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Review article

Oxidative stress generation by microcystins in aquatic animals: Why and how

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ABSTRACT

Microcystins (MICs) are potent toxins produced worldwide by cyanobacteria during bloom events. Phosphatases inhibition is a well recognized effect of this kind of toxins as well as oxidative stress. However, it is not fully understood why and how MICs exposure can lead to an excessive formation of reactive oxygen species (ROS) that culminate in oxidative damage. Some evidences suggest a close connection between cellular hyperphosphorylation state and oxidative stress generation induced by MICs exposure. It is shown, based on literature data, that MICs incorporation *per se* can be the first event that triggers glutathione depletion and the consequent increase in ROS concentration. Also, literature data suggest that hyperphosphorylated cellular environment induced by MICs exposure can modulate antioxidant enzymes, contributing to the generation of oxidative damage. This review summarizes information on MICs toxicity in aquatic animals, focusing on mechanistic aspects, and rise questions that in our opinion needs to be further investigated.

Contents

1. Introduction

Anthropogenic activities along with deficient water management have led to the enhancement of eutrophication in water bodies all over the world (Svrcek and Smith, 2004; Carmichael, 2007). Eutrophication processes plus specific environmental conditions such as high temperature and pH values, low turbulence and high nutrients input, can lead to cyanobacterial blooms, which are characterized by excessive proliferation of cyanobacterial cells (de Figueiredo et al., 2004).

Cyanobacteria (blue-green algae) are capable of producing a wide range of toxins including hepatotoxins, neurotoxins, cytotoxins, dermatoxins and irritant toxins (lipopolysaccharides) (Wiegand and Pflugmacher, 2005). Globally, the most frequently found cyanotoxins in fresh and brackish water are the cyclic peptide toxins of microcystins (MICs) family and nodularins (Chorus and Bartram, 1999). Microcystins (MICs) are potent hepatotoxins produced by a number of planktonic cyanobacteria genera, such as *Anabaena*, *Anabaenopsis*, *Nostoc*, *Planktothrix* and *Microcystis* (Chorus and Bartram, 1999). There are more than 75 isoforms of MICs varying by degree of methylation, hydroxylation, epimerization, peptide sequence and toxicity. Besides this great variability, MICs collectively may be described as monocyclic heptapeptides containing both D- and L-amino acids plus N-methyldehydroalanine (Mdha) and a unique β-amino acid side-group, 3-amino-9-methoxy-2-6,8-trymethyl,10-phenyldeca-4,6-dienoic acid (Adda) (Svrcek and Smith, 2004).

The concentration of dissolved toxin in the environment varies from traces up to 1800 μg/L or higher, immediately after the collapse of a highly toxic bloom (Chorus and Bartram, 1999; Svrcek and Smith, 2004). The intact cells as well as the toxins released after cellular lysis can be responsible for the toxic effects observed in many organisms,

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from microalgae to mammals (de Figueiredo et al., 2004; Jos et al., 2005).

Aquatic organisms are exposed to MICs by several different routes. They can come into direct contact with toxins after the senescence and lysis of cyanobacterial cells. In addition some phytoplanktivorous and omnivorous species can ingest cyanobacterial cells as food (Li et al., 2004; Xie et al., 2004). It is also important to consider that in natural environments, surface aggregations of some cyanobacteria (complex cyanobacterial samples) may accumulate to scum with high cell density and toxin concentrations, thus increasing the potential toxicity to aquatic animals in direct contact with it (Chorus and Bartram, 1999). The fact that MICs have been found to accumulate in species of zooplankton (Mohamed, 2001), mussels (Eriksson et al. 1989; Amorim and Vasconcelos 1999), crustaceans (Chen and Xie, 2005), marine biota consumed as "seafood" (Ibelings and Chorus, 2007), several fish species (Soares et al., 2004; Cazenave et al., 2005; Chen et al., 2006a) and other vertebrates (Chen et al., 2009) indicates that another route of exposure to the toxin, both for aquatic and terrestrial species, including humans, is the consumption of aquatic organisms which had previously accumulated microcystin in their tissues (Zhang et al., 2009). For example, MICs content in the muscle of Nile tilapia exposed to the toxin through food exceeded the upper limit of the tolerable daily intake (TDI) suggested by the WHO (0.04 μg/kg body weight/day), suggesting that Nile tilapia fed on toxic cyanobacteria is not suitable for human consumption (Zhao et al., 2006).

Due to its high molecular weight (varying from 900 to 1100 Da), MICs are unable to easily penetrate biological membranes and bioconcentrate (Svrcek and Smith, 2004). However, some kinds of cells express specific membrane transporters that enable toxin accumulation.

Consequences of MICs intoxication in mammals include disruption of liver cytoskeleton, apoptosis, necrosis and internal hemorrhage in liver that may lead to death by hemorrhagic shock in acute intoxication (Dawson, 1998). MICs chronic ingestion can cause increased liver weight, hepato-histological damage (Heinze, 1999), liver cancer (Hu et al., 2008; Hernández et al., 2009; Li et al., 2009) and kidney damage (Milutinovi et al., 2003). In juvenile and adult fish species, analogous, but not identical effects were observed when they were exposed to purified MICs or cyanobacterial material (Fischer et al., 2000). Studies with different fish species have shown that chronic MICs exposure mainly affects growth (Bury et al., 1995; Kamjunke et al., 2002), blood indices (Li et al., 2004, 2008a; Qiu et al., 2009), ionic regulation (Gaete et al., 1994; Bury et al., 1996) histopathology (Fischer and Dietrich, 2000a; Fischer et al., 2000), heart rate (Best et al., 2001), locomotor activity and behaviour (Baganz et al., 2004; Cazenave et al., 2008). Early life stages are more susceptible to the toxin than adult fish, because of the thinner epithelial layer combined with a relative large body surface, high metabolic rate and limited mobility. Several laboratory and field studies describe the effects induced by chronic MICs exposure in early life stages of fish as interferences with developmental processes and organ functions, which can decrease the survival rate of enough individuals to effectively affect at the population level (Malbrouck and Kestemont, 2006).

In aquatic invertebrate species the effects of MICs exposure are less well known (Martins and Vasconcelos, 2009). Sessile species may be the most obvious group of organisms threatened by the presence of toxic cyanobacteria. In fact, some studies have demonstrated altered larvae survival and development, changes on feeding behaviour and energy balance in a mussel species (*Dreissena polymorpha*) feeding on phytoplankton (Pires et al., 2003). However, insensitivity to MICs has also been reported in bivalves, where expulsion via pseudo-faeces has been described as a primary defense mechanism. For example, *Dreissena polymorpha* produces large quantities of mucous, called 'pseudodiarrhoea', periodically expelled through the pedal gape by shell valve adduction when exposed to highly toxic strains of cyanobacteria (Juhel et al., 2006). Contardo-Jara et al. (2008) demonstrated that exposure of *D. polymorpha* to MIC-LR was related to an immediate increase in P-glycoprotein (P-gp) activity, suggesting multi-xenobiotic-resistance as a possible explanation for the insensitivity of bivalves towards cyanobacterial toxins.

At the molecular level, the classical mechanism of MIC toxicity is the irreversible inhibition of serine/threonine protein phosphatases PP1 and PP2A, an effect described in zooplankton (DeMott and Dhawale, 1995), amphibians (Fischer and Dietrich, 2000a), fish (Fischer and Dietrich, 2000b; Fischer et al., 2000) and mammals (Chen et al. 2006b). However, several recent reports have argued that oxidative stress is also a toxicological consequence of the exposure to MICs in different organisms.

Oxidative stress is classically defined as a disturbance in the pro-oxidant/antioxidant balance in favor of the former, leading to potential molecular damage (Halliwell and Gutteridge, 2007). However, the major cellular thiol/disulfide systems, including GSH/GSSG, thioredoxin-1 (-SH_2/-SS-), and Cys/CySS, are not in redox equilibrium and respond differently to chemical toxicants and physiologic stimuli. Thus, from a mechanistic standpoint, oxidative stress may be better defined as a disruption of redox signaling and control (Jones, 2006). So, the aim of the present review is to make a compilation of recent information on MICs toxicity, with regard mainly to aquatic animals. Also, some topics that we believe need to be investigated are highlighted.

2. Why microcystins induce oxidative stress in aquatic organisms?

In the last years, as shown in Table 1, several evidences indicate that MICs can alter the antioxidant system and/or induce oxidative stress in diverse aquatic species and organs, a fact that prompt us to analyze and review such important toxicological issue. MICs uptake has been related to the production of reactive oxygen species (Ding et al., 2000a, 2001; Li et al., 2003), leading to an increase in lipid peroxidation (Pinho et al., 2005; Jos et al., 2005; Prieto et al., 2007), DNA damage (Zegura et al., 2003, 2008; Votto et al., 2007), DNA–protein crosslink (Leão et al., 2008), mitochondrial damage (Ding and Ong, 2003) and alteration of the antioxidant defense system (Vinagre et al., 2003; Pinho et al., 2005; Cazenave et al., 2006a,b; Prieto et al., 2007; Amado et al., 2009). However, the causes and the mechanisms involved in these responses have been analyzed in lesser depth.

A cyanobacterial bloom can promote oxidative stress simply by generating hyperoxia/anoxia cycles through photosynthetic and respiratory processes, as previously observed under a cyanobacterial bloom dominated by the genera *Microcystis* and *Anabaena*, among others (Seki et al., 1980). A cycle of low/high oxygen levels in the water column was suggested by Rosa et al. (2005) to explain the extremely high levels of lipid peroxidation in the estuarine worm, *Laeonereis acuta* (Nereididae) collected under a bloom event dominated by cyanobacteria genera such as *Anabaena*, *Aphanocapsa*, *Merismopedia* and *Snowella*. Hypoxia/hyperoxia cycles occurring in nature, resemble the ischemic/reperfusion process. After a reduction of oxygen flow (ischemia), the return of oxygen in the reperfusion causes an increase in the production of reactive oxygen species (ROS), leading to oxidation of cellular components including proteins, membrane lipids and DNA. The electron carriers of the mitochondrial respiratory chain are reduced during ischemia, whereas immediate re-oxygenation of these carriers takes place after the reperfusion, leading to oxyradical overproduction (Halliwell and Gutteridge, 2007).

In vitro studies, however, have shown that the ROS production is also a metabolic response to MICs exposure and not only an effect analog to ischemia/reperfusion process. Ding and Ong (2003) reported elevated ROS levels in rat hepatocytes just 5 min after exposing them to 1 μM

Table 1
Some evidences of oxidative stress or alteration in antioxidant system of aquatic animals exposed to microcystins.

Species	Organ	Exposure	Antioxidant response analyzed	Oxidative stress parameters analyzed	Main observed effects	Reference
Laeonereis acuta (P)	Whole animal	Immersion in *Microcystis aeruginosa* extract (~2 μg MIC/mL) during 48 h	CAT, GST and GR activities	LPO	Lower CAT activity and no alteration on GST activity; higher LPO levels and DNA–protein cross-links content	Leão et al. (2008)
Chasmagnathus granulatus[a] (C)	Gills (anterior and posterior)	Injected with *Microcystis aeruginosa* extract (39.2 μg MIC/L) during 48 h	Total antioxidant capacity (TOSC assay[b]) and GST activity.	LPO	Higher TOSC and GST in posterior gills.	Vinagre et al. (2003)
Chasmagnathus granulatus[a] (C)	Hepatopancreas	Injected with *Microcystis aeruginosa* extract (17.6 μg MIC/L) during 72 h and one week of exposure	CAT, SOD and GST activities	LPO	Higher CAT and GST activities in crabs exposed for 7 days. No effect on SOD activity and LPO levels	Pinho et al. (2003)
Chasmagnathus granulatus[a] (C)	Hepatopancreas	Forced ingestion (~1 and 5 μg MIC/kg) during 168 h	CAT, GST and SOD activities	LPO and protein carbonyl groups	Biphasic alteration of CAT; Augmented GST activity; Higher LPO levels	Pinho et al. (2005)
Chasmagnathus granulatus[a] (C)	Hepatopancreas	Gavage with *Microcystis aeruginosa* extracts (34, 172, 860 μg MIC/kg) during 6, 12 and 72 h.	GST activity	LPO	Higher GST activity in crabs exposed to 860 μg MIC/kg for 12 h Higher LPO levels in crabs exposed to all doses after 72 h of exposure	Dewes et al. (2006)
Danio rerio (F)	Fish embryos	Immersion in REKO medium containing 0.1, 0.5, 1, 2 and 5 μg MIC-LR/L over ontogenetic development and after hatch (3 and 5 days)	sGST, mGST and GPx activity	None	Dose dependent increase in sGST and mGST activity over ontogenetic development (from 0.1 to 2 μg MIC-LR/L). The higher dose suppressed soluble GST activity. GPx increased in 0.5 μg MIC-LR/L exposure dose	Wiegand et al. (1999)
Hypophthalmichthys molitrix (F)	Hepatopancreas	Immersion in a cyanobacterial bloom (*Microcystis ichthyoblabe* 60% and *M. aeruginosa* 40%, 4–116 × 10^6 cells/mL	GSH	LPO	Higher GSH and no LPO effect	Bláha et al. (2004)
Oreochromis sp (F)	Liver, kidney and gills	Cyanobacterial cells mixed with a commercial fish food or crushed into a commercial fish food through 14 and 21 days.	SOD, CAT, GPx, GR activities	LPO	In general, antioxidant enzymes and LPO were increased after 21 days of exposure to crushed food in almost all organs, but liver was the most affected.	Jos et al. (2005)
Misgurnus mizoleps (F)	Liver	Orally exposed to 75 mg of dry cells/kg body weight (equal to 10 μg MIC-RR/kg body mass), for 28 days	SOD, CAT, GPx activities	LPO	Activity of antioxidant enzymes was increased and LPO remained stable.	Li et al. (2005)
Danio rerio (F)	Fish embryos	Immersion in REKO medium with 25 μg MIC-RR/L or 25 μg MIC-LF/L during 24 h	sGST, mGST, CAT, POD, GPx, GR	None	Higher sGST, mGST and CAT activity; no effect in the other analyzed enzymes	Cazenave et al. (2006a)
Corydoras paleatus (F)	Liver, gill, intestine and brain	MIC-RR dissolved in water (up to 1 μg/L) during 24 h	CAT, GPx, GR, GST, POD activities	TBARS	Decreased GST activity in all organs; the other antioxidant enzymes augmented in liver; higher LPO levels in brain	Cazenave et al. (2006b)
Oreochromis sp (F)	Liver, kidney and gill	I.p. injection of a single dose of 500 μg MIC-LR/kg or 500 μg MIC-RR/kg and killed after 7 days.	SOD, CAT, GPx; GR	LPO	In general, antioxidant enzymes, mainly SOD and CAT, and LPO levels were increased	Prieto et al. (2006)
Oreochromis niloticus (F)	Liver, kidney and gills	Food pellets with 1350 μg/g of pellet during 24 h	CAT, GPx, GR and SOD activities	LPO and protein carbonyl groups	In general, a decreased activity of antioxidant enzymes; higher LPO levels in all organs. Higher protein oxidation in liver.	Prieto et al. (2007)
Tinca tinca (F)	Liver and kidney	Orally exposed to cyanobacterial cells dosing 5, 11, 25 and 55 mg MC-LR/fish mixed with the food	SOD, CAT, GSH, GSH/GSSG	LPO and protein carbonyl groups	SOD activity decreased in a dose dependent manner in liver and kidney; decrease in CAT activity in the liver in the two higher doses and no effect in CAT activity in kidney; no effect on GSH and GSH/GSSG levels; LPO increased in the two higher doses; no effect in protein oxidation	Atencio et al. (2008)
Carassius auratus (F)	Liver, kidney and intestine	One injection (50 and 200 μg MIC/kg) and then followed up to 168 h	Expression of several GST genes	None	In general decreased transcription of several GST isoforms (including α, π and θ)	Li et al. (2008a,b)
Jenynsia multidentata (F)	Liver and brain	Food pellets with MIC-RR (up to 1 μg/g of pellet) and analyzed during 24 h	GST activity	None	Higher GST activity at the highest dose	Cazenave et al. (2008)
Jenynsia multidentata (F)	Liver, brain, gills and muscle	*Microcystis aeruginosa* cells (RST9501) extracts dissolved in water to reach 5 and 100 μg/L toxin	Total antioxidant competence against peroxyl radicals (ANCOMROS)	Protein oxidation (carbonyl groups)	Increased ANCOMROS in liver and decreased antioxidant capacity in brain and gills. There was no protein oxidation in liver.	Amado et al. (2009)

CAT: catalase. GPx: glutathione peroxidase. GST: glutathione-S-transferase. sGST: soluble glutathione-S-transferase. mGST: microssomal glutathione-S-transferase. LPO: lipid peroxidation. MIC: microcystin. MIC-RR: microcystin RR isoform. MIC-LF: microcystin LF isoform. MIC-LR: microcystin LR isoform. POD: guaiacol peroxidase. SOD: superoxide dismutase. TOSC: total oxyradical scavenging capacity. P, C and F refer to polychaeta, crustacean and fish species, respectively.

[a] Now cited as *Neohelice granulata*.

[b] Winston et al. (1998).

MIC-LR. High levels of ROS generation were also reported for fish hepatocytes and lymphocytes (Li et al., 2003; Zhang et al., 2007), after 30 and 90 min of MIC exposure, respectively.

Along with the increased level of ROS production, several studies have evidenced the alteration in cytosolic glutathione (GSH) concentration due to MICs exposure. GSH is the most abundant

non-protein thiol, being found at the milimolar range in most cells, playing a key role as antioxidant (Dickinson and Forman, 2002; Maher, 2005). A biphasic response in terms of glutathione (GSH) levels has been reported by Ding et al. (2000b) in rat hepatocytes during exposure to a microcystic cyanobacteria extract (125 μg/mL lyophilized algae cells). The authors considered that the initial increase of intracellular GSH is probably because its conjugation with microcystin, as previously shown by Pflugmacher et al. (1998), triggering the synthesis of new GSH. The subsequent GSH depletion, was considered to be related to cell membrane damage and consequent GSH efflux (Ding et al., 2000b). In contrast, Runnegar et al. (1987) and Li et al. (2003) described only a decrease in GSH concentration, 10 and 15 min after rat and fish hepatocytes exposure to the toxin, respectively. In both studies purified MIC-LR was used and could explain the different response observed by Ding et al. (2000b) as they used cyanobacteria extract. It has been manifested already that other components of cyanobacteria cells than the toxin, such as lipopolysaccharides, can have influence in toxic responses (Best et al., 2002).

MICs were suggested to disrupt the mitochondrial electron transport chain (ETC), thus favoring ROS generation (Ding et al., 2002). However, high ROS levels were observed prior to the landmark effects of dysfunctional mitochondria, including membrane potential depolarization and mitochondrial permeability transition (MPT) (Ding et al., 2000a; Ding and Ong, 2003). Mitochondria are not only the main site of production of ROS, but also the main target of such toxic molecules, so the maintenance of its antioxidant capacity is vital for cell integrity (Aon et al., 2007). It has been attested already that mitochondria do not have the enzymatic pool associated with GSH synthesis and that mitochondrial GSH is of cytoplasmatic origin (Meister, 1995). So depletion of cytosol GSH could reflect in a decreased GSH concentration inside mitochondria, a situation that favors the described increase in ROS production and ETC disruption induced by MICs. We also have to consider that mitochondrial permeability transition pore (PTP) is under redox control (Aon et al., 2007). Oxidative stress through oxidation of intracellular GSH and other critical sulfhydryl groups favors the PTP opening (Chernyak, 1997), which leads to membrane potential depolarization and mitochondrial permeability transition (MPT). As mentioned above, these are the effects described in hepatocytes exposed to MICs. Mikhailov et al. (2003) showed that MIC-LR can bind to the beta subunit of ATP-synthase, what can contribute to the intensification of the mitochondrial membrane depolarization, disruption of ETC and ROS generation. Mitochondrial membrane depolarization leads to the release of cytochrome *c*, signaling to apoptosis, a microcystin effect that has been shown in several studies using cell lines from different organisms, including fish (Ding et al., 2002; Zhang et al., 2006, 2007, 2008).

In the context of the GSH biphasic response or depletion after MICs exposure, other possible consequences of MICs and ATP-synthase interaction can be postulated, since any ATP synthesis impairment should affect ATP-consuming reactions, including GSH synthesis, also contributing to the lowering of GSH inside the cell. Lowering of intracellular GSH concentration posseses dramatic consequences for cellular well-living and also should promote a loss of microcystin detoxification capacity. As shown by Pflugmacher et al. (1998), the glutathione-S-transferase (GST) enzyme catalyzes the conjugation of MICs with glutathione in several aquatic organisms. Metcalf et al. (2000) showed that the conjugate of the LR form of microcystin (MIC-LR) and MIC-YR with both cysteine and GSH is lesser toxic than MICs alone (in about 3–10 times). So it confirms the statement of Pflugmacher et al. (1998) that the conjugation catalyzed by GST is the first step for detoxification.

Besides the loss of detoxification capability, the alteration in GSH concentration can also have effects in several signaling pathways that are modulated by alterations in the redox status of the cell. Dickinson and Forman (2002) hypothesized that many environmental agents exert their deleterious effects by altering, either directly or indirectly, the cellular redox status through manipulation of thiols metabolism such as glutathione.

Table 2
Predicted phosphorylation sites in antioxidant enzymes in the aquatic model zebrafish (*Danio rerio*, Cyprinidae).

Protein	NCBI or GenBank accession number	Predicted phosphorylation sites in zebrafish sequences	Experimental evidences (biological model)	Reported phosphorylation effect	References
Superoxide dismutase (soluble)	NP_571369 (NCBI)	S at position 60 T at position 40	Phosphorylation by ERK-1 (NFS-60 myeloid murine cells)	Unknown	Csar et al. (2001)
Glutamate cysteine ligase (catalytic subunit)	NP_954971 (NCBI)	S at positions 132, 172, 259, 292, 304, 317, 328, 332, 338, 382, 419, 455, 465, 571 and 595 T at positions 15, 105, 113, 163, 188, 213 and 350 Y at positions 53, 100, 238, 265, 330, 336, 344, 442 and 591	Phosphorylation at S and T by PKA, PKC (Sprague–Dawley rats)	Reduction of *Vmax*, with no modification of *Km* (male *Drosophila melanogaster* and C57BL/6 mice)	Sun et al. (1996) Toroser et al. (2006)
Thioredoxin reductase	AAO65267 (GenBank)	S at positions 15 and 106 T at positions 34 and 166 Y at positions 30, 114, 139, 154 and 187	None	Unknown	–
Glutathione peroxidase	AAO86703 (GenBank)	S at positions 45, 123 and 161 T at positions 33, 38 and 166 Y at positions 37 and 79	Phosphorylation at Y 96 by c-Abl e Arg (SH-SY5Y cells)	Phosphorylation increases GPx1 activity	Cao et al. (2003a)
Glutathione-S-transferase (alpha-like)	NP_998559 (NCBI)	S at positions 18, 122, 152 and 200 T at positions 36, 112 and 213 Y at position 210	None	Unknown	–
Glutathione-S-transferase (mu)	NP_997841 (NCBI)	S at positions 124, 125 and 138 T at positions 26 and 205 Y at positions 7, 28, 33, 41, 62 and 126	None	Unknown	–
Glutathione-S-transferase (pi)	NP_571809 (NCBI)	S at positions 88, 165, 167, 174, 177 and 183	Phosphorylation at S42 and S184 by PKA and PKC (human glioma cells).	Phosphorylation enhances the metabolic function of the GSTP1 protein.	Lo et al. (2004)
		T at position 114	Phosphorylation at Y7 and Y198 (human glioma and breast cancer cells) by epidermal growth factor receptor (EGFR)	Phosphorylation increased GSTP1 catalytic efficience by reducing 3.8-fold the *Km* for EA (ethacrynic acid)	Okamura et al. (2009)

Some evidences suggest that GSH depletion could be also related to cellular MICs uptake. Usually, the paradigm of MICs-induced cellular toxicity considered that it was determined by the cell ability in taking up the toxin or, in other words, the presence of toxin carriers in cell membrane. As acute effects are observed in liver, almost all studies that analyzed MICs uptake were done in mammalian hepatocytes. Runnegar et al. (1995) showed that the MICs incorporation was inhibited by cholate, taurocholate and bromosulfophtalein, suggesting the participation of one or more of the multi-specific organic anion/bile acid transporters in MICs-induced toxicity. Furthermore, studies from Fischer et al. (2005) and Meier-Abt et al. (2007) showed that MICs transport into the cell is carrier-mediated, involving members of Organic Anion Transporting Polypeptides (animals: Oatps; human: OATPs), present both in mammals liver and brain (Fischer et al., 2005) and also described in a fish species liver (*Leucoraja erinacea*) (Meier-Abt et al., 2007). Oatps/OATPs are a group of membrane carriers which mediate a multi-specific substrate uptake in various organs of vertebrate animal species (Hagenbuch and Meier, 2003). Interestingly, there are experimental evidences showing that the activity of Oatps/OATPs is modulated by phosphorylation (Miyazaki et al., 2004). Both inhibition by protein kinase C (PKC) phosphorylation in serine residues of the transporters molecules (Uwai et al., 1998; You et al., 2000; Guo and Klaassen, 2001; Wolff et al., 2003) and stimulation by epidermal growth factor (EGF) via mitogen-actived protein kinases (MAPKs) (Sauvant et al., 2004) were already demonstrated, suggesting that the hyperphosphorylation state induced by MICs incorporation into the cell can alter, in some way, the activity of Oatps/OATPs.

The driving force for OATPs-mediated transport has not been investigated in detail for all transporters, but for Oatp1 and Oatp2,

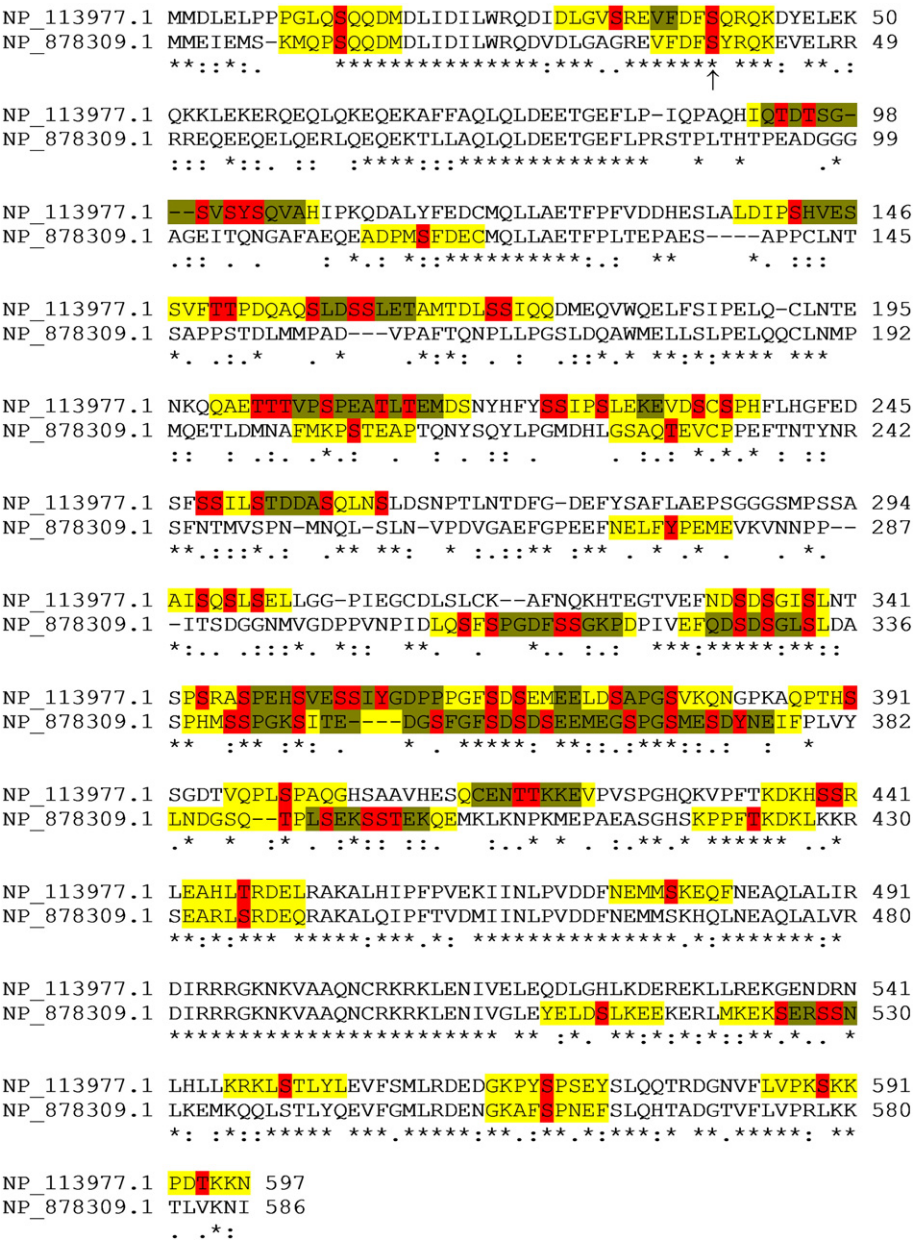

Fig. 1. Comparison of rat (*Rattus norvegicus*; NP_113977.1) and zebrafish (*Danio rerio*; NP_878309.1) Nrf2 phosphorylation sites in primary protein sequences predicted by NetPhos (http://www.cbs.dtu.dk). Highlighted in yellow is the consensus sequence for phosphorylation. In green are highlighted consensus sequences for phosphorylation that overlap in the two sequences. Indicated in red is the amino acid residue target for phosphorylation. The arrow indicates the amino acid residue shown experimentally to be phosphorylated (rat S40). Note that in zebrafish sequence there is also a serine residue in the same position. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

described by Fischer et al. (2005) to be involved in MICs uptake, exist experimental evidence indicating that intracellular GSH plays an important role (Hagenbuch and Meier, 2003). Thus, other possible cause of GSH depletion due to MICs exposure could be its exchange with the toxin. From this point of view, the simple entrance of MICs in the cells triggers the efflux of GSH through the same transport system, a mechanism useful for the toxin, since not only its entrance is allowed, but also the capacity of the cell to perform the detoxification reaction through GST activity is lowered because of the GSH efflux.

3. How microcystins induce oxidative stress in aquatic organisms?

Several hypotheses can be postulated at present about the "how" issue. Some of them are probably related to the link between the hyperphosphorylation state of the cell after MICs exposure and how it can affect the antioxidant system. Although some researchers have stated that antioxidant enzymes are mainly regulated at transcriptional level (Gehringer et al., 2004), increasing evidences are suggesting that post-transcriptional modifications can also modulate the activity of antioxidant enzymes (Rhee et al., 2005). A search of phosphorylation sites of important antioxidant defenses was performed by using NetPhos, version 2.0 (http://www.cbs.dtu.dk) (Blom et al., 1999), a kinase-specific prediction tool of protein phosphorylation sites. This computer tool was used on protein primary sequences of the zebrafish (*Danio rerio*, Cyprinidae), a well-known model of aquatic organism. As shown in Table 2, several possible phosphorylation sites do exist in the antioxidant system of zebrafish. Under this scenario two points should be considered: (1) the reliability of NetPhos prediction and (2) the effects of phosphorylation in antioxidants enzymes activities. The comparison of the data found in NetPhos with experimental evidences reported in literature for different biological models shows the considerable precision of the computer tool. For example, Huang et al. (2002) experimentally demonstrated the phosphorylation of Ser 40 of the transcriptional factor Nrf-2 from rats by PKC. Kwak et al. (2004) showed that phosphorylation induce an increase in mean life of rat Nrf2, a transcription factor involved in the expression of a number of antioxidant and phase II detoxifying enzymes (Kobayashi et al., 2009). Applying the NetPhos to the Nrf-2 sequence from rat used by Huang et al. (2002) (NP_113977.1; NCBI), among several other possible sites, Ser 40 appears as a predicted one for phosphorylation with a high score (0.919). Interestingly, the alignment of zebrafish Nrf-2 sequence (NP_878309.1; NCBI) with the rat Nrf-2 sequence indicates similar phosphorylation sites, including the same site experimentally demonstrated in rat (Ser 40) that is also a phosphorylation target in zebrafish, as shown in NetPhos prediction (Fig. 1). The same kind of comparison was done using sequences of zebrafish and human peroxiredoxin 1 (Prx1). Peroxiredoxins are a family of peroxidases that reduce H_2O_2 and alkyl hydroperoxides to water and alcohol, respectively, with the use of reducing equivalents provided by thiol-containing proteins (Hofmann et al., 2002). Chang et al. (2002) found that phosphorylation of Prx1 on Thr90 by CDKs reduced the peroxidase activity of this protein by 80% in HeLa cells. The analysis of human Prx1 sequence (NP_859048.1; NCBI) in NetPhos also predicted this threonine residue as a site for phosphorylation with a high score (0.966). The analysis of zebrafish Prx1 (CAP09310.1; GenBank) sequence in NetPhos shows that Thr89 has a high score for phosphorylation (0.976). When the two sequences were aligned, it became evident that Thr90 of human sequence corresponds to Thr89 of zebrafish sequence, thus confirming similarities in the phosphorylation sites in the rat and zebrafish models (Fig. 2). From these data, it is possible that MICs exposure in aquatic organisms can augment the mean life of Nrf2 (triggering an antioxidant response) and diminish the peroxidase activity through phosphorylation of Prx1.

Besides the prediction indicated by NetPhos for several phosphorylation sites in different antioxidant enzymes (Table 2), experimental evidences also indicate that these enzymes are subjected to post-transcriptional regulation, i.e., phosphorylation. Sun et al. (1996) and Toroser et al. (2006) showed that phosphorylation of catalytic subunit of glutamate cysteine ligase (GLC), triggered by PKA and PKC, affected the enzymatic activity, mainly by lowering Vmax. GCL activity is the rate-limiting step in the *de novo* synthesis of GSH and its expression can be modulated by a number of different factors, including GSH depleting agents, reactive oxygen and nitrogen species, cytokines, and hormones (White et al., 2003). Gehringer et al. (2004) demonstrated that GCL transcription was induced 8 h after a single dose (75% LD) of MICs i.p. injection in rats, what contributed to the returning of GSH concentration to the control levels 24 h post MICs exposure. The same study indicated that GST and glutathione peroxidase (GPx) presented transcriptional increases after MICs exposure, responses that should contribute to cellular detoxification.

If we consider that MICs induce a cellular hyperphosphorylation state, the results found by Gehringer et al. (2004) are in agreement with Kwak et al. (2004) who described an increase in GCL, GST and GPx expression when the transcription factor Nrf2 presented an augmented mean life after phosphorylation. However, the same cellular hyperphosphorylation state would lead to the phosphorylation of the new transcripted GCL, reducing its efficiency in promoting GSH synthesis (Sun et al., 1996; Toroser et al., 2006). For GST and GPx, there are experimental evidence that phosphorylation increases its activities (Lo et al., 2004; Okamura et al., 2009; Cao et al., 2003a,b). Yet

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NP_859048.1 MSSGNAKIGHPAPNFKATAVMPDGQFKDISLSDYKGKYVVFFFYPLDFTF 50
CAP09310    MSAGNAKIGQPAPQFKATAVV-DGQFKDIQLSDYRGKYVVLFFYPLDFTF 49
            **:*******:***:******: ********.****:*****:********* 

NP_859048.1 VCPTEIIAFSDRAEEFKKLNCQVIGASVDSHFCHLAWVNTPKKQGGLGPM 100
CAP09310.1  VCPTEIIAFSERAAEFRKIGVELIAASTDSHFSHLAWINTPRKQGGLGSM 99
            **********:** **:*:. ::*.**.****.****:***:******.* 
                                                  ↑

NP_859048.1 NIPLVSDPKRTIAQDYGVLKADEGISFRGLFIIDDKGILRQITVNDLPVG 150
CAP09310.1  NIPLVADLTQSISRDYGVLKEDEGIAYRGLFVIDDKGILRQITINDLPVG 149
            *****:* .::*::****** ****::****:***********:****** 

NP_859048.1 RSVDETLRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVQKSKEYFSKQK 199
CAP09310.1  RSVDETLRLVQAFQHTDKYGEVCPAGWKPGSDTIVPDVQKSKEFFSKQ- 197
            **************.***:*************** ********:**** 
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Fig. 2. Comparison of human (*Homo sapiens*; NP_859048.1) and zebrafish (*Danio rerio*; CAP09310.1) Prx1 phosphorylation sites in primary protein sequences predicted by NetPhos (http://www.cbs.dtu.dk). Highlighted in yellow is the consensus sequence for phosphorylation. In green are highlighted consensus sequences for phosphorylation that overlap in the two sequences. Indicated in red is the amino acid residue target for phosphorylation. The arrow indicates the amino acid residue shown experimentally to be phosphorylated (human Thr90). Note that in zebrafish sequence there is also a threonine residue in the same position. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

both enzymes need GSH as substrate. Therefore, besides all other MICs toxic effects described, it could be summed up the futile expense of ATP in the synthesis of proteins that probably do not have the optimum cellular concentration of its co-susbtrate GSH. Moreover, it should be remembered that another via of ATP stores depletion in MICs exposed cells could be through the mitochondrial damages exerted by this toxin (see Section 2), that can lead exposed cells to energy failure. In fact, Zhang et al. (2007) demonstrated that fish lymphocytes incubated with MIC-LR had ATP content depleted with the increasing of exposure time. In this study, after 4 h of incubation with MIC-LR (10 nM) ATP content decreased about 50%.

4. Chemoprevention strategies against oxidative stress generated by microcystins

As MICs generate oxidative stress mainly by inducing an increase in ROS production associated with a depletion of antioxidant defenses, a chemical which is capable to induce an increase in antioxidant molecules synthesis could provide protection of organisms exposed to this toxin. A potential useful molecule, lipoic acid (LA) and its reduced form, dihydrolipoic acid (DHLA), seems to fulfill several criteria that an antioxidant must possess: they are ROS scavengers, they have metal-chelating activity, participate in the recycle of other antioxidants and in the repair of damaged molecules induced by oxidative stress (Packer et al., 1995). Also, the redox potential of DHLA/LA ($E0' = -0.32$ V) is more negative than that of the reduced/oxidised glutathione (GSH/GSSG) and cysteine/cystine (CSH/CSSC) couples ($E0' = -0.24$ V and -0.22 V, respectively) (Hermes-Lima, 2004). Due to its redox potential, the pair DHLA/LA can reduce CSSC to CSH and GSSG to GSH and in this last case, could be an alternate system to glutathione reductase activity. Previous studies have shown that LA ameliorates the toxic effects of compounds like arsenic in terms of brain lipid peroxidation (Shila et al., 2005).

Furthermore, valuable features of lipoic acid may be related to the transcription factor Nrf2 which, as previously mentioned, controls the expression of genes that are important to the antioxidant defense system (Kwak et al., 2004; Lee and Surh, 2005). The migration of transcription factors like Nrf2 from the cytoplasm to nucleus is the key process for the expression of important genes to the antioxidant defense, including those coding for the catalytic subunit of GCL and several GST isoenzymes (Lee and Surh, 2005). Authors as Suh et al. (2004) clearly showed that rats exposed to LA augmented the nuclear concentration of Nrf2 factor, promoting higher proteins levels of the catalytic subunit of GCL. In fish species, the study of Monserrat et al. (2008) reported that adding LA in a commercial diet reduced protein oxidation in liver and muscle, and augmented GCL activity in brain and liver.

Chemopreventive strategies must be also considered from a more ecotoxicological point of view. In a natural condition, intoxication by cyanobacteria is not necessarily only due to toxic effects of MICs. Several findings indicate that cyanobacterial blooms contain as-yet-unidentified compounds and other known components that can evoke more pronounced toxic effects than MICs or other well recognized toxins (Oberemm et al., 1997, 1999; Pietsch et al., 2001; Burýsková et al., 2006). Burýsková et al. (2006) exposed *Xenopus laevis* embryos to five different fractions of complex cyanobacterial blooms. Fractions containing complex biomass or aqueous extract

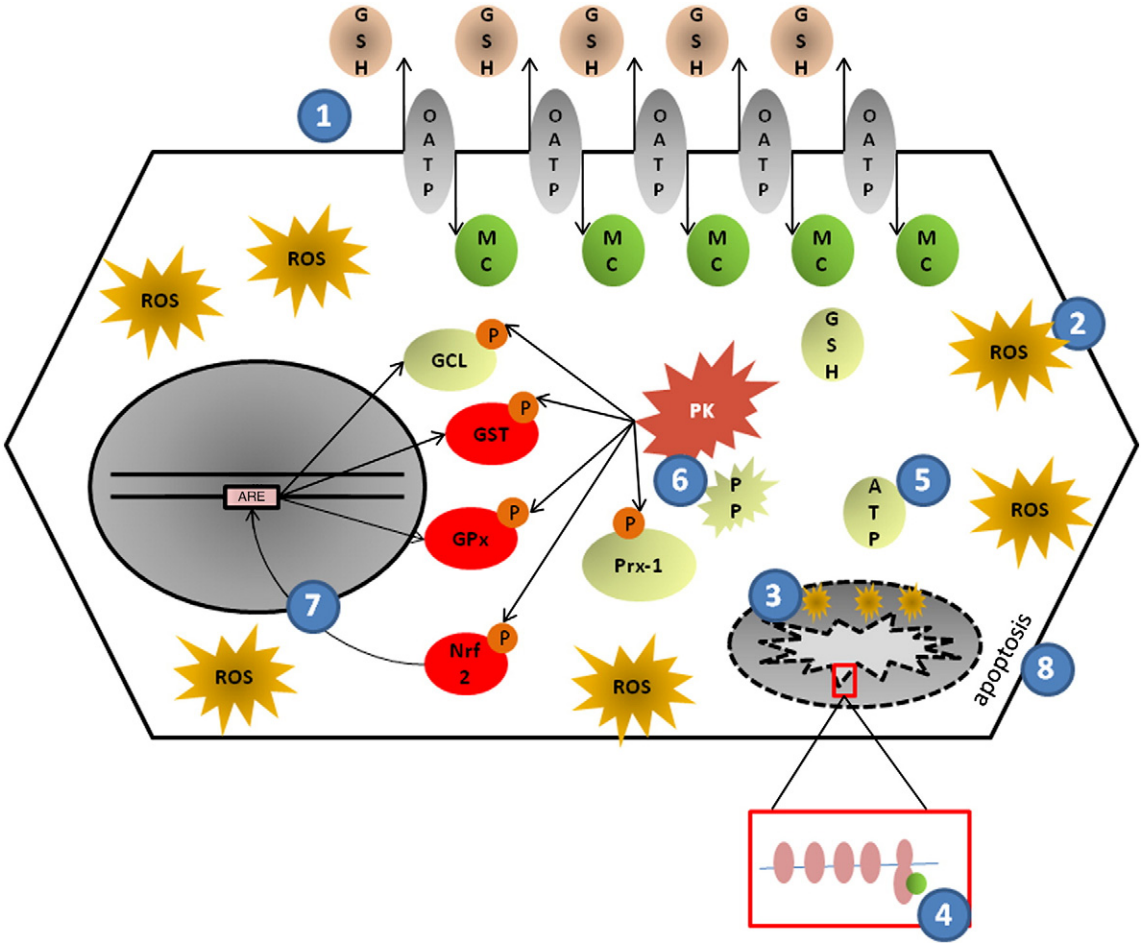

Fig. 3. Hypothetical model of MIC induced toxicity based in literature data. For a detailed explanation see the text. MC: microcystins; GSH: reduced glutathione; OATP: organic anion transporting polypeptide; ROS: reactive oxygen species; PK: protein kinases; PP: protein phosphatases; GCL: glutamate cysteine ligase; GST: glutathione-S-transferase; GPx: glutathione peroxidase; Nrf-2: NF-E2-related factor-2; ARE: antioxidant responsive element.

were generally more toxic in terms of embryos mortality and growth inhibition whereas eluates, containing MICs were generally less toxic. Also, MICs had no significant effect on glutathione pool and enzymatic activity of GPx, GST and GR. These results indicate that MICs are not the only or major toxic component in complex cyanobacterial samples. In fact, Best et al. (2002) demonstrated that lipopolyssacharides (LPS) of cyanobacteria and heterotrophic bacteria that can be associated to cyanobacterial blooms, can reduce GST activity. This effect could have implications for both animal and human health, as GSTs participate not only in the MICs detoxication pathway (Pflugmacher et al., 1998) but also in the *in vivo* metabolism and detoxication of numerous other toxic compounds.

5. Conclusions

In light of literature data and hypothesis discussed herein, we suggest some important events (Fig. 3) that could occur in MICs intoxication and still need experimental corroboration:

1. MICs are incorporated into the cell via OATPs which uses GSH as driving force for the exchange with MICs. In this way, MICs incorporation *per se* could induce loss of intracellular GSH reducing the detoxification capability of the cell;
2. As GSH is the first line of defense against ROS, its cell efflux should increase ROS generation.
3. As mitochondrion depends on cytoplasmatic GSH, the depletion of cytosol GSH should reflect in a decreased GSH concentration inside mitochondria, a situation that favors ROS production and ETC disruption. Oxidative stress through oxidation of intracellular GSH favors the PTP opening, leading to membrane potential depolarization and mitochondrial permeability transition (MPT);
4. MICs can bind to the beta subunit of ATP-synthase, what could reduce ATP synthesis and contribute to the intensification of the mitochondrial membrane depolarization, disruption of ETC and ROS generation;
5. Mitochondrial disruption leads to an impairment of ATP production, affecting all cellular process that depend on ATP, such as *de novo* GSH synthesis;
6. Besides all this effects MICs inhibit protein phosphatases, leading to a cellular hyperphosphorylation state;
7. Hyperphosphorylation state together with the pro-oxidative environment inside the cell should favor the Nrf2 migration to the nuclei, promoting the transcription of genes involved in antioxidant response, such as GST, GPx and GCL. Phosphorylation and ATP depletion inhibits GCL activity, favoring the continuous depletion of GSH; phosphorylation activates GST and GPx, but the low GSH concentration limits their activities impairing even more cellular detoxification for MICs and H_2O_2. Finally, Prx inhibition by phosphorylation contributes to the lowering of ROS detoxification capability;
8. All these events together cause alteration in redox status of the cell and mitochondrial disruption what could lead to the release of cytochrome *c* which, in turn, activates signaling cascades to apoptosis.

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