Neurotoxicity induced by Microcystins and Cylindrospermopsin: A review

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Abstract

Microcystins (MCs) and cylindrospermopsin (CYN) are among the most frequent toxins produced by cyanobacteria. These toxic secondary metabolites are classified as hepatotoxins and cytotoxin, respectively. Furthermore, both may present the ability to induce damage to the nervous system. In this sense, there are many studies manifesting the potential of MCs to cause neurotoxicity both *in vitro* and *in vivo*, due to their probable capacity to cross the blood-brain-barrier through organic anion transporting polypeptides. Moreover, the presence of MCs has been detected in brain of several experimental models. Among the neurological effects, histopathological brain changes, deregulation of biochemical parameters in brain (production of oxidative stress and inhibition of protein phosphatases) and behavioral alterations have been described. It is noteworthy that minority variants such as MC-LF and -LW have demonstrated to exert higher neurotoxic effects compared to the most studied congener, MC-LR. By contrast, the available studies concerning CYN-neurotoxic effects are very scarce, mostly showing inflammation and apoptosis in neural murine cell lines, oxidative stress, and alteration of the acetylcholinesterase activity *in vivo*. However, more studies are required in order to clarify the neurotoxic potential of both toxins, as well as their possible contribution to neurodegenerative diseases.

Keywords: cyanotoxins, MCs, CYN, nervous system, ecotoxicology, environmental risk.

1. Introduction

Cyanobacteria are a group of Gram-negative prokaryotes capable of growing under almost every environmental condition (Chorus and Bartram, 1999). Due to climate change and anthropogenic activities, their presence is increasing (Davis and Gobler, 2016). As a consequence, there is an enhancement of the production of toxic secondary metabolites of great importance for the ecotoxicology known as cyanotoxins (Duy et al., 2000). These toxins are classified as hepatotoxins (e.g. microcystins, nodularins), cytotoxins (e.g. cylindrospermopsin), neurotoxins (e.g. anatoxin-a, homoanatoxin, saxitoxins), dermatotoxins (e.g. lungbyatoxin) or irritant toxins (e.g. lipopolysaccharides) (Testai et al., 2016). There are different exposure routes for cyanotoxins, being the most important the oral route. In fact, many aquatic organisms are able to live in presence of cyanotoxins, and some of them have proved to bioaccumulate these secondary metabolites, acting as a reservoir for animals higher up the trophic chain, and also for humans (Berry and Lind, 2010; Gutiérrez-Praena et al., 2013). However, dermal, inhaling or even parenteral exposures are also possible (Buratti et al., 2017). Thus, the variety of targets and exposure routes together with the rise of cyanobacterial proliferations make of cyanotoxins a serious concern for animal livestock, human activities and public health (Testai et al., 2016).

In the last decades, the toxic effects of cyanotoxins on the nervous system have been widely studied, not only those caused by the so-called neurotoxins with well-defined mechanisms of action in this system such as anatoxins (ATX) and saxitoxins (STX), but also, by other cyanotoxins with different target organs (Florczyk et al., 2014). Neurotoxicity could be described as ‘any adverse effect on the central or peripheral nervous system caused by chemical, biological or physical agents’ (Costa et

al., 2008). The keys to the brief communication within the nervous system are the generation of a potential of action as a quick response of dendrites to the neurotransmitters released from contiguous neurons, and its fast travelling for the neuronal axon for its release afterwards (Kem, 2000).

In this sense, among all the cyanotoxins, microcystins (MCs) and cylindrospermopsin (CYN) have proven to exert damage in the nervous system as well, in spite of not being considered as neurotoxins *per sé*. These are very common cyanotoxins (Table 1) able to put health at risk due to their ubiquity (Gutiérrez-Praena et al., 2013), as previously demonstrated in different human poisoning cases. The most serious episode associated with human exposure to MCs occurred when 126 people were intoxicated at a haemodialysis clinic in Caruaru (Brazil), causing the death of almost half of them. All patients presented malaise, weakness, dizziness, vertigo, tinnitus, mild deafness and, in severe cases, visual disturbance and blindness, grand mal convulsions, and gastrointestinal and hepatic symptoms (Pouria et al., 1998; Carmichael et al., 2001). Most of these symptoms have a neuronal origin, standing out the possible MCs-crossing the blood-brain barrier (BBB) as several authors have reported (Feurstein et al., 2009; 2010; 2011; Zhao et al., 2015a), causing their toxic effects.

In the case of CYN, the most important outbreak occurred in Palm Island (Australia) in 1979, when 146 people were hospitalized with symptoms of malaise, vomits, anorexia, and hepatomegaly after drinking from a water supply that contained a CYN-producing *Cylindrospermopsin raciborskii* strain (Bourke et al., 1983; Griffiths and Saker, 2003). However, it is important to mention that CYN was also present in the Caruaru outbreak, possibly contributing to the neurological affectation reported (Bláha et al., 2009) although it is hard to differentiate the effects caused for each toxin in the

symptoms observed, as both toxins are often present together in nature (Gkelis and Zaoutsos, 2014; Trainer and Hardy, 2015; Loftin et al., 2016; Buratti et al., 2017). Due to the low molecular weight of CYN, it might be able to cross the BBB. In fact, CYN was detected in brains of two fish species (Guzmán-Guillén et al., 2015; da Silva et al., 2018). Thus, although not being considered as neurotoxins, both cyanotoxins have demonstrated its neurotoxic potential in different *in vitro* and *in vivo* experimental models, increasing the interest of the scientific community in this matter. Taking into account all these facts, the aim of the present work was to gather the existent knowledge about the potential to exert neurotoxic effects of both toxins from 1998 to 2018.

2. Microcystins

Microcystins (MCs) are cyclic heptapeptides molecules containing a hydrophobic C_{20} D-amino acid commonly known as ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), crucial for the toxicity of these cyanotoxins due to their interaction with protein phosphatases (Song et al., 2006) (Fig. A). More than 246 isoforms of MCs have been detected (Spoof and Catherine, 2017), mainly differing in the L-amino acids at positions 2 and 4, causing differences in toxicokinetic and toxicodynamic properties (Rinehart et al., 1994). These compounds are the most widespread cyanobacterial toxins detected in freshwaters (Spoof and Catherine, 2017), being many the cyanobacteria genera capable of synthesize them: *Microcystis, Plankthotrix, Anabaena, Nostoc, Aphanizomenon, Anabaenopsis*, *Rivularia and Fisherella*, among others (Sivonen and Jones, 1999; Rao et al., 2002; Carey et al., 2007; Bittencourt-Oliveira et al., 2014; Cirés et al., 2014).

The most known mechanism of action of MCs is the protein serine/threonine phosphatases inhibition, able to cause phosphoprotein-deregulation, which leads to

tumor promotion and apoptosis (MacKintosh et al., 1990; Vichi et al., 2016). Furthermore, the potential of MCs to increase reactive oxygen species (ROS) and to reduce glutathione (GSH) levels, causing oxidative stress and, therefore, apoptosis, has already been demonstrated (Puerto et al., 2011; Wang et al., 2013; Li et al., 2015; Liu et al., 2016; Qian et al., 2018). Although being considered as hepatotoxins, MCs can damage other organs such as intestines, heart or kidneys (Moreno et al., 2003; Atencio et al., 2008; Qiu et al., 2009; Li et al., 2011a; Zeng et al., 2014). In this sense, it has been demonstrated that MCs require organic anion transporting polypeptides (OATPs for humans/ Oatps for rodents) in order to cross cell membranes (Chen and Xie, 2016). The OATP1B1 and OATP1B3 are common in liver cells, while OATP1A2 is thought to be the responsible for the transport of MC-LR, across the BBB and the kidneys, for example (Fischer et al., 2005; Feurstein et al., 2009). This means that significant amounts of MCs could reach the brain across the BBB and induce brain pathology, depending on the type and expression of OATPs/Oatps at the BBB, the blood-cerebrospinal fluid barrier, and the neuronal cell membrane (Bronger et al., 2005; Huber et al., 2007; Westholm et al., 2009). Most of the existent studies have been carried out using MC-LR, due to its major presence and its wide demonstration of causing neurotoxic effects in several experimental models, although other more toxic congeners such as MC-LW and MC-LF have also been studied (Feurstein et al., 2009; 2010; 2011; Rozman et al., 2017). This can be due to the hydrophobicity of MC-LF and MC-LW. Structure variations and differences in molecular properties such as hydrophilicity/hydrophobicity can lead to a modification on molecular interactions with lipid membranes (Vesterkvist and Meriluoto, 2003) modifying PP-inhibitory activity (Díez-Quijada et al., 2019).

Concerning to their effects in the nervous system, Florcyk et al. (2014), Hu et al. (2016) and Mello et al. (2018) have reviewed the main mechanisms of neurotoxicity of MCs at different levels. Firstly, neurotransmission, by causing effects on GABAergic neurons. Secondly, neurochannels, by affecting the ionic concentrations in and outside the cells. Linked to this, signal transduction, as a consequence of the deregulation of Ca^{2+}, which, by activating calcineurin leads to apoptosis. Moreover, the production of oxidative stress, by deregulating several antioxidant enzymes such as catalase or superoxide dismutase (SOD). And finally, cytoskeleton disruption, by alteration of structural brain proteins such as Tau. However, important contributions have been made lately, confirming these mechanisms using mostly *in vivo* experimental models. In this sense, the studies carried out using different animal models (mice, fish) revealed an important effect on the neurotransmission induced by MC-LR (Wu et al., 2016; Qian et al., 2018; Shin et al., 2018; Wang et al., 2018), together with an enhancement in oxidative stress in mice (Shin et al., 2018; Wang et al., 2018), and cytoskeleton disruption in the case of rats (Zhang et al., 2018) (Fig. B).

2.1. Neurotoxicological *in vitro* studies performed with microcystins

Table 2 shows the different *in vitro* assays performed with MC-LR and some other congeners in different neuronal cell lines and primary cultures. The *in vitro* studies are relatively recent, comprising a range of ten years (2009-2018) (Table 2). This fact demonstrates the importance that MCs have lately acquired concerning their neurotoxicity nowadays. Thus, it is possible to find different studies carried out in permanent cell lines (PC12, BV-2, N2a, GT1-7, and SH-SY5Y) and in several primary cell cultures. It is also important to remark that all the toxins used in these studies are commercial standards with a purity >95%, which guarantees that the results reported are

due to the MC itself and not to other potential bioactive compounds that can be present in cyanobacterial extracts (Falconer, 2007). Furthermore, it is important to highlight that no studies have been performed using extracts *in vitro*.

2.1.1 Cell viability studies after exposure to MCs

Cell death caused by MCs in neuronal cells has been studied by different assays. Occupying an important place in these studies are the cytotoxicity assays. As it can be observed in Table 2, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay, the lactate dehydrogenase (LDH) release assay, and the cell counting kit-8 (CCK-8) test have been used to explore the cytotoxicity of MC congeners in several neuronal cell lines. In primary cell lines, Feurstein et al. (2009) found that MC-LF, -LW, and -LR induced a concentration-dependent decrease of primary murine WBC when exposed to 0-5 μM MCs for 48 hours, being MC-LF the most potent toxin. Rozman et al. (2017) also evidenced the different cytotoxicity induced by the MC-LW and MC-LF congeners in primary rat astrocytes exposed to 0-10 μM MCs for 24 hours. However, these authors did not find any significant reduction of viability in cells exposed to MC-LR. On the contrary, Cai et al. (2015) found a concentration-dependent reduction of cell viability in primary hippocampal neurons, although the MC-LR concentrations used were higher (0-30 μM) than in the previous study. Despite this, Li et al. (2015a) used lower concentrations of MC-LR (0-3 μM) in the same cellular model, remarking that they only found a reduction of cell viability at the highest concentration assayed after 48 hours of exposure. These same authors also evaluated the LDH release, showing that this release increased with the MC-LR concentration. However, Zhang et al. (2018) observed that only the highest concentration (10 μM MC-LR) induced a significant loss of viability in SH-SY5Y cells exposed for 24 hours. This

186 fact would indicate that the cellular model could play a role in the MC-LR toxicity,
187 being more sensitive those cells derived from the hippocampus.

188 Concerning permanent cell lines, different patterns have been observed. Thus,
189 Takser et al. (2016) found that murine microglial BV-2 cell line suffered a decrease in
190 cell viability when exposed to 0-10 µM MC-LR during 72 hours. Furthermore, these
191 same authors revealed that the N2a cell line presented an even more significant
192 reduction of viability after 72 hours of exposure, establishing possible differences
193 between cells from different origins. The results obtained by Ding et al. (2017) were
194 especially remarkable, finding that MC-LR induced a concentration-dependent
195 reduction of viability in GT1-7 cells exposed up to 1 µM MC-LR during 48 hours. In
196 this study, the MC-LR concentrations used were pretty lower than those used by the rest
197 of the authors. Thus, the main target of MC-LR in the nervous system seems to be the
198 limbic system, since cells from hypothalamus and hippocampus have proven to be the
199 most sensitive.

200 2.1.2 Effects of MCs in different proteins

201 Many of the presented studies deal with the fact that MCs need to enter the
202 neuronal cells to exert their toxic effects. It is well known that MCs use OATP/Oatp to
203 get into cells. In this sense, Feurstein et al. (2009) stated that primary murine whole
204 brain cells (WBC) presented, at least, five Oatps, and demonstrated the role of these
205 transporters in the toxicity induced by different MC congeners. Lately, the same authors
206 employed primary murine neurons and cerebellar granule neurons (Feurstein et al.,
207 2010; 2011), and demonstrated that MC-LF, -LW, and –LR produced a significant PPs
208 inhibition at different concentrations, being MC-LF the most potent toxin and MC-LR
209 the least one. Concerning MC-LR, Meng et al. (2011), in differentiated rat

neuroendocrine PC12 cells, and Zhang et al. (2018), in human neuroblastoma SH-SY5Y cells, found that this toxin inhibited the PP2A in a concentration-dependent manner. However, MCs uptake has been also shown by imaging techniques. Thus, Rozman et al. (2017) confirmed the uptake of different MCs congeners by immunochemistry in primary rat astrocytes. Moreover, Ding et al. (2017) and Zhang et al. (2018) used the western-blot technique to demonstrate the penetration of MC-LR in hypothalamic neuronal mouse cells 1-7 (GT1-7) and SH-SY5Y cells, respectively, analyzing the PP1 and PP2A catalytic subunits, which appeared reduced as the toxin concentration increased.

Inhibition of PP2A activity has been described as the main toxic mechanism of MCs (Yoshizawa et al., 1990), which is related to the selective destruction of microtubules, leading to cell death. Different proteins are involved in cellular organization, and among them, one of the most relevant is Tau. This abundant microtubule-associated protein which main function to stabilize the microtubules assembly, is less effective the more phosphorylated Tau is (Buée and Delacourte, 2001), being associated with microtubule dysfunction and cell death (Feurstein et al., 2011). These last authors found, in primary murine cerebellar granule neurons (CGNs), that MC congeners induced Tau hyperphosphorylation at lower concentrations than the needed for PP2A inhibition, which could evidence that specific proteins from the nervous system display more sensitive response to MCs. However, these concentrations did not lead to significant cell death by apoptosis (activation of caspase-3/7 was absent); although disruption of the neurite network was observed, which is in agreement with the findings of Rozman et al. (2017) in primary rat astrocytes. Meng et al. (2011) also established the connection between the inhibition of PP2A and Tau protein hyperphosphorylation in differentiated PC12 cells. Furthermore, these authors studied

afterwards Tau phosphorylation through the p38-mitogen-activated protein kinase (p38-MAPK), reporting that MC-LR exposure induced p38-MAPK activation, although at higher concentrations than those required for the inhibition of PP2A. Thus, they established that this could be an indirect mechanism of Tau hyperphosphorylation. In addition, they also found that the heat-shock protein 27 (HSP27), responsible of actin cytoskeleton remodeling, was also increased due to the activation of p38-MAPK, contributing to the cell disruption caused by MC-LR. Related to this, Meng et al. (2013) demonstrated that the previously described activation of p38-MAPK by MC-LR in PC12 cells was downstream of ROS-dependent signaling cascades. More recently, Zhang et al. (2018) confirmed the activation of the p38-MAPK in SH-SY5Y cells exposed to MC-LR. Moreover, these authors also found that MC-LR activates the c-Jun N-terminal kinase (JNK), a protein associated with the induction of cell death by apoptosis. Besides, MC-LR induced the phosphorylation of the glycogen synthase kinase-3 (GSK-3β), contributing to the dissociation of the regulatory subunit B55α from the PP2A and its degradation, facilitating Tau hyperphosphorylation.

2.1.3 Involvement of MCs in the $[Ca^{2+}]i$ levels:

Intracellular calcium ($[Ca^{2+}]_i$) levels are crucial for cell survival. In this sense, Ding et al. (2001) indicated that MC-LR is implicated in Ca^{2+} release from mitochondria and the activation of Ca^{2+}/calmodulin-dependent protein kinase, which triggers cell death by apoptosis in hepatocytes. Thus, Cai et al. (2015) found a concentration-dependent Ca^{2+} mobilization in primary hippocampal neurons exposed to 0-30 μM MC-LR. These authors demonstrated that the increase of $[Ca^{2+}]_i$ levels could be due mainly to its mobilization from the endoplasmic reticulum. Mitochondria seemed not to play an important role in the cascade of $[Ca^{2+}]_i$. This fact is in agreement

with the results obtained in the previously described MTT assays (Feurstein et al., 2009, 2011; Takser et al., 2016), since authors described a concentration-dependent loss of cell viability, but only a few observed significant differences against the control groups. In addition, Li et al. (2015a) reported that MC-LR participated in the activation of the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin (CaN), through the mobilization of $[Ca^{2+}]_i$ levels, leading to the activation of an apoptotic caspase cascade. In this sense, Feurstein et al. (2011) found that MC-LF and MC-LW induced a concentration-dependent increase of caspase-3/7 activity in primary murine CGNs. Furthermore, Rozman et al. (2017) also observed apoptosis in primary rat astrocytes exposed to different MC congeners. However, these authors did not propose any theory about the apoptosis pathway. These findings could be in agreement with the reports of Cai et al. (2015) and Li et al. (2015a) concerning Ca^{2+} mobilization and apoptosis.

Summarizing, MC-LW and –LF have proven to exert higher neurotoxic effects *in vitro* than MC-LR. However, since MC-LR is the most abundant congener in nature, all the studies presented in Table 2 have been carried out using this cyanotoxin. The way these toxins reach the nervous system is not fully elucidated yet, although several authors demonstrated the participation of OATPs/Oatps in their transport, together with an inhibition of the protein phosphatase. Once inside neuronal cells, MCs have shown to disrupt several proteins participating in the cellular structure (PP2A, Tau, p38 MAPK, HSP27, GSK-3β, etc.), inducing cytoskeleton remodeling and cell death. In addition, cellular disruption has been demonstrated as well by cytotoxicity and apoptosis assays. Both mechanisms could be associated with the increment of $[Ca^{2+}]_i$ levels. However, it is noteworthy that cells affected by MCs are mainly those present in the limbic system, pointing out this system as a possible target for MCs.

2.2. Neurotoxicological *in vivo* studies performed with microcystins in aquatic animals

Several works have investigated so far MCs potential neurotoxicity in different fish species, mainly in zebrafish (*Danio rerio*) (Table 3). The first studies reporting the chronic effects of dissolved MC-LR on the fish behavior were performed by Baganz et al. (1998, 2004). Behavioral studies are important to establish the lowest level of disturbance. In this sense, these authors observed that MC-LR induced a decrease of daytime and nighttime activity in *D. rerio* after their exposure to high concentrations, while at low ones, that reduction at night was compensated by a rise in their daytime activity. On the contrary, at high concentrations, *Leucaspius delineatus* reduced its activity during the daytime, increasing at night, whereas a rise was reported during both day and night at low concentrations. These compensative responses could be explained as an escape strategy or as a consequence of some changes in the spatial orientation to deal with alterations in the medium conditions, represented by the presence of MCs. However, the decreased motility observed at high MC-LR concentrations may be interpreted as an attempt to save energy, needed maybe to biotransform the toxin, which is a possible reason why glutathione-S-transferase (GST) activity appeared enhanced. *L. delineatus* showed greater sensitivity than *D. rerio*, as it responded earlier and for a longer period of time (Baganz et al., 2004).

Neurotoxicity of pure MC-LR at the proteomic level was firstly demonstrated in zebrafish brains after chronic exposure (30 days) by Wang et al. (2010) and in developing zebrafish larvae after 96 hours post-fertilization exposure by Li et al. (2011b). Furthermore, chronic exposure seemed to interfere concomitantly with signal transduction, leading to apoptosis, transport and protein degradation, and increasing the

PP activity at higher toxin concentrations by PP2Cα2 overexpression (Wang et al., 2010). Li et al. (2011b) suggested a potential involvement of creatine kinase (CK) and dihydropyrimidinase-like 2 (DRP2) in the neurotoxicity induced by MC-LR, which were upregulated in larvae of zebrafish. The CK seemed to be correlated with increased energy requirements, and DRP2 with axonal outgrowth, cell migration, neuronal growth, and pathfinding. In this sense, a decreased expression of DRP2 has been reported in schizophrenia, Alzheimer disease, the Down syndrome, and affective disorders (Johnston-Wilson et al., 2000; Lubec et al., 1999).

Pavagadhi et al. (2012) studied the influence of sub-lethal concentrations of dissolved MC-LR and MC-RR (0-10 µg/L) on several oxidative stress parameters in the brain of zebrafish adults such as GST, glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD) activities. Generally, most of the parameters followed a bell-shaped curve for both toxins, with peaks at different concentrations. Most of these enzyme activities rose at lower concentrations and decreased at the highest (5 and 10 µg/L). However, discrepancies between GPx and GR activities were observed as the effects of MC-LR were more prominent in GPx activity while GR activity was more enhanced after exposure to MC-RR. These variations could probably be due to the biochemical adaptive response of the organisms to MCs exposure depending on their specific toxicity.

A more recent study has shown that accumulation of MC-LR in zebrafish larvae led to hypoactivity with alteration of the cholinergic system, showed by decreased dopamine (DA) and ACh levels, and increased AChE activity, which could also yield to hypoactive muscular contraction and behavioral responses (Wu et al., 2016). In addition, and similar to previous works, their proteomic analysis suggested that this

331 neurotoxicity could be related to neuron maturation, axon growth, and cytoskeleton
332 regulation. Nevertheless, if these effects induced by MC-LR could be of parental
333 transmission or not was later clarified by chronic exposures of adult zebrafish to
334 environmentally relevant concentrations of MC-LR (1-25 µg/L), demonstrating, for the
335 first time, the toxin accumulation and developmental neurotoxicity in offspring (Wu et
336 al., 2017). The mechanisms by which these transgenerational effects are exerted could
337 be by interrupting the neuronal development and/or by hampering the neurotransmitter
338 systems (as shown by decreases in DA and serotonin levels, and in AChE activity).
339 Moreover, exposure of zebrafish embryos to similar concentrations of MC-LR for 90
340 days led to several histopathological damages in the brain (Yan et al., 2017). Despite
341 lacking the clear cerebral cortex of higher vertebrates, fish cerebra rule complex
342 behavior such as escaping from predators, swimming, and feeding modulation. Thus, it
343 would make sense that the ultrastructural changes detected in this study could have
344 impaired the function of nerve fibers in zebrafish exposed to MC-LR. These authors
345 suggested that the disruption of the GABA pathway might be also implicated in the
346 mechanism of MC-LR-induced neurotoxicity (Yan et al., 2017). The stress response in
347 fish is regulated by the hypothalamic-pituitary-adrenal (HPI) axis, which modulates
348 cortisol levels (Yan et al., 2012; Chen et al., 2016), having both important functions in
349 behavior and development. In addition, cross-talk among the nervous, endocrine, and
350 immune systems have been previously reported in fish (Steenbergen et al., 2011). In this
351 sense, Liu et al. (2015), Zhao et al. (2015b), Su et al (2016) and Chen et al. (2018)
352 observed altered transcription of genes along the HPI axis in zebrafish, mostly of
353 gonadotropin hormone, which is also a modulator of the reproductive behavior.
354 Moreover, Chen et al. (2018) observed, for the first time, that MC-LR altered cortisol

355 levels. Thus, neurotoxicity of MCs could have an impact on endocrine disruption,
356 influencing the autonomic nervous system activity.

357 Apart from *D. rerio* and *L. delineatus,* the effects of pure MC-LR have been also
358 described in whitefish (*Coregonus lavaretus*). Thus, MC-LR induced an up-regulation
359 of the protein expression of the glial fibrillary acidic protein (*gfap*), suggesting neuronal
360 toxicity, although no changes were observed in the expression of MiR124-3p (Florczyk
361 et al., 2018). Thus, after damage to the central nervous system (CNS), astrocytes
362 normally act by reaction with a quick synthesis of *gfap*, whereas the most abundant
363 microRNA in the nervous system, MiR124, is involved in brain development and
364 neuronal regulation. These results provide new information to understand the role of
365 microRNAs in the mechanisms of MC-LR-induced neurotoxicity, and they suggest that
366 MiR124-3p cannot be considered as a biomarker of MC-LR-induced brain injury.

367 In agreement with the findings *in vitro*, Fischer et al. (2005) demonstrated, in
368 oocytes of the frog *Xenopus laevis*, that human OATP1A2, expressed in endothelial
369 cells of the BBB, mediates the transport of MC-LR into the brain. Furthermore, they do
370 not rule out that other transporters, as Oatp1c1/OATP1C1, may be also involved in this
371 function.

372 Studies conducted with lyophilized cyanobacterial cultures containing MCs are
373 scarcer compared to those performed with pure MCs. In this regard, Fischer and
374 Dietrich (2000) detected, for the first time, MC protein-adducts in the brain of carp
375 (*Cyprinus carpio*) acutely exposed to a freeze-dried culture of *M. aeruginosa* containing
376 MC-LR, although no pathological changes were observed in brain. Later, Gélinas et al.
377 (2012) studied several antioxidant parameters and AChE activity in brain after exposure
378 of juvenile rainbow trout (*Oncorhynchus mykiss*) to crude extract from *M. aeruginosa*

379 containing MC-LR (0-5 µg/L) for 96 hours. No significant changes were observed in
380 GST activity or in LPO levels, and a decrease in AChE activity only occurred at the
381 highest concentration assayed. However, an evident reduction of the protein-bound
382 phosphate at all concentrations assayed was found, which could lead to a diminishment
383 of protein phosphatase activity. Contrarily, after acute exposure to MC-LR isolated
384 from *M. aeruginosa* by dissolving the toxin in water and intraperitoneally, Kist et al.
385 (2012) demonstrated that zebrafish brain suffered an increase of AChE activity only
386 when dissolved, being relevant as its over-expression can promote apoptosis. According
387 to the authors, AChE effect in brain may be indirectly caused by the calcineurin, present
388 in the zebrafish brain. In agreement with Gélinas et al. (2012) but in discordance with
389 Kist et al. (2012), Qian et al. (2018) reported a decrease in AChE levels in larvae of the
390 same species after exposure to a *M. aeruginosa* culture containing MC-LR. This could
391 have, as a consequence, a reduction of the gene transcription of *ache*, together with a
392 concentration-dependent decline of the nicotinic acetylcholine receptor a-7 (*chrna7*)
393 transcription, being this, at least, one of the possible causes of the slowing down of the
394 swimming speed. Besides, neuronal development and differentiation effect, impaired
395 synapse formation, astroglia effect and a concentration-dependent reduction of
396 dopamine were observed; together with an effect of the dopaminergic system in the
397 zebrafish larvae. Differences in locomotion were observed in the embryos of the same
398 species exposed to *Planktothrix agardhii* containing MC-LR and MC-YR, and to *M.*
399 *aeruginosa* containing MC-LR (Jonas et al., 2015).

400 The neurotoxic effects of pure MC-RR on aquatic organisms have been far less
401 investigated in comparison to pure MC-LR, and they are somehow contradictory.
402 Although Cazenave et al. (2006) reported the brain of *Corydoras paleatus* as the most
403 affected organ after exposure to dissolved MC-RR by increases on lipid peroxidation

(LPO) levels and decreases in GST activity, they were not able to detect the toxin in brain of this species (Cazenave et al., 2005). In agreement with this study, Cazenave et al. (2008) found that exposure of *Jenynsia multidentata* to MC-RR led to oxidative stress and altered locomotor activity. The hyperactivity observed at low doses suggests an escaping from the stress of MC-RR exposure, while the reduced swimming activity together with the increased detoxification at higher doses may represent a reallocation of energy (Cazenave et al., 2008), response that was obtained as well in previous studies carried out, in this case, with MC-LR (Baganz et al., 1998, 2004). In addition, fish hyperactivity could be also a result of the alert reaction caused by the presence of MC-RR in the fish brain, showing for the first time that MC-RR, although being more hydrophilic than MC-LR, is able to cross the BBB in *J. multidentata* (Cazenave et al., 2005).

Up to date, only one study has evaluated the effect on the fish brain after exposure to MC-RR extracted from freeze-dried crude algae (Okogwu et al., 2014). *Carassius auratus* showed a reduction of the total antioxidant capacity in brain combined with a hypoxia-reoxygenation process. A decrease in the SOD and GPx levels was observed during reoxygenation, as myoglobin and neuroglobin were upregulated both during hypoxia and reoxygenation, which might help to the detoxification process of reactive nitrogen species and ROS, being of use in the fight against oxidative stress.

Generally, the effects of both pure MCs and those from cyanobacterial blooms have been shown in the central and peripheral nervous systems of several fish species, although different sensitivity was observed among them. Main observations were changes in behavior, oxidative stress parameters, genes involved in energy requirements and axonal growth, and in cholinergic and dopaminergic systems, together with

428 disruption of the GABA pathway. These, together with MC-LR accumulation in fish
429 brain and offspring, could explain the observed transgenerational changes and
430 developmental neurotoxicity of MC-LR. Compensation responses in the circadian
431 rhythm of fish have been also reported, with a generally increased activity at low doses
432 and the opposite at high doses. In any case, the neurotoxic effects of MC-RR have been
433 less investigated than those of MC-LR, in spite of being one of the most common
434 congeners. More studies are needed to clarify the ability of MC-RR to cross the BBB in
435 other aquatic species, given its differential detection in the two fish species studied.
436 Moreover, comparative studies of the neurotoxicity induced by exposure to pure MCs or
437 to cyanobacterial extracts could help to clarify MCs crossing of the BBB in aquatic
438 organisms. The potential energy reallocation in the brain of MCs-exposed organisms
439 also deserves further research, together with its effect on the endocrine system because
440 of the damage caused in the HPI axis. Furthermore, investigating the inhibition of
441 OATP-mediated MCs transport could be of interest to provide an option for
442 neurotoxicity prevention.

443 **2.3. Neurotoxicological *in vivo* studies performed with microcystins in terrestrial**
444 **animals**

445 Nowadays, several *in vivo* studies have been carried out focusing on the
446 neurotoxic potential MCs can exert in terrestrial animals (Table 4). Many of them have
447 been performed in nematodes (Li et al., 2009a, b; Ju et al., 2013, 2014; Moore et al.,
448 2014; Saul et al., 2014), mice (Shin et al., 2018; Wang et al., 2018) and rats (Li et al.,
449 2012a, b; Wang et al., 2013; Li et al., 2014; Li et al., 2015b; Zhang et al., 2018) using
450 pure MC congeners, mainly MC-LR. This is probably due to the fact that, although a
451 total of 246 variants of MCs have been described so far (Meriluoto et al., 2017), MC-

LR has demonstrated to be one of the most toxic structural variants, contributing on 46-99.8% of the total MCs in natural waters (Ufelmann et al., 2012). Considering that cyanotoxins are not found isolated in nature but together with other substances produced in cyanoblooms, very few studies have been conducted using cyanobacterial biomass cultures or their extracts for terrestrial animal exposure (Pašková et al., 2008; Wang et al., 2008; Ju et al., 2014; Zhao et al., 2015).

Approximately a third part of these studies have been performed using the nematode *Caernorhabditis elegans* as experimental model and almost under the same experimental conditions. This may be due to its short lifespan and its usage as an environmental bio-indicator, reacting to a variety of environmental stimuli (Mutwakil et al., 1997; Graves et al., 2005). Moreover, *C. elegans* only presents 302 neurons, and the complete writing diagram for chemical and electrical connections is available (White et al., 1986). It is also important to highlight that, as a liver-lacking animal, the neurotoxic effects were more obvious (Saul et al., 2014).

The first study performed in *C. elegans* exposed to pure MC-LR reported a decrease in the chemotaxis to NaCl and diacetyl and in the thermotaxis in a concentration-dependent manner, suggesting damage on the corresponding sensory neurons (Li et al., 2009a). These effects were probably caused by the disruption of ASE and AWA sensory neurons, responsible for the chemotaxis, while an impairment of sensory neurons AFD and interneuron AIY, responsible for the thermotaxis, was reported as well (Satterlee et al., 2001; Li et al., 2009a), demonstrating a genetic control of these neurons by MC-LR. According to these results, Li et al. (2009b) reported a significant decrease of lifespan and body size after exposure to the highest concentrations of MC-LR assayed, together with a decrease of the head thrash and body

476 bend after exposure to low concentrations. Moreover, effects on generation time, brood
477 size and stress parameters were also observed. Ju et al. (2013) reported that low
478 concentrations of MC-LR produced a significant decrease of body bend and head thrash
479 frequency after 8 hours of exposure while, after 24 hours, all concentrations did,
480 showing a time-dependent response. Moreover, the morphology effects caused by
481 different neurotransmitters after exposure to MC-LR were evaluated and, although no
482 structural alterations were observed in the cholinergic, serotonergic, dopaminergic and
483 glutamatergic systems, a GABAergic neuronal loss and aberrant neuronal morphology
484 were observed after exposure to the highest concentration of MC-LR. Furthermore, this
485 study revealed that MC-LR induced 1) adverse effects on the transportation and location
486 of GABA altering unc-47, unc-46, and unc-30 gene expression and 2) alteration of both
487 the inhibitory and excitatory GABA receptors decreasing unc-49 and exp-1 expression
488 levels. This effect on GABA could lead to the effects previously observed in the
489 locomotor behavior. In agreement with these results, Ju et al. (2014) reported a
490 significant decrease of different autonomic functions, such as body bend and touch
491 response, move length, pharyngeal pumping frequency and defecation period interval
492 (only after 24 hours of exposure to the highest concentration of MC-LR). These authors
493 demonstrated, exposing to a filtrate of *M. aeruginosa* culture containing MCs, that the
494 response opposed to the one obtained with pure MC-LR, observing an increase in
495 locomotive behavior and pumping activity and no alteration of sensory functions. These
496 differences could be due to 1) the higher concentration present in the biomass compared
497 to pure MC-LR used (300 vs 100 μg/L), 2) the presence of other active substances, and
498 3) the presence of several MC congeners, such as MC-RR and MC-YR. In addition,
499 Moore et al. (2014), demonstrated alteration to diacetyl after exposure to MC-LR,
500 showing an alteration of the function of the AWA sensory neuron. However, the effects

501 on the chemotaxis to benzaldehyde after exposure to MC-LR, regulated by AWC
502 neurons, was not observed, highlighting the fact that AWC and AWA neurons act as
503 independent targets. Moreover, these effects were compared to the ones caused by the
504 exposure to MC-LF, suggesting a more potent effect by MC-LF than MC-LR. Up to
505 date, only this neurotoxicity study has been carried out with this congener in nematodes,
506 despite MC-LF is transported more efficiently into the neurons (Feurstein et al., 2010).
507 Furthermore, Saul et al. (2014) obtained a significant decrease in all life trait variables,
508 measured at different periods of the nematode life cycle, only at the highest
509 concentration of MC-LR assayed. They investigated widely the variation in the gene
510 expression, reporting an enhancement of 125, among which was unc-30, related to the
511 GABAergic response, and a decrease of 76. Although these results may seem
512 contradictory to the ones obtained by Ju et al. (2013), as they described a diminish of
513 unc-30 gene expression, it is important to highlight that the duration of the stress
514 exposure is essential for their regulation, being the possible cause for their discordance
515 (Nadal et al., 2011). Moreover, Saul et al. (2014) also reported a down-regulation in *let-*
516 *7* expression, which could play a role in the development and the reproductive
517 processes, contributing, therefore, to the effects observed in the brood size and growth.
518 Their results manifested that many of the affected genes by MC-LR are involved in
519 neurogenesis, signaling or neurological behavior processes, reinforcing those results
520 previously obtained by Li et al. (2009a) and Ju et al. (2013), where MC-LR played an
521 important role in the neuromodulating action.

522 In general, the different behavioral studies agree that MC-LR produced a
523 decrease in autonomic (body bend, head thrash, move length, pharyngeal pumping,
524 touch response) and sensory (chemical, thermal) functions reflecting an alteration in the
525 nervous system functions to generate appropriate behaviors from sensory signals in

nematodes (Li et al., 2009a;b; Ju et al., 2013, 2014; Moore et al., 2014). Therefore, MC-LR at environmentally relevant concentrations, could affect the nervous system regulation to receive, process, integrate and interpret sensory signals, as suggested by the gene expression results (Li et al., 2009a;b; Ju et al., 2013; Saul et al., 2014; Hu et al., 2016). It is important to point out that not always a variation in the gene expression can be translated to a change in protein levels, being required complementary studies in order to assure the neurotoxic role of this toxin (Saul et al., 2014). Although previous studies confirmed the suitability of the *C. elegans* test as a neurotoxicity screening test for MCs (Ju et al., 2014), it should be taken into account that this experimental model is much simpler than the mammals-nervous system.

The only neurotoxicity study performed in birds was carried out in Japanese quail exposed to *Microcystis* biomass containing MC-LR, MC-RR, MC-YR and MCs-similar compounds (Pašková et al., 2008). This study focused on the determination of oxidative stress, where a significant enhancement was reported in cytochrome P-450-dependent 7-ethoxyresorufin O-deethylase (EROD) levels in the brain after acute and sub-chronic exposure at medium concentrations of MCs. The LPO levels were also enhanced after acute and sub-chronic exposure, so did the GSH levels, decreasing, nonetheless, after acute exposure. Howbeit, no significant changes were observed in GST activity in this organ. In general, a rise in the oxidative stress parameters was described by these authors in brain (Pašková et al., 2008). Oxidative stress as a mechanism of toxic action of MCs has been widely studied in other organs such as liver or kidney in different species (Li et al., 2003; Jos et al., 2005; Skocovska et al., 2007; Weng et al., 2007; Prieto et al., 2009); however, these investigations are very scarce in brain. The increase of ROS could be involved in the mitochondrial dysfunction and

activation of calpain and $Ca^{2+/}$ calmodulin-dependent protein kinase II (Ding and Nam Ong 2003), generating damage in the brain structure and neurological functions.

Mice exposed to pure MC-LR showed differences in the effects on hippocampus and cortex after oral exposure by drinking water with 1-40 µg/L MC-LR for a year (Wang et al., 2018). Histopathological changes were observed in the hippocampus (bright eosinophil-like angular shape and nuclear fragments) and in the cortex (shrunken bodies and pyknotic nuclei) dose-dependently. Likewise, MC-LR produced different impacts on mRNA transcription genes and in their protein expression (ATP6, COX3, CYTB, DNA polymerase ɣ (POLG), mitochondrial single-stranded DNA-binding protein (mtSSB) and mitochondrial transcription factor A (TFAM)), mainly affecting the hippocampus. In accordance with these results, Shin et al. (2018) described a dose-dependent neuronal loss in the same hippocampal cells due to several morphological changes, but in this case, after exposure to a cyanobacterial extract containing MC-LR. Moreover, several behavioral studies demonstrated memory impairment after Morris water maze (MWM) and passive avoidance tests. However, these effects were only observed after exposure to 4 µg/mL MC-LR, suggesting that the neuronal loss is not the main cause for these toxic effects in mice. After exposure to the same doses, no effects on spatial working and visual recognition memory were detected by Y-maze and novel object recognition tests, respectively. These effects were patent only in non-transgenic (non-Tg) mice compared to those overexpressing glutathione peroxidase (GPx Tg). Besides, in non-Tg group, these authors observed significant changes in oxidative stress biomarkers such as increased protein oxidation, LPO and ROS, together with a decrease of the GSH/Glutathione disulfide (GSSG) ratio. Moreover, the rise in SOD enzyme activity was more evident in non-Tg compared to the increase observed in GPx-1 Tg, while the enhancement of GPx enzyme activity was more visible in this last group of

mice. Furthermore, no proinflammatory tumor necrosis factor-α (TNFα) and allograft inflammatory factor-1 (Iba1) levels were affected after the MC-LR exposure. All of the results obtained in this study suggest that memory impairments in mice exposed were due to oxidative stress in spite of by neuroinflammation process, which could be confirmed by the enhancement of nuclear factor erythroid-derived 2 (Nrf2) observed in both exposed groups. In addition, the reduced responses of GPx-1 Tg compared to non-Tg mice suggest a possible prevention of memory impairment by compounds implied in antioxidant activity (Shin et al., 2018).

Furthermore, it is important to highlight that, although not being neurotoxicological studies *per sé*, some studies exposing mice to pure MC-LR have proven its capacity to cause effects in the HPI axis at hypothalamic level, altering the neurohormonal control of reproduction (Wang et al., 2012; Xiong et al., 2014; Chen et al., 2016).

In rats exposed to MC-LR after infusion into hippocampus presented longer periods of time searching the platform and shorter swimming distance in the target zone, but no significant differences in swimming speed were appreciated compared to the control group (Li et al. 2012a). This would point out the spatial learning and memory impairment caused by the exposure to the toxin. Furthermore, some neuronal injury was observed by shrunk nuclei and cellular edema or dissolved cell organelles, diminishing significantly the number of CA1 pyramidal cells in the hippocampus. Nonetheless, after exposure to the lowest dose, some morphological changes were appreciated in the neurons, like swollen and degranulated endoplasmic reticulum or puffed periplast. In fact, a more significant rise in oxidative stress parameters was reported after exposure to the highest concentration (LPO, CAT, GPx, and SOD) versus

599 the lowest (LPO and CAT). In agreement with these results, Li et al. (2012b) reported
600 that chronic exposure produced neither changes in intake, body weight and overall
601 mobility, nor visual and locomotor deficits, although they demonstrated the presence of
602 this toxin in brain. However, treated rats did take longer to find the platform, mainly
603 over the late days, spending less time in the target zone, which implies effects on spatial
604 learning and memory as well. This impairment was also confirmed by the degeneration
605 and apoptosis of hippocampal cells in rats exposed to MC-LR for 50 days. Furthermore,
606 the authors observed the presence of proteins involved in neurodegenerative diseases
607 such as septin 5, a-internexin and a-synuclein, and a PPs inhibition after exposure to 10
608 μg/kg MC-LR, which may lead to Tau hyperphosphorylation, implied in the generation
609 of Alzheimer's disease. This is the first scientific study correlating MC-LR exposure to
610 an age-associated neurodegenerative disorder.

611 Additionally, Wang et al. (2013) reported a significant PPs activity enhancement
612 after exposure to pure MC-LR in rats, in disagreement with the results obtained by Li et
613 al. (2012b). This effect could be the cause for the reduction, at all concentrations, of the
614 phosphorylation of GSK-3β in the hippocampus and, consequently, for the described
615 long-term potential concentration-dependent effects, leading to a loss of neuronal
616 plasticity. Moreover, this study showed, for the first time, the prevention of the
617 neurotoxic effects caused by MC-LR by simultaneous treatment with a GSK-3β
618 inhibitor.

619 In agreement with Li et al. (2012a, b), an investigation of the effects of MC-LR
620 on learning and memory ability in rats was performed by Li et al. (2014), obtaining that
621 the rats exposed to the highest dose presented prolonged escape latencies on the third
622 day of training, while those exposed to lower doses had shorter frequencies entering the

623 enlarged platform. Despite no significant differences in the number of damaged neurons
624 were observed, an increase of astrocyte cells density in the hippocampus was reported
625 after the exposure to the highest dose. This could be related to the increase of nitrogen
626 reactive species, an inflammatory indicator, reported in the hippocampus at the same
627 dose, playing a role in the central neuron system inflammatory reactions and affecting
628 spatial memory impairment.

629 The only study evaluating the transmission of the toxic effects of MC-LR in
630 female rats to offspring was performed by Li et al. (2015b). In maternal rats, a decrease
631 in the mean body weight gain was significant only at the highest dose of exposure.
632 Respecting the behavior of the offspring, a significant reduction of the ability in the cliff
633 avoidance test was observed, although no differences were perceived after the surface
634 righting reflex and the negative geotaxis tests. However, no significant alterations in the
635 locomotor activity were observed. In the MWM test, the frequencies in reaching the
636 platform zone decreased dose-dependently in male offspring at all exposure doses,
637 while in the case of female offspring, the diminishment of frequency was produced only
638 after exposure to the highest doses, together with the effects on the swimming speed.
639 Furthermore, although no evident pathological alterations in the hippocampus were
640 observed, a significant increase of LPO and SOD levels were reported in male and
641 female offspring after exposure to the highest dose, and an increase of LPO levels after
642 5 μg/kg MC-LR exposures only in male subjects.

643 Recently, Zhang et al. (2018) indicated an accumulation of MC-LR in the
644 hippocampus after 24 hours of injection, causing demethylation of PP2Ac (inhibition of
645 PP2Ac) and phosphorylation of GSK-3β (activation of GSK-3β). This could lead to the
646 hyperphosphorylation of Tau, being in agreement with Li et al. (2012b) and Wang et al.

(2013). These results confirm the effects obtained, as mentioned above, in the SH-SY5Y *in vitro* model in the same study (Zhang et al., 2018). Moreover, going along with the results obtained in the MWM test by Li et al. (2012a, b, 2014, 2015b), a reduction of the swimming distance spent in the target zone was observed as well, in this case, after day 8 compared to day 6, producing, consequently, memory impairments.

Although they represent a more realistic scenario, only two neurotoxicity studies have been carried out with MCs contained in extracts of cyanoblooms. In this sense, Maidana et al. (2006) used the step-down inhibitory avoidance test by injection of MC-LR containing raw extract. They reported a significant effect on long-term memory and the impairment of its retrieval at both doses assayed, while no significant changes were produced in short-term memory at any dose. Furthermore, using the radial arm maze to test the spatial memory, the number of working and reference memory errors increased only at day 8 of exposure at both concentrations, being probably caused by the accumulation of previous extracts-administrations. Surprisingly, an increase of the time spent to consume all the baits was reported in the same test only at the lowest dose. Moreover, these authors also studied different oxidative stress parameters, obtaining higher GST activity after exposure to the lowest dose compared to the highest. In the case of LPO levels, higher levels were obtained after exposure to the highest MCs dose, although the lower dose also caused lipid peroxidation. These parameters could be the cause for the increase of DNA damage observed after exposure to MCs in the comet assay, corroborating the role of oxidative stress in the neurotoxic effects produced by MC-containing extracts, as was previously demonstrated with pure MCs (Li et al., 2012a, 2015b). In agreement with these oxidative stress results, Zhao et al. (2015a) obtained an increase in the LPO levels in the brain of the pups after maternal exposure

to MCs-extract, together with a decrease in the GSH levels and in AChE activity in the cerebral cortex. Moreover, although these authors verified the presence of MC-LR in the offspring brains, no changes were obtained in the PP activity after maternal exposure, which would be in disagreement with the PP activity enhancement reported by Wang et al. (2013) and its decrease reported by Li et al. (2012b) and Zhang et al. (2018). This could be due to the experimental subjects since in both cases the parameter was measured in a direct object, the adult rats, versus an indirect object, their pups; or the discordance in the administration route, being, in this case, subcutaneous. Furthermore, similar ultrastructural changes were obtained in brain offspring by Li et al. (2012a). Likewise, an alteration of proteins involved in neurodevelopment was detected as well, in agreement with Li et al. (2012b).

Taken together, all the experiments conclude that MCs both pure and contained in cyanoblooms extracts produced important neurotoxic effects in several species by different exposure routes. Mostly, MCs caused oxidative stress and alteration of biochemical chains that ended up leading to huge effects such as hyperphosphorylation of Tau. In fact, most of them demonstrate the spatial learning and memory impairment by several behavioral tests. Howbeit, very few studies have been performed using other MC congeners besides MC-LR, isolated and contained in the mixture in a cyanobacterial extract, although some of them have demonstrated to exert more severe neurotoxic effects, being the case of MC-LF for instance.

3. Cylindrospermopsin

Cylindrospermopsin consists of a tricyclic guanidine group combined with a hydroxylmethyl uracil group (Ohtani et al., 1992). Its structure presents a zwitterionic nature and a low molecular weight (415 Da) (Falconer and Humpage, 2006). This

cyanotoxin is produced by several cyanobacterial genera such as *Cylindrospermopsis*, *Aphanizomenon*, *Umezakia*, *Chrysosporum*, and *Anabaena*, among others (Harada et al., 1994; Banker et al., 1997; Shaw et al., 1999; Schembri et al., 2001) (Fig. C).

Despite being the liver its main target, many other organs such as kidneys, lungs, thymus, marrow bone, adrenal gland, gastrointestinal tract, immune and nervous systems, and heart have been described as potential targets as well (Hawkins et al., 1985; Terao et al., 1994; Falconer et al., 1999; Humpage et al., 2000; Guzmán-Guillén et al., 2015). The most well-known mechanism of action for CYN is the protein and GSH synthesis-inhibition (Terao et al., 1994; Runnegar et al., 1995; Froscio et al., 2003). In addition, due to its ability to enhance ROS production, this toxin can lead to DNA damage, causing cell death by apoptosis (Roos and Kaina, 2006; Gutiérrez-Praena et al., 2011; Puerto et al., 2011; Gutiérrez-Praena et al., 2012; Guzmán-Guillén et al., 2013). Moreover, some studies have demonstrated the importance of its previous metabolic activation by the enzymatic complex cytochrome P-450, since it is able to exert genotoxic potential (Runnegar et al., 1995; Norris et al., 2002; Froscio et al., 2003; Humpage et al., 2005; Zegura et al., 2011; Puerto et al., 2018). As a cytotoxin, these effects could be also caused in the nervous system. Besides, it is important to notice that the chemical structure of CYN is more alike to neurotoxins than to hepatotoxins, as it was classified at first, not being unexpected for this cyanotoxin to also cause neurological disorders (Kiss et al., 2002). Furthermore, although it is not likely for CYN to cross the BBB by passive diffusion due to its hydrophilic properties (Banks et al., 2009), its low molecular weight might play a role in its entrance to the nervous system. There are some studies pointing out its neurotoxicity in different *in vitro* and *in vivo* models, although the mechanisms for which CYN could exert neurotoxic effects in the brain remain unknown.

3.1. Neurotoxicological *in vitro* studies performed with cylindrospermopsin

Up to date, in comparison with MCs, very few studies have brought to light the potential neurotoxicity CYN can exert (Table 5). Furthermore, most of them have been performed using extracts or cultures of *Cylindrospermopsis raciborskii* or *Aphanizomenon ovalisporum*. In this sense, the first study suggesting its neurotoxic effect was performed by Kiss et al. (2002), who exposed CNS neurons of two species of snail, *Helix pomatia* L. and *Lymnaea stagnalis* L., to a *C. raciborskii* purified fraction and ATX-a. They suggested that the purified fraction could be CYN, and although it had no direct effect on the membrane of the neurons, it decreased the ACh-induced membrane response, suggesting a neuroactive effect on the cell membrane for the first time. On the contrary, Vehovszky et al. (2013) reported that application of a CYN-producing strain to CNS preparations of *H. pomatia* (at 20 mg/mL) did not display the same cholinergic inhibitory effects, although these were observed after exposure to a non-CYN-producing *C. raciborskii* bloom, which authors attribute to some ATX-a like compound.

In the case of CYN, contrary to MCs, there is only one work with pure toxin, performed by Takser et al. (2016). These authors evaluated *in vitro* the individual and combined effects of CYN, MC-LR and ATX-a, at environmentally relevant low concentrations (10 µM alone and 3.3 µM in mixture), in brain cell lines. Their findings revealed that CYN individually and the mixture containing CYN were 3-15 times more potent than the individual toxins, inducing apoptosis and inflammation in murine BV-2 microglia cells and N2a murine neuroblasts cells. Besides, the latest were more sensitive to the mixture than BV-2 cells, causing a meaningful pro-inflammatory response to CYN and the mixture, demonstrating that low concentrations of CYN are

745 highly relevant for neurodegeneration. These outcomes could have potential
746 implications in future research on neurodegenerative diseases. Nevertheless, care should
747 be taken in the extrapolation of these *in vitro* results to *in vivo* circumstances, including
748 human health effects, mainly concerning the developing brain where there is no BBB
749 yet.

750 **3.2. Neurotoxicological *in vivo* studies performed with cylindrospermopsin**

751 Studies concerning CYN neurotoxicity *in vivo* are scarce (Table 5), although
752 they provide interesting results. In this regard, White et al. (2007) reported that 7 day-
753 exposure of *Bufo marinus* tadpoles to whole cell extracts or live cultures of
754 *C. raciborskii* at 400 or 232 µg/L, respectively, appeared to decrease their activity
755 levels, mostly swimming behavior, which could make them more vulnerable to prey,
756 but also be used as an avoidance strategy from visually-oriented hunters. This effect,
757 however, might have been caused by damage in some other organs. It is worth to
758 mention that live *C. raciborskii* cultures contained a mixture of intra- and extracellular
759 CYN, whereas the cell extracts only had extracellular CYN, and they also reported the
760 presence of deoxy-CYN. This work by White et al. (2007) was the first one using
761 amphibians as experimental model, whose changes in behavior gain relevance as they
762 are usually the first indication of sublethal exposure (Henry, 2000), being a possible
763 indicator of CYN neurotoxicity. In agreement with these results, Kinnear et al. (2007),
764 using the same model and conditions, but nearly half the concentrations (200 and 107
765 µg/L, for the cell extracts and the live cultures, respectively), reported a reduction in the
766 swimming ability and un-coordination in tadpoles of *B. marinus.* They suggested that it
767 could be due to the disintegration of the brain, as the encephalon had a loosely arranged
768 matrix and brain cells were disintegrated and sometimes necrotic, showing a mix of the

769 outer matrix and inner cells, together with general organ failure. Besides, authors also
770 hypothesized that degeneration of the gill epithelia could have led to suffocation, and
771 finally to the consequently reduced activity.

772 To our knowledge, there are only two studies concerning the neurotoxicity of
773 CYN in fish. CYN was detected by ELISA in the brain of all tilapia fish (*Oreochromis*
774 *niloticus*) exposed subchronically (14 days) by immersion to repeated concentrations
775 (10 µg/L) of an *A. ovalisporum* culture containing CYN and deoxy-CYN (Guzmán-
776 Guillén et al., 2015). As a result, a marked increase in LPO levels, and a reduction in
777 AChE activity in tilapia brains was observed, although the inhibition of AChE activity
778 was too low to induce neurological symptoms. In addition, signs of necrosis,
779 vacuolization, chromatin condensation, cytoplasmic edema and mitochondrial swelling
780 were reported as well. Recently, detection of CYN has also been reported in brains of
781 the fish *Hoplias malabaricus* exposed by a single i.p. injection (50 µg/kg b.w.) to
782 purified CYN or to extracts of a CYN-producing strain of *C. raciborskii*, even 7 and 14
783 days after exposure (da Silva et al., 2018). In addition, detected CYN levels were higher
784 after exposure to the extracts, which could point out the importance of other compounds
785 in the extract (i.e. lipopolysaccharides) that might also affect CYN crossing of the BBB.
786 Nonetheless, no significant effects were noticed on AChE activity after CYN exposure
787 in any form tested, contrary to the results obtained by Guzmán-Guillén et al. (2015),
788 which could be due to differences in the exposure concentrations and times (subchronic
789 versus acute exposure) or in the fish species, although both studies agree on the rise of
790 LPO levels. Moreover, GSH levels did not vary in *H. malabaricus* after exposure to
791 CYN, but different responses were obtained for GST activity for extracts and pure
792 CYN. To exert neurotoxic effects, toxins must be transported into cells or interact with

channels or receptors of the cell membrane (Stillwell, 2013), suggesting the interference of other compounds present in the extract (da Silva et al., 2018).

Some neurological symptoms after exposure of alligators (Schoeb et al., 2002) and mice (Saker et al., 2003, Zagatto et al., 2012) to *C. raciborskii* strains have been attributed to CYN (Poniedzialek et al., 2012). However, it is important to clarify that neither of these studies proved the presence of CYN in those strains, so the reported effects might be due to different compounds present in the extracts or different secondary metabolites, such as STX.

4. Conclusions

This review summarizes, as far as we know, the reports available on the scientific literature dealing with the neurotoxicity assays performed *in vitro* and *in vivo* to elucidate the toxic effects that MCs and CYN can exert in the nervous system. In the case of MCs, they have proven to cause neurotoxicity by their crossing using the OATPs, which are present in the BBB and in most neural cells, leading to a rise in the $[Ca^{2+}]_i$ levels and, therefore, apoptosis. These cyanotoxins have demonstrated to exert neurotoxic effects mostly in the limbic system. In fact, some histopathological studies have described important damages in the hippocampus and in the cortex, together with global biochemical alterations, being especially relevant Tau hyperphosphorylation, characteristic of some neurodegenerative diseases such as Alzheimer's disease. On the other hand, these toxins have proven to cause damage in the hypothalamus as well, having an impact in other systems of the organism such as the reproductive or the endocrine. Furthermore, MCs have exerted a rise in oxidative stress and lipid peroxidation, together with neurotransmission alterations (DA, ACh and GABA levels), leading to autonomic and sensory responses. Thus, MCs not only cause effects in the

CNS but also in the peripheral nervous system. Furthermore, some other minor variants such as MC-LF or MC-LW require attention as well, since both have demonstrated to be even more toxic in neural cells, in spite of being less environmentally abundant. Special attention should be paid to the fact that very little studies have been carried out *in vivo* using one of the major congeners in nature, MC-RR. In the case of CYN, the number of studies performed is even scarcer, reporting deregulation of some oxidative stress parameters was observed together with alteration of AChE activity, which could be linked to the histological changes observed. Thus, although neurotoxicity mechanisms for CYN are still unknown, it seems to be caused by damage in the CNS. For all mentioned above, further research is required in order to clarify the neurotoxic potential of several MC congeners and CYN, as well as their possible contribution in neurodegenerative diseases.

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Table 1.

Toxin	Chemical structure	Molecular properties				Environmental concentrations	
		Molecular weight $[M+H]^+$	**Molecular composition**	**Kow**	**BCF Plants**	**In surface waters (µg/L)**	**In mollusks and fish samples (ng/g d.w)**
MC-LR	Cyclo(-D-Ala-L-**Leu**-D-erythro-β-methylAsp(iso-linkage)-L-**Arg**-Adda-D-Glu(iso-linkage)-*N*-methyldehydro-Ala	995.5561	$C_{49}H_{75}N_{10}O_{12}$	2.16 (Ward and Codd, 1999)	Up to 680.05±40.88 (Romero-Oliva et al., 2014)	Up to 2100 (Faasen and Lurling 2013)	Up to 130 in fish muscle (Roy-Lachapelle et al., 2015)
MC-LF	Cyclo(-D-Ala-L- **Leu** -D-erythro-β-methylAsp(iso-linkage)-L-**Fe**-Adda-D-Glu(iso-linkage)-*N*-methyldehydro-Ala	986.5234	$C_{52}H_{72}N_7O_{12}$	3.56 (Ward and Codd, 1999)	nf	Up to 51 (Graham et al., 2010)	Up to 300 in common carp (Gurbuz et al., 2016)
MC-LW	Cyclo(-D-Ala-L- **Leu** -D-erythro-β-methylAsp(iso-linkage)-L-**Trp**-Adda-D-Glu(iso-linkage)-*N*-methyldehydro-Ala	1025.5343	$C_{54}H_{73}N_8O_{12}$	3.46 (Ward and Codd, 1999)	nf	Up to 260 (Faasen and Lurling 2013)	Up to 15.5 in bivalves (Preece et al., 2015)
MC-RR	Cyclo(-D-Ala-L- **Arg** -D-erythro-β-methylAsp(iso-linkage)-L- **Arg** -Adda-D-Glu(iso-linkage)-*N*-methyldehydro-Ala	1038.5731	$C_{49}H_{76}N_{13}O_{12}$	1.54 (Liang et al., 2011)	Up to 54.09±17.01 (Romero-Oliva et al., 2014)	Up to 16000 (Graham et al., 2010)	Up to 463000 in silver carp (Xie et al., 2007)
MC-YR	Cyclo(-D-Ala-L-**Tir**-D-erythro-β-methylAsp(iso-linkage)-L- **Arg** -Adda-D-Glu(iso-linkage)-*N*-methyldehydro-Ala	1045.5353	$C_{52}H_{73}N_{10}O_{13}$	nf	nf	Up to 343 (Simiyu et al., 2018)	Up to 20000 in bivalves (Kim et al., 2017)
CYN	2,4(1*H*,3*H*)-Pyrimidinedione, 6-[(*R*)-hydroxy[2a*R*,3*S*,4*R*,5a*R*,7*S*)-2,2a,3,4,5,5a,6,7-octahydro-3-methyl-4-(sulfooxy)-1*H*-1,8,8b-triazaacenaphthylen-7-yl]methyl]-, rel-(-)- (9CI)	416.1234	$C_{15}H_{21}N_5O_7S$	Highly water-soluble	Up to 3.88±0.33 (Cordeiro-Araújo et al., 2017)	Up to 800 (Shaw et al., 2000)	Up to 200 in crayfish (Saker ans Eaglesham, 1999)

Abbreviations: BCF: bioconcentration factor; d.w: dry weight; Kow : octanol/water partition coefficients; nf: not found;

Table 2.

Toxin	Experimental model	Experimental conditions	Assays Performed	Relevant results	LC_{50}	References
Pure MC-LR Pure MC-LW Pure MC-LF	Primary murine WBC	0, 0.2, 0.4, 0.6, 0.8, 1, 3 and 5 µM for 48 hours	MTT assay PPI assay	At 5 µM, complete loss of cell viability by MC-LF, decrease of cell viability by MC-LR and MC-LW (54% and 33%, respectively). Decrease of cell viability after exposure to -LF, -LW and -LR to ≥ 200 nM, ≥ 400 nM and ≥ 600 nM, respectively.	>10 µM 3 µM aprox 3 µM aprox	Feurstein et al. (2009)
Pure MC-LR Pure MC-LW Pure MC-LF	Primary murine neurons	0, 0.31, 0.63, 1.25, 2.5, and 5 µM for 48 hours	PPI assay	20% inhibition of PP activity at low MCs concentrations. Decrease of activity at 2.5 µM by 25% (-LR), 30% (-LW), and 60% (-LF). Decrease of PP activity by 65% at 5 µM -LF.	-	Feurstein et al. (2010)
Pure MC-LR Pure MC-LW Pure MC-LF	Primary murine CGNs	0, 0.2, 0.4, 0.5, 0.6, 0.8, 1, 3, 5 and 10 µM for 48 hours	MTT assay Apoptosis Morphology PPI assay Tau phosphorylation	At 5 µM, decrease of cell viability by -LR (to 70%), -LW (to 50%) and -LF (to 8%). 3-5 µM -LF caused the highest level of apoptosis. Apoptotic nuclei at 5µM -LW while -LR did not induced them at any concentration assayed. Enhance of caspase-3/7 activity for -LF and -LW, and no changes for -LR. -LF caused a complete disintegration of the neurite network, whereas -LR induced a slight impairment. No statistical differences in PPI of -LR, while -LF induced a concentration-dependent inhibition from 2.5 µM. Tau phosphorylation fast and potent for -LF, being less evident for -LW, and with a low constant signal for -LR.	>10 µM 5 µM 1.5 µM aprox	Feurstein et al. (2011)
Pure MC-LR	Differentiated PC12 cells	0, 0.1, 0.5, 1, 5 and 10 µM for 6 hours	PPI assay Tau phosphorylation p38-MAPK activation Morphology	MC-LR caused a concentration-dependent significant inhibition of PP2A by 27.4% at low concentrations, by 36.5% at 5 µM and by 60.5% at 10 µM, leading to Tau hyperphosphorylation. Drastic enhance of p38-MAPK phosphorylation with 10 µM MC-LR. Loss of the regular filamentous distribution and decrease of tubulin and actin fibers in the cytosol, enhancing in the periphery.	-	Meng et al. (2011)
Pure MC-LR	Differentiated PC12 cells	0, 1, 2.5, 5, 7.5 and 10 µM for	ROS Tau phosphorylation	MC-LR induced a concentration- and time-dependent alteration of intracellular ROS until 6 hours of exposure, recovering to the	-	Meng et al. (2013)

		24 hours	p38-MAPK activation	baseline at 18 hours. A Tau phosphorylation was observed from 1 hour of exposure, reaching the highest effect at 3 hours, and gradually decreasing to basal levels. Enhance of p38-MAPK activation from 1 to 24 hours of exposure.		
Pure MC-LR	Primary hippocampal neurons	0, 0.1, 0.3, 1, 3, 10 and 30 µM for 24 hours	MTT assay Calcium mobilization	Decrease of cell viability by MC-LR in a concentration-dependent way. Enhance of apoptotic and necrotic neurons number with 1 µM MC-LR. The toxin induced a concentration-dependent intracellular calcium mobilization.	10 µM aprox	Cai et al. (2015)
Pure MC-LR	Primary hippocampal neurons	0, 0.3 and 3 µM for 48 hours	Proteome analysis CaN activity MTT assay LDH release	Alteration of 45 proteins implied in calcium-ion signal transduction, apoptosis, oxidative stress response, and cytoskeleton structure. Enhance of CaN levels. Decrease of cell viability at the highest MC-LR concentration assayed. Enhance of LDH release with the increment of the concentration.	-	Li et al. (2015a)
Pure MC-LR	BV-2 cells N2a cells	0, 0.1 and 10 µM for 24, 48 and 72 hours	MTT assay	BV-2 cells exposed to MC-LR never reached LD_{50} levels at any of the exposure times, but significant decrease of viability after 24 hours at both MC-LR concentrations, and only at 10 µM after 48 and 72 hours. Decrease of cell viability after exposure to both concentrations assayed after 24, 48 and 72 hours in N2a cells.	>10 µM 10 µM aprox	Takser et al. (2016)
Pure MC-LR	GT1-7 cells	0, 0.01, 0.05, 0.1, 0.5 and 1 µM for 48 hours	Toxin uptake CCK-8 test	Uptake of MC-LR into cells was confirmed by western-blot, since it covalently bound to the PP1 and PP2A catalytic subunits. Decrease of cell viability in a concentration-dependent way. No affectation when deprived from the Oatp1a5 transporter.	-	Ding et al. (2017)
Pure MC-LR Pure MC-LW Pure MC-LF	Primary rat astrocytes	0, 0.5, 2 and 10 µM for 24 hours	MTT assay Apoptosis Immunocytochemistry Morphology	Intracellular localization of MCs using immunocytochemistry. Cytoskeletal disruption, decrease of cell viability and enhance of number of apoptotic cells after MC-LW and MC–LF exposure. MC-LR did not cause any of the alterations above.	-	Rozman et al. (2017)
Pure MC-LR	SH-SY5Y cells	0, 5 and 10 µM for 24 hours	Toxin uptake Tau phosphorylation	Uptake of MC-LR into cells confirmed by western-blot, using PP1 and PP2A catalytic subunits-antibodies.	-	Zhang et al. (2018)

			PPI assay LDH release	Enhance of Tau phosphorylation the concentration of accumulated MC-LR. The PP2A activity was inhibited in a concentration-dependent way. The highest MC-LR concentration caused cell dead, related to Tau phosphorylation.		

Abbreviations: BV-2: cellosaurus cell line; CaN: calcineurin; CCK-8: cell counting kit-8 test; CGNs: cerebellar granule neurons; GT1-7: hypothalamic neuronal mouse cells 1-7); LDH: lactate dehydrogenase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N2a: fast-growing mouse neuroblastoma cells; Oatp: organic-anion-transporting-polypeptide; p38-MAPK: P38 mitogen-activated protein kinases; PC12: pheochromocytoma of rat adrenal medulla; PP: protein phosphatase; PPI assay: protein phosphatase inhibition assay; ROS: reactive oxygen species; SH-SY5Y: *Homo sapiens* bone marrow neuroblast; WBC: whole brain cells.

Table 3.

Microcystin congener/Cyanobacteria	Experimental model	Experimental conditions	Assays performed	Relevant Results	References
Aquatic animals					
Pure MC-LR	Zebrafish *(Danio rerio)*	0.5, 5 or 15 µg/L for 25 days and 50 µg/L for 6 days, by oral and transdermal route	Behavioral study	The motility showed a dose-effect relationship and changes in the circadian rhythm	Baganz et al. (1998)
Pure MC-LR	Zebrafish *(Danio rerio)* Sunbleak *(Leucaspius delineatus)*	0.5, 5 or 15 µg/L for 17 days and 50 µg/L for 6 days, by oral and transdermal route	Behavioral study	Lower concentrations increased motility, whereas the highest one decreased the activity of both species. *D. rerio* was less sensitive. Despite *D. rerio* remained diurnally active, the swimming activity of *L. delineatus* was altered, reversing diurnal and nocturnal activity	Baganz et al. (2004)
Pure MC-RR	Peppered catfish *(Corydoras paleatus)*	0.5, 2, 5 or 10 µg/L for 24 hours, by oral and transdermal route	Oxidative stress parameters (GR, POD, GPx, CAT and LPO) and detoxification system (GST activity)	Increased LPO levels in brain of exposed fish, and a general activation of the antioxidant enzymatic system	Cazenave et al. (2006)
Pure MC-RR	Onesided livebearer *(Jenynsia multidentata)*	0.01, 0.1 or 1 µg/g for 24 hours, by oral route	Swimming activity and detoxification system (GST activity)	Low doses increased swimming activity, while the highest dose headed to small reduction after 20 hours	Cazenave et al. (2008)
Pure MC-LR	Zebrafish *(Danio rerio)*	2 or 20 µg/L for 30 days, by oral and transdermal route	Protein expression	Oxidative stress, dysfunction of cytoskeleton assembly and macromolecule metabolism, and interference with signal transduction and other functions in brain. The PP activity rose with MC-LR concentration	Wang et al. (2010)
Pure MC-LR	Zebrafish	0.2, 0.5, 2 and 5 mg/L at 96	Protein and gene	Upregulation of CKs and DRP2	Li et al.

	(Danio rerio)	hpf, by oral and transdermal route	expression		(2011b)
M. aeruginosa containing MC-LR	Rainbow trout (*Oncorhynchus mykiss*)	0.75, 1.8 and 5µg/L for 96 hours, by oral and transdermal route	Oxidative stress parameters (GST activity, LPO levels), PBP and AChE activity	Neither GSH activity nor LPO altered. Lower levels of PBP and AChE	Gélinas et al. (2012)
M. aeruginosa containing MC-LR	Zebrafish *(Danio rerio)*	50 or 100 µg/L for 24 hours, by branchial and oral route	AChE activity and protein and gene expression of whole brain	Enhancement of the AChE activity depending on the exposure route	Kist et al. (2012)
Pure MC-LR and MC-RR	Zebrafish *(Danio rerio)*	0.1, 0.5, 1, 5 or 10 µg/L for 4, 7 and 15 days, by oral and transdermal route	Antioxidant enzymatic activities (GST, GPx, GR and SOD)	A bell shaped curve of response for most of the parameters	Pavagadhi et al. (2012)
Crude algae containing MC-RR	Goldfish *(Carassius auratus)*	0, 50 or 200 µg/kg b.w., tested at 6, 12, 24 and 48 hours, by intraperitoneal injection	Glucose levels and antioxidant enzymatic activities (TAOC, SOD, CAT and GPx), histopathological study and protein and gene expression of globin proteins	The injection before hypoxia and reoxygenation reduced antioxidant capacity in most organs. Myoglobin and neuroglobin mRNAs were induced in the brain	Okogwu et al. (2014)
Planktothrix agardhii containing MC-YR and MC-LR *M. aeruginosa* containing MC-LR	Zebrafish *(Danio rerio)*	0.3, 1, 3 or 10 g d.w./L for 96 hours, by transdermal and oral route	Behavioral study	Slight increase of movement in zebrafish embryos	Jonas et al. (2015)
Pure MC-LR	Zebrafish *(Danio rerio)*	0.8, 1.6 or 3.2 µg/L for 120 hpf, by transdermal and oral route	Developmental toxicity and locomotor study, ACh and DA levels, protein and	Hypoactivity of larvae and alteration of the cholinergic system	Wu et al. (2016)

			gene expression related to development, AChE activity		
Pure MC-LR	Zebrafish *(Danio rerio)*	0.3, 3 or 30 µg/L for 90 days, by transdermal and oral route	Histopathological study and protein and gene expression of GABA and glutamate	Edematous and collapsed myelinated nerve fibers, distention of endoplasmic reticulum and swelling mitochondria in brain	Yan et al. (2017)
Pure MC-LR	Zebrafish *(Danio rerio)*	1, 5 or 25 µg/L for 60 days, by transdermal and oral route	Behavioral study, protein and gene expression, levels of MC-LR, DA, GABA, serotonin, ACh and DOPAC, and AChE activity	Parental exposure resulted in MC-LR accumulation and developmental neurotoxicity in offsprings	Wu et al. (2017)
M. aeruginosa containing MC-LR	Zebrafish *(Danio rerio)*	0.02, 0.04 or 0.08 OD values, for 4 days, by transdermal and oral route	Locomotor behavioral study, gene expression, AChE and DA levels	Affectation of both cholinergic and dopaminergic systems changes in the gene transcription of the nervous system, and a decrease of the locomotor activity in larval zebrafish	Qian et al. (2018)

Abbreviations: ACh: acetylcholine; AChE: acetylcholinesterase; b.w.: body weight; CAT: catalase; CKs: creatine kinases; DA: dopamine; DOPAC: dihydroxyphenylacetic acid; DRP2: dihydropyrimidinase-like 2; d.w.: dry weight; GABA: gamma-aminobutyric acid; GPx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione-S-transferase; hpf: hours post-fertilization; LPO: lipid peroxidation; OD: optical density; PBP: protein-bound phosphate; POD: guaiacol peroxidase activity; SOD: superoxide dismutase; TAOC: total antioxidant capacity.

Table 5.

CYN/Cyanobacteria	Experimental model	Experimental conditions	Assays performed	Relevant Results	References
In vitro					
Crude extracts of *C. raciborskii*	Neurons of *Helix pomatia*	Extracts of both the bloom sample and the laboratory isolate of the bloom were diluted in physiological *Helix* saline and applied by perfusion at a constant flow rate	Electrophysiological experiments	No cholinergic alteration was observed with the CYN-producing strain	Vehovszky et al. (2013)
Pure CYN	N2a murine neuroblastoma derived cells	0.001, 0.1 and 10 µM for 24, 48 and 72 hours	MTT assay Apoptotic cell death TNF-α measurement	Concentration and time-dependent decrease of cell viability after all time exposures to both 0.1 and 10 µM. Significant rise in proapoptotic caspases after exposure to 10 µM	Takser et al. (2016)
	BV-2 microglia murine cells			Concentration-dependent decrease of cell viability after all exposure times to both 0.1 and 10 µM. Significant rise in proapoptotic caspases after exposure to 10 µM	
In vivo					
Whole cell extracts of *C. raciborskii* and live cultures of *C. raciborskii*	*Bufo marinus* tadpoles	0-200 and 0-107µg/L, respectively, for 7 days, by transdermal route	Histopathological study	No mortality observed. Several histopathological changes in the encephalon	Kinnear et al. (2007)
Whole cell extracts of *C. raciborskii*	*Bufo marinus* tadpoles	0-400 µg/L for 7 days, by transdermal route	Behavioral studies Toxin analysis	Decrease in behavior scores Neither mortality nor growth rates were affected	White et al. (2007)
Live cultures of *C. raciborskii*		0-232 µg/L for 7 days, by transdermal route		Decrease in behavior scores Time-dependent increase in mortality Negative growth rates	
A. ovalisporum culture containing CYN	Tilapia fish (*Oreochromis nicotilus)*	10 µg/L for 14 days, by transdermal and oral route	AChE activity, LPO, histopathological study and ELISA	Inhibition of the AChE activity Rise in LPO levels Necrosis, hyperemia, haemorrhagia and edema CYN detection in all brain samples	Guzmán-Guillén et al. (2015)

Purified CYN (CYNp) and extract of *C. raciborskii* containing CYN (CYNex)	Trahira (*Hoplias malabaricus*)	Single dose of 50 µg/kg b.w. for 7 and 14 days by intraperitoneal injection	AChE activity, GST activity, LPO and ELISA	Increase of AChE activity after 7 days of exposure to CYNex, decreasing after 14 days. Decrease of GST after 7 days of exposure to CYNex and increase after 7 days of exposure to CYNex and after 14 days of exposure to CYNp and CYNex. Rise in LPO levels after 7 and 14 days of exposure to CYNp and CYNex. Detection of CYN in brain	da Silva et al. (2018)

Abbreviations: *A. ovalisporum*: *Aphanizomenon ovalisporum*; AChE: Acetylcholinesterase; BV-2: cellosaurus cell line; b.w.: body weight; *C. raciborskii*: *Cylindrospermopsis raciborskii*; CYN: cylindrospermopsin; CYNp: purified cylindrospermopsin; CYNex: extract containing cylindrospermopsin; GST: glutathione-S-transferase; LPO: lipid peroxidation; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N2a: fast-growing mouse neuroblastoma cells.

Table 4.

Microcystin congener/Cyanobacteria	Experimental model	Experimental conditions	Assays performed	Relevant Results	References
Nematodes					
Pure MC-LR	*Caenorhabditis elegans*	1, 10, 20, 40, 80 or 160 µg/L for 24 hours in sterile culture plates	Behavioral study and gene expression	Decrease of chemotaxis to NaCl and diacetyl from 40µg/L Decrease of thermotaxis from 20µg/L Decrease of expression patterns of sensory neurons (ASE, AWA, AFD and AIY)	Li et al. (2009a)
Pure MC-LR	*Caenorhabditis elegans*	1, 10, 20, 40 or 80 µg/L in different periods of time, in sterile culture plates	Behavioral study, morphological changes, gene expression and life-cycle indices	Decrease of head thrash, body bends Decrease of body size Enhance of *gfp* gene expression Decrease of life span, brood size, generation time	Li et al. (2009b)
Pure MC-LR	*Caenorhabditis elegans*	0.1, 1, 10 or 100 µg/L for 8 or 24 h, in 12-well sterile culture plates	Behavioral studies, morphologic changes and gene expression	Decrease of locomotion behavior Enhance of neuronal loss of GABAergic neurons, presenting aberrant neuronal morphology at 10-100 µg/L No changes in cholinergic, serotonergic, dopaminergic and glutamatergic neurons Decrease of Gene expression affecting GABAergic neurons	Ju et al. (2013)
Pure MC-LR *Microcystis aeruginosa* culture containing MC-LR, MC-RR and MC-YR	*Caenorhabditis elegans*	0.1, 1, 10 or 100 µg/L for 24 or 72 hours, in sterile culture plates 300 µg/L for 24 or 72 hours, in sterile culture plates	Behavioral study	Decrease of body bends, move length, pharyngeal pumping frequency and touch response Alteration of the thermotactic behavior after 72 hours of exposure to 100 µg/L Enhance of motile and pumping activity	Ju et al. (2014)

Pure MC-LR Pure MC-LF	*Caenorhabditis elegans*	1, 10, 40, 80, 160, 320, 500, or 1000 µg/L for 24 hours, in sterile culture plates 1, 10, 100, 160 or 320 µg/L for 24 hours, in sterile culture plates	Function of sensory neurons	Affectation of AWA sensory neurons, but not of AWC sensory neurons: MC-LF >MC-LR	Moore et al. (2014)
Pure MC-LR	*Caenorhabditis elegans*	1, 50 or 100 µg/L in different periods for life cycle, in sterile culture plates	Life-cycle indices and gene expression	Decrease of lifespan, body length and brood size after 100 µg/L of exposure Alteration of genes expression after 100 µg/L of exposure	Saul et al. (2014)
Birds					
Cyanobacterial biomass containing MC-LR, MC-RR, MC-YR and MCs similar compounds	Japanese quail (*Coturnix coturnix japonica*)	0.045, 0.459, 4.605 or 46.044 µg/day for 10 or 30 days, by oral route	Oxidative stress parameters	Alteration in brain, after acute exposure: Decrease of GSH, Enhance of TBARS, Enhance of EROD. After subchronic exposure: Enhance of GSH, Enhance of GPx Enhance of TBARS, Enhance of EROD	Pašková et al. (2008)
Mammals					
MC raw extracts containing mainly [D-Leu^{1}]MC-LR	Rats	1 µL of extracts containing 0.01 or 20 µg/L (equivalent to 0.045x10E-6 and 9.1x10E-5 µg/kg) by intrahippocampal injection	Behavioral study, oxidative stress parameters and DNA damage	Enhance of latency of long-term memory in rats exposed to 20 µg/L Decrease of latency of memory retrieval Enhance of working and reference memory errors after 8 days of exposure Enhance of GST activity in brain rats exposed to 0.01 µg/L Enhance of LPO content in brain rats exposed to 20 µg/L DNA damage in brain of both MCs doses treated rats	Maidana et al. (2006)
Extracted and purified MC-LR and MC-RR from blooms	Rats	80 µg MC-LReq/kg b.w. injected i.v. The analysis was performed 1, 2, 4, 6, 12 and 24 hours post-injection	Determination of MCs content in different tissues by LC-MS	MCs contents in brain (0.2%): 2 > 24 > 1 > 12 > 6 ≈ 4 hours post-injection kidney>lung>stomach> liver> small	Wang et al. (2008)

				intestine> gonad> spleen> muscle> heart>**brain**	
Pure MC-LR	Rats	1 µL containing 1 or 10 µg/L MC-LR (equivalent to 5x10E-6 or 5x10E-5 µg/kg), bilaterally injected into hippocampal. Parameters were measured 15 days post-injection	Behavioral study, histopathological study and oxidative stress parameters	Enhance of latencies to find the platform Decrease of swimming distance in the target zone Swimming speed did not change Decrease of total hipoccampal neurons Highest MC-LR dose: Enhance of LPO, Enhance of CAT, Enhance of GPx, Enhance of SOD Lowest MC-LR dose: Enhance of LPO, Enhance of CAT	Li et al. (2012a)
Pure MC-LR	Rats	1 or 10 µg/kg day i.p. injected for 50 days	Behavioral study, histopathological study, protein expression, MC-LR content analysis	Enhance of latencies to find the platform Decrease of swimming distance in the target zone Enhance of degeneration and apoptosis of hippocampal cells Hyperphosphorylation of tau 41.6±8.45 ng/g d.w. of MC-LR was detected in brain of rats exposed to 10 µg/kg day	Li et al. (2012b)
Pure MC-LR	Rats	10 µL containing 5 or 25 µg/L (equivalent to 2.5x10E-4 or 1.25x10E-3 µg/kg), by i.c.v. injection MC-LR + LiCl and SB216763 inhibitors of GSK-3β	Electrophysiologycal studies	Enhance of PPs activity Decrease of phosphorilated GSK-3β Decrease of LTP Inhibitors avoid effects produced by MC-LR	Wang et al. (2013)
Pure MC-LR	Rats	0.2, 1 or 5 µg/kg every 2 days for 8 weeks, by intragastric route	Behavioral study, histopathological study and immunohistochemistry staining	Enhance of escape latencies in 5 µg/kg MC-LR-treated rats Decrease of frequencies entering the enlarged platform in 1 and 5 µg/kg MC-LR-treated rats No significant differences in the number of damaged neurons	Li et al. (2014)

				Enhance of astrocyte density and NO concentration in hippocampus exposed to 5.0 µg /kg	
Pure MC-LR	Rats	1, 5 or 20 µg/kg every 2 days for 8 weeks, by intragastric route. Later the rats became pregnant of a non-exposed male	Maternal toxicity and reproductive outcome, simple motor and locomotor activities, behavioral study and oxidative stress parameters	Decrease of mean body weight gain in maternal rats. Decrease of number of pregnant rats Alteration of behavior and neurodevelopment in rat offsprings Enhance of MDA and SOD in hippocampus of offsprings	Li et al. (2015b)
Extracted and purified MC-LR from blooms	Pregnant rats and pups	10 µg/kg daily from day 8 to postnatal day 15	Oxidative stress parameters, determination of MC-LR, histopathological study and protein expression	Enhance of MDA, Decrease of GSH andAChE activity No significant PPs changes 3.75±0.94 ng/g d.w. were detected in brain of pup rats Morphological changes Alteration of proteins involved in neuronal processes in pup rats	Zhao et al. (2015)
Extracted and purified MC-LR from blooms	Mice	1 µL containing 1-20 ng/µL, by i.c.v. route. All parameters measured 3 hours, 1 day, 3 day and 7 day after exposure	Behavioral study, histopathological study and oxidative stress parameters	Decrease of memory impairment Morphological changes in hippocampal neurons from 10 ng/µL Enhance of protein oxidation, LPO, ROS, SOD, GPx and Nrf2 Decrease of GSH/GSSG	Shin et al. (2018)
Pure MC-LR	Mice	1, 5, 10, 20 or 40 µg/L 12 weeks, by oral route	Histopathological study and protein expression	Pathological changes in hippocampus and cortical cells in a dose-dependent way. Differences between hippocampus and cerebral cortex in the affectation of mRNA and proteins expression: ATP6, COX3, CYTB, POLG, mtSSB and TFAM	Wang et al. (2018)
Pure MC-LR	Rats	3 µL of 0.1 µg MC-LR/µL (equivalent to 1.5 µg/kg) via hippocampal injection. All parameters measured 24 hours, before and after	Protein expression and behavioral study	Enhance of desmethylation of PP2Ac, phosphorylation of GSK-3β and tau, spatial memory deficit.	Zhang et al. (2018)

		exposure			

Abbreviations: AChE: acetylcholinesterase; ATP6: adenosine triphosphate-6 ; b.w.: body weight; CAT: catalase; COX3: cyclooxygenase-3 ; CYTB: Cytochrome B ; EROD:cytochrome P-450-dependent 7- ethoxyresorufin O deethylase; GLU: glucose; GPx: glutathione peroxidase; GSH: reduced glutathione; GSK-3β: Glycogen synthase kinase 3 beta; GSSG: oxidized gluthatione; GST: Glutathione-S-transferase; i.c.v.: intracerebroventricular; LPO: lipid peroxidation; LTP: long term period; MDA: malondialdehyde; mtSSB: mitochondrial single-stranded DNA binding protein; NO: nitric oxide; POLG: DNA polymerase g; PP: protein phosphatase; PP2Ac: catalytic subunit of protein phosphatase 2A; ROS: reactive oxygen species; SOD: superoxide dismutase; TBARS: total thiobarbituric acid reactive species; TFAM: mitochondrial transcription factor A.

Table captions

Table 1. Properties and environmental concentrations of some MCs congeners and CYN.

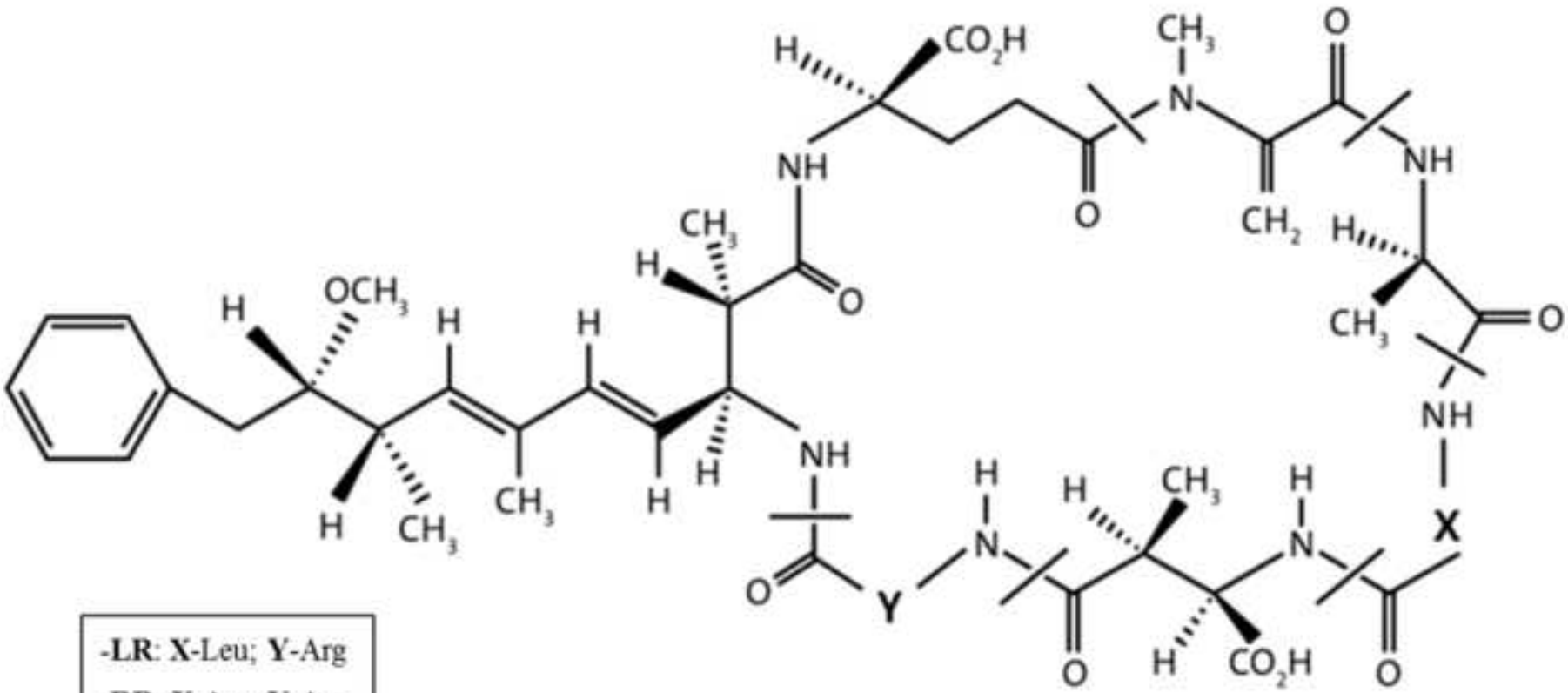
Table 2. *In vitro* neurotoxicity studies after exposure to MCs.

Table 3. *In vivo* neurotoxicity studies in several aquatic animal models exposed to MCs.

Table 4. *In vivo* neurotoxicity studies in different terrestrial models exposed to MCs.

Table 5. Neurotoxicity studies performed with CYN.

Figure A.



-**LR**: **X**-Leu; **Y**-Arg

-**RR**: **X**-Arg; **Y**-Arg

-**YR**: **X**-Tyr; **Y**-Arg

-**LF**: **X**-Leu; **Y**-Phe

-**LW**: **X**-Leu; **Y**-Trp

Figure B.

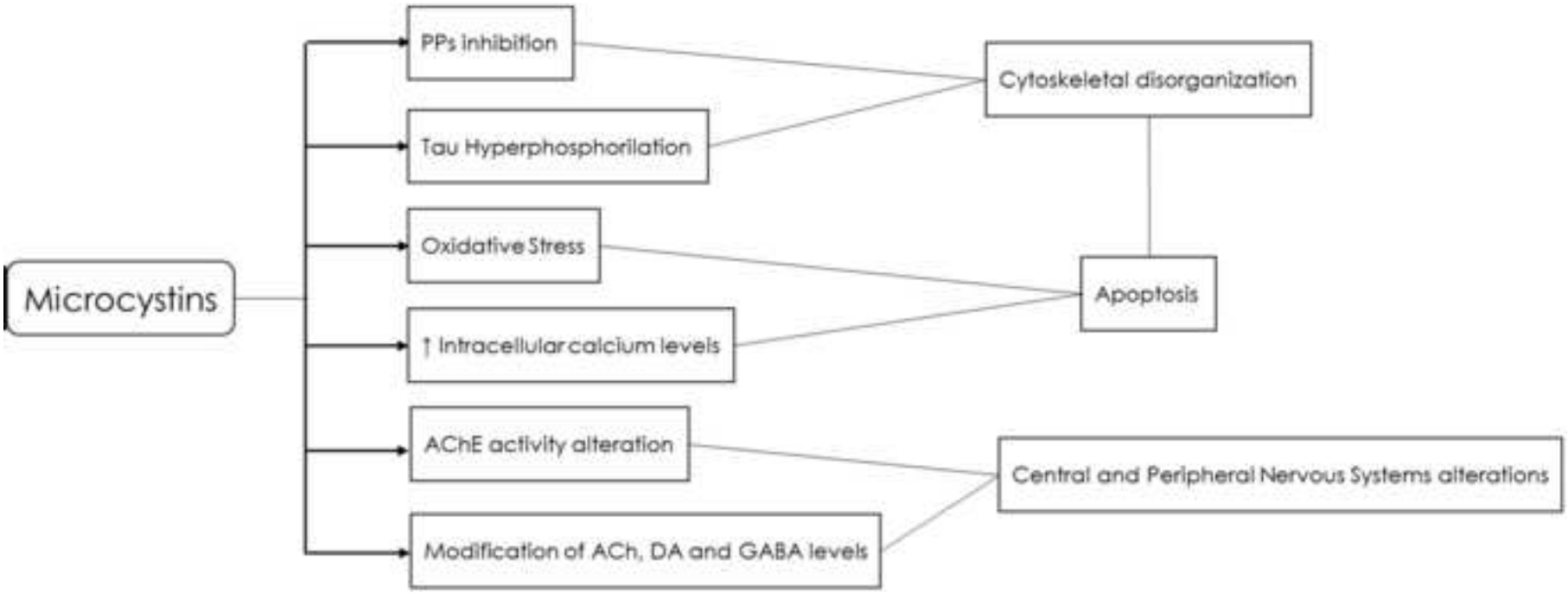

Microcystins
PPs inhibition
Tau Hyperphosphorilation
Oxidative Stress
↑ Intracellular calcium levels
AChE activity alteration
Modification of ACh, DA and GABA levels
Cytoskeletal disorganization
Apoptosis
Central and Peripheral Nervous Systems alterations

Figure C.

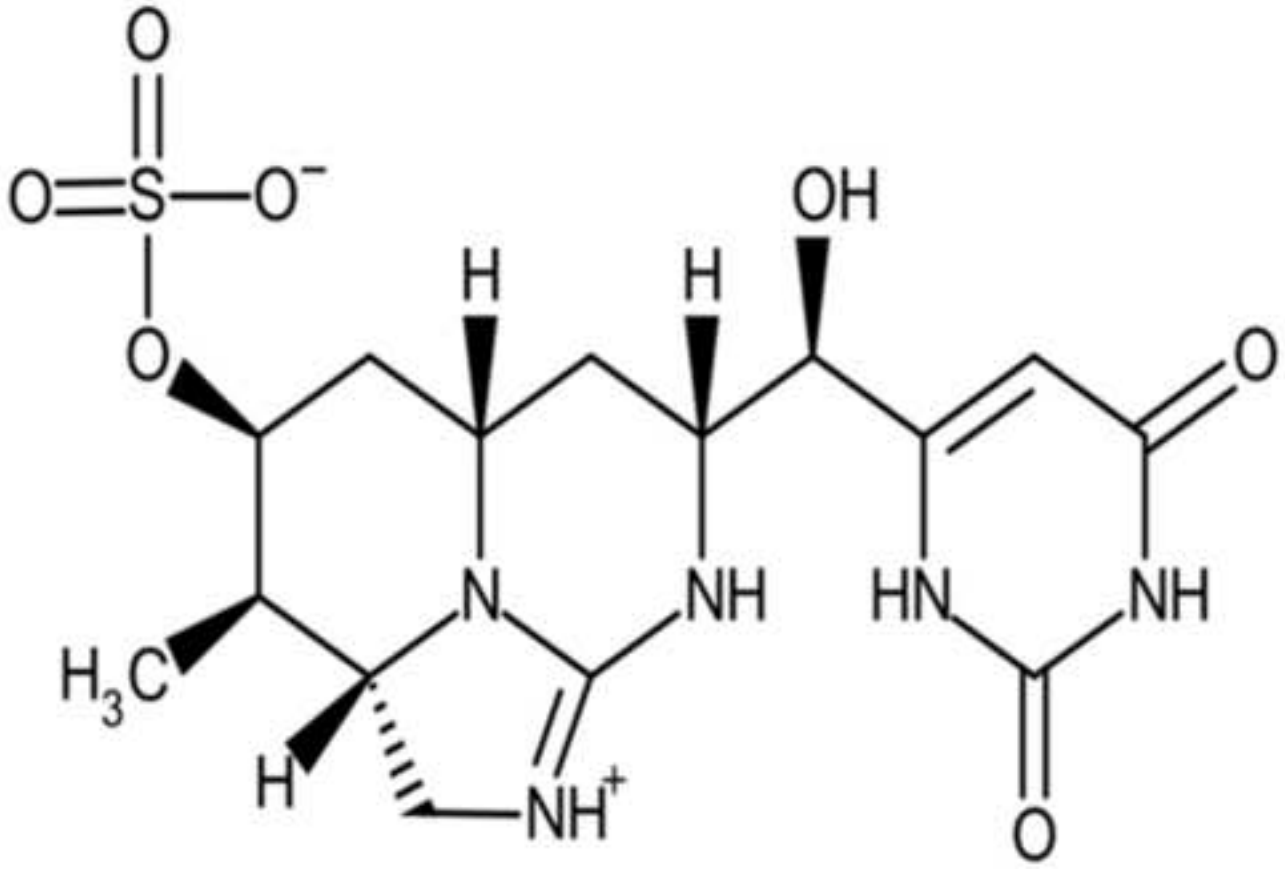


Figure D.

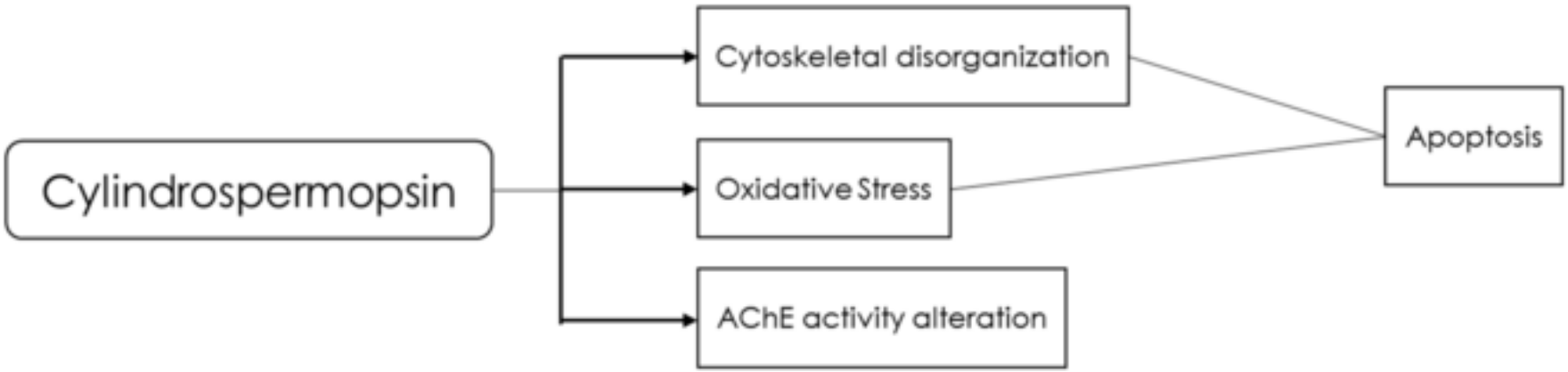

Cylindrospermopsin
Cytoskeletal disorganization
Oxidative Stress
AChE activity alteration
Apoptosis

Figure captions:

Figure A. Structure of MCs.

Figure B. Main mechanisms of neurotoxic action of MCs.

Figure C. Structure of CYN.

Figure D. Main mechanisms of neurotoxic action of CYN.