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Microcystin-producing blooms—a serious global public health issue ☆

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Abstract

The investigation on microcystin topics is increasing due to the related ecological and public health risks. Recent investigation confirms a gap in establishing global patterns relating a particular environment to the bloom occurrence of a species and the production of certain microcystin variants. All the results concerning the environmental effects on the microcystin synthesis of one species must be checked in the light of genome diversity. Thus, the poisoning risks of a bloom depend on the strain causing toxicity. To be more effective, specific water treatment methods are required for blooms of different microcystin producing species (such as colonial and filamentous cyanobacteria found in stratified and unstratified water bodies, respectively). With the increasing number of new microcystin variants discovered, the development of new rapid, inexpensive and sensitive enough monitoring methods to promptly screen simultaneously a great diversity of toxins and also check their toxic effects is becoming necessary.

Keywords: Cyanobacterial blooms; Microcystin; Synthesis; Toxicity; Bioaccumulation; Occurrence; Monitoring; Removal and elimination

1. Introduction

The increase of human population and the consequent intensification of agricultural and industrial activities along with deficient water management have led to the enhancement of eutrophication in superficial freshwater bodies used for recreational purposes and as drinking water sources. The occurrence of phytoplanktonic blooms is also becoming more frequent worldwide. Environmental conditions such as higher temperature and pH values, low turbulence, and high nutrient inputs (particularly phosphorus (P), as well as nitrogen (N)) enhance the development of planktonic cyanobacteria in lakes and reservoirs, leading to formation of surface blooms that may accumulate as scum. The dominance of certain cyanobacteria at the surface is due to some advantageous characteristics such as lower nutrient (particularly nitrogen) requirements and buoyancy regulation in the water column for achieving better light and nutrient level conditions (Oliver and Ganf, 2000). The development of cyanobacterial blooms has become a serious problem because in the past decades many cyanobacteria have been reported to be able to produce secondary metabolites toxic to many organisms, including humans (Gorham and Carmichael, 1988; Codd et al., 1995; Codd, 2000; Briand et al., 2003; Haider et al., 2003; WHO, 2003). Cyanotoxins are very diverse in their chemical structure and toxicity (Dow and Swoboda, 2000; Kaebernick and Neilan, 2001; Briand et al., 2003), usually being classified as dermatotoxins (lipopolysaccharides, lyngbyatoxin-a, and aplysiatoxins), neurotoxins (anatoxin-a, homoanatoxin-a, anatoxin-a(s), and saxitoxins), and hepatotoxins (microcystins, nodularin, and cylindrospermopsin), according to the toxic effects

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on animals. Microcystins are hepatotoxins to which special attention has been given not only due to their ability to cause acute poisonings but also due to their cancer promotion potential by chronic exposure of humans to low microcystin concentrations in drinking water (Ueno et al., 1996; Zhou et al., 2002), making production of these toxins a serious public health issue.

The present study reviews some recent work made on microcystin toxicity on diverse organisms (including humans), factors influencing their production, and processes to eliminate them from drinking water. A retrospective concerning the occurrence of microcystin-producing blooms worldwide in the past 2 decades was also made.

2. Microcystin structure and synthesis

Microcystins are cyclic heptapeptides with the general structure cyclo(-D-Ala-L-X-erythro-β-methyl-D-isoAsp-L-Y-Adda-D-isoGlu-*N*-methyldehydro-Ala). The aminoacid Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) is considered responsible for the molecules hepatotoxicity (Dawson, 1998). There are more than 60 microcystin isoforms (Codd, 2000; Dow and Swoboda, 2000) in part due to the variable L-amino acids X and Y, but the most frequent and studied variant is microcystin-LR (MC-LR) with the variable amino acids leucine (L) and arginine (R). Other variants that also occur more frequently are MC-RR, MC-YR and MC-LA.

These toxins occur in freshwaters worldwide and are mainly produced by colonial *Microcystis* spp. and filamentous *Anabaena (An.)* spp., *Planktothrix*/*Oscillatoria* (*P. agardhii* and *P. rubescens*), *Anabaenopsis* spp., *Nostoc* (*N. rivulare*), *Aphanizomenon* (*Aph. flos-aquae*) but also species belonging to the terrestrial genus *Hapalosiphon* (Codd et al., 1995; Dow and Swoboda, 2000; Kaebernick and Neilan, 2001). MC-LR, in particular, is known to be produced by species belonging to the genera *Anabaena*, *Microcystis*, *Nostoc* and *Anabaenopsis* (Dow and Swoboda, 2000; WHO, 2003) and MC-YR is produced by *Microcystis aeruginosa*, *Microcystis viridis* and *Hapalosiphon* spp. (Dow and Swoboda, 2000; WHO, 2003). MC-RR has been isolated from *Oscillatoria agardhii*, *Microcystis aeruginosa* and *M. viridis*, and MC-LA from *Microcystis aeruginosa* (Dow and Swoboda, 2000). There are no conclusive studies about the purpose of microcystins (secondary metabolites) synthesis but some results indicate that it may act as a chemical defence against grazing (Kurmayer and Jüttner, 1999; Henning et al., 2001) or have an allelopathic effect over algal competitors (Kearns and Hunter, 2001) in addition to regulating endogenous protein phosphatases or being used as nitrogen reserve.

Microcystins are produced nonribosomally through a microcystin synthetase complex (Kaebernick and Neilan, 2001) and their synthesis is an energy (ATP)-dependent process (Bickel and Lyck, 2001). The synthesis enzymatic complex is codified by an *mcy* genes cluster composed by two operons (*mcyA–C* and *mcyD–J*) (Kaebernick and Neilan, 2001) and it is present in toxic strains of the genus *Microcystis* but also in microcystin-producing strains of *Anabaena*, *Nostoc* and *Planktothrix* (Neilan et al., 1999), allowing the development of rapid and sensitive *polymerase chain reaction* (PCR) methods for its detection directly from environmental samples (Tillett et al., 2001; Pan et al., 2002). Despite many contradictory studies, there are some factors that have been shown to influence microcystin synthesis. In general, microcystin synthesis increases with light intensity or photosynthetically active radiation (Rapala et al., 1997; Rapala and Sivonen, 1998; Kaebernick et al., 2000; Hesse and Kohl, 2001; Kaebernick and Neilan, 2001; Wiedner et al., 2003) but light quality is also a determinant factor (red light favors toxin production while blue light does not) (Kaebernick et al., 2000). Moreover, there are maximum irradiance values above which microcystin production is inhibited (Wiedner et al., 2003). Yet, some recent studies (Böttcher et al., 2001; Hesse and Kohl, 2001) concluded that light intensity variation in natural environments has little or no significant effect on microcystin cellular content and the differences found in between toxicity of blooms of one same species are mainly due to the growth rates and toxic characteristics of different strains. Temperature has been shown to influence the type of toxin produced with high temperatures ($>25^{\circ}C$) enhancing MC-RR production and lower temperatures favoring MC-LR synthesis (Rapala et al., 1997; Rapala and Sivonen, 1998). In the non-nitrogen-fixing cyanobacterium *M. aeruginosa*, microcystin content increases at higher N:P ratios (Utkilen and Gjølme, 1995; Lee et al., 2000) but Long et al. (2001) reported that fast cell growth of *M. aeruginosa* under N-limited conditions is associated with smaller cells and consequent by higher intracellular microcystin production. The growth of *Microcystis* spp. is also enhanced by increasing phosphorus concentrations (Utkilen and Gjølme, 1995; Rapala and Sivonen, 1998; Kotak et al., 2000; Oh et al., 2000) as well as the microcystin content in *Microcystis aeruginosa* (Jacoby et al., 2000; Kotak et al., 2000), although Oh et al. (2000) documented higher values of microcystin content in *M. aeruginosa* under more P-limited conditions. There are many controversial results concerning the effects of nitrogen and phosphorus concentrations on microcystin content of cyanobacteria but microcystin production in *Microcystis* strains seems to be influenced by variation in nitrogen and phosphorus concentrations with different responses depending on the considered strain (Vézie et al., 2002).

Microcystin content in *Anabaena* spp. has shown an increase at higher phosphorus and nitrogen concentrations (Rapala et al., 1997), although in nitrogen-free medium N-fixing cyanobacteria could still produce more microcystin than the non-nitrogen-fixing cyanobacteria, probably due to the fact that N-fixing cyanobacteria are less dependent on N concentrations. Lukac and Aegerter (1993) found that zinc (Zn) enhanced growth and microcystin production in *M. aeruginosa*, and low iron (Fe) concentrations decreased growth but increased toxin synthesis. Utkilen and Gjølme (1995) obtained contradictory results (probably due to the use of a different strain) in which a decrease in the iron concentration decreased the microcystin content, and microcystin synthetase should be actively controlled by the amount of available free Fe^{2+}. Bickel and Lyck (2001) suggested that if microcystin synthesis requires energy (as ATP), the variation of toxin production should be mostly explained by the energetic state of the cyanobacterial cells, with nutrient limitation (P, N and Fe) and light variation having only an indirect influence, since cell energetic state changes under stress conditions. Under circumstances of low levels of energetic charge, available energy in cells is primarily applied in essential protein synthesis and not in microcystin (secondary metabolite) synthesis (Bickel and Lyck, 2001). Recent approaches pose genotype diversity between strains as the main factor determining the variability in toxicity levels in blooms of the same species (Rohrlack et al., 2001; Kurmayer et al., 2002), with the development and success of strains better adapted to certain environmental conditions. The genotypes may differ in growth strategy, plasmid content, interaction with zooplankton, microcystin content (Hesse and Kohl, 2001) and microcystin synthetase genes cluster, originating different variants of the toxin with different toxicities (Mikalsen et al., 2003).

3. Microcystin toxicity and bioaccumulation

Microcystins are known to affect many organisms, from microalgae to mammals. MC-LR, in particular, is able to paralyze the motile green alga *Chlamydomonas reinhadtii*, enhancing its settlement and creating a lake zone free of competitors for microcystin-producing cyanobacteria (Kearns and Hunter, 2001). The lack of alternative phytoplankton for food when cyanobacteria dominate may contribute to unfavorable nutritive conditions for zooplankton (Kurmayer and Jüttner, 1999) despite the ingestion of *Microcystis* colonies by zooplankton, which can also be affected by colony size and/or mucilage and toxin content (Henning et al., 2001). While calanoid copepods avoid cyanobacteria that possess microcystins, daphnid cladocerans are less selective (Kurmayer and Jüttner, 1999), being able to ingest both toxic and nontoxic *Microcystis* (Rohrlack et al., 1999) under depletion of edible food (green algae and diatoms), accumulating the microcystins (Mohamed, 2001) and potentially transferring them to higher trophic levels through the food chain. But toxic effects have also been observed in *Daphnia* spp. after cell-bound microcystin ingestion (Rohrlack et al., 2001) including the inhibition of protein phosphatases PP1 and PP2 (Henning et al., 2001). The brine shrimp *Artemia salina*, a crustacean, was shown to be sensitive to MC-LR (Delaney and Wilkins, 1995), leading to an increased detoxification system glutathione *S*-transferase (GST) activity and conjugation of the toxin to glutathione via this GST, as the first step to microcystin detoxification (Beattie et al., 2003).

Macrophytes such as *Phragmites australis* (Pflugmacher et al., 2001), *Ceratophyllum demersum* and *Elodea canadensis* (Wiegand and Pflugmacher, 2001) have been shown to absorb MC-LR. In *P. australis* the higher values for absorbed MC-LR were found in the stem and rhizome, with an increase in soluble glutathione *S*-tranferases (sGST) (Pflugmacher et al., 2001). In the bryophyte *Vesicularia dubyana*, MC-LR absorption was higher than that in the two macrophytes (Wiegand and Pflugmacher, 2001). Microcystins also cause a reduction in the number and mass of fronds in the water plant *Spirodela oligorrhiza* (Romanowska-Duda and Tarczynska, 2002) and MC-LR is known to affect the physiology (including growth) of the white mustard *Sinapis alba* seedlings (McElhiney et al., 2001; Hamvas et al., 2003). Crop plants (for human consumption) that are irrigated with microcystin-contaminated water may suffer growth and development effects—in addition to accumulating the toxins and therefore posing the potential risk of toxin transference to humans through the food chain. The salad lettuce (*Lactuca sativa*) grown with spray irrigation of water containing microcystin-producing *M. aeruginosa* also retains microcystins (Codd et al., 1999). Under laboratory conditions, microcystins proved to be both inhibitors of growth and development in potato shoots and mustard seedlings (McElhiney et al., 2001) and inhibitors of plant protein phosphatases.

Mussels, crayfish, and fish used for human consumption may also accumulate microcystins and pose intoxication hazards to human consumers in such a way that microcystins should always be monitored during and after the occurrence of estuarine cyanobacterial blooms. The mussel *Mytilus edulis*, fed on *Microcystis aeruginosa* (with high microcystin content) for 3 days, accumulated microcystins in its tissues (Williams et al., 1997). In another mussel, *Mytilus galloprovincialis*, microcystins were quickly accumulated but its depuration was not a very rapid process with microcystin persistence even after the bloom disappearance, probably due to recontamination by feces containing

the toxins (Amorim and Vasconcelos, 1999). Microcystins have also been shown to be retained in some gastropods through grazing activity (Kotak et al., 1996). The crayfish *Procambarus clarkii* accumulates microcystins in the intestine and hepatopancreas (Vasconcelos et al., 2001). With regard to fish, it has been documented that low concentrations of microcystins cause hepatopancreas and kidney damage in european carp (*Cyprinus carpio*) (Fischer and Dietrich, 2000) and the rainbow trout (*Oncorhynchus mykiss*) suffers hepatotoxicosis by accumulating MC-LR that leads to changes in cellular morphology, protein phosphatases inhibition, and liver necrosis (Fischer et al., 2000). A study using embryos of zebrafish (*Danio rerio*) showed that MC-LR is absorbed by the embryos (Wiegand and Pflugmacher, 2001). In another study, embryos and larvae of the loach (*Misguruns mizolepis*), a small freshwater fish, were shown to be affected by toxicity of MC-LR which targets their liver and heart (Liu et al., 2002). Oberemm also reported that the young life stages of fish were more sensitive to microcystin hepatotoxic effects than adults or juveniles (Oberemm, 2001a). The freshwater fish *Oreochromis niloticus* accumulates microcystins in the guts, liver and kidneys (Mohamed et al., 2003) but there are fish that can also accumulate these toxins in the muscle tissue, posing high risks to humans that consume contaminated fish (Magalhães et al., 2001). From an ecological point of view, probably to allow adaptability, some fish, for example, the zebrafish (*Danio rerio*), show changes in their behavior (reduced motility, increased rates of activity at night, reduced activity during the spawning period, and reduced reaction on feeding) when exposed to long-term sublethal doses of MC-LR (Baganz et al., 2001). These changes can have reproductive effects with substantial ecological consequences such as reducing population growth and changing species composition of the water body (Baganz et al., 2001).

With regard to terrestrial animals, the African locust *Locusta migratoria migratorioides* has been shown to be sensitive to MC-LR (Hiripi et al., 1998) with an LD_{50} value of 0.2 μg per animal or 130 mg kg^{-1}. Microcystins are also known to cause liver necrosis in birds (ducks) (Matsunaga et al., 1999).

In mammals, microcystins are selective for hepatic cells, irreversibly inhibiting serine/threonine protein phosphatases PP1 and PP2A (Dawson, 1998) and causing disintegration of hepatocyte structure, apoptosis, liver necrosis, and internal hemorrhage in liver that may lead to death by hemorrhagic shock (Dow and Swoboda, 2000). MC-LR seems to bind also to ATP synthetase, potentially leading to cell apoptosis (Mikhailov et al., 2003). Orally ingested microcystins are transported across the ileum into the bloodstream via a bile-acid transporter that exists in hepatocytes and cells lining the small intestine. Microcystins bind specifically to hepatocytes (the reason why they concentrate in the liver) and are actively absorbed to hepatic cells (Dawson, 1998; Dow and Swoboda, 2000). In the hepatocytes, they form adducts with PP1 and PP2A from cytoplasm and nuclei, inhibiting them and leading to disruption of liver cell structures, intrahepatic hemorrhage and death if a high dose is administrated (Fitzgerald, 2001). Microcystins seem not to be hydrolyzed by stomach peptidases and MC-LR appears to be absorbed by the intestine (Dow and Swoboda, 2000). In mice, the intraperitoneal (i.p.) LD_{50} value for MC-LR is usually 50 μg kg^{-1} of body weight (Dow and Swoboda, 2000) but it can range from 25 to 125 μg kg^{-1} (Dawson, 1998; WHO, 1998). Although the inhalation toxicity of MC-LR is also high (Dawson, 1998), this toxin is much less toxic by oral ingestion (Ito et al., 1997), with LD_{50} of 5000 μg kg^{-1} in mice (WHO, 1998). MC-YR has an LD_{50} (i.p., in mice) of 70 μg kg^{-1} and MC-RR has an LD_{50} (i.p., in mice) of 300–600 μg kg^{-1} (WHO, 2003). In swine, the lethal (i.p.) dose of MC-LR is 72 μg kg^{-1} and acute toxicosis results from severe intrahepatic hemorrhage with the blood flow being obstructed through the liver causing hypovolemic shock, severe hypoglycemia and/or terminal hyperkalemia (Beasley et al., 2000). Some of the symptoms characteristic for microcystin poisoning are weakness, anorexia, cold extremities, pallor, apathy, respiratory problems, gastroenteritis, vomiting and diarrhoea (Codd et al., 1995; Codd, 2000; Dow and Swoboda, 2000) with necrosis of the liver that may lead to death by hemorrhagic shock or liver failure after some hours or days, depending on the species (Gorham and Carmichael, 1988). Nevertheless, by inhibiting PP1 and PP2A, two important enzymes involved in tumor suppression, microcystins chronically administered have shown to promote liver cancer in mammals (Ito et al., 1997) by inducing oxidative DNA damage (Zegura et al., 2003). Mice exposed to a sub lethal dose of MC-LR by intraperitoneal (i.p.) injections developed multiple neoplastic nodules in the liver (Ito et al., 1997) despite the finding that oral administration evidenced no chronic injuries. Yet, chronic effects (increased liver weight and hepatohistological damage) have been detected in rats after treatment with low concentrations of microcystins in drinking water for 28 days (Heinze, 1999) and a recent study added that microcystins chronically administered may also induce kidney damage in rats (Milutinovi et al., 2002).

4. Public health risks

Human exposure to microcystins may occur through a direct route such as drinking water (Ueno et al., 1996; WHO, 1998; Zhou et al., 2002), recreational water (WHO, 2003) or hemodialysis (Pouria et al., 1998), or through an indirect route such as food (Williams et al.,

1997; Amorim and Vasconcelos, 1999; Codd et al., 1999; Schaeffer et al., 1999; Magalhães et al., 2001). The knowledge about microcystin effects on humans is based on epidemiologic data, but there are also reports of intoxications and toxicological studies made on laboratory animals. The symptoms observed for laboratory mammals are thought to be similar to those felt by humans, despite the lack of studies in this area. Thus, epidemiological studies are the basis for human poisoning assessment and from the many worldwide cases reported until now it is demonstrated that microcystins cause acute (WHO, 1998) and chronic (Ueno et al., 1996; Zhou et al., 2002) effects on humans and even death (Pouria et al., 1998). Acute intoxication by microcystins coincides frequently with the lysis of the bloom-forming cells (by natural senescence or water treatment processes) and liberation of toxins to the water. The inhalation of dry cyanobacteria cells or contaminated water is more dangerous than oral ingestion of contaminated water indicating the hazardous potential of practising aquatic sports in recreational waters that suffer a microcystin producing bloom (WHO, 2003). As shown before, MC-LR is a potent cancer promoter in laboratory animals. Thus, chronic exposure to low concentrations of microcystins in drinking water can be a serious problem to public health, contributing to promotion of cancer in humans. Epidemiological studies have already related the presence of microcystins in drinking water to an increase in the incidence of colorectal cancer (Zhou et al., 2002) and primary liver cancer (Ueno et al., 1996). There are groups more sensitive to microcystin poisoning that require special attention such as B-hepatitis patients but also children and old people (Fitzgerald, 2001).

Due to the rapid, irreversible and severe damage that microcystins cause in the liver, therapy is difficult and prophylaxis is complicated. In 1988, Gorham and Carmichael referred immediate gastric lavage as the possible treatment if effective antidotes were unavailable. However, in the past 15 years, several experimental studies about attenuation of animal and human intoxication by microcystins, showing interesting results, were made (Dawson, 1998; Fitzgerald, 2001). Some are based on monoclonal antibodies against MC-LR (Nagata et al., 1995) and others on hepatic uptake blockers such as the immunosupressant Cyclosporine A and the antibiotic rifampin (Dawson, 1998). Recent studies such as at by Gehringer et al. (2003) show that the membrane-active antioxidant vitamin E, taken as a dietary supplement, may protect against toxicity of MC-LR by chronic exposure.

5. Guidelines for MC-LR

The danger of tumor promotion by chronic exposure of microcystins in drinking water was the main reason for the definition of guidelines for these toxins by the World Health Organization (WHO). The lifetime safe consumption level proposed was of $1\,\mu g\,L^{-1}$ for MC-LR (WHO, 1998; Fitzgerald, 2001) and it was based on animal studies of MC-LR orally administered to pigs and mice (Fitzgerald, 2001). Many countries (such as Brazil, New Zealand, and UK) have adopted this value as a guideline for drinking water but Canada proposed the value $1.5\,\mu g\,L^{-1}$ and Australia proposes values ranging from 1.3 to $10\,\mu g\,L^{-1}$ (USEPA, 2001). In Canada, there was also proposed a value of $10\,\mu g\,L^{-1}$ for short-term exposure (Fitzgerald, 2001). For recreational waters with cyanobacterial blooms, WHO has established three health hazard alert levels, depending on the risk of adverse health effects (WHO, 2003) and these are based on cyanobacterial densities. For cyanobacterial food supplements, there is a MC-LR proposed guideline of $10\,\mu g\,g^{-1}$ (Schaeffer et al., 1999) and, in Oregon, USA, there has been established a maximum value of $1\,\mu g\,g^{-1}$ for food (USEPA, 2001).

6. Monitoring methods

Methods based on high pressure liquid chromatography (HPLC) (Poon et al., 2001; Spoof et al., 2001) are the most widespread quantitative and sensitive methods for detection of microcystins and other cyanotoxins, allowing the distinction between microcystin variants and also its isolation. Yet, they are expensive and time consuming, and require a considerable sample volume for low concentrations; also there are few available certified standards for MC variants and usually purification or concentration of the sample is required (Tsutsumi et al., 2000; Nicholson and Burch, 2001). The recently developed method MALDI-TOF-MS (matrix assisted laser desorption/ionization—time of flight mass spectrometry) has been used for the analysis of many peptides, including cyanobacterial secondary metabolites (e.g., antibiotics or toxins such as microcystins (Fastner et al., 2001; Welker et al., 2002)). It requires only microgram quantities (not milligram quantities like HPLC or bioassays) of cell material and the detection is rapidly made, without the need for time-consuming extraction or purification processes, allowing the identification of known microcystin variants and other unknown metabolites which can be further characterized (Fastner et al., 2001; Welker et al., 2002).

Enzyme linked immunosorbent assays (ELISA) are based on monoclonal (Zeck et al., 2001) and polyclonal (Metcalf et al., 2000; Yu et al., 2002) antibody actions against microcystin structure. They have low equipment requirements and allow rapid, easy, effective and sensitive detection of microcystins (particularly MC-LR) in water samples (Nicholson and Burch, 2001), microorganisms, and animal tissues, but toxicity is not

assessed. Thus, these assays can be used only as a semiquantitative screening tool. The problem of cross-reactivity with nontoxic compounds (leading to false positives) has been minimized with competitive ELISA methods which may have detection limits of $0.07\,\mu g\,L^{-1}$ (Zeck et al., 2001) or even less, making ELISA suitable for assessing microcystin concentrations below the WHO guideline of $1\,\mu g\,L^{-1}$ in drinking water. Protein phosphatase inhibition assays (PPIA) are based on immunodetection and on the toxic effects of microcystins at a molecular level, i.e., on the ability of microcystins to specifically inhibit the PP1 and PP2A, despite that toxin transport into the cells is neglected and that there is no direct relationship with mammalian toxicity. Many PPIA were shown to overestimate the toxin concentrations and, for that, they are presently used just as a screening method. The colorimetric PPIA assay is a rapid, easy and sensitive screening method that does not require much equipment and that is less expensive than ELISA or radiolabeled PPIA (which uses both PP1 and PP2A) although in this assay only PP1 is used. Nevertheless, it is an assay which correlates positively with HPLC (Wirsing et al., 1999; Metcalf et al., 2001) and there are recent options for detecting MC-LR in drinking water with detection limits below the WHO guideline of $1\,\mu g\,L^{-1}$ (Bouaícha et al., 2002). Competitive binding assays based on blockage of the active site of PP2A have also been developed for microcystins (Serres et al., 2000) and there are immunoblotting procedures based on anti-microcystin-LR monoclonal antibodies to monitor the formation of microcystin-PP1 adducts in vitro and in vivo (Liu et al., 2000).

Bioassays based on *Aeromonas hydrophila, Bacillus cereus* and *B. subtilis* have been shown to be sensitive and suitable for assessing toxicity of *M. aeruginosa* extracts (Ostensvik et al., 1998). There are many plants such as *Spirodela oligorrhiza* (Romanowska-Duda and Tarczynska, 2002), *Solanum tuberosum* (McElhiney et al., 2001) and *Sinapis alba* (McElhiney et al., 2001; Hamvas et al., 2003) that have been shown to be sensitive to microcystins and that may be used to assess toxicity of these toxins. Bioassays using *Daphnia* spp. (Tarczynska et al., 2001; Kim et al., 2003) and *Artemia salina* (Delaney and Wilkins, 1995; Sabour et al., 2002) have also become frequently used to assess microcystin toxicity. Test kit bioassays using larvae of the freshwater crustacean *Thamnocephalus platyurus* (Torokne et al., 2000) or using the African locust (*Locusta migratoria migratorioides*) (Hiripi et al., 1998) showed reaction to microcystins despite the toxic responses not being specific. Fish embryo tests using *Danio rerio* (zebrafish) have been demonstrated to be sensitive against cyanobacterial metabolites, in relation to adults or juveniles, probably due to their thin epithelia, large ratio of body surface to volume of embryos and vulnerability of developmental processes (Oberemm, 2001b). Mouse bioassays are the most used bioassays for determination of LD_{50} values and symptoms and effects of microcystins in mammals, and they allow distinguishing between hepatotoxins and neurotoxins. Adult mice are usually injected intraperitoneally with the sample and according to sample toxicity different intoxication symptoms are observed usually within 24 h. There are many studies using this kind of bioassay (Ito et al., 1997; Sedmak and Kosi, 1997; Vasconcelos and Pereira, 2001; Oudra et al., 2002; Sabour et al., 2002). Rat (Sekijima et al., 1999) and swine (Beasley et al., 2000) bioassays have also been used to assess microcystin toxicity. Nevertheless, these bioassays do not detect low microcystin levels nor distinguish between microcystin variants (Nicholson and Burch, 2001). In response to the inherent ethical questions of the *in vivo* mammal bioassays, the *in vitro* studies (Heinze et al., 2001; Zegura et al., 2003) have been adopted as a more ethical and sensitive alternative for toxicity bioassays. The use of freshly prepared rat hepatocyte bioassays as an *in vitro* test system (with semiquantitative microscopic assessment of cell damage) seems to be promising in assessing toxicity of the cyanobacterial bloom samples, showing a strong correlation with the analytical data from HPLC (Heinze et al., 2001), despite the operational requirements such as the preparation of cell suspensions (Nicholson and Burch, 2001). Along with the analytical methods such as HPLC, bioassays are still an important tool for assessing the toxicity level of the known cyanotoxins or the presence of additional unknown toxic substances. Thus screening methods such as ELISA, PPIA or bioassays should always be combined with more sophisticated methods such as HPLC or MALDI-TOF (Nicholson and Burch, 2001).

Methods based on *Polymerase chain reaction* (PCR) are a recent approach to the detection of pathogen microorganisms in natural environments and they are being proposed as means both to rapidly determine whether a cyanobacterial bloom or a determined species is potentially toxic and to quantify toxic cyanobacteria by designing primers based on *mcy* genes (Rudi et al., 1998; Tillett et al., 2001; Pan et al., 2002).

7. Microcystin removal and elimination processes

The removal of cyanobacterial cells by flocculation or filtration methods has proven to be an effective method to reduce toxin levels in water but only if there is no cell lysis and liberation of microcystins to the water. If toxins are released, other methods such as activated carbon adsorption and ozonation are required to effectively eliminate dissolved microcystins from drinking water. Hence, methods that lead to cells lysis are not advisable and should be avoided in drinking water

treatment plants. For the removal of cell-bound microcystins, flocculation by ferric chloride seems not to cause cyanobacterial lysis nor an increase in dissolved microcystin concentrations for *M. aeruginosa* and *An. circinalis* (Chow et al., 1998). Slow sand filtration has also been shown to efficiently remove cell-bound microcystins from drinking water (Grutzmacher et al., 2002). Microcystins show stability in deionized water, in sterilized water and under irradiation by sunlight (Dawson, 1998) or under extreme temperatures ($>300°C$) and pH (WHO, 1998). Thus, in natural environments, microcystins must be instable due to biodegradation and indirect photodegradation. For the dissolved microcystin elimination, some of the bacteria known to degrade microcystins are gram-negative and oxidase-positive with low catalase activity (Welker et al., 2001). *Sphingomonas* sp. is a bacterium that degrades MC-LR through microcystinase, a constitutively expressed metallo-protein that is produced even in absence of the toxin (suggesting its hydrolytic activity over other peptides) (Bourne et al., 2001). Other *Sphingomonas*-like bacteria can also degrade MC-YR and MC-RR and MC-LR (Park et al., 2001). In natural environments, photodegradation of microcystins occurs indirectly via pigments or humic substances that absorb the sunlight (Welker et al., 2001). Microcystins may be also photodetoxificated by UV irradiation (Kaya and Sano, 1998) and their rapid photocatalytic degradation can be achieved through a reactor with immobilized titanium dioxide catalyst (Shephard et al., 2002). In the environment, microcystin detoxification seems to be enhanced by adsorption on sediments (Tsuji et al., 2001) but the elimination of microcystins in slow sand filtration filters is probably due to biodegradation rather than adsorption (Grutzmacher et al., 2002). Presently, most drinking water treatment plants have methods such as ozonation, activated carbon filtration and chlorination that allow the removal of the majority of microcystins (but not all) in superficial waters (Tsuji et al., 1997). Yet, ozonation effectiveness in microcystin destruction has been shown to be reduced by high levels of total organic carbon and high cyanobacterial densities (cells lyse with ozonation, increasing the dissolved toxin level) (Hoeger et al., 2002). Particularly wood-based activated carbons efficiently adsorb microcystins from aqueous solutions (Pendleton et al., 2001) but clay material also effectively removes microcystin-LR from water by adsorption of the toxin (Morris et al., 2000). Chlorination, using adequate sodium hypochlorite doses after cell removal, seems to be very effective for the elimination of microcystin-LR in raw water with no formation of noxious products from the process (Tsuji et al., 1997). Microcystins may be efficiently decomposed and removed from waters with high total organic carbon by ferrate oxidation–coagulation (Yuan et al., 2002) and Fenton oxidation of MC-LR by Fenton reagent has been shown also to be a promising method for rapid degradation of this kind of hepatotoxins (Gajdek et al., 2001).

8. Occurrence of microcystin-producing blooms

Since the past century many hepatotoxic blooms have been documented and have been recently reviewed by several authors such as Gorham and Carmichael (1988), Codd et al., (1995), WHO (1998, 2003), Codd (2000), Briand et al. (2003) and Haider et al. (2003). The cases discussed here are restricted to the past 20 years.

In Europe, during the past decade, several Portuguese freshwater bodies (including lakes, reservoirs and rivers), used for recreational or drinking purposes, have been found to have hepatotoxic blooms with production of diverse microcystins (MC-LR, MC-RR, MC-YR, and others) mainly associated with the dominance of *M. aeruginosa* (Vasconcelos et al., 1996; Vasconcelos, 2001; Vasconcelos and Pereira, 2001). In France, microcystins have been detected in Lake Grand-Lieu (Vézie et al., 1998) and Saint-Caprais reservoir (Maatouk et al., 2002) and were produced by *M. aeruginosa* and *Aph. flos-aquae*, respectively. In 1995, near Liège, Belgium, three adjacent ponds suffered a *M. aeruginosa* bloom with microcystin production related to bird deaths (Wirsing et al., 1998). Although cyanobacterial blooms usually accumulate as surface scum in eutrophic water bodies, there may be also the formation of dense mats of benthic cyanobacteria such as the *Oscillatoria* and *Phormidium* bloom that has been reported to occur in the oligotrophic, cold and turbid alpine waters of southeastern Switzerland, showing hepatotoxic (by microcystins) and neurotoxic effects in mice (Mez et al., 1997). Germany has studies from recent years that indicate that many German water bodies used for recreational or drinking water purposes were in their majority dominated by cyanobacteria from genera *Planktothrix*, *Microcystis*, *Anabaena* and *Aphanizomenon*, with microcystin and anatoxin-a production (Hummert et al., 2001; Wiedner et al., 2001; Frank, 2002) and having implications on the growth of fish (Ernst et al., 2001). In the past decade, many Czech recreational and drinking water reservoirs and fish ponds were found to be dominated by *Microcystis* spp., *P. agardhii* and *Aph. flos-aquae* that produced microcystins (Marsálek et al., 2001). High microcystin levels were detected as well in raw waters and some treated waters from drinking water treatment plants, endangering the consumers' health (Bláha and Marsálek, 2001). During 1995 and 1996, three eutrophic Latvian lakes (Lakes Mazais, Lielais Balterzers and Sekitis) had summer blooms of potentially toxic *M. aeruginosa* (with production of microcystins), *Aph. flos-aquae* and *An. flos-aquae*, leading to a decrease in drinking water quality and health problems resulting

from the recreational use of the lakes (Eynard et al., 2000). Northeastern Slovene freshwaters have suffered many blooms with *M. aeruginosa* dominance and microcystin production (Sedmak and Kosi, 1997). In the past 20 years, the Loch Leven, in Scotland, had several hepatotoxic blooms of *M. aeruginosa* and *Anabaena flos-aquae* with the report of more than 1000 dead fish (with liver necrosis) that accumulated in the shores after the senescence of an *A. flos-aquae* bloom (Codd et al., 1995). In the United Kingdom, *Microcystis* blooms in lakes and reservoirs have been associated with the death of sheep and dogs and with human illness due to microcystin production (Dow and Swoboda, 2000; WHO, 2003). In Sweden, between 1991 and 1997, microcystins were detected in some lakes with dominance of *M. aeruginosa*, *M. viridis* and *P. prolifica* (Willén et al., 2000) and in a water treatment plant (Codd et al., 1995), and the MC found in a river with a *P. agardhii* bloom caused the intoxication of pets and 121 persons (WHO, 2003). A study based on dozens of south Norwich water bodies revealed the occurrence of many microcystin-producing blooms of *Anabaena* spp., *Microcystis* spp. and *Oscillatoria* (*Planktothrix*) spp. (Utkilen et al., 2001). In the past decade, an intensive study on hundreds of freshwater bodies from Denmark reached the conclusions that the majority of blooms had microcystin production by *Microcystis* spp., *Anabaena* spp., *P. agardhii* and *Aph. flos-aquae* (Henriksen, 2001). Moreover, the deaths of thousands of fish and a cow were related to hepatotoxic blooms of *Anabaena flos-aquae* and *P. agardhii*, respectively (Henriksen, 2001).

In North America, Canada has frequent microcystin occurrence and drinking water is the main poisoning exposure route with lakes used as drinking water sources suffering from *M. aeruginosa* blooms which have MC-LR concentrations higher than the WHO guideline, in both raw and treated tap waters (Gupta et al., 2001). In the United States of America there have been reported hepatotoxic blooms of *M. aeruginosa* (Puschner et al., 1998; Jacoby et al., 2000) related to animal deaths (24 heifers) (Puschner et al., 1998), and food supplements made on natural *Aph. flos-aquae* blooms were found to have high microcystin levels (Schaeffer et al., 1999).

With regards to South America, in 1996, Caruaru, Pernambuco state, Brazil, the deaths of 60 patients from a hemodialysis unit were related to microcystin intoxication due to the use of water from a reservoir suffering a bloom of *Anabaena* spp. and *Microcystis* spp., along with insufficient treatment to eliminate the microcystins from that water (Pouria et al., 1998). In January 2000, the Cargalheiras Reservoir, one of the man-made reservoirs of the semiarid region of Rio Grande do Norte State (Northeast Brazil), suffered a massive bloom of *Pseudanabaena schimdlei* (with the presence of *M. aeruginosa* also) which led to the death of almost all fish in the reservoir (Chellappa et al., 2000). This bloom was related to the increasing eutrophication of the reservoir due to the intensification of aquaculture practice (Chellappa et al., 2000). The state of Paraná, Brazil has frequent occurrence of microcystin-producing *Microcystis* spp. blooms in freshwater lakes and reservoirs used for recreational and animal farming purposes but also in some used as drinking water supplies (Hirooka et al., 1999). The Patos Lagoon estuary, Rio Grande do Sul, southern Brazil, suffers regular blooms of *Microcystis* and microcystin is synthesized during their occurrence (Matthiensen et al., 2000). In Conception, Chile, *Microcystis* spp. blooms with the presence of microcystins have been reported in different lakes (Campos et al., 1999; Neumann et al., 2000).

In Oceania, specifically at Swan-Canning estuary, in Western Australia, during February 2000 there was a dense and severe *M. aeruginosa* bloom (Atkins et al., 2001). Another hepatotoxin (cylindrospermopsin) occurrence seems to be very frequent in Australia (Fitzgerald, 2001).

With regard to the Asian countries, various brackish and freshwater bodies in South Korea including dams and lagoons used as drinking water sources showed dominant species belonging to *Microcystis* genus, but also to *Anabaena* and *Planktothrix*/*Oscillatoria* genera, with production of microcystins and anatoxin-a (Oh et al., 2001; Park, 2001). In China, lakes suffering from *Anabaena* and *Oscillatoria* blooms revealed the presence of microcystins (Xu et al., 2000) and a significant correlation between microcystin-producing bloom occurrence in the superficial drinking water sources (ponds and rivers) and primary liver cancer incidence has been reported (Ueno et al., 1996). Over the past 20 years, in Japan, microcystins were detected in various naturally occurring *Microcystis* blooms (Tsuji et al., 1996; Park et al., 1998; Matsunaga et al., 1999) and in one case they have caused the death of dozens of ducks (Matsunaga et al., 1999). Microcystins have also been found in several strains of *M. aeruginosa* isolated from eutrophic aquaculture ponds and water reservoirs in Taiwan (Lee et al., 1998). In the Philippines, during past years, Laguna de Bay suffered periodic blooms of *M. aeruginosa* and many variants of microcystins were detected (Cuvin-Aralar et al., 2002).

From Africa, in July of 1995, in Egypt, River Nile (used as drinking water source) at Sohag province suffered an *Oscillatoria tenuis* bloom with production of microcystins (Brittain et al., 2000). In Morocco, many ponds, lakes, and reservoirs proved to have several cyanobacterial microcystin producing strains belonging to the genera *Microcystis*, *Synechocystis*, *Pseudanabaena*, and *Oscillatoria* (Oudra et al., 2002; Sabour et al., 2002). In South Africa, there has been reported a contamination of drinking water by the presence of *M. aeruginosa* (with confirmed MC-LR synthesis) and

its relation to a livestock poisoning outbreak (Van Halderen et al., 1995).

9. Concluding remarks

After reviewing the more than 100 reports on microcystin investigation cited in this review paper, some main ideas should be highlighted and kept. The relationship between the worldwide increasing eutrophication of water bodies and the intensification of the worldwide occurrence of microcystin-producing blooms mainly dominated by *M. aeruginosa* strains is well established, although other species of *Microcystis* and of other genera such as *Aphanizomenon*, *Anabaena*, and *Planktothrix* may codominate with *M. aeruginosa*. Nevertheless, this review also included reports on microcystin-producing blooms occurrence in oligotrophic water bodies and rivers (dominated by cyanobacteria belonging to the genera *Oscillatorial Planktothrix* and *Phormidium*), illustrating the diversity of ecological features among toxic cyanobacteria, which makes difficult the implementation of general cyanobacterial blooms control strategies. Moreover, from the most recent scientific results one may conclude that there is no global pattern in the blooms occurrence and the toxicity of a certain species. Rather than being due to the environmental parameters variation, the explanation for the differences found between the toxicity of blooms of one same species relies on the genotypic diversity of different strains and on the various growth and toxic characteristics of each one of those strains. However, despite all the controversial results, microcystin variants are secondary metabolites whose synthesis is known to be somehow regulated by factors such as light intensity and quality, temperature and nutrients and trace metal concentrations.

The occurrence of hepatotoxic microcystin producing blooms may lead to serious poisoning episodes such as those ones reviewed. Microcystins are known to affect microalgae, zooplankton, aquatic and terrestrial plants, fish and terrestrial insects, birds and mammals, among other organisms. Moreover, microcystins bioaccumulation has been reported for several organisms including crop plants (irrigated with contaminated water) and mussels, crayfish, and fish (grown in estuarine contaminated water) used for human consumption. This poses a great concern for public health safety due to the cancer promotion potential of microcystins (MC-LR, in particular) by the chronic ingestion of trace amounts of this toxin indirectly through food or directly through the drinking water. Thus, world organization WHO has established the guideline of $1\,\mu g\,L^{-1}$ for MC-LR in drinking water as the life time safe consumption level and many countries have followed (with slight changes) this guideline for drinking water and food as well. With such a low guideline value several monitoring methods had also to be developed in order to achieve the necessary sensitivity level and the most currently used include the expensive and sensitive HPLC and MS methods and the rapid, screening and less expensive ELISA and PPIA methods. However, new detection methods such as the *in vitro* bioassays (as a more ethical and sensitive alternative to the *in vivo* bioassays) are becoming necessary due to the enormous diversity of cyanotoxins and the need to test their potential toxic effects.

Despite of all the new technology, microcystin removal and elimination processes are greatly dependent on the producing cyanobacteria type. Flocculation or slow sand filtration steps are effective for the removal of cell-bound microcystins (by removing the cells from the water without their lysis) but for the dissolved microcystins, ozonation and activated carbon adsorption are efficient methods to eliminate these hepatotoxins from the water. The recently developed PCR methodology is also a promising methodology to detect the potentially toxic cyanobacteria directly from environmental samples, allowing the development of specific bloom control and toxin elimination strategies for that particular strain with a higher probability of success. Thus, investigation should become more and more interdisciplinary in its approache with regard to the toxic cyanobacterial blooms occurrence and toxicity, in order to achieve better results in the mitigation of the microcystin-poisoning episodes. It is very important that the characterization of the ecotoxicological features of a certain bloom is done in relation to the specific strain found as dominant. Only then one may establish specific patterns for blooms occurrence of that strain worldwide and define specific strategies for its growth control, toxin elimination and lessening of toxic effects on organisms.

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