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Review

The Natural Degradation of Microcystins (Cyanobacterial Hepatotoxins) in Fresh Water – the Future of Modern Treatment Systems and Water Quality Improvement

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Abstract

The increasing occurrence of hepatotoxic cyanobacterial blooms in fresh waters is one of the most serious risks to human health in the 21st century. Therefore, proper management of waters contaminated with cyanobacteria requires elaboration of the appropriate methods to remove cyanobacterial hepatotoxins-microcystins (MCs) from the environment. Many chemical and physical procedures have been proposed to remove MCs from fresh waters, but more attention is now paid to more environmentally friendly and cost-efficient techniques for MCs reduction. Therefore, this paper presents a summary of the current knowledge concerning the possible use of natural processes with the participation of environmental bacteria for MCs degradation in fresh water.

Keywords: microcystins, bacterial degradation, *Sphingomonas* sp., fresh water

Introduction

Increasing environmental degradation, including changes in biogeochemical cycles due to increased anthropopressure and climate change, has contributed to accelerated eutrophication on a global scale. An increasing occurrence of toxic algal and cyanobacterial blooms, commonly known in the scientific community as Harmful Algal Blooms (HABs), is one of the negative consequences of this condition. In the global perspective, cyclic hepatotoxins-microcystins (MCs) – represent the most common group of cyanobacterial toxins (cyanotoxins) [1]. They are produced by a diverse range of cyanobacteria, but *Microcystis*, *Planktothrix* (Oscillatoria), and *Anabaena* represent the genera that most frequently occur in freshwater environments [2-4]. MCs pose a risk to the environment because they are able to affect vegetation growth, animal and human metabolism, deteriorate health, and can even threaten life [5]. Therefore, the first step toward reducing the hazard of HABs should include the development of monitoring systems and methods to determine MCs-producing cyanobacteria and their hepatotoxins. The literature provides many descriptions of proposed monitoring schemes, including biological, chemical, biochemical, and molecular methods [6-14]. Another step in proper management of toxic cyanobacterial blooms should include elaboration of appropriate methods to remove cyanobacterial

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toxins from the environment. Many chemical and physical procedures have been proposed for removal of MCs from fresh water (drinking water bodies in particular), such as adsorption on active carbon, chlorination, and oxidation using permanganate or hydrogen peroxide, photolysis, and ozonation [4]. However, they have certain limitations, both in terms of:

1) efficiency of MCs removal and formation of toxic intermediates
2) investment and operating costs that restrict their application in production.

Therefore, more attention should be paid to environmentally friendly and cost-efficient techniques to reduce the eutrophication process and incidence of HABs [15-18]. Such a systematic approach is promoted by Ecohydrology, which is a sub-discipline of hydrology that focuses on ecological processes occurring within the hydrological cycle and strives to utilize such processes for enhancing environmental sustainability [19]. Reduced eutrophication and its consequences, such as occurrence of toxic cyanobacteria, can be achieved by decreasing nutrient availability through reduction of nutrients load from both point sources (e.g. sewage treatment plants) and non-point (diffuse) sources in the river basin. Methods using natural processes to control and reduce intensity of eutrophication are currently being developed and tested, including within the ecohydrological research performed under a UE Project: "ECOtones for Reducing Diffusion Pollution" (EKOROB LIFE08 ENV/PL/000519). However, we are not always able to stop the eutrophication process at a satisfactory rate. Therefore, the second element of the HAB management system should include development of methods that can facilitate elimination of cyanotoxins that have already formed in the aquatic environment. According to some literature reports, microorganisms play an important role in the removal of cyanobacterial hepatotoxins. For this reason, in this publication we would like to summarize current knowledge concerning the possible use of natural processes with the participation of environmental bacteria to degrade MCs in fresh waters.

Microcystins – Cyanobacterial Hepatotoxins

Environmental Properties

Heptapeptide hepatotoxins-microcystins (MCs) represent the type of cyanobacterial toxins that are most frequently found in fresh and brackish waters worldwide. They are produced by multiple genera of cyanobacteria: *Microcystis* spp., *Anabaena* sp., *Oscillatoria* spp., *Planktothrix* spp., *Chroococcus* sp., *Nostoc* sp., *Hapalosiphon* sp., *Anabaenopsis* sp., *Aphanocapsa* sp., *Pseudoanabaena* sp., and *Phormidium* spp. [20-22].

Cyanobacteria responsible for MCs production are characterized by a wide range of adaptation in different climates and environments. Moreover, global climate change, which has contributed to increases in water temperature and severe droughts, results in massive cyanobacterial blooms in many water bodies in which they previously had not been observed [23, 24]. Production of MCs is also stimulated by other abiotic environmental factors, such as nitrogen (N) and phosphorus (P) concentrations, pH, or sunlight. However, decisive parameters are specific to individual water bodies [25-29]. The effects of N and P on MCs production are highly variable and sometimes contradictory. For instance, Codd and Poon [30], Oh et al. [27], and Kameyama et al. [31] found that decreasing concentrations of P increased MCs production by *Microcystis* sp. On the other hand, the environmental studies of Izydorczyk et al. [32] and a laboratory study of Giani et al. [33] revealed a positive trend between weight-specific MCs content and increasing $P\text{-}PO_4$. Based on culture experiments, some authors have shown that the MCs content of *Microcystis aeruginosa* was positively correlated with N content in the growth medium. However, Kotak et al. [36], based on the preliminary laboratory study, suggested that MCs production is not a response to N limitation stress.

Additionally, MCs production also depends on biotic parameters. One of them influencing MCs production is zooplankton grazing, which was suggested by Jang et al. [37]. In the experiment performed by Jang et al. [37], direct exposure to zooplankton caused mass-specific MCs productions in *Microcystis* strains (61.5-177.3 μg/g dry cell), which was up to five times greater than the production in the controls. This finding confirms the hypothesis that MCs may act as compounds that prevent grazing [38].

Nevertheless, changes in MCs production by cyanobacteria in the context of natural ecosystems are still not fully understood.

Chemical Properties

MCs have a basic cyclic structure of cyclo-(D-Ala(1)-X(2)-D-MeAsp(3)-Z(4)-Adda(5)-D-Glu(6)-Mdha(7)) (MW= 994.55 g/mol), in which X(2) and Z(4) are variable L amino acids (Fig. 1). The amino acid Adda (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) is a unique structure in the group of hepatotoxins [1]. Additionally, the Adda moiety was reported to be crucial for biological activity of MCs molecules, which lose their toxic properties without it [39]. Until now, more than 90 MCs variants have been isolated and characterized [21]. The MCs isoforms differ primarily due to two L-amino acids and, secondarily, due to the presence or absence of the methyl groups on D-erythro-methylaspartic acid (D-MeAsp) and/or N-methyldehydroalanine (Mdha) [39]. Some nontoxic variants have also been identified, such as the compound containing the 6Z-stereoisomer of Adda [1]. Variation of amino acids at positions 2 and 4 (X and Y) provide the basis for MCs nomenclature, for instance MC-LR comes from leucine (L) at position 2 and arginine (R) at position 4 (Fig. 1). The variation in chemical structure of MCs influences diversity of their biological activity (toxicity). MC-LR is a variant that is most frequently found in the environment and therefore has been intensively examined in order to elucidate the toxicological mechanism for MCs [40-41]. It is also considered to be the most toxic variant reported as it was established that the LD50 reached 50 μg per kg

of body weight in mice when pure MC-LR was injected intraperitoneally, whereas in the example of the MC-RR, the LD50 reached 600 μg [21]. Hydrophobic properties of MC-LR molecules also have a positive impact on its biological activity by increasing mobility of MC-LR by cell membranes [41]. The remaining respondents also represent other hydrophobic variants, such as MC-LW and MC-LF.

Function

Many authors have discussed specific MCs function in the metabolism of various types of cyanobacteria. However, knowledge concerning the biological roles of these cyanotoxins and the specific reason for their production remains insufficient. Production of MCs is very energy-consuming, which may suggest that they perform important functions in cyanobacterial metabolism [42]. Some of the MCs functions proposed include: defence mechanism against plankton grazers [37, 43], ferrous iron (Fe^{2+}) chelation [44, 45], and allelopathic function related to cyanobacterial interactions with phytoplankton, zooplankton, and bacteria [46]. MCs are located in thylakoids, and due to this reason Young et al. [47] suggested that they may also play a part in photosynthesis. It was also proposed that MCs may play a part in gene regulation [48, 49]. Some authors also suggested that MCs may act as extracellular signalling molecules [50-52]. Their presence in water bodies may be sensed as a symptom indicating environmental stress and cell disintegration, and stimulate MCs biosynthesis in the remaining cells.

Biological Activity and Toxicity

Due to their high chemical stability and water solubility, MCs have important implications for their environmental persistence and exposure to the human population. MCs are inhibitors of eukaryotic protein serine/threonine phosphatases 1 and 2A (PP1 and PP2A). Inhibition of these enzymes leads to hyperphosphorylation of cytoskeletal proteins, which results in deformation of hepatocytes. Massive intrahepatic centrilobular hemorrhaging may contribute to liver enlargement [53], which may lead to intensive bleeding into the liver and cause death due to hemorrhagic shock [54]. MCs are able to get into the human body through consumption of food and water (plants, fish, shellfish etc.) and, to a lesser extent, through inhalation and skin contact [55, 56]. Generally, there are three points at which MCs in drinking water may have adverse effects on human health:

(i) acute or chronic toxic injury to the liver and other tissues
(ii) tumor promotion leading to liver or intestine cancer
(iii) the possibility of genotoxicity and carcinogenesis [57-60]

In small doses administered as a result of recreational activity in MC-containing water, they can cause: diarrhea, gastrointestinal disorders, dizziness, and vomiting, as well as skin rashes.

Additionally, MCs have been shown to affect growth and physiological functions due to their bioaccumulation in organisms co-occurring with MC-producing cyanobacteria, both of animals (e.g. fish *Cyprinus carpio*, *Oncorhynchus mykiss*, *Perca flavescens*, *Oreochromis niloticus*, mollusks *Unio*, *Anodonta*, *Dreissena*) and plants (e.g. *Lemna minor*, *Wolffia arrhiza*) [61-67]. MCs were found in phytoplankton, zooplankton, and gastropods (*Lymnaea stagnalis*, *Helisoma trivolvis,* and *Physa gynna*), as well as in copepods or crab larvae [5, 68]. The presence of MCs in irrigation waters may cause plant growth inhibition (accumulation of MCs in: radish, arugula, lettuce, dill, parsley, and cabbage) and therefore economic losses [69].

Due to a serious risk caused by the presence of MCs in drinking water, the WHO proposed a provisional guideline for drinking water for MC-LR of 1 μg/L (free plus cell bound) [70] and guidelines for safe recreational water environments [56].

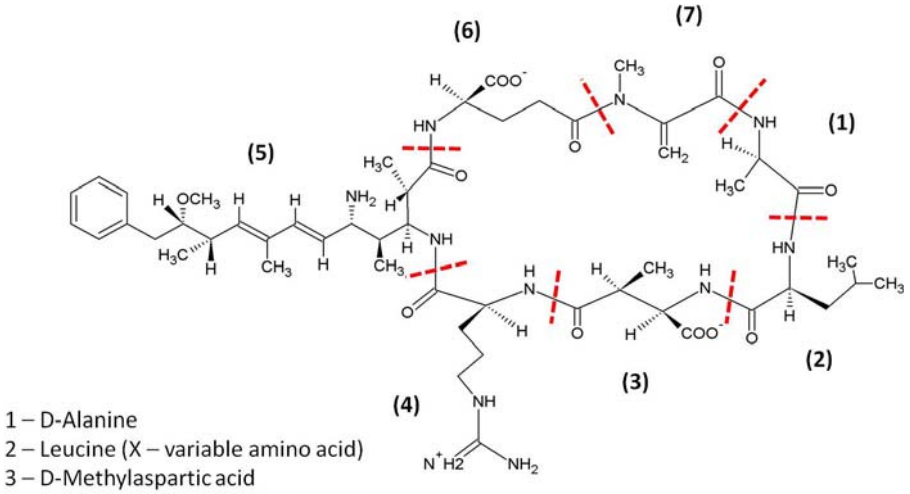

Fig. 1. The chemical structure of microcystin-LR (adapted from Bourne et al. [87]).

Stability and Decomposition

MCs are soluble in water, methanol, and ethanol, and insoluble in acetone, ether, chloroform, and benzene. The cyclic structure of MCs is probably responsible for their toxicity and stability in a wide range of the following factors: pH, temperature, and common proteases [71]. A series of laboratory experiments was conducted to determine their resistance to the above-mentioned factors. It was reported that they may survive even in extreme temperatures (>300ºC) for several hours. Additionally, if they are not exposed to sunlight and dryness, they may survive in low temperatures for many years [42, 72]. MCs were also reported to be resistant to irradiation by sunlight, but Tsuji et al. [73] showed the possibility of MC-LR and MC-RR degradation under UV light (238-254 nm) in less than 10 days. Another experiment also was conducted to check the stability of MCs under extremely low and high pH. The half-time of MC-LR at pH 1 reached 3 weeks, and at pH 10 took more than 3 times longer [74]. That experimental research showed that those hepatotoxins were stable compounds and only extreme physical and chemical conditions could lead to their rapid decomposition. Nevertheless, these experiments did not provide information concerning the fate of MCs in the natural environment, where such extreme conditions do not commonly occur.

In 1996 Cousins et al. [75] found that decomposition of MCs at a concentration of 10 μg/L took more than 27 days in deionized water, but in sterilized reservoir water it took only 12 days. However, after using water obtained directly from the lake, the MCs degradation rate was accelerated further and reached 7 days. That discovery raised the interest of researchers in the natural process of MCs decomposition.

Natural Degradation of Microcystins

Generally, the fate of MCs in natural aquatic environments may be categorized according to: dilution, adsorption, bioaccumulation, and degradation, which is sub-divided into:

(i) physico-chemical decomposition (e.g. photolysis)
(ii) biodegradation (e.g. bacterial degradation) [76]

The above-mentioned biodegradation and photodegradation (photolysis) seem to have the highest capability of complete inactivation of MCs in the natural aquatic environment, as dilution, adsorption, and bioaccumulation only reduce the level of toxins in water and do not eliminate their toxic properties. Moreover, when MCs are bioaccumulated, they can be transferred along the food chain from water into the terrestrial environment and intoxicate other organisms, including humans (see chapter "Biological Activity and Toxicity") [77]. Therefore, it is important to understand the natural processes occurring in the course of MCs biodegradation in order to elaborate methods to:

(i) remove MCs from the environment, including drinking and bathing waters
(ii) prevent their accumulation and transfer along the food chain

Table 1. Intermediate products of microcystin-LR photolysis with different sources of light (adapted from de la Cruz et al. [4]).

Source of light	Intermediate Product	MW	Source
UVC	4(Z)-Adda MC-LR	995.5	[72, 73]
	4(Z), 6(Z)-Adda MC-LR	995.5	
	6(Z)-Adda MC-LR*	995.5	
Sunlight	6(Z)-Adda MC-LR*	995.5	[72]
Sunlight and Pigments	6(Z)-Adda MC-LR*	995.5	[72]
	(OH)2Adda MC-LR (A)	1,029.5	
	(OH)2Adda MC-LR (B)	1,029.5	

*reversible process

Photodegradation

The effect of solar radiation has been considered as a potential source of MCs degradation [72]. In general, MCs are resistant to irradiation by sunlight due to the fact that the solar spectrum consists of wavelengths longer than 295 nm, while the absorbance of MCs occurs at wavelengths of λ=238-240 nm [71]. The compound needs to adsorb radiation at the same wavelength as the one emitted from the source to facilitate the process of photolysis [71]. Therefore, sunlight alone may not lead to significant MCs degradation. However, it may cause the isomerization of ex. MC-LR molecules to a less toxic 6(Z)-Adda MC-LR variant (Table 1). Unfortunately, it was proved that this reaction was reversible [72, 78, 79]. Nevertheless, photolysis of MCs can occur in the presence of other compounds, which can be photoexcited by the emitted solar radiation [71]. Tsuji et al. [73] found that the natural degradation of MCs by photodegradation with sunlight occurred in the presence of pigments and humic substances (which was confirmed by Welker et al. [80]) (Table 1). Pigments can act as photosensitizers through adsorption of sunlight in the range of λ=290 nm to visible light, forming ex. hydroxyl radicals and singlet oxygen, which are able to degrade MCs [73]. The half-life of MCs under exposure to sunlight with the presence of 1 mg/L of pigments was reported to reach 10 days under natural conditions [73]. However under UV irradiated with 1350 μW/cm^2, the half-life for MCs (10 mg/L) significantly decreased to only 1 minute [73]. This provided an excellent basis for the use of photodegradation methods by UV in water treatment stations [4]. However, in the natural environment, the degradation caused by solar radiation can be largely reduced, for example due to the turbidity of water reservoirs or self-shading caused by a large amount of organic matter, dense blooming, or the presence of macrophytes and higher plants. Therefore, the mechanism for removal of MCs from water must be based on other co-occurring processes. In this context, removal of MCs by microorganisms, indigenous bacteria in particular, seems the most likely process. Biodegradation of MCs with the partic-

ipation of indigenous environmental bacteria populations represents a recently studied new and promising method for removing MCs from fresh water without harming the environment.

Biological Degradation by Bacteria

Biodegradation with the participation of environmental bacteria is most likely to become the dominant process of MCs removal and detoxification in the water environment [77, 81]. One of the first reports was delivered by Lam et al. [82], who reported the degradation of MC-LR using a sewage effluent as inoculum. Jones et al. [83] and Cousins et al. [75] also found a method for natural removal of MC-LR in lake water. Jones and Orr [84] reported that the removal of MCs in the natural environment was most likely associated with a complex community of indigenous aquatic bacteria, which were using these molecules as a source of carbon (C) and nitrogen (N). Those initial results indicated that further studies should be conducted to:

(i) determine which bacteria were responsible for biodegradation
(ii) identify the mechanism of MCs degradation

This knowledge will help to elaborate solutions to remove MCs from different water sources.

The first case of identifying MCs degrading bacterial strain was reported from Australia, at the Murrumbidgee River by Jones et al. [83] (Table 2). *Pseudomonas* sp. was identified as the bacterial strain capable of degrading almost all the MC-LR and MC-RR in 3 days, following the initial lag phase (2-8 days). The bacteria from the genus *Pseudomonas* were reported later in Japan, in a reservoir managed by the Ohi water purification facility – Munakata, Fukuoka [85], and in China, in water from Lake Taihu used for an experiment with artificial media [86] (Table 2). In 1996, Bourne et al. [87] re-classified the *Pseudomonas* sp. strain described by Jones et al. [83] as *Sphingomonas* sp. ACM 3962 (MJ-PV) (Table 2).

In fact, the bacteria from the *Sphingomonadaceae* class represent the largest group of all the species that have been identified so far. Most reports concerning the presence of *Sphingomonas* sp. strains in the study water bodies came from Japan (Table 2). In 2001 and 2004 two *Sphingomonas* sp. strains (Y2 and 7CY) were isolated from Lake Suwa [88, 89] (Table 2). Subsequently, the Y2 strain was re-classified as a separate genera and species: *Sphingosinicella microcystinivorans* [90]. In 2003, Saitou et al. [91] identified the MD-1 strain in the water of Lake Kasumigaura (Table 2). The same strain was further isolated from the biofilm collected from the water treatment plant facilities, which also drew water from Lake Kasumigaura [92] (Table 2). The B-9 strain isolated from Lake Tsukui was another *Sphingomonas* sp. strain found in Japan [93] (Table 2). Other reports on the *Sphingomonas* genera have come from Argentina (San Roque Reservoir) and New Zealand (Lake Rototti) [81, 94] (Table 2). Nevertheless, bacteria from the other genera, which are classified as *Sphingomonadaceae*, were also reported. The new strains from *Sphingopyxis* genera were isolated from a biological sand filter, in which the water from Myponga Reservoir in Australia was filtrated (LH21 strain), and two Chinese lakes: Hongfeng (C-1 strain) and Dianchi (USTB-05 strain) [95-97] (Table 2). *Novosphingobium* sp. THN1 was the strain that was reported last, and was also found in Lake Taihu, China [98] (Table 2). Apart from MCs-degrading bacterial species, classified as *Sphingomonadaceae*, other were also found in Lake Taihu: *Methylobacillus* sp. (in sludge) [99] and *Stenotrophomonas* sp. EMS strain (in water) [76] (Table 2). Two other bacterial strains found in Asia included *Ralstonia solanacearum*, a plant-based pathogen grown on biocompatible (non-toxic) carbon nanotubes, which contributed to a significant removal of MCs from water [100], and *Bacillus* sp. AMRI-03 isolated from Lake Tendaha in Saudi Arabia [101] (Table 2).

In Europe, the first information on MCs disappearance from water in Lake Tuusulanjärvi in Finland came from Lahti et al. [102]. Nevertheless, the first bacterial strain was isolated from Lake Vihnusjärvi sediment in 2005, and was reported to be the novel bacterium *Paucibacter toxinivorans* [103] (Table 2). In Scotland three new bacterial strains, *Arthrobacter* sp., *Brevibacterium* sp., and *Rhodococcus* sp., were isolated form Loch Rescobie, Forfar Loch and River Carron [104, 105] (Table 2). Since then, no other strains (to the best of our knowledge) have been published in Europe.

Reports on the occurrence of MC-degrading bacteria also appeared in both Americas. In the USA, three species capable of MC-LR degradation were identified. The first one, *Morganella morganii*, which occurs in open environments and in the intestinal tracts of animals and people, was isolated from Lake Mead and from the biofilm originating from the active anthracite biofilter of the treatment plant receiving water from this lake [106] (Table 2). *Microbacterium* sp. and *Rhizobium gallicum* were two other novel strains observed in Lake Okeechobee, reported by Ramani et al. [107] (Table 2). Finally, in Patos Lagoon, Brazil, the genus *Burkholderia* was reported to degrade MCs, but the products of biotransformation maintained toxic properties [108, 109] (Table 2).

In addition to environmental research concerning MCs degradation, some strains of probiotic bacteria were also found capable of removing MC-LR from aqueous solutions [110]. Commercially available probiotic strains isolated from the dairy products: *Lactobacillus rhamnosus* GG and LC-705, *Bifidobacterium longum* 46, *Bifidobacterium lactis* 420, and *Bifidobacterium lactis* Bb12, as well as the *Lactobacillus plantarum*, i.e. IS-10506 and IS-20506 isolated from Indonesian traditional fermented milk (dadih), were found to be very effective in MCs removal [110, 111].

All these findings demonstrate that the process of bacterial degradation of MCs is widespread, but depending on a given environment it can be facilitated by various species of bacteria and is not limited to one route of degradation [4, 87].

Mechanism of Biodegradation

The process of MCs biodegradation by bacteria is now studied in detail (Fig. 2). It seems that the degradation pathway and enzymes that facilitate degradation can be differ-

Table 2. Microcystins-degrading bacteria (updated from de la Cruz et al. [4]).

Bacterium	Degraded MCs variants	Source	Country	References
Sphingomonas sp. ACM-3962 (MJ-PV)	LR, RR	lake water	Murrumbidgae River, Australia	[83, 87, 113]
Pseudomonas aeruginosa	LR	lake water	reservoir managed by Ohi water purification facility, Munakata, Fukuoka, Japan	[85]
Sphingomonas sp. Y2 (*Sphingosinicella microcystinivorans*)	LR, RR, YR, 6(Z)-Adda_MCLR	lake water	Lake Suwa, Japan	[88, 90]
Sphingomonas sp. MD-1	LR, RR, YR	lake water, biofilm from the water treatment plant	Lake Kasumigaura, Japana	[91, 92, 121]
Sphingomonas sp. B9 (*Sphingosinicella* sp.)	LR, RR, 3-DMMCLR, DHMCLR, MCLR-Cys	lake water	Lake Tsukui, Japan	[93, 114]
Sphingomonas sp. 7CY	LR, LY, LW, LF, NOD in the presence of RR	lake water	Lake Suwa, Japan	[89]
Ralstonia solanacearum	LR, RR	isolated plant pathogen	Japan	[100]
Paucibacter toxinivorans	LR, RR, NOD	lake sediments	Lake Vihnusjärvi, Finland	[103]
Sphingomonas sp. CBA4	RR	lake water	San Roque reservoir, Argentina	[94]
Sphingopyxis sp. LH21	LR, LA	lake water	Myponga Reservoir, Australia	[95]
Burkholderia sp.	LR	lake water	Patos Lagoon, Brazil	[109]
Arthrobacter sp.	LR, LF, LY, LW, RR	lake water	Loch Rescobie, Forfar Loch, River Carron, Scotland	[104, 105]
Brevibacterium sp.	LR, LF, LY, LW, RR	lake water	Loch Rescobie, Forfar Loch, River Carron, Scotland	[104, 105]
Rhodococcus sp.	LR, LF, LY, LW, RR	lake water	Loch Rescobie, Forfar Loch, River Carron, Scotland	[104, 105]
Methylobacillus sp.	LR, RR	sludge from cyanobacteria-salvaged yard	Lake Taihu, China	[99]
Pseudomonas sp.	LR, RR	lake water	Lake Taihu, China	[86]
Sphingopyxis sp. C-1	LR	lake water	Lake Hongfeng, China	[96]
Stenotrophomonas sp. EMS	LR, RR	lake water	Lake Taihu, China	[76]
Bacillus sp. AMRI-03	RR	lake water	Lake Tendaha, Saudi Arabia	[101]
Morganella morganii	LR	lake water and anthracite media from the Los Angeles filtration plant	Lake Mead, USA	[106]
Sphingopyxis sp.USTB-05	RR	lake water	Lake Dianchi, China	[97]
Sphingomonas sp. NV-3	RR, dME-RR, YR, LR, [Dhe7] LR	lake water	Lake Rotoiti, New Zeland	[81]
Microbacterium sp.	LR	lake water	Lake Okeechobee, USA	[107]
Rhizobium gallicum (AY972457)	LR	lake water	Lake Okeechobee, USA	[107]
Novosphingobium sp. THN1	LR	lake water	Lake Taihu, China	[98]

ent, depending on the bacterial strain. Until now (to the best of our knowledge), the degradation products were closely described for MC-LR and MC-RR (Fig. 2A). The first pathway of MC-LR degradation by the *Sphingomonas* sp. (MJ-PV) strains and genes involved in this process was described by Park et al.[88] and Bourne et al. [113] (Fig. 2). Bourne proposed four intracellular hydrolytic enzymes:

(i) "microcystinase," a putative metalloprotease (MlrA)
(ii) a putative serine peptidase 2 (MlrB)
(iii) a putative metalloprotease 3 (MlrC)
(iv) (MlrD) – a putative oligopeptide transporter, which takes part in the uptake of MCs into the cell

These enzymes were encoded by the 5.8 Kb gene cluster, including *mlrA*, *mlrB*, *mlrC,* and *mlrD* genes [113]. The MlrA enzyme, encoded by the gene *mlrA,* catalyzes the hydrolysis and opening of the ring at the Adda-Arg peptide bond (Fig. 2A). The product, i.e. MC-LR linearized (acyclo-) MC-LR, was reported to be 160-fold less reactive than the cyclic MC-LR. The MlrB encoded by the *mlrB* gene catalyzes the hydrolysis at the Ala-Leu peptide bond in linearized MC-LR (Fig. 2A). The MlrC encoded by the *mlrC* gene catalyzes the further hydrolysis to smaller peptides and amino acids (Fig. 2A). However, Bourne et al. [87] did not describe these smaller peptides in detail. Later, Harada

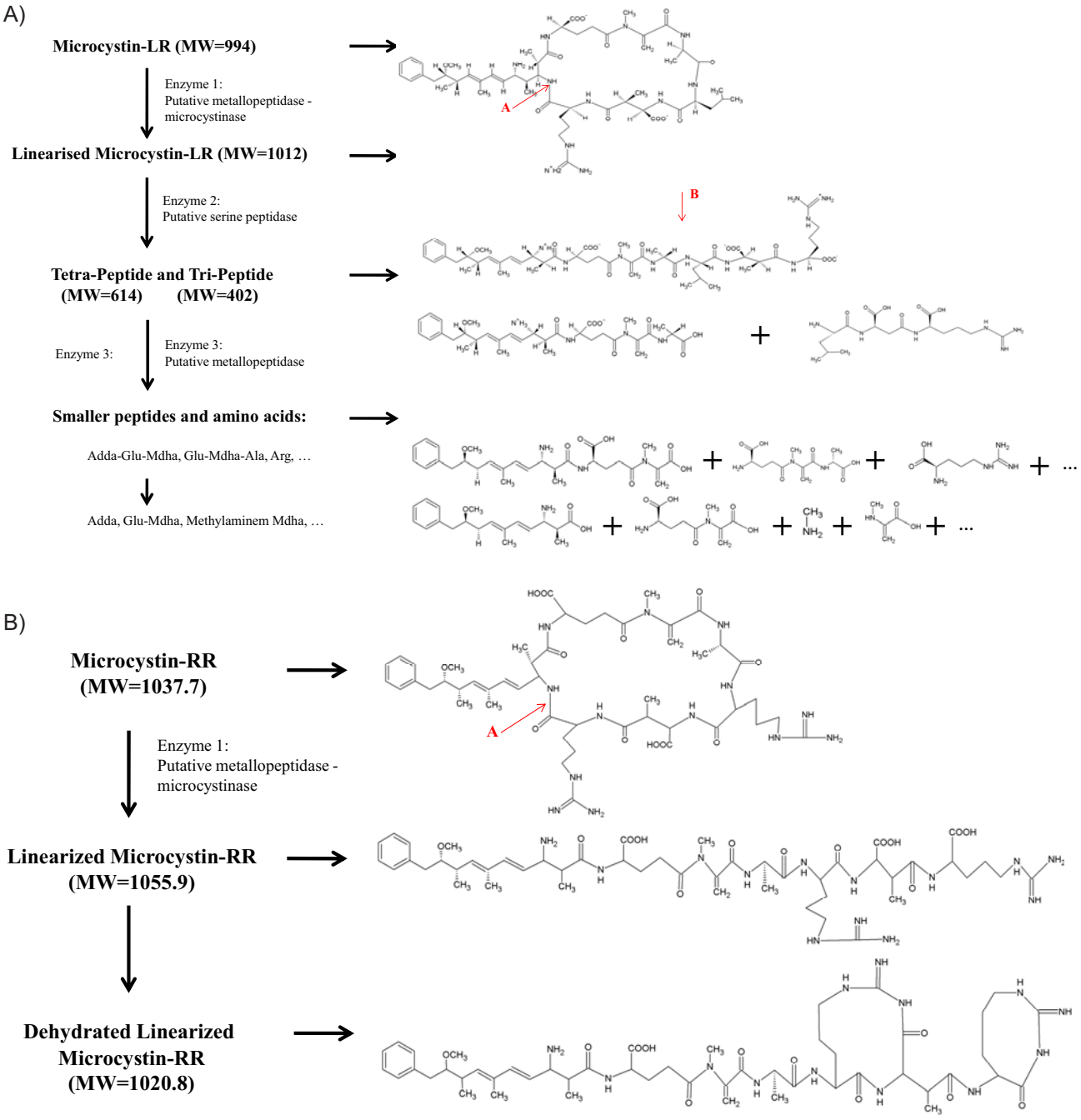

Fig. 2. Degradation schemes for microcystins: A) -LR and B) -RR variants, (adopted from [87, 97, 113, 115]). The large black arrows indicate the route of degradation. Small black arrows marked with A and B indicate the sites of peptide hydrolysis. MW – molecular weight.

et al. [93] and Imanishi et al. [114] found similar products of MC-LR degradation by *Sphingomonas* sp. B-9 strain, to those described by Bourne et al. [87]. However, they also managed to isolate Adda moiety, which was a product of the MC-LR degradation. Nevertheless, so far it has been Hashimoto et al. [115] who gave the most accurate description of the main degradation products of MC-LR (Fig. 2A). The degradation of MC-LR started at the Arg-Adda peptide bond with the ring opening to produce a linearized MC-LR (Adda-Glu-Mdha-Ala-Leu-MeAsp-Arg) (Fig. 2A). Hydrolysis at the Ala-Leu peptide bond at the same time provided tetrapeptide (Adda-Glu-Mdha-Ala) and tripeptide (Leu-MeAsp-Arg) (Fig. 2A). The successive cleavage of the tetrapeptide led to the formation of two tripeptides (Glu-Mdha-Ala and Adda-Glu-Mdha), followed by (Glu-Mdha), Mdha and Adda, with Methylamine in the end [115] (Fig. 2A). Subsequently, the cleavage of the tripeptide should lead to the formation of the dipeptides (Leu-MeAsp) and (MeAsp-Arg). However, only Arg was detected as a further breakdown product of the dipeptides (Fig. 2A).

Methods for Detection and Analysis of Microcystin-Degrading Bacteria

Experiment with Water or Bacterial Culture and Microcystin Standard

Experiments aimed at identifying bacteria capable of MCs degradation were mainly conducted in the laboratory and they involved material collected from water bodies (directly from water or sediments) [75, 84, 116, 117] and/or treatment plants (biofilm from sand filters, treated effluent, activated sludge) [91, 92, 118-122]. The experiments were usually performed as batch laboratory tests, in flasks or bottles containing:

(i) a medium such as raw or sterile lake water [77], or microbiological media: NB [88], LB [107], MS [76], Powell and Errington's [106], Sakurai [123], or M9 [104]
(ii) with study bacterial population
(iii) spiked with a known MCs concentration

The concentration of MCs used at the beginning of the experiment ranged from low concentrations, which usually occur in the environment (~1-100 µg/L) [86, 95, 106, 124] to high, reaching 50 mg/L [81, 85, 88, 97].

The MCs variants used in the assays, separately or combined, were commercially available standards and variants isolated from the environmental samples or cyanobacterial cultures: MC-LR, -RR, -LW, -LF, -LY [84, 85, 87, 91, 95, 104, 105, 113], 6(Z)-Adda MC-LR [88], 3-DMMCLR, DHMCLR, MCLR-Cys, [93], or dME-RR and [Dhe7] MCLR [81].

In order to determine the concentration of remaining MCs in the batch experiment, samples were collected at regular intervals. The concentrations of the remaining MCs were established/quantified with the aid of screening methods using enzyme-linked immunosorbent assay (ELISA) or protein phosphatase inhibition assay (PPIA) [95, 122, 125], and/or by more accurate analytical methods such as high-performance liquid chromatography (HPLC) [76, 87, 88, 103]. Subsequently, the products of MCs degradation were mainly described with the use of liquid chromatography/mass spectrometry (LC-MS) [93, 87, 115]. Then the isolated bacteria have undergone a series of molecular studies (polymerase chain reaction PCR, fluorescent in situ hybridization FISH and sequencing) in order to classify them in appropriate taxonomic groups by analysis of their 16S rRNA gene sequence.

In general, the optimal physical and chemical conditions, such as temperature, pH, initial MCs concentration, quantity of bacterial isolates, and presence of additional sources of C and N, which were used in the experiments and reported by various authors, were similar to those prevailing in the natural environment of isolated bacterial strains. The range of the study temperatures for MCs removal was 4-37ºC [76, 95], but the temperature that was most often used was similar to natural conditions and ranged between 20-27ºC [76, 85-87, 93, 94, 98, 103, 104, 106, 107, 126]. The pH in which MCs degradation was tested ranged from 6-11, and the value of 7 was found to be optimal [96]. Most studies concerning MCs biodegradation were performed with the presence of additional C and N sources in the medium [75, 83-85, 88, 89, 101, 116, 122, 127]. Park et al. [88] reported that MCs degradation occurred several times faster where no nutrients were present in the medium. Nevertheless, as reported by Christoffersen et al. [127] bacteria are able to degrade MCs along with other organic compounds commonly found in the environment. Therefore, the presence of an additional carbon source in the medium should not inhibit MCs degradation. Additionally, Holst et al. [120] found that the addition of supplementary carbon sources in the form of glucose had a positive effect on the anoxic biodegradation of MCs in sediment slurries. Studies conducted by Holst et al. [120] reported another important factor influencing the rate of MCs degradation in the natural environment. Authors observed that the addition of NO_3-N (5 mM) significantly stimulated MCs degradation under anoxic conditions, and suggested that this process was associated with de-nitrification. Aerobic biodegradation has been often studied in different water bodies and filtration systems. However, the reports indicate that high MC concentrations can be found at the sediment-water interface characterized by anoxic conditions [76]. Studies have reported that anoxic biodegradation was comparable to aerobic conditions, but that it was highly dependent on temperature [76, 120].

The time period in which the total biodegradation was observed during that experiment generally ranged between 4 and 15 days after the initial lag-phase [88, 95, 101, 104, 106, 108, 109]. However, quick degradation, usually facilitated by bacterial isolates, was reported to occur between 3 and 24 hours [76, 91, 96, 99, 114, 122, 124, 128].

Biolog MT2 Plates

Manage et al. [104] described another method of identifying MCs degrading strains. Bacteria isolated from water were tested for MC-LR degradation using the Biolog MT2

Table 3. Primes and probes used for detection of the *mlrA* (1008 bp) gene needed for determination of microcystin-degrading bacteria.

Primer/Probe	Sequence (5'-3')	Size	References
MF	gacccgatgttcaagatact	807 bp and 453 bp fragments	[121]
MF2	tcgccatttatgtgatggctg		
MR	ctcctcccacaaatcaggac		
qmlrAf	agccckggcccrctgc	120 bp fragment	[139]
qmlrAr	atgccargcccaccacat		
qmlrA-tm (probe)	fam-tgccscagctsctcaagaagtttg-bhq1		
QMF	agacgcacgctcacctcaa	350 bp fragment	[92]
QMR	gagcagttcacgaaatcc		
QMT (probe)	atacgctcttactgtttccggccgcc		

plates. Metabolism of the MC-LR, the wells in particular, resulted in the formation, of formazan, producing a color change of the tetrazolium dye present in the wells. Manage et al. [104] concluded that Biolog MT2 plates were useful as a quick and easy method for screening active isolates utilizing MC-LR as a sole source of carbon and energy. Therefore, the use of Biolog MT2 plates allows the selection of a microbial system adapted to metabolite-conscripted chemicals as their sole carbon source. However, until now this method was reported only on two occasions [104, 105], which suggests that the traditional way of performing in batch laboratory tests continues to be preferred.

Genetic Analysis of mlrA Gene

Moreover, bacterial strains capable of MCs degradation can be distinguished only on the basis of genetic analysis. *MlrA* gene (responsible for the synthesis of metallopeptidase – MlrA) is best-known, and so far is the most often analyzed gene in terms of monitoring the ability of bacteria to degrade MCs (Table 3).

The MlrA enzyme is considered to be the most important enzyme for MCs removal and detoxification as it facilitates opening the ring, which is equivalent to reducing their toxicity [113]. The *mlrA* gene, which encodes the MlrA enzyme synthesis, was reported to have a rare sequence and only few homologous genes [73, 113]. In 2003 Saito et al. [121] reported amplification of the *mlrA* gene homologous with specifically designed primers (MF, MF2, and MR) for hemi-nested PCR reaction in two *Sphingomonas* sp. strains, i.e. MD-1 and Y2, which were isolated from Lake Kasumigaura and Lake Suwa in Japan (respectively) (Table 3). Moreover, the amplification was not successful when non-MCs-degrading bacteria isolates were used. Further work of Jimbo et al. [92] also revealed the presence of the *mlrA* gene in *Sphingopyxis* sp. C-1 strain isolated from Lake Hongfengh (China). On the other hand, no amplification products were obtained from *Paucibacter* sp. strain (isolated from sediments samples in Finland), which were also reported as MCs degraders [92, 103]. Similar results were reported also by Manage et al. [104], who did not find *mlrA* amplification product in three strains proven for MCs degradation isolated from Scottish water bodies. Those results suggested that not all MCs-degrading bacteria had the *mlrA* gene and thus these strains may contain an unknown mechanism for MCs degradation [92]. Therefore, the *mlrA* gene may not be a perfect molecular marker and further studies are required to explain the degradation mechanism of MCs by bacteria. To the best of our knowledge, so far the *mlrA* gene has been found also in the following strains: *Sphingopyxis* sp. LH21 [95], *Sphingomonas* sp. NV-1 [81], *Bacillus* sp. AMRI-03 [101], *Stenotrophomonas* sp. EMS [76], and *Novosphingobium* sp. THN1 [98].

Bacterial Methods for Treating Water Containing Microcystins

Numerous studies have been conducted to assess the removal of MCs or their transformation to non-toxic products by chemical methods using: chlorination, permanganate, chlorine dioxide or recently advanced oxidation under appropriate conditions [4, 129-135], or physical methods, such as UV photolysis [136]. Each method has been found to remove MCs. However, they present the following limitations:

(i) doses that must be applied
(ii) price to be paid
(iii) by-products that may form during the process of purification

Due to limitations, these methods may be insufficient for smaller treatment plants dealing with water contaminated with MCs. Chlorination, for instance, is dependent on chlorine dose and pH, as the presence of other compounds, which may be more reactive than MCs, can decrease actual removal of these hepatotoxins [129, 137]. Moreover, chlorine dioxide significantly reduces its performance in the presence of NOM (natural organic matter), and therefore fewer MCs decompose [129]. Another example concerns the use of permanganate, which may cause problems in treated drinking water due to the formation of toxic par-

ticulate manganese dioxide [134]. As reported by Song et al. [18], other conventional treatment methods, such as coagulation, sedimentation, and simple sand filtration, may also be less effective in degradation of MCs dissolved in water.

The above examples stimulated the search for new solutions to cope with MCs in drinking water and in wastewater, which in the face of global shortages of water is considered to be an alternative source of water. Following the discovery of bacteria's capacity to remove MCs, attention was drawn to possibilities of using them in specially constructed systems designed for water purification.

The use of sand filters in water treatment plants was the first reported natural way of purification of MCs-containing water by bacteria [95, 117, 122, 125]. Ho et al. [125] was the first scientist who actually reported biological degradation of MCs in rapid sand filtration. He performed an experiment in columns filled with sand obtained from filter beds at the Morgan Water Treatment Plant. The Authors proved that under study conditions, rapid biological sand filtration with natural indigenous bacteria was an effective treatment process for the complete removal of at least two MCs analogues, -LR and -LA (in the target concentration of 20 μg/L). *Sphingopyxis* sp. LH21 was the bacterium that mainly degraded MCs in the sand filter [95].

Bourne et al. [117] also reported for the first time the possibility of applying characterized cultured bacterium *Sphingomonas* sp. MJ-PV strain in slow sand filtration columns (containing sand, rocks, and pebbles). The complete removal of MC-LR (target concentration of app. 50 μg/L) was observed within 6 days.

Another method of MCs removal was described by Ji et al. [86]. A meso-scale experiment was performed in Mailing Bay of Taihu Lake (China) with three kinds of artificial media (assembled medium, elastic medium, and non-woven fabric medium) submerged in the flowing water collected from the lake. The indigenous bacterial population, including MCs-degrading *Pseudomonas* sp., which created a biofilm in all three artificial media, were able to degrade the MCs from water (max. 8.93 μg/L) at a rate of 40-67%.

As mentioned above, several authors proposed the use of MCs-degrading bacteria as a useful tool in water purification facilities. However, their reports mainly focused on laboratory experiments. An example of real-life application of bacteria in active and controlled water purification was proposed in a patent by Sumino et al. [138], which described the methods and equipment for MCs removal in closed water areas (Patent No. US 7,425,267 B2). The patent comprises a step, during which MCs-containing water is dosed with *Sphingomonas* sp. MDB1 strain (FERM O-19480), i.e. with a bacterium that is considered not to affect the environment and ecosystem. The use of *Sphingomonas* sp. bacterium enables us to detoxify MCs rapidly and the treatment process does not require much effort. It may be used in various circumstances. Three methods for bacteria application are proposed. Under the first method, properly prepared bacterial suspension of MDB1 strain is sprayed over the closed-water area (108 cells/m^2 for 4 m^2). As far as efficiency of this method is concerned, it is possible to reduce 18 μg of MCs/L to less than 1 μg/L in just 5 days. The second method involves entrapping and immobilizing MDB1 strain by polymerization in pellets (3 mm diameter) containing 10% polyethylene glycol diacrylate and 0.25% potassium persulfate. The MCs-containing water is pumped from the closed-water area into a vessel filled with the pellets, where the process of degradation occurs. During the 30-minute retention time the concentration of MCs may be reduced from 10 μg/L to less than 1 μg/L. The third treatment method involves the use of attachment immobilization of MDB1 strain in MCs-containing water treatment equipment located in the closed water area. Bacteria are transferred from the culture tank container to the treatment section with net rings fixed to linking members floating at the water surface. The 100 L of water with MCs concentration of 40 μg/L was successfully purified to contain less than 1 μg of MCs/L in 20 days. The MDB1 suspension was supplied in a flow of 10 L/day.

Conclusions

The presence of MCs in fresh water is an increasing problem and poses a potential risk to human and animal health on a global scale. Some reports have shown that MCs are vulnerable to breakdown by indigenous bacteria [4, 5, 83, 104, 117]. The bacterium *Sphingomonas* sp. ACM-3962 (MJ-PV) strain was one of the first MCs-degrading bacteria identified [83]. Since then the number of known MCs-degrading bacteria has increased. So far, it has been established that these bacteria belong to the genera of *Sphingomonas* (7 strains), including *Sphingosinicella* (1 strain), *Pseudomonas* (2 strains), *Sphingopyxis* (3 strains), *Paucibacter* (1 strain), *Burkholderia* (1 strain), *Ralstonia* (1 strain), *Arthrobacter* (1 strain), *Brevibacterium* (1 strain), *Rhodococcus* (1 strain), *Methylobacillus* (1 strain). *Stenotrophomonas* (1 strain), *Bacillus* (1 strain), *Microbacterium* (1 strain), *Rhizobium* (1 strain), and *Novosphingobium* (1 strain) (Table 2). These bacteria occur in various types of environments such as lakes, ponds, rivers, sediments, and sewage treatment plants, as well as drinking water purification systems. This may qualify them as alternative water treatment strategies [76, 120]. Some strains, such as *Sphingomonas* sp. bacterium MDB1 strain (Patent No. US 7,425,267 B2), have been proposed for commercial use to purify water contaminated with MCs. Experiments with sand filters embedded with MCs-degrading bacteria (i.e. *Sphingomonas* sp. MJ-PV strain, *Sphingopyxis* sp. LH21) indicated successful removal of MCs [117, 125]. This offers a cost-efficient and sufficient treatment process. Until now, most studies concerning identification of MCs-degrading bacteria and, above all, their implementation in the biodegradation process in specific aquatic systems, were performed in Asia. Therefore, knowledge and implementation of natural methods of removing cyanobacterial hepatotoxins should be developed for other continents, including identification of specific indigenous bacteria for individual regions.

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