction assay was the most sensitive endpoint, showing that mitochondrial function can be involved in cytotoxic effects (Ufelmann et al., 2012). However, in contrast to previous studies which evidenced cytotoxic effects of minority MCs, Lundqvist et al. (2017) showed contradictory results in HepG2 cells exposed to MC-LY for 24 h, as they found a cell viability higher than 80%. As it is the only study that investigated this particular variant in hepatic cells it is not possible to make comparisons. Sorichetti et al. (2014) also tested MC-LY, but in RTgill-W1 cells, and again no cytotoxicity was observed.

With regard to other cell lines, Puerto et al. (2009) showed that undifferentiated Caco-2 cells were more sensitive than differentiated cells when exposed to MC-YR. Moreover, they suggested the following sequence of cytotoxicity: MC-YR ≥ MC-LR > MC-RR. The main morphological changes observed were hydroponic degeneration and loss of cells. In concordance with these results, Vesterkvist et al. (2012), observed toxic effects induced by MC-LR, -LW, and -LF in Caco-2 cells, MC-LF being the most toxic MC congener. Morphological changes were also observed in a concentration- and time-dependent manner with alterations in cell shape, with a high loss of cells and loss of adhesion among cells. Again, more hydrophobic MC variants were more toxic than MC-LR to Caco-2 cells.

Minority MC congeners have also been shown to induce cytotoxic effects on human cell lines from kidney (Fischer et al., 2010; Piyathilaka et al., 2016). These two studies show contradictory results, because the first one showed that MC-LF and -LW were more toxic than MC-LR, whereas in the second one MC-LR was more toxic than those variants. This could be due to the different exposure time and endpoints used in the two assays.

Three papers also explored the toxic effects of various MC variants on neuronal cells (Feurstein et al., 2009, 2011; Bulc Rozman et al., 2017). They all focused on MC-LW and MC-LF (and MC-LR), and they showed similar results: higher cytotoxicity of these minority MCs and evidence of the mediated transport of the toxins in these experimental models. Moreover, morphological changes such as cytoskeletal disruption and apoptosis were observed. Finally, cell lines representative of other organs have been used more rarely. Kujbida et al. (2008) found that human neutrophil viability was not altered by MC-YR treatment and was increased after MC-LA exposure. Heussner et al. (2012) observed high cytotoxic effects in alveolar cells (A549) after exposure to an algal product containing MC-LA (among other variants).

In plants, only one study assessed the toxicity of MC congeners. In this study, an extract containing MC-XR was evaluated in *Allium cepa*,

although no cytotoxicity was observed at the concentrations assayed (Laughinghouse et al., 2012).

With respect to the various biomarkers used, most articles employed cell viability and cytotoxicity assays (such as MTS and MTT assays, Neutral Red Uptake, and Protein content), although others assessed morphological changes (Pichardo et al., 2007; Puerto et al., 2009; Feurstein et al., 2011, etc.) and cell death, especially apoptosis (Feurstein et al., 2011; Vesterkvist et al., 2012; Bulc Rozman et al., 2017). Furthermore, loss of cells, hydropic degeneration, and necrotic cells were observed (Pichardo et al., 2007; Puerto et al., 2009; Feurstein et al., 2011).

In general, the analysis of the cytotoxicity assays performed so far evidences a congener-specific toxicity and the importance of a congener-specific transport in the various cell types, which explains the toxicity observed. However, further efforts are needed as the number of known structural variants of MC is growing faster than knowledge about their toxicity profiles.

4.2.2. Oxidative stress studies

The production of reactive oxygen species (ROS) is one of the well-known mechanisms of the action of MCs (Campos and Vasconcelos, 2010). However, as can be seen in Table 3, there are very few *in vitro* studies that assess the ability of various MC variants to cause oxidative stress. Only two studies evaluated the ability of MCs other than MC-LR and MC-RR to cause oxidative stress (Kujbida et al., 2008; Lundqvist et al., 2017). Kujbida et al. (2008) used flow cytometry to assess the capacity of MC-YR, MC-LA, and also MC-LR to produce extracellular and intracellular ROS in human and rat neutrophils. The results showed that only the highest concentration evaluated (1000 nM) produced an increase in extracellular ROS levels. Additionally, Lundqvist et al. (2017) reported that the nuclear erythroid 2-related factor 2 (Nrf2) was affected in HepG2 cells after exposure to MC-LY. Nrf2 is a mechanism of protection to oxidants (Ma, 2013). These authors showed that MC-LY produced an increase in Nrf2 activity in HepG2 cells, which proved the ability of MCs to induce oxidative stress.

More studies are required to evaluate the ability of minority MC variants to induce oxidative stress. Given that MC variants show different capacities to inhibit PP, another well-known toxicity mechanism of MCs, it is of interest to know whether they also show different capacities to induce oxidative damage.

4.2.3. Genotoxicity studies

It is known that MCs can cause DNA damage and produce genotoxicity, and they are considered tumor promoters (Žegura et al., 2011). However, *in vitro* assays that evaluate genotoxicity of MCs other than MC-LR and MC-RR are scarce (Table 3).

Kujbida et al. (2008) assessed genotoxicity damage in rat and human neutrophils exposed to MC-YR, MC-LA, and MC-LR for 24 h. Rat neutrophils treated with MC-LA (1 and 1000 nM) and MC-YR (1000 nM) showed an increased percentage of cells with fragmented DNA compared with the control group after 24 h of treatment. Moreover, only human neutrophils incubated with MC-LR (1000 nM) resulted in a decreased percentage of cells with fragmented DNA compared with control conditions. This is in agreement with the results obtained by Abramsson-Zetterberg et al. (2010), who did not observe genotoxicity when human lymphocytes were exposed to an extract containing MC-YR. However, studies in *Allium cepa* exposed to an extract containing MC-XR showed that MC-XR could produce micronuclei (MN) and chromosomal aberrations in this experimental model (Laughinghouse et al., 2012). These results indicated that the *Allium cepa* test is a good biomarker of chromosomal alterations, and therefore an indicator of genotoxicity.

In any case, these 3 reports are still insufficient to draw conclusions about the potential genotoxicity of MC variants. Genotoxicity is a key component in hazard/risk assessment of chemicals in general, and EFSA made recommendations on strategies for genotoxicity testing that include a basic test battery comprising two or more *in vitro* tests (EFSA, 2011). Further research is therefore required on this topic.

4.3. In vivo (laboratory) toxicity studies

4.3.1. Animals

Several *in vivo* laboratory studies have been carried out to elucidate the toxic effects produced by minority MC congeners, such as those caused by MC-YR, MC-LA, MC-LY, MC-LW, and MC-LF, in a great variety of experimental models such as protozoa, mayfly, Japanese quail, various aquatic animals, mice, and rats (Table 4). Some of these minority MCs were included in the literature search conducted for the EFSA report: MC-YR, MC-LF, MC-LA, MC-FR, MC-WR, MC-dMeLR, [Asp^3]MC-LR, [Asp^3, AcAdda^5]MC-LR, and MC-(H4)YR (Testai et al., 2016). MC-YR was the congener most tested in various experimental models such as mice (Gupta et al., 2003), rats (Kujbida et al., 2009), Japanese quail (Skocovska et al., 2007; Damkova et al., 2009, 2011), and aquatic animals (Oberem et al., 1999; Blom et al., 2001; Blom and Jüttner., 2005; Lecoz et al., 2008; El Ghazali et al., 2010). However, only some of the studies were performed with this pure toxin (Oberem et al., 1999; Blom et al., 2001; Gupta et al., 2003; Blom and Jüttner., 2005; Kujbida et al., 2009), showing the toxic effects produced only by this congener. There are also three studies showing the toxic effects produced by pure minority congeners of MCs in various experimental models such as MC-LF in zebrafish (Cazenave et al., 2006), MC-LA in rats (Kujbida et al., 2009), and MC-LW in mayfly (Liarte et al., 2014). Most studies have been performed using extracts of cyanobacterial cultures, which could be due to the high price of pure toxins, an important limitation for the advancement of MC toxicological knowledge, and consequently these results can be useful only as supportive data. On the other hand, only the studies conducted in birds have been performed using cyanobacterial biomass containing minority congeners of MCs such as MC-YR, evidencing a more realistic scenario. In any case, the study of natural MC mixtures and extracts does not reflect the toxicity of the individual MC because there can be other bioactive compounds that can have a role on the toxicity observed (Falconer, 2007).

4.3.1.1. Lower organisms/Insects. In lower organisms, a single study showed that more hydrophobic MC congeners such as MC-LF, MC-LW, and MC-LY were significantly more toxic to *Tetrahymena pyriformis* cells than hydrophilic MC-LR (Ward and Codd, 1999). But on the contrary, the MC-LW congener proved to be less toxic than MC-LR to mayfly (*Ecdyonurus angelieri* Thomas), showing a lesser mortality range and histopathological lesions.

4.3.1.2. Aquatic animals. Reports dealing with the toxic effects induced in aquatic organisms by minority congeners of MC include crustaceans (Blom et al., 2001; Blom and Jüttner., 2005), zebra mussel (Juhel et al., 2006a,b; 2007), experiments with embryos or larvae of various fish or amphibian species (Oberem et al., 1999; Cazenave et al., 2006; Lecoz et al., 2008; El Ghazali et al., 2010), and fish (Da Silva et al., 2011; Le Manach et al., 2018). There are other works investigating the effects of MCs in the aquatic invertebrate *Daphnia magna* (Dao et al., 2010) and in carp (Li et al., 2004, 2005, 2007b). However, in these studies the most abundant congeners present in the cyanobacterial extracts employed are MC-LR and −RR, which are not the objective of the present review. Nevertheless, they are cited here as these authors also report the presence, although scarce, of other minority MC congeners that could also be of interest.

According to EFSA (Testai et al., 2016), specifically with regard to the data gaps in risk assessment, the characterization of the toxicological profile of different MC congeners should not be limited to acute exposure but also related to short-term and chronic effects, looking at other effects besides hepatotoxicity, and preferring the oral route of exposure, which is more representative of actual exposure for

Table 4
In vivo (laboratory) studies in several experimental models exposed to various minority MCs.

Experimental Model	Cyanotoxin/Cyanobacteria	Exposure conditions	Signs and symptoms/Histopathology	Reference
Lower organisms				
Protozoan (*Tetrahymena pyriformis*)	Extracted and purified MC-LY, MC-LW, MC-LF or MC-LR, from culture strains of *Microcystis* PCC7813	50 μg mL^{-1} of each toxin added in the culture medium (Tris-HCl buffer) for 24 h of exposure	Effects on growth rate inhibition, maximum culture density and respiration rates from 10 min to 24 h of exposure to MCs were: MC-LF ≈ MC-LW > MC-LY > MC-LR.	Ward and Codd (1999)
Insects				
Mayfly (*Ecdyonurus angelieri*)	Pure MC-LW or MC-LR	5 μg/L of MC-LR or MC-LW in Petri dishes for 3 h	MC-LW (60% survival) < MC-LR (40% survival). MC-LW produced slight depigmentation in the tracheal system epithelium. MC-LR produced necrosis in fat body and disintegration of the tracheal system.	Liarte et al. (2014)
Aquatic animals				
Thamnocephalus platyurus	Commercial pure MC-YR standard (also MC-LR, −RR and Nodularin), purified [D-Asp^3, (E)-Dhb^7] MC-RR from *Planktothrix rubescens*.	0–100 μM for 24 h	Mortality data obtained were the following: LC_{50} value of 6.1 μM for MC-YR, 8.6 μM for MC-LR, 8.3 for MC-RR, 3.6 for [D-Asp3, (E)-Dhb7]MC-RR and 1.4 μM for Nodularin.	Blom et al. (2001)
Thamnocephalus platyurus	Commercial pure MC-YR standard (also MC-LR, −RR and Nodularin), purified [D-Asp3, (E)-Dhb7]MC-RR from *Planktothrix rubescens*, [D-Asp3]MC-LR from *M. aeruginosa*.	0.004–400 nM for 24 h	A LC_{50} of 4.7 μM was obtained for MC-YR in the acute toxicity assay. The lowest LC_{50} was 1.4 μM for Nodularin, and the highest 10.8 μM for MC-LR. Data obtained in PP1A and PP2A assays don't correlate with the results obtained in acute toxicity bioassay.	Blom and Jüttner. (2005)
Zebra mussel (*Dreissena polymorpha*)	*M. aeruginosa* strains containing MC-LF (and MC-LR)	2-h exposures by feeding 2 strains of *M. aeruginosa* with different toxin profile: a) 7.4 μg L^{-1} MC-LR (the less toxic); b) 82.9 μg L^{-1} MC-LF and 23.8 μg L^{-1} MC-LR (the most toxic)	Suspensions containing MC-LF had a more severe effect on the feeding behaviour and energy balance of mussels, compared to the less toxic strain (only MC-LR). MC-LF induced mucous pseudofaeces in mussels	Juhel et al. (2006a)
Zebra mussel (*Dreissena polymorpha*)	*M. aeruginosa* strains containing MC-LF (and MC-LR)	90-min exposures by feeding 2 strains of *M. aeruginosa* with different toxin profile: a) 7.4 μg L^{-1} MC-LR (the less toxic); b) 82.9 μg L^{-1} MC-LF and 23.8 μg L^{-1} MC-LR (the most toxic)	The acute irritant response induced by MC-LF was the production of mucous pseudofaeces and pseudodiarrhoea, affecting the opening of valves and siphons.	Juhel et al. (2006b)
Zebra mussel (*Dreissena polymorpha*)	*M. aeruginosa* strains containing MC-LF (and MC-LR)	Daily exposures (21 d) by feeding 3 strains of *M. aeruginosa* with different toxin profile: a) 7.4 μg L^{-1} MC-LR; b) 82.9 μg L^{-1} MC-LF and 23.8 μg L^{-1} MC-LR; c) 95.8 μg L^{-1} MC-LF and 19.3 μg L^{-1} MC-LR	The three toxic strains induced DNA damage in the haemocytes of exposed mussels.	Juhel et al. (2007)
Embryos of 11 species of fish (cyprinids) and amphibians	Pure MC-YR Purified MC-LF or MC-LR from *M. aeruginosa*	Exposure in Petri dishes to solutions with: - Low concentrations: 0.5, 5, and 50 μg/L pure MC-YR. - High concentrations: 0.1, 1, 5 and 10 mg/L pure MC-YR, or purified MC-LF or MC-LR	- Reduced timing of hatching in rainbow trout and delayed feeding in axolotl with low MC-YR concentrations.	Oberem et al., 1999
Embryos of zebra fish (*Danio rerio*)	Pure MC-LF	Embryos were incubated for 48 h in Petri dishes with solutions containing 25 μg L^{-1} MC-LF or MC-RR	- Overall, MC-LF resulted more teratogenic than MC-RR: impaired blood circulation, retardation in development and deformations. - Oxidative stress was more evident with MC-LF than with MC-RR. -Embryos exposed to MC-LF consumed more lipids	Cazenave et al. (2006)
Embryos of medaka fish (*Oryzias latipes*)	Crude extract of *Planktothrix agardhii* containing MCs (MC-YR and MC-dMeLR were detected)	Microinjection (2 nL) of the *Planktothrix agardhii* crude extract at different concentrations (from 5×10^{-6} to 10 mg mL^{-1}). Fish embryos were observed until hatching for developmental studies at 1, 4 and 11 d post-injection	- Reduced survival rates with 10 mg mL^{-1}. - Histopathological alterations in the liver and digestive tracts of surviving treated embryos.	Lecoz et al. (2008)

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Table 4 (*continued*)

Experimental Model	Cyanotoxin/Cyanobacteria	Exposure conditions	Signs and symptoms/Histopathology	Reference
Common carp (*Cyprinus carpio* L.) larvae	*M. aeruginosa* extracts with different MCs profiles: a) MC-YR, MC-(H4)YR, MC-FR, MC-WR, MC-LR and MC-RR. b) dominance of MC-LR (74.05%)	Fish were daily fed for 12 d with *Artemia salina* nauplii that had been preexposed to 100 MC-LReq μg L^{-1} from extracts of two *M. aeruginosa* natural blooms (a and b)	- Growth inhibition in fish larvae, more severe by exposure to bloom a, compared to bloom b.	El Ghazali et al. (2010)
Tilapia fish (*Oreochromis niloticus*)	*Microcystis* spp extract containing MC-LA (97.9 μg mL^{-1}) and MC-LR (41.0 μg mL^{-1})	- Single i.p. injection of 6.90 or 13.80 μg kg^{-1} b.w. (72 h) - Immersion in aquaria containing 5 or 103.72 μg/L (72 h)	Effects in peripheral erythrocytes: - i.p. injection induced DNA damage. - immersion induced DNA damage and micronucleus. - apoptosis at low concentrations and necrosis at high concentrations.	Da Silva et al., 2011
Medaka fish (*Oryzias latipes*)	*M. aeruginosa* living culture containing MC-LA, [Asp^3]MC-LR, [Asp^3, $AcAdda^5$]MC-LR and MC-LR *M. aeruginosa* extract containing [Asp^3]MC-LR, [Asp3, $AcAdda^5$]MC-LR and MC-LR	Fish were sub-chronically exposed (21 d) to a MC-producing strain of *M. aeruginosa* (both the living culture and the extract, at 10 μg eq. MC-LR L^{-1})	Induction of enzymatic oxidative defenses and histopathological changes in liver, and apoptotic processes were different in males and females. Direct exposure to the living culture caused more pronounced acute effects compared to the MC-extract.	Le Manach et al. (2018)
Birds				
Japanese quail (*Coturnix coturnix japonica*)	Cyanobacterial biomass containing 33.7 μg of MC-YR, 141.7 μg of MC-LR, 141.8 μg of MC-RR and 51.1 μg of unidentified compound similar to MCs per g d.w.	10 mL of *Microcystis* biomass containing 0.045, 0.459, 4.605 or 46.044 μg MCs/d per bird was orally administered for 10 (acute exposure) or 30 d (subchronic exposure).	At the histopathological level, the following changes were observed: In testicles: vacuolar degeneration of the germinative epithelium. In liver: cloudy swelling of hepatocytes, hyperplasia of lymphatic centers, vacuolar dystrophy and steatosis. ↑ LDH and ↓ GLU in blood.	Skocovska et al. (2007)
Japanese quail (*Coturnix coturnix japonica*)	Cyanobacterial biomass containing 33.7 μg of MC-YR, 141.7 μg of MC-LR, 141.8 μg of MC-RR and 51.1 μg of unidentified compound similar to MCs per g d.w.	10 mL of *Microcystis* biomass containing 0.045, 0.459, 4.605 or 46.044 μg MCs/d per bird was orally administered for 10 (acute exposure) or 30 d (subchronic exposure).	Alteration of antioxidant parameters in liver after acute exposure: ↑GSH, ↑GPx, ↑GR, ↑TBARS; and after subchronic exposure: ↑GSH, ↑GST, ↓ GPx. In the heart, after acute exposure: ↑GSH, ↑GR, ↑TBARS, ↑EROD and after subchronic exposure: ↑GST, ↑TBARS. In brain, after acute exposure: ↓GSH, ↑TBARS, ↑EROD and after subchronic exposure: ↑GSH, ↑GPx ↑TBARS,↑EROD.	Pasková et al., 2008
Japanese quail (*Coturnix coturnix japonica*)	Cyanobacterial biomass containing MC-YR MC-LR and MC-RR.	Cyanobacterial biomass containing 7.62 μg MC-YR, 27.39 μg MC-LR and 26.54 μg MC-RR mixed with 26.79 g food daily for 8 weeks.	Increased fertilization rates (viability) and decreased eggs weight. A significant ↑ LDH in blood.	Damkova et al. (2009)
Japanese quail (*Coturnix coturnix japonica*)	Cyanobacterial biomass containing MC-RR, MC-YR and MC-LR.	Cyanobacterial biomass containing 7.62 μg MC-YR, 27.39 μg MC-LR and 26.54 μg MC-RR mixed with 26.79 g food daily for 8 weeks.	Decreased numbers of developmental stadia of spermatozoa, vacuolar dystrophy of the epithelium of the seminiferous tubules and numeric atrophy of the seminiferous tubular epithelium. Cyanobacterial exposed group showed ↓LPO, ↓GPx, ↑CAT and no differences in GSH level, GR, GST and SOD activity compared to control group in the testes.	Damkova et al. (2011)
Mammals				
Mice	Extracted and purified MCs from culture strains of *M. aeruginosa* were assayed in isolated forms: MC-RR, MC-LY or MC-LA; in binary mixtures such as MC-LR and MC–RR, MC-LR and MC–LA or MC-RR and MC–LA and in ternary mixture: MC-LR, -LA and MC–RR	Toxins were injected i.v. in a dorsal tail vein. The dose used for each MC congener was the equivalent to 1.7–1.8 LD_{50}: 200 ng g^{-1} b.w. for MC-RR, 160 ng g^{-1} b.w. for MC-LY, 60 ng g^{-1} b.w. for MC-LR and 70 ng g^{-1} b.w. for MC-LA. Moreover, a single dose of CyA (0.2 mg/mouse) was given 1 h before a lethal dose of toxin or its mixture	Death. Lethal dose: MC-LA ≈ MC-LR > MC-LY > MC-RR CyA decreased the acute lethality of MCs in a range of 0%–90%. The degree of protection provided by CyA was: Complete protection: MC-RR = MC-LR and MC–RR mixture = MC-LR, MC-LA and MC −RR mixture > MC-RR and MC–LA mixture > MC-LY > MC-LR and MC–LA mixture > MC-LA: No protection.	Stoner et al. (1990)
Mice	MC-YR, MC-FR, MC-AR, MC-WR, MC-M(O)R, MC-LR, MC-RR, [$DMAdda^s$]MC-LR, [Dhar]MC-LR,[$Mser^7$] MC-LR or ($C_3H_7O_2$) MC-LR extracted and isolated from cyanobacterial cells	The mice were dosed with 0.1 mL of 0.09% saline solution i.p. containing a dose of 1 mg MC kg^{-1} b.w.	Death, except ($C_3H_7O_2$)MC-LR. Toxicity depending on the DL_{50}: MC-LR > [$DMAdda^s$]MC-LR > MC-YR = MC-WR > MC-FR = MC-AR > [Dhar]MC-LR > [$Mser^7$] MC-LR > MC-RR > MC-M(O)R Clinical signs of toxicosis and enlargement and lesion of the liver, except ($C_3H_7O_2$)MC-LR.	Stotts et al. (1993)

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Table 4 (*continued*)

Experimental Model	Cyanotoxin/Cyanobacteria	Exposure conditions	Signs and symptoms/Histopathology	Reference
Mice	Pure MC-YR, MC-LR or MC-RR	Dixon's up and down methods was applied to determine LD_{50} for each toxin. Acute 24 h LD_{50} via i.p. of MC-YR, MC-LR or MC-RR.	LD_{50}: MC-YR (110.6 μg/kg) > MC-LR (43.0 μg/kg) MC-YR (110.6 μg/kg) < MC-RR (235.4 μg/kg) MTD: MC-YR < MC-LR < MC-RR. Restlessness, labored ventilation, motor incoordination, spasms, convulsions and sudden change in respiratory variables prior to death. Increase of liver body weight index at MTD. Severe hepatic and pulmonary lesions such as congestion, hemorrhage, portal mononuclear cell infiltration and obliteration of chromatin material in hepatic tissues; and bronchial epithelial hyperplasia and necrosis, occlusion bronchiole, hemorrhage, congestion, stenosis of bronchial lumen, BALT necrosis, inflammatory reaction, edema and accumulation of fibroid debris in lung. ALT ↑, AST ↑, g-GT ↑, LDH ↑, DNA fragmentation ↑, GSH↓ at 30 min and MTD post-treatment in blood	Gupta et al. (2003)
Rats	Pure MC-LA, MC-YR or MC-LR	10 μL of MC-LA, MC-YR or MC-LR (1 nM) by topical application on the postcapillary venules	The interaction between leukocytes and vessel walls not increased in rats exposed to MC-LA or MC-YR compared to control group. Both events were increased in animals exposed to MC-LR.	Kujbida et al. (2009)

Abbreviations: ALT: alanine transaminase; AST: aspartate transaminase; b.w.: body weight; CAT: Catalase; CyA: Cyclosporine A; d.w.: dry weight; EROD: cytochrome P-450-dependent 7- ethoxyresorufin O deethylase; GLU: glucose; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione; GST: Glutathione-S-transferase; LDH: lactate dehydrogenase; MTD: mean time to death; SOD: superoxide dismutase; TBARS: total thiobarbituric acid reactive species.

aquatic animals than i.p. injection (the preferred administration in acute toxicity studies). In this context, the works reviewed are either acute or (sub)chronic over a period of days or months, and most of them were carried out by the oral route.

Only four experimental works are available in the scientific literature concerning the toxic effects of pure MC-YR or MC-LF on aquatic fauna. The studies on the crustacean *Thamnocephalus platyurus* evaluated acute toxicity using Thamnotoxkit F, usually employed for environmental risk assessment. Blom et al. (2001) observed the following ranking of toxicity (LC_{50}) for the MC variants evaluated: [D-Asp^3, (E)-Dhb^7]MC-RR > MC-YR > MC-RR > MC-LR. However, these results did not correlate with PP inhibition assays performed by Blom and Jüttner (2005). The authors suggested that there may be mechanisms other than PP inhibition (i.e. uptake, transport, detoxification, or other target sites) that could have a strong modulating effect on the toxicity of a MC congener for a particular animal. Oberem et al. (1999) exposed embryos from fish and amphibians by immersion in solutions containing pure MC-YR, -LR, or −RR, and few acute toxic effects were recorded at low concentrations. In an attempt to determine the toxic thresholds of different MCs, zebrafish embryos were exposed to higher concentrations of pure MC-YR or −RR, or purified MC-LF or -LR; however, no effects due to MC-LF exposure were detected. These authors also tested the effects of crude cyanobacterial extracts on zebrafish embryos and found that the effects were much more pronounced than those obtained with pure toxins in all species. However, they make no mention of any specific MC variant in the extract apart from MC-LR, so its effects cannot be compared with those of pure MC-YR or purified MC-LF reported in the same work (Oberem et al., 1999). Cazenave et al. (2006) investigated the toxicity of pure MC-LF in zebra fish embryos by immersion at a concentration lower than the one tested by Oberem et al. (1999). Generally, MC-LF exerted more pronounced effects than MC-RR, also tested in the same study, at different levels: teratogenicity, enzymatic status, and energy costs (Cazenave et al., 2006). The teratogenic effects were observed mainly at the early developmental stages. MC-LF showed a greater detoxication activity than MC-RR, by increased glutathione *S*-transferase (GST) activity, as well as oxidative stress, shown by various biochemical parameters. They also tested natural organic matter (NOM) and it was useful only to attenuate the oxidative effects induced by MC-LF, but not by MC-RR. A possible explanation for this may be the differential ability of NOM to attach structurally different MC variants.

As commented earlier, the possibility of conducting toxicity studies on minority MC variants may have been hampered by the restricted availability of pure toxins and their price; as this is normally a drawback, the use of cyanobacterial extracts prevails over pure toxins. However, these extracts are sometimes poorly characterized and therefore are not useful for an accurate risk assessment (Testai et al., 2016). Consequently, the conclusions drawn in these papers usually cannot be easily attributed to the toxins themselves, or to the less common variants of MCs, as the toxicological profile of the extracts used in some studies shows that MC-LR and −RR are the most abundant variants (Dao et al., 2010; Li et al., 2004, 2005, 2007b). The vast majority of the studies on aquatic organisms that have been reviewed were conducted by exposure to bloom scums, aqueous crude or freeze-dried extracts, in which one or more rare congeners of MC were detected. As MC-LR has the highest acute toxicological properties, MC-LR concentration equivalents have usually been used as the default value for the total concentration of all MC variants. However, the extrapolation from the acute toxicity ranking among MC congeners to the chronic toxicity remains to be demonstrated (Manganelli et al., 2012).

Survival and growth parameters on the crustacean *Daphnia magna* decreased in a dose-dependent way after exposure to a *M. aeruginosa* extract containing MC-LA, MC-YR and MC-LF, detecting malformations in the offspring for the first time (Dao et al., 2010). Juhel et al. (2006a,b) demonstrated the stressful effect and acute irritant response of MCs (particularly MC-LF) on the feeding behaviour and energy

balance of zebra mussels, *Dreissena polymorpha*, fed *M. aeruginosa* strains. They observed reductions in absorption and ingestion rates and in clearance, large quantities of mucous pseudofeces and pseudodiarrhea and closure of shell valves and siphons. These results suggest that zebra mussels are able to preferentially ingest non- or low-toxic species rather than suspensions containing MC-LF, and they may not accumulate and depurate this MC congener because of its high toxicity. Other authors also suggested that the gills and labial palps of bivalves may select the most "palatable" food according to its toxicity (Baker et al., 1998, 2000; Bastviken et al., 1998), and this could have happened in this case with MC-LF. More studies were needed in this regard, due to the high toxicity shown by this variant in the exposed mussels. For the first time, the genotoxicity of MC-LF was proved to be higher and more persistent than that from MC-LR, both from *M. aeruginosa* strains, by the comet assay (Juhel et al., 2007). This difference may be due either to a greater genotoxicity or to the higher concentrations of MC-LF, compared to MC-LR, or to a combination of both conditions. The increase in DNA damage could be due to insufficient repair of DNA strand breaks induced by MC-LF and/or because MC-LF may also act as an inhibitor of this repair, as suggested by the authors. In this respect, it seems that higher inhibitory potencies were shown with hydrophobic residues in position 2, and with hydrophilic ones in position 4 (Rinehart et al., 1994; Fontanillo and Köhn, 2018).

Little is known about the effects of rare variants of MCs on embryos or larvae of fish. In this regard, an 81% reduction in survival rates, hepatic hemorrhage with loss of glycogen in hepatocytes and cellular edema were evidenced in the liver and digestive tracts of surviving juveniles of medaka fish embryos injected with crude extracts of *Planktothrix agardhii* containing MC-YR and MC-dMeLR (Lecoz et al., 2008). The MC profile in natural blooms could exert differential toxicity on larvae of common carp (*Cyprinus carpio*) (El Ghazali et al., 2010) as shown by the greater affectation detected in individuals exposed to the bloom containing various MC congeners (MC-YR, MC-(H4)YR, MC-FR, MC-WR, MC-LR, and MC-RR), compared to the bloom with dominance of MC-LR. This is in agreement with the previous studies in mussels (Juhel et al., 2006a; b, 2007), and follows the toxicity order previously shown by Ward and Codd (1999) in *Tetrahymena pyriformis* cells, indicating a positive correlation between hydrophobicity of MCs and toxicity.

Tilapia fish (*O. niloticus*) were exposed to a *Microcystis* spp. extract containing mainly MC-LA, by immersion and i.p. routes, detecting DNA damage by the comet and micronucleus (MN) assays. However, the MN test was only positive with immersion, possibly because i.p. injection was so toxic that it inhibited cell division, impeding the visualization of micronuclei. Therefore, the highest levels of MC genotoxicity were revealed by the comet assay after i.p. injection. The first study to evaluate the effects of cyanobacteria and their bioactive compounds through a combined approach of oxidative stress, histopathology, and "omics" was performed recently on medaka fish (*Oryzias latipes*) subchronically exposed by immersion to a living culture or to an extract of *M. aeruginosa*, with a slightly different toxin profile, as two variants of MC-LR were detected in both options, but MC-LA was present only in the biomass and not in the extract (Le Manach et al., 2018). Differential oxidative stress responses in male and female fish were highlighted by the proteomic analysis; exposure to the biomass induced enzymatic oxidative defenses in liver, whereas the extract caused down-regulation of proteins involved in detoxification. Histopathological observations revealed loss of glycogen content in female fish, whereas other liver damage (disorganization of hepatic parenchyma, inter-hepatocyte space disjunction, etc.) was more pronounced in males. Although both treatments evidenced the metabolic and potent reproductive disorders in fish, the differential toxicity profile of the culture/extract was revealed, as seen by the more pronounced acute effects induced by direct exposure to the living culture compared to the MC extract treatment. The differences may have arisen from MC-LA itself and/or other compounds able to cause this damage.

There are still some gaps that deserve further research. For example, the majority of the published works have focused on MC-YR or -LF, but in the list included in the EFSA report there are no studies available yet regarding toxic effects on aquatic organisms produced by MC-AR, MC-LW, MC-deLW, MC-deLF, MC-(O)Y, or MC-m[Glu(OCH_3)]-LR (Testai et al., 2016). Proteomic and metabolomic analysis are also warranted, in both males and females, to prove the reproductive disorders possibly induced by these congeners. Furthermore, as some of these minor MC congeners are more lipophilic, it could be hypothesized that some of them are able to cross the blood–brain barrier, leading to neurotoxicity in aquatic organisms, and there are no studies in the literature reporting the induction of this effect by minority MCs. In order to make a more accurate assessment of the environmental risk of cyanobacteria on fish, further investigations of the potential toxicological effects of other cyanobacterial components, such as microginins, cyanopeptolins, and aeruginosins, are still needed.

4.3.1.3. Birds. Up to now, no studies have been carried out with pure minority MC congeners in birds, so the isolated toxic effects produced by these congeners have not been evaluated per se.

In birds, all the research work has been performed under similar laboratory conditions on Japanese quail orally exposed to cyanobacterial biomass containing several MC congeners including MC-YR (Skocovska et al., 2007; Pasková et al., 2008; Damkova et al., 2009, 2011). Although MCs are widely recognized as hepatotoxins (Buratti et al., 2017), only two studies focused on the toxic effects produced by MCs in bird liver (Skocovska et al., 2007; Pasková et al., 2008), being the reproductive organs the most studied in this animal model (Skocovska et al., 2007; Damkova et al., 2009).

Skocovska et al. (2007) demonstrated for the first time vacuolar degeneration in the testes of exposed animals, which was later confirmed by Damkova et al. (2011), even though the different doses and exposure times used. Histopathological alterations in liver and changes in biochemical parameters in blood were observed (Skocovska et al., 2007), and alterations in oxidative stress biomarkers were also reported for the first time in several organs after acute or subchronic exposure (Pasková et al., 2008). Damkova et al. (2009) reported that eggs from MC-exposed birds presented the highest viability and survival rates. However, egg weight and body mass of chicks (14 days after hatching) in MC-exposed animals were lower from control. Although negative effects may be expected after exposure to cyanobacterial biomass, it is worth mentioning that some biologically active compounds might be contained in complex biomass, which could potentially stimulate reproduction. In agreement with previous results, a significant increase in lactate dehydrogenase enzyme activity (LD) was observed in blood samples of birds exposed to cyanobacterial biomass, suggesting a low degree of hepatocellular damage and reproductive stimulation (Damkova et al., 2009). Later, these authors demonstrated that the involvement of the antioxidant defense system to protect the reproductive organs was insufficient after chronic MCs exposure, reporting histopathological alterations in these organs (Damkova et al., 2011).

4.3.1.4. Mammals. Very few studies have been conducted in mammals to evaluate the toxic effects produced by minority congeners of MC, among which pure MC-YR is the congener that has been studied most (Gupta et al., 2003; Kujbida et al., 2009). In view of the similarity of this experimental model to the human species, more research with minority MC congeners is needed. The studies that have been carried out describe various toxic effects produced by MCs with various exposure conditions, such as different administration routes (i.p. or topical route), so they show complementary rather than comparable results. In nature, cyanotoxins do not occur in isolation but are included in blooms containing other unknown substances. In addition to investigating the toxicity of minority MCs per se, it is important to determine the possible influence of these unknown substances on the

toxic effects produced by them. For this reason, it is also necessary to carry out studies in mammals with cyanobacterial biomass, because at present no such research exists.

The first study conducted in mammals investigated the lethal dose values for MC-LA, MC-LY, MC-LR, and MC-RR, and demonstrated the high toxicity of the MC-LA congener, similar to that of MC-LR (Stoner et al., 1990). The authors also proved that MC-LY is more toxic than MC-RR but less than MC-LA and MC-LR in mice injected i.v. They focused on testing the effectiveness of substances with potential protective effects against the acute lethality produced by MCs, such as cyclosporine A (CyA). This compound decreased the acute lethality of MCs in a range of 0%–90%, indicating that the efficacy of possible prophylactic treatments could depend on the MC congener involved in the intoxication.

Stotts et al. (1993) observed that MC-YR, MC-WR, MC-FR, and MC-AR were more toxic than the MC-RR congener but less than MC-LR, demonstrating that when hydrophobic Leu amino acid was replaced by other hydrophobic amino acids such as Phe, Ala, or Trp the LD_{50} value increased slightly (from 97 μg/kg to 171–249 μg/kg). Also, there was a larger decrease in the hepatotoxicity when Leu was changed to Arg or sulfoxide of methionine. Replacement of the second amino acid, Leu, in MC-LR with Tyr (MC-YR) or Arg (MC-RR) produced quantitative differences in toxic effects; however, the already known hepatotoxic effect, as described previously by other researchers, was maintained. Moreover, the fourth amino acid, Arg, can be modified without eliminating hepatotoxicity. Gupta et al. (2003) obtained similar LD_{50} results in mice exposed i.p., showing that pure MC-YR was more toxic than pure MC-RR, but not than pure MC-LR, although the mean time to death in MC-YR-intoxicated mice was less than for MC-LR (76.2 versus 97.5 min, respectively). MC-YR produced restlessness, labored ventilation, motor incoordination, spasms, convulsions, and sudden changes in respiratory variables in mice. In liver and lungs, MC-YR produced more severe histopathological alterations than MC-LR or MC-RR, but the lesions were qualitatively identical. Structural differences can modify the hydrophobicity/hydrophilicity of MCs and therefore their interaction with lipid membranes, which can modify their organotropism and toxicokinetics, and the bioaccumulation of these toxins (Vesterkvist and Meriluoto, 2003). Also, MC-YR led to alteration of biochemical serum variables such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (G-GT), total protein, albumin, globulin; decreased hepatic glutathione (GSH), and increased hepatic DNA fragmentation. However, there was no significant change in serum total protein profile, albumin, and albumin/globulin ratio after MC-YR exposure. In general, toxicological effects caused by MC-YR in mice were similar to those induced by more popular congeners such as MC-LR or MC-RR. By contrast, Kujbida et al. (2009) reported different toxicological patterns in rats exposed to 10 μL of different MC congeners (1 nM of pure MC) by topical application. Neither MC-YR nor MC-LA produced an increase in the interaction between leukocytes and vessel walls, whereas MC-LR did.

The present review highlights the fact that the toxic effects produced by different congeners of MC can vary both qualitatively and quantitatively, depending on the exposure conditions (route, dose, and experimental model), so the toxicological effects of the best-known MC congeners cannot be extended to minority MC congeners.

4.3.2. Vegetables

Studies investigating the effects of pure MCs alone (Pflugmacher et al., 2001), and of both pure MCs and extracts containing MCs in vegetables (Pflugmacher et al., 2006, 2007; Peuthert et al., 2007) are very scarce (Table 5). The influence of pure toxins has been evaluated in ≤24 h or 7 d, whereas most of the studies that exposed plants to cyanobacterial extracts normally lasted longer, mostly 7 or 30 d, the time needed to observe the potential alterations in plant morphology and development.

With regard to the effects of pure MCs, short-term exposure to pure MC congeners resulted in cellular damage via oxidative stress. In reed (*Phragmites australis*) exposed to pure MC-YR or MC-LY, it seems that sGST activity in the stems is highly sensitive to MC-YR, which could be connected with higher uptake of the toxin into this part and therefore the need for a good detoxification system (Pflugmacher et al., 2001). Later, Peuthert et al. (2007) tested eleven agricultural plants exposed to pure MC-LF for 24 h. The highest LPO values were reported in *P. sativum* roots and shoots, and the lowest were detected in red *V. radiata*. However, the authors pointed out that the short exposure time may not be enough for a good correlation of cellular damage with toxin uptake, and exposures of several days may give a better understanding of the effects caused by the contact of MC-LF with the plants.

Studies conducted with 7 d exposures induced morphological and developmental changes in plants, as well as alterations in their oxidative status. For example, a reduction of root development was observed in alfalfa (*M. sativa*) seedlings after exposure to pure MC-LW or MC-LR (Pflugmacher et al., 2006). Increases in LPO levels were observed, mainly with pure MC-LR, followed by MC-LW. Activities of superoxide dismutase (SOD), CAT, peroxidase (POD), GST and GR were also altered by toxin exposure in general, and MC-LW managed to induce higher increases than MC-LR in the cases of POD, GST and GR activities (Pflugmacher et al., 2006). The effects of pure MC-YR, -LF, -LA, -LW, and MCHCyR were also reflected in seeds from corn (*Zea mays*), with pure MC-LA (88%) inducing the highest reduction in germination rate (Pflugmacher, 2007). Exposure to pure MCs had significant effects on primary root and shoot length. Activity of POD in the roots and shoots showed a significant increase in most seeds, mainly after exposure to MC-LF or MC-LW, in agreement with Pflugmacher et al. (2006), whereas MC-LA induced a decrease in this enzymatic activity, possibly inhibited owing to the high amount of ROS (Pflugmacher, 2007).

By contrast, the majority of the studies, including the most recent ones, were performed with cyanobacterial extracts. In the studies reviewed, these extracts contained a variety of minority MC congeners, mainly MC-YR and -LY, and most of the rare variants only appear once. The frequency with which these congeners appear is, in decreasing order: MC-YR > MC-LY > MC-FR > MC-WR = MC-(H4)YR = DM-LR > MC-LF > MC-LA = MC-AR = MC-LW = MC-LM = DM[Asp^3]-LR = [DMAdda^5]MC-LR = [L-$MeSer^7$]MC-LR = [DM]MC-LF = [DMAdda]MC-LF = [DM]MC-LW = [DMAdda]MC-LW = DM-RR. As commented earlier, in the case of cyanobacterial extracts it is difficult to establish the toxic effects due specifically to one MC congener, and this is especially important in studies where MC-LR or −RR are the main constituent of the extracts, in which case the main toxic effects should be attributed to them, not to the rare and less abundant variants of MCs (Chen et al., 2004, 2010; Saqrane et al., 2007; El Khalloufi et al., 2011, 2012; Corbel et al., 2015; Romero-Oliva et al., 2015; Cao et al., 2017, 2018). The main results of these studies are described below.

Growth of *Sinapis alba* seedlings was inhibited after a 7 d exposure to purified MC-LF, MC-LR, or MC-RR from a *M. aeruginosa* bloom. In the case of MC-LF, 2.5 μg mL^{-1} was needed to cause this effect, whereas with the other two variants, inhibition occurred at lower concentrations; MC-RR was more toxic than MC-LR and -LF (McElhiney et al., 2001). Similarly, exposure of seeds of rape (*Brassica napus*) and rice (*Oryza sativa*) to various concentrations of a culture containing MC-YR, MC-LR, and MC-RR for 10 d caused seedling growth inhibition in both species (Chen et al., 2004), and phytotoxic effects, mainly on *B. napus*, with lower germination and dry mass percentages; however, the proportion of MC-YR in the extract was only 3%, so the effects might not be due to this congener.

A realistic exposure through MC-contaminated lake water was performed by Crush et al. (2008) with lettuce (*Lactuca sativa*), rape (*B. napus*), ryegrass (*Lolium perenne*), and white clover (*Trifolium repens*) grown in sand. Analysis of the lake water revealed the presence of 10 MC congeners. The authors compared the effects of 3 and 6 applications of the water either to the soil or to the shoots, generally detecting more obvious effects with fewer applications, suggesting a plant

Table 5
Studies on vegetables exposed to minority MCs.

Experimental Model	Cyanotoxin/Cyanobacteria	Exposure conditions	Main outcomes	Reference
Mustard (*Sinapis alba*)	MC-LF, -LR or -LR, purified from a *M. aeruginosa* bloom	Seeds were placed onto the surface of solidified Hoagland mediums containing 0.1, 0.5,1, 2.5, 5, 10, 25, or 50 μg mL^{-1} of each toxin separately, and let to grow for 7 d	Inhibited seedlings growth, following the potency: MC-RR > MC-LR > MC-LF.	McElhiney et al. (2001)
Reed (*Phragmites australis*)	Pure MC-YR or MC-LY	Extracts of rhizome, stem and leaves of *P. australis* were incubated with the pure toxins (concentration of 15 μg per test with less than 3% methanol) separately shaking for 2 h	Increases in sGST activity after incubation with MC-YR in rhizome, stem and leaves. MC-LY only manages to increase sGST activity in rhizome and stem.	Pflugmacher et al. (2001)
Alfalfa (*Medicago sativa*)	Pure MC-LW	Seedlings were exposed to 5 μg MC-LW L^{-1} for 7 d	After 7 d of exposure, morphological changes in the sprouts of alfalfa and increased antioxidant capacity were detected.	Pflugmacher et al. (2006)
11 agricultural plants	Pure MC-LF	Seeds were exposed for 24 h to 5 μg/L pure MC-LF, by immersion in a glass tube with the medium containing the toxin	LPO was detected in roots and shoots of all the seedlings exposed to pure MC-LF, with highest values in *P. sativum.*	Peuthert et al. (2007)
Corn (*Zea mays*)	Pure MC-YR, -LF, -LA, -LW or MCHCyR.	Seedlings were exposed separately to the different MCs variants (5 μg/L) or to the crude extract for 7 d (5 μg/L)	- Reduction in germination: MC-LA induced a reduction of 88%; the rest of MCs variants reduced germination in the range 17–32%. - Reduction in root and shoot length in general. - Increased POD activity mainly in roots and shoots of seedlings exposed to MC-LF or -LW. Decreased POD activity detected after exposure to MC-LA.	Pflugmacher, 2007
Lettuce (*Lactuca sativa*), rape (*B. napus*), ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*)	Lake water with *M. aeruginosa* and *Anabaena* c.f. *smithii* blooms, producing −FR (15%), -WR (15%), -LR (25%), −RR (31%), - and -LA, -AR, -desMeLR, -LY and –desMeRR (5–0.3%)	Plants were irrigated 3 or 6 times with the contaminated water applied either directly to the sand surface, or to the shoots, watering every 2–3 d for a total of 12 d. Each plant received per application 170 μg of the mixture of MCs	Altered growth of plants was more evident after 3 applications, compared to 6 applications.	Crush et al. (2008)
Wheat (*Triticum durum*), maize (*Z. mays*), pea (*Pisum sativum*) and lens (*Lens esculenta*)	*M. aeruginosa* extract containing MC-YR (16%), -(H4) YR (14.2%), -WR (6.6%), −FR (4.6%), −RR (31.6%) and -LR (27%)	Plants in sand were irrigated every 3 days for a total of 30 d with a solution containing the diluted extract to get: 0.5, 1.05, 2.1 and 4.2 μg equivalent MC-LR mL^{-1}	Effects dependent on the plant species, concentration and time of exposure. - Decrease in Photosystem II activity (Fv/Fm fluorescence) - Enhanced accumulation of Na^{+},K^{+},Ca^{2+}, P and N	Saqrane et al. (2009)

Abbreviations: (s)GST: (soluble) Glutathione S-transferase; LPO: lipid peroxidation; *M. aeruginosa: Microcystins aeruginosa;* POD: peroxidase.

adaptation to the toxins. The ability of high concentrations of MCs to inhibit protein phosphatases could lead to a disruption of nutrient absorption or root functions, stimulating root growth to compensate, at the expense of shoot growth.

Saqrane et al. (2009) exposed two cereals and two leguminosae to various MC concentrations by irrigation with a solution of a *M. aeruginosa* extract and reported a significant negative effect on growth and development. *T. durum* was the most sensitive plant and *L. esculenta* the most resistant, but in general all the effects were plant species-, dose-, and time-dependent. Moreover, irrigation of *M. sativa* plants with water contaminated with a *M. aeruginosa* extract containing MC-FR, -LY, -(H4)-YR, and DMC-LR, as well as MC-LR, affected physiological and metabolic parameters of the plants, causing inhibition of germination of alfalfa seeds and growth reduction (El Khalloufi et al., 2011). Contrary to the results of El Khalloufi et al. (2012), no inhibition of germination was observed in tomato seeds following exposure to a *M. aeruginosa* culture containing a mixture of 13 MC variants at similar concentrations (Corbel et al., 2015), although in this case the seeds were exposed for a shorter period (7 d versus 30 d). However, wheat seed germination was inhibited in the study conducted by Corbel et al. (2015), possibly owing to alteration of metabolic activities of the seeds during the germination process. Although no significant effects were found in tomato or lettuce seeds at low MC concentrations, they increased the growth and affected the morphology of those radicles, whereas high concentrations of MCs (20 mg eq. MC-LR L^{-1}) reduced the length of tomato radicles. All this may indicate that the sensitivity to MCs can vary within plants, also depending on the doses employed, as was also found by Chen et al. (2004) and Saqrane et al. (2008) in several plant species. Increased root membrane permeability was detected for the first time in rice plants exposed to a MC mixture in a hydroponic system (Cao et al., 2017). Growth inhibition of the plant was evidenced, the root biomass being more sensitive at low MC concentrations, possibly owing to metabolic imbalance and cellular metabolic disorder induced by excessive ROS production.

As demonstrated in the studies reviewed, oxidative stress seems to be involved in the toxicity of less common congeners of MCs in plants exposed for 7–30 d periods, generally by enhanced MDA levels and phenolic compounds and increases in the activity of antioxidant enzymes. Altered SOD and POD activities with increasing concentrations of MCs have been reported in rape and rice (Chen et al., 2004) and in apple shoots (Chen et al., 2010). The aquatic plant *Lemna gibba* showed increased POD activity at the lowest MC concentration when exposed to a *Microcystis* extract containing MC-WR and −RR (Saqrane et al., 2007). Increases in POD activity and in phenolic compounds were also found in alfalfa and tomato plants after irrigation for 30 d with water contaminated with a *M. aeruginosa* extract containing MC-FR, -LY, -(H4)-YR, DMC-LR, and –LR, in a concentration-dependent way (El Khalloufi et al., 2011, 2012). On the basis of the differential adverse effects on plants induced by MCs, the response of the antioxidant system of plants as a result of toxin exposure presumably depends on plant species, MC variants, type and time of exposure, etc. Most of the earlier studies investigated the effects in macrophytes exposed to individual MCs (Campos et al., 2013; Pflugmacher, 2007), but Romero-Oliva et al. (2015) recently suggested that hydroponic exposure to a cyanobacterial extract containing a mixture of MCs, including MC-YR, led to a strong activation of antioxidant defense systems and to an effort by the plant to biotransform MCs through activation of GST activity, suggesting an adaptive potential of these macrophytes to adverse conditions. CAT activity also increased, together with SOD activity and MDA levels, in rice exposed for 30 d to an extract containing MC-YR, -LR, and −RR, owing to ROS production in most tissues of the plant (Cao et al., 2017, 2018).

Some studies suggested that various congeners of MCs impaired photosynthetic activity in several plant species (El Khalloufi et al., 2012; Romero-Oliva et al., 2015; Saqrane et al., 2009). This could be partially due to damage to PSII reaction centers, deleterious effects on PSII activity, and changes in the function of various proteins involved in the photosynthetic process related to chloroplast ATP synthesis, carbon fixation, photosynthesis, and metabolism of carbohydrates, chlorophyll concentrations, and chlorophyll fluorescence (Guzmán-Guillén et al., 2017). Sometimes these studies have been performed in hydroponic conditions; however, when plants are cultivated in sand, differences could be found in the effects exerted. Decreased photosynthetic activity, measured by Fv/Fm fluorescence, was found by Saqrane et al. (2009) in wheat, maize, pea, and lentil grown in sand and exposed to various MC concentrations by irrigation with a *M. aeruginosa* extract containing MC-YR, -(H4)YR, -WR, −FR, −RR, and -LR, although no significant changes were recorded in chlorophyll ($a+b$) content.

With regard to mineral uptake by plants, exposure of soil-grown wheat, maize, pea, and lentil to *M. aeruginosa* extract containing MC-RR, -LR, -YR, -(H4)YR, -WR, and −FR led to enhanced accumulation of some minerals such as Na^+, K^+, Ca^{2+}, P, and N (Saqrane et al., 2009). According to these authors, the possible impairment of growth and development would probably imply defects in nutrient allocation and utilization. In fact, nutrient elements play important roles in plants, and excessive or deficient nutrient concentrations can cause negative impacts on the plant (Cao et al., 2017). Later, El Khalloufi et al. (2012) showed increased levels of Na^+ and Ca^{2+} in tomato plants (*Lycopersicon esculentum*) irrigated with water contaminated with a *M. aeruginosa* extract containing MC-FR, -LY, -(H4)-YR, and DMC-LR, as well as MC-LR, and these alterations were concentration-dependent. Recently, Cao et al. (2017) showed that uptake of microelements (Fe, Mn, Cu, and Zn) in rice was generally not affected after a 30-day exposure to MCs, whereas root uptake of macroelements (Na, Ca, Mg, etc.) was stimulated. In all cases, Ca^{2+} in roots increased especially. The increase shown in the uptake of nutrients in the study conducted by Cao et al. (2017) was not expected, because of the inhibition of plant growth. The authors suggested that exposure of rice roots to MCs leads to root damage and loss of membrane integrity, producing greater permeability and nutrient uptake, as also suggested by El Khalloufi et al. (2012) to explain greater nutrient accumulation. More recently, it was suggested that these changes in rice root permeability were responsible for the effects of MCs on rice root exudation, such as increases in dissolved organic carbon (DOC) and carbohydrate (Cao et al., 2018). However, most of the organic acids and amino acids in root exudates were not affected, possibly because of the hydroponic conditions, compared to soil conditions, where organic acids often increase in root exudates in response to various environmental stresses (Jones, 1998).

Therefore, taking all the available information into account, it is recommended that studies are performed to elucidate the implication of natural soil–plant systems, not only hydroponic ones, in the effects of minority MCs on vegetables. More research is also needed to compare the effects of realistic MC concentrations for longer exposure periods (more than 14 d), and the impact on the nutritional value of edible vegetables (vitamin and mineral contents). More studies are necessary to compare all these effects in different plant species.

4.4. Field studies and case reports

Few field studies in animals showing the presence and toxic effects of minority congeners of MCs have been reported in the scientific literature (Table 6). This may be due to low recognition of the impact of these cyanotoxins on domestic and wild animals, as many cases are misdiagnosed, few cases are biochemically confirmed, and even fewer are reported in the scientific literature or in animal health surveillance systems (Zaias et al., 2010). In addition, the animal species involved are varied, including dogs, heifers, roe deer, birds, flamingos, ducks, and otters. Among all of them, special attention has been paid to cyanotoxin poisoning affecting dogs. In fact, a review reporting the cases that took place in the United States between 1920s and 2012 has already been published (Backer et al., 2013). The minority congeners of MCs most frequently detected in animal samples are MC-YR (Krienitz et al., 2003;

Table 6
Field studies or case reports in various animals exposed to minority MCs.

Animals/Place	Detected Cyanotoxins	Exposure conditions	Signs and symptoms/Histopathology/Analytical parameters	Other relevant findings	Reference
2 Flamingos/Kenya (Lake Bogoria)	MC-LF, MC-YR, MC-LR, MC −RR and Anatoxin-a.	Natural exposure.	Death.	Cyanobacteria mats contained 177–375 μg/g d.w. of MC-LF, 14–352 μg/g d.w. of MC-YR, 23–198 μg/g d.w. of MC-LR and 35–58 μg/g d.w. of MC-RR.	Krienitz et al. (2003)
1 Roe deer (*Capreolus capreolus*)/Norway	MC-YR, MC-LR and MC-RR.	Natural exposure.	The animal presented general weakness, apathy and stupor. Heart with hemorrhage. The lungs were congested. The liver was enlarged, presented pale in color, ascites, diffuse hepatocellular dissociation, degeneration and necrosis.	MCs were detected in liver sample by LC-MS. Liver presented 1255 ng/g of MC-YR, 68 ng/g of MC-LR and 38 ng/g of MC-RR.	Handeland and Østensvik (2010)
21 Otters/California	MC-LA, MC-LF, MC-YR, MC-RR, MC-LR and MC-Desmethyl-LR.	Natural exposure.	Death. The liver samples presented hepatocellular vacuolation, apoptosis, necrosis and hemorrhage. Moreover, livers appeared enlarged, bloody and friable.	MCs were detected in liver and feces samples by LC-MS/MS.	Miller et al. (2010)
1 Dog/Montana	MC-LA.	Natural exposure by algal bloom in a community lake. Supportive therapy applicated: fluids, mucosal protectants, vitamins, antibiotics, nutritional supplements and, a bile acid sequestrant, cholestyramine.	Anorexia, vomiting and depression. Lymphopenia, thrombocytopenia, ↑bilirubin, ↑ alanine, ↑ ALT and ↑ ALP. Rapid clinical improvement.	Feces samples contained 217 ppb of MC-LA.	Rankin et al. (2013)

Abbreviations: ALP: alkaline phosphatase; ALT: alanine aminotransferase; b.w.: body weight; d.w.: dry weight; f.w.: fresh weight; i.p.: intraperitoneal; MCs: microcystins.

Handeland and Østensvik, 2010; Miller et al., 2010; Lürling and Faassen, 2013), MC-LF (Krienitz et al., 2003; Miller et al., 2010; Lürling and Faassen, 2013), and MC-LA (Miller et al., 2010; Rankin et al., 2013).

In general, individual MC congeners do not appear in isolation in nature. For this reason, it is very difficult to attribute the toxic effects to a specific congener of MC. So far, there is only one study in which a single MC congener, MC-LA, was detected in a dog sample after its hospitalization with severe symptoms of intoxication. MC-LA produced alteration of several biochemical parameters measured in blood, and severe hepatocellular injury and cholestasis in the dog. Moreover, the animal's previous contact with an algal bloom containing MC-LA was corroborated. Similar symptoms of vomiting and lethargy have been described in many cases of cyanotoxicosis caused by natural exposure to MCs in various dog species (Lürling and Faassen, 2013; Rankin et al., 2013; Moore et al., 2016), even though different MC congeners were involved. Up to seven congeners of MC (MC-YR, MC-LY, MC-LW, MC-LF, MC-RR, dm-7-MC-LR, MC-LR) were identified in the vomit sample of an intoxicated canine by LC-MS/MS (Lürling and Faassen, 2013), MC-LR being the most abundant MC identified (73%), followed by MC-LW (6.6%), and dm-7-MC-LR (6.0%). Although the relative abundance of MC-LW and MC-LF was about 12% compared to that of MC-LR, the contribution of these minority MC congeners to toxicity could be of the same order of magnitude as that displayed by MC-LR, taking into account their higher toxicity (> 7 times more than MC-LR) reported by Fischer et al. (2010) in both primary human hepatocytes and OATP-transfected HEK293 cells. In agreement with these results, Moore et al. (2016) described a similar symptomatology in a dog that had swum in a lake containing MC-LA, MC-LF, MC-LR, and MC-RR congeners. The authors suggested that the dog died from MC toxicosis, although they could not prove the involvement of specific congeners.

Important decreases have occurred in the population density of flamingos in Kenyan lakes since 1999, although the reasons have not been fully elucidated yet (Krienitz et al., 2003). In fact, two dead flamingos appeared in a lake where four structural variants of MC were detected (MC-LF, MC-YR, MC-LR, and MC-RR). The authors reported the presence of isolated MC-YR in fecal content, whilst MC-LF and MC-YR were found in the stomach. As minority congener concentrations (MC-YR and MC-LF) detected in water were similar, the non-detection of the MC-LF congener in fecal samples could be due to toxicokinetic differences between the two congeners. The authors suggested the possibility of the contribution of these minority MC congeners (MC-YR and MC-LF) to these deaths (Krienitz et al., 2003).

The first report confirming cyanobacterial intoxication in wild mammalian species described a case involving a roe deer (Handeland and Østensvik, 2010). The highest MC concentration in liver corresponded to MC-YR (1255 ng/g), whereas other MCs were found in much smaller concentrations (68 ng/g for MC-LR and 38 ng/g for MC-RR). Histopathological damage was observed in liver, but not in other vital structures, such as brain and kidney (Handeland and Østensvik, 2010). In light of the MC concentration detected in the liver sample, it could be assumed that the toxic effects described were mainly produced by MC-YR, indicating the need to extend toxicity studies to other lesser-known minority MC congeners.

Another recognized environmental problem is the possibility of cyanotoxins flowing from the rivers to the ocean. For example, significant concentrations of freshwater-derived MCs such as those reported in the Pinto lake (up to 2100 ppm of MC-LA out of a total of 2900 ppm, together with MC-RR, MC-LR, MC-Desmethyl-LR, MC-LF, and MC-YR), are intermittently polluting the land–sea interface in central California (Miller et al., 2010). Marine animals such as otters died showing signs of liver poisoning, and liver and feces of the dead otters tested positive for MCs. However, although MC-LA was the most detected variant in water samples, the authors did not detect the presence of minority congeners of MC in these animal samples. Consequently, it cannot be confirmed whether these deaths were due to

minority MC congeners, to other better-known congeners such as MC-LR or MC-RR, or to the combined effect of them all.

In summary, higher concentrations of congeners such as MC-YR and MC-LF were detected in some field studies, instead of MC-LR and MC-RR. This fact, together with the scarce number of studies, highlights the importance of extending toxicological research and monitoring efforts not only to MC-LR but also to these minority MC congeners, mainly MC-YR MC-LF and MC-LA, and MC mixtures, in order to increase our understanding of their roles in biological systems for a more realistic risk assessment.

5. Conclusions and recommendations

Despite the increasing number of MC congeners identified to date, most of the toxicological information is focused on MC-LR. Occurrence data show that minority MCs are also distributed worldwide and can even become the predominant cyanotoxins. Reports about their toxicokinetic and toxicodynamic profiles are scarce, but they describe important findings, such as higher toxicity than MC-LR for specific variants and new potential target tissues such as the nervous system. Globally, further studies are required in all aspects (water monitoring, food occurrence, biodistribution, toxicity mechanisms, toxic effects, etc.) with special focus mainly on MC-YR and MC-LA, for its occurrence and abundance and on MC-LF for its toxicity. Moreover, attention should be paid to MC mixtures, which may lead to synergistic/antagonistic effects.

Particularly, priorities for the requirement of additional data could include: to develop and validate sensitive and robust analytical methods in several matrices (water, different food commodities), as well as appropriate reference materials and commercial standards, in order to perform correct identification/quantification procedures and also to characterize their toxicity profiles. In food, monitoring studies are needed to know potential seasonal variations of minority MC variants accumulation. Moreover, further *in vitro* bioaccessibility and bioavailability assays, including the effects of different cooking procedures, are required to evaluate the real contribution of these MC variants to the exposure. In relation to toxicological aspects, it would be interesting to investigate the expression of individual OATPs involved in the toxicokinetic of minority MC congeners in several organs, experimental models, and through different administration routes, even at molecular level. Only a few studies with pure minority MC variants have been performed so far (apart from PP inhibition assays) to have a complete picture of their toxicological profile, and mechanisms of action such as oxidative stress or genotoxicity and targeted toxicity such as nervous system and reproductive toxicity should be further explored. Proteomics, metabolomic and transcriptomic analysis could contribute to the hazard characterization of these minor MC congeners. Moreover, taking into account the MC transfer to the diet by vegetables and even the deleterious effects on plants this experimental model is also of interest. Finally, additional field studies could increase our understanding of their roles in biological systems. All these data will contribute to perform more realistic risk assessments both for humans and the environment.

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Appendix A. Supplementary data

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Transparency document

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