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Biodegradation of Cyanobacterial Toxins

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1. Introduction

Water is an essential natural resource, necessary for drinking, agriculture and industrial activities, and providing the human population with safe drinking water is one of the most important issues in public health. Cyanobacteria produce toxins that may present a hazard for drinking water safety. These toxins are structurally diverse and their effects range from liver damage, including liver cancer, to neurotoxicity. Toxic cyanobacteria have been reported in lakes and reservoirs around the world. The World Health Organization (WHO) has set a provisional drinking water guideline of 1 µg/L for microcystin-LR, one of the most commonly occurring cyanotoxin worldwide [1].

The occurrence of cyanobacteria and their toxins in water bodies used for the production of drinking water causes a technical challenge for water treatment and cleaning. Drinking water should be pure enough to be consumed or used with low risk of immediate or long term harm. The presence of toxins in drinking water creates a potential risk of toxin exposure for water consumers. Conventional water treatment procedures are in some cases insufficient in the removal of cyanobacterial toxins. Besides the chemical and physical methods used, biological degradation could be an efficient method of water detoxification. Therefore there is a need for simple, low-cost and effective water treatment procedures.

This review describes problems related to cyanobacterial toxins and safe drinking water, compares already existing methods of water treatment and cyanotoxin-removal and proposes novel methods of water decontamination. The majority of cyanotoxin-biodegradation studies so far have focused on bacteria isolated from water sources exposed to microcystin-containing blooms. The use of probiotic bacteria is proposed and discussed as a new and efficient means of cyanotoxin-degradation. The removal of cyanobacterial toxins and other environmental contaminants from drinking water is of great importance and probiotic bacteria show promising results in this respect. There is a high demand for effective and low-cost approaches for

removing cyanotoxins from potable water due to the significant health risk and inadequate access to safe drinking water.

2. Cyanobacterial toxins

Cyanobacteria have a long evolutionary history and are among the oldest organisms in the world. There is evidence of the organisms even from around 3500 million years ago [2]. Cyanobacteria carry out oxygen-evolving photosynthesis. In eutrophic water, cyanobacteria recurrently form mass occurrences, so-called water blooms. Mass occurrences of cyanobacteria can be toxic. They have caused a number of animal poisonings and may also pose a threat to human health.

Cyanobacteria produce many different classes of biologically active compounds, including hepatotoxic cyclic peptides, microcystins and nodularins, cytotoxic cylindrospermopsins, neurotoxic anatoxin-a and -a(S), saxitoxins, neurotoxic amino acid β -N-methylamino-L-alanine (BMAA) and non-toxic irritating lipopolysaccharides [3]. Although both neurotoxins and hepatotoxins are distributed worldwide [4,5], it appears that hepatotoxic blooms of cyanobacteria are more commonly found than neurotoxic blooms, and neurotoxins are considered to be of lower risk as they are less stable [6]. In contrast, hepatotoxins are highly stable and exposure to these toxins has resulted in significant toxicity to both animals and humans.

Cyanobacteria are ubiquitous in their distribution in both fresh and marine waters. Toxic cyanobacterial blooms have been reported in most parts of the world, reviewed in [7]. Cyanobacterial blooms are a result of the increasing eutrophication in waterbodies [7]. Most of these cyanobacteria are harmful to animals and humans because of their production of toxins. Over the past several centuries, human nutrient over-enrichment in water, particularly nitrogen and phosphorus, associated with urban, agricultural and industrial development, has promoted eutrophication, which favours algal and cyanobacterial bloom formation. Decay of these excessive blooms results in decreased dissolved oxygen and the release of cyanotoxins in the water, which can result in mortality of animals and even humans [7].

2.1. Microcystins

Globally, the most frequently reported cyanobacterial toxins are cyclic heptapeptide hepatotoxins, microcystins (MC). These can be found primarily in some species of the freshwater genera *Microcystis*, *Anabaena*, *Planktothrix*, *Nostoc*, and *Anabaenopsis*. Microcystins are named after *Microcystis aeruginosa*, the cyanobacterium in which the toxin was first isolated and described [8].

Microcystins are cyclic heptapeptides with variable amino acids and a general structure of cyclo(-D-Ala(1)-L-X(2)-D-MeAsp(iso-linkage)(3)-L-Z(4)-Adda(5)-D-Glu(iso-linkage)(6)-Mdha(7), in which amino acid residues at 2 and 4 are variable L-amino acids, D-MeAsp is D-erythro- β -methylaspartic acid, and Mdha is N-methyldehydroalanine, while the amino acid

Adda is (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid (Figure 1). The Adda component of microcystins is contributing to their toxicity [4,9]. There are around 100 structural variants of microcystins described in the literature (listed in [3,10,11]). The most widely-distributed [4] and studied microcystin variant is microcystin-LR (MC-LR), with the amino acid residues leucine and arginine in positions 2 and 4, respectively, and a molecular weight of 994. Production of MC-LR is dependent on various factors like strain specificity, genetic differences and metabolic processes required for toxin production [9]. A single bloom can have both toxigenic and non-toxigenic strains within it [7]. The toxins are generally bound to the cell membrane and are released as cells age and die, and under stress. They can also passively leak out of cells or be released by lytic bacteria [4].

MC-LR is hepatotoxic and a potent tumour promoter. The primary target organ of MC-LR is the liver [12,13] although it also affects the kidney, gastrointestinal tract and colon [14]. Microcystins are potent and specific inhibitors of serine/threonine-specific protein phosphatases 1 and 2A [15]. Microcystins are distributed in waterbodies worldwide, and the toxicity on exposure to microcystins has been reported worldwide in fish, animals and humans (reviewed in [16]). The World Health Organization has set a provisional drinking water guideline of 1 µg/L for MC-LR [1]; new edition in [17].

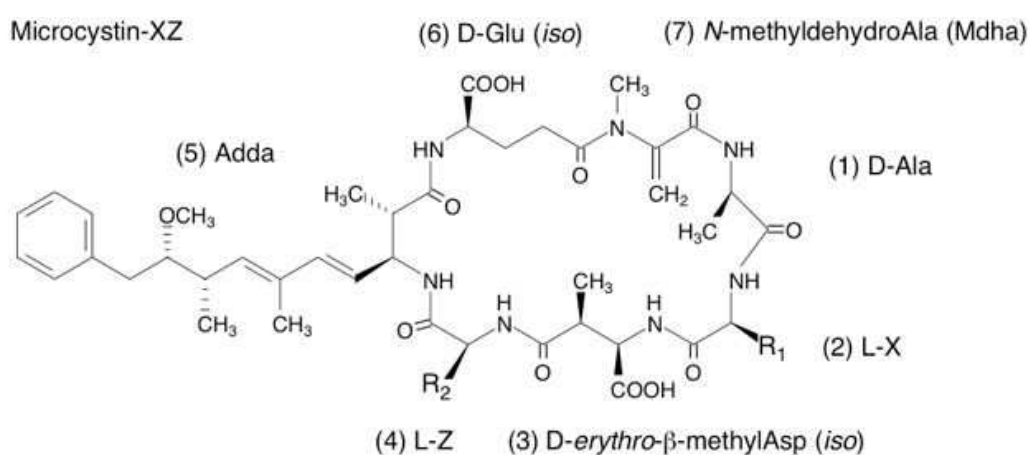


Figure 1. General structure of the hepatotoxic cyclic peptides, microcystins.

2.2. Other cyanobacterial toxins

The cyclic pentapeptide nodularin (NOD) is common in brackish water. It occurs in the Baltic Sea as well as in saline lakes and estuaries. In the Baltic Sea, marine blooms of *Nodularia spumigena* are among some of the largest cyanobacterial mass events in the world. Cylindrospermopsin (CYN), originally isolated from the cyanobacterium *Cylindrospermopsis raciborskii*, is an alkaloid cytotoxin with the structure of a tricyclic guanidine moiety attached to a hydroxymethyluracil [18] and a molecular weight of 415. Cylindrospermopsin inhibits protein synthesis and mainly affects the liver [19], but can also affect the kidney, spleen, thymus, and

heart. It is a cyanotoxin occurring in tropical or subtropical regions that has recently been detected also in temperate regions.

Cyanobacterial neurotoxins belong to a diverse group of heterocyclic compounds called alkaloids. Three types of cyanobacterial neurotoxins, anatoxin-a, anatoxin-a(S), and saxitoxins, are known. A mild neurotoxin, BMAA, has been found in a variety of cyanobacteria [20,21]. Anatoxin-a is a small alkaloid with a molecular weight of 165, and it mimics the effect of acetylcholine and causes rapid death by respiratory arrest. Homoanatoxin-a (MW 179) is an anatoxin-a homologue. Anatoxin-a is perhaps the most common cyanobacterial neurotoxin, especially in North America and Europe, and has caused numerous animal poisonings. Anatoxin-a(S) is an irreversible acetylcholine esterase inhibitor and its characteristic signs of poisonings in mice include salivation. Anatoxin-a(S) was first reported in North America where it has caused animal poisonings and later also in Denmark [22].

Saxitoxins, also known as paralytic shellfish poisons (PSP toxins) were originally isolated and characterised from marine dinoflagellates [23]. Saxitoxins are sodium channel blocking agents causing paralysis and have caused human poisonings due to their ability to concentrate in shellfish [23].

Lipopolysaccharide endotoxins are generally found in the outer membrane of the cell wall of Gram-negative bacteria, also in cyanobacteria. Bacterial lipopolysaccharides are pyrogenic and toxic [24]. It is often the fatty acid component of lipopolysaccharides that elicits an irritant, pyrogenic or allergenic response in humans and mammals. Cyanobacterial lipopolysaccharides may contribute to human health problems via exposure to mass occurrences of cyanobacteria.

3. Occurrence and levels of cyanobacteria and hepatotoxins

Toxic cyanobacteria are found worldwide both in inland and coastal water environments. Cyanobacteria occur in various environments including water, such as fresh and brackish water, oceans, hot springs, moist terrestrial environments such as soil, and in symbioses with plants, lichens and primitive animals. Some environmental conditions, including sunlight, warm weather, low turbulence and high nutrient levels, can promote growth. A high density of suspended cells may lead to the formation of surface scums and high toxin concentrations.

The toxins are not actively secreted to the surrounding water; most of the toxin is intracellular in growing cells. The release of toxin occurs during senescence of the cultures and when cultures shift from growth phase to stationary and death phases. Under field conditions, the majority of microcystin is intracellular during active growth of the cells [25]. There are reports of hepatotoxic blooms from all continents around the world [7]. Some of the highest reported cyanotoxin concentrations in bloom samples (measured by HPLC) have been 7300 µg/g dry weight microcystin in a *Microcystis* bloom from China [26], 18000 µg/g dry weight nodularin in a *Nodularia* bloom from the Baltic sea [27] and 5500 µg/g dry weight cylindrospermopsin from Australia [3]. Toxic and non-toxic strains from the same cyanobacterial species cannot be

separated by microscopic identification. To confirm that a particular cyanobacterial strain produces toxins, it is important to isolate a culture of that strain, and to detect and quantify toxin concentrations in the pure culture.

4. Human health effects caused by cyanobacterial toxins

Many cyanobacteria produce potent toxins. As reported in literature, problems caused by cyanobacteria are encountered around the world and problems related to safe drinking water production are common (reviewed in e.g. [7]). The human health effects caused by cyanobacterial toxins vary in severity from mild gastroenteritis to severe and sometimes fatal diarrhoea, dysentery and hepatitis. Microcystins, including the most common variant MC-LR, are hepatotoxic and potent tumour promoters. Acute symptoms reported after exposure to microcystin-containing cyanobacteria include gastrointestinal disorders, nausea, vomiting, fever and irritation of the skin, ears, eyes, throat and respiratory tract, abdominal pain, kidney and liver damage. There are several reports of human health effects associated with ingestion of water containing microcystins, with effects ranging from gastroenteritis [28] to liver damage [12] and even death [29,30].

Humans can be exposed to a range of cyanotoxins contained either in cyanobacterial cells or released into the water. The dissolved toxins are stable against low pH and enzymatic degradation and will therefore remain intact within the digestive tract. As microcystins do not readily penetrate the cell membrane [31], they enter the body from the intestine via the organic anion transporting polypeptides [32]. From the blood microcystins are then concentrated in the liver as a result of active uptake by hepatocytes [33]. The toxins are covalently bound to protein phosphatases in the hepatocyte cytosol [34]. Human health problems are often associated with chronic exposure to low microcystin concentrations in inadequately treated drinking water, contaminated food (such as fish, mussels and prawns) or with the consumption of algal supplements contaminated with cyanotoxins. Exposure routes include the oral route, through inhalation, through dermal exposure or the nasal mucosa [35,36].

4.1. Risk assessment

Poisonings caused by cyanotoxins produced during heavy blooms have affected both humans and wild and domestic animals. Both hepatotoxic and neurotoxic poisonings have been associated with mass occurrences of cyanobacteria [7]. Many reported incidents of human health effects have involved inappropriate treatment of water supplies. The health risk caused by cyanotoxin exposure is difficult to quantify, since the actual exposure and resulting effects have not been conclusively determined. The most likely route for human exposure is the oral route via drinking water [37], and from recreational use of lakes and rivers [36].

Due to the growing concern about health effects of cyanotoxins especially via drinking water, WHO has adopted a provisional guideline value of 1.0 µg/L for MC-LR in 1998 [1]. The newest 4th edition to the drinking water guideline was published in 2011 [17]. Assessment of different water treatment procedures has shown that many of the treatment methods result in a

reduction of cyanotoxin concentrations to below acutely toxic levels and below the WHO guideline value of 1.0 µg/L MC-LR in drinking water. During a cyanobacterial bloom the treatment procedures may however be insufficient, and also when different water treatment procedures are not used in combination. Therefore it is important to observe the water treatment efficiency during cyanobacterial blooms.

5. Treatment of drinking water containing cyanotoxins

Water is an essential natural resource, necessary for drinking, agriculture and industrial activities. Contamination of water can therefore influence humans, agricultural livestock and irrigated field crops, as well as wildlife drinking the water or living in the aquatic environment. Drinking water should be pure enough to be consumed or used with low risk of immediate or long term harm. In large parts of the world, the population has inadequate access to safe potable water and use sources contaminated with disease vectors, pathogens or unacceptable levels of toxins and other harmful substances.

Prevention of bloom formation is naturally the most efficient method for avoiding cyanobacterial toxin contamination of drinking water. Cyanotoxins are produced within the cyanobacterial cells and thus toxin removal involves procedures to destroy or avoid the cells. Cyanotoxins are also water soluble and therefore chemical or biological procedures reducing the toxicity or completely removing the toxins from the drinking water are needed. If high extracellular toxin concentrations are present in the raw water, problems will occur for drinking water treatment plants. Under natural circumstances high toxin concentrations appear during the breakdown of a cyanobacterial bloom. Cyanobacterial cells are also lysed in the presence of chemicals, such as potassium permanganate or chlorine [38].

In cyanotoxin-removal from drinking water there is a need for knowledge of the physical and chemical properties of the toxin, such as the hydrophobicity, molecular size, and functional groups, the nature of the toxin, i.e., intracellular or extracellular, cyanobacterial growth and bloom patterns, and effective treatment processes [7]. However, these treatments may not be sufficient during cyanobacterial blooms or when a high organic load is present, and toxin levels should therefore be monitored during all steps of water treatment processes. Some of the existing methods of drinking water treatment are shortly described in the following section.

5.1. Water treatment processes

Most drinking water plants use conventional treatment methods that are unable to yield complete removal of microcystins or are too expensive [39]. Conventional surface drinking water treatment utilises coagulation, flocculation, sedimentation, filtration and disinfection as basic methods. However, conventional treatment may need to be optimised for cyanotoxin-removal, relating to the form of the toxin to be removed (intra- or extracellular), the background water matrix, and possible dissolved toxin release during the treatment process [40]. Alternative processes, such as granular activated carbon, powdered activated carbon, and membrane filtration have been proven efficient for the removal of microcystins [41]. However, these

methods are sometimes considered too expensive to exclusively remove a contaminant that is irregularly occurring.

Coagulation or flocculation involves the aggregation of smaller particles into larger particles using chemicals, such as ferric chloride or aluminium sulphate. Coagulation can be an efficient method for eliminating cyanobacterial cells from water, but soluble cyanotoxins are not very efficiently removed by this method [42]. Coagulation may also cause additional problems such as lysis of cyanobacterial cells leading to release of toxins. The activated carbon approach uses either powdered activated carbon, which can be added occasionally when there is a need, or granular activated carbon adsorbers, which are used continuously [43]. Both microcystins and cylindrospermopsin can be absorbed by activated carbon [43]. The disposal of the carbon containing cyanobacterial toxins may present a challenge for this type of treatment.

Rapid filtration is a method usually used after a coagulation step in conventional water treatment, but does not effectively remove cyanobacterial cells from water. Conventional water treatment requires regular backwashing of the filters, but if the washing process is inadequately performed, lysis of cyanobacterial cells on the filters can lead to release of toxins into the water [7]. Two types of membrane filtration, microfiltration and ultrafiltration, are commonly used to remove contaminants from drinking water. Both microfiltration and ultrafiltration have been shown to be effective in removal of intact cyanobacterial cells [44].

The most common chemical oxidants used in drinking water treatment are ozone, hydroxyl radicals, chlorine, chlorine dioxide, chloramine and permanganate. Chlorination and ozonation are effective for the removal of microcystins [43]. However, there are concerns regarding the release of toxin when cyanobacteria are chlorinated and with the formation of undesirable chlorination by-products [45]. Ozonation has been shown to be a very effective method for destroying microcystins and nodularins. In recent years, many water treatment plants have included a two-stage ozonation treatment [46].

Removal and inactivation of cyanobacteria and intracellular and extracellular cyanotoxins most often requires a combination of treatment processes or a multiple barrier approach. Furthermore, biological treatment of water is a method used for cyanotoxin-removal from drinking water. Biologically active filtration in the form of river bank filtration and both slow and rapid filtration have been reported to remove or to inactivate microcystins in drinking water (e.g. [47,48]) and are discussed more in detail in the following section.

6. Biodegradation of cyanotoxins

Biodegradation is a chemical disruption of organic materials by microorganisms or other biological agents. Microbial degradation of chemicals in the environment is an important route for the removal of these compounds. Biodegradation is also one of the essential processes for the reduction of microcystins in natural eutrophic lakes and reservoirs. Cyanotoxin-degrading bacteria are distributed all over the world. Of all the cyanotoxin-biodegradation studies, most have focused on microcystins as a consequence of their biodegradability in drinking water

sources. This section mainly describes biodegradation studies of microcystins, but studies on nodularin, cylindrospermopsin, saxitoxins and anatoxin-a have also been performed to some extent.

People are frequently exposed to cyanobacterial toxins as well as other microbial contaminants through drinking water. Conventional water treatment procedures discussed in the previous section are in some cases insufficient in the removal of cyanobacterial toxins from drinking water, especially during cyanobacterial blooms. If the cyanobacterial cells are not removed by traditional water treatment methods, the cells and therefore the toxins remain in the drinking water and must be degraded to non-toxic compounds. Since microcystins have been released into the water body, the toxins can persist for weeks [20] before they are adequately degraded by for example bacteria.

6.1. Bacterial degradation of microcystins

Different biological methods have been applied to remove cyanobacteria and their toxins. One type of these methods is the use of microorganisms or biofilms capable of degrading microcystins. Biological treatment for removal of toxin contaminants is becoming more useful as toxins can be removed without the addition of chemicals that may have the potential to produce undesirable by-products. Biodegradation of microcystins in water has been proven to be very effective as they can be used as a carbon source by heterotrophic bacteria [25,38,49,50]. Methods utilizing microcystin-degrading microorganisms can be classified into two groups. One is the use of biofilms grown on the surface of substrates within bioreactors, such as biological sand [48,51,52], biofilm-reactors based on immobilised microorganisms [53], biological treatment facilities combined with conventional treatment processes [54], and granular activated carbon filters [55]. The other group depends on specific microorganisms efficient in microcystin-degradation, such as bacteria of the *Sphingomonas* sp. [56,57] and *Sphingopyxis* sp. [58].

Different variants of microcystins have been demonstrated to be degraded after incubation with water from a lake in Japan, which is frequently contaminated with cyanobacteria [59]. A more effective degradation was observed after adding bed sediment or mud from the lake. Christoffersen *et al.* found out that bacteria can efficiently degrade microcystins in natural waters with previous cyanobacterial contamination and that the degradation process is rapid and without lag phase [60].

Many other studies have also reported biological degradation of microcystin in natural waters from lakes and reservoirs, particularly those containing toxic cyanobacterial blooms [25,50,61,62]. Several strains of the genus *Sphingomonas* have been reported to degrade microcystins [49,57,63–67]. Table 1 lists strains reported to degrade different variants of microcystins and nodularin. A part of the recognised microcystin-degraders so far belonging to the family *Sphingomonadaceae* are closely related and possess homologues of the *mlrA* gene. Seventeen strains of Gram-negative bacteria with the ability to degrade microcystins were isolated by Lahti *et al.* [47]. Other reported microcystin-degrading bacteria include *Pseudomonas aeruginosa* [68], *Paucibacter toxinivorans* [69] and *Sphingosinicella microcystinivorans* [70]. In a study of Rapala *et al.* thirteen bacteria capable of degrading microcystins and nodularin were isolated from lake sediment [61]. Genomic characterisation of these strains indicated that they formed a single microdiverse species and a novel genus and species (*Paucibacter toxinivorans*

gen. nov., sp. nov.) was proposed. A bacterium isolated from water samples in Brazil showed high homology with the *Burkholderia* genus, belonging to the beta subdivision of proteobacteria [71], which was the first reported bacterium from the genus *Burkholderia* as a cyanobacterial toxin degrader.

Bacterial strain	Degradable toxins	Reference
<i>Arthrobacter</i> sp.	MC-LR	[72]
<i>Bacillus</i> sp. strain EMB	MC-LR, MC-RR	[74]
<i>Brevibacterium</i> sp.	MC-LR	[72]
<i>Burkholderia</i> sp.	MC-LR, [D-Leu ¹]MC-LR	[71]
<i>Lactobacillus rhamnosus</i> GG and LC-705, <i>Bifidobacterium longum</i> 46	MC-LR, MC-RR, MC-YR, MC-LF, MC-LY, MC-LW	[79,80]
<i>Methylobacillus</i> sp. strain J10	MC-LR, MC-RR	[75]
<i>Microbacterium</i> sp.	MC-LR	[78]
<i>Morganella morganii</i>	MC-LR	[77]
<i>Paucibacter toxinivorans</i> sp. nov.	MC-LR, MC-YR, NOD	[69]
<i>Poteroiochromonas</i> sp.	MC-LR	[81]
<i>Pseudomonas aeruginosa</i>	MC-LR	[68]
<i>Rhizobium gallicum</i>	MC-LR	[78]
<i>Rhodococcus</i> sp.	MC-LR	[72]
<i>Sphingomona stygia</i>	MC-LR, MC-RR, MC-YR	[65]
<i>Sphingomonas</i> sp. 7CY	MC-LR, MC-RR, MC-LY, MC-LW, MC-LF	[66]
<i>Sphingomonas</i> sp. ACM-3962	MC-LR, MC-RR	[25,63,82]
<i>Sphingomonas</i> sp. B9	MC-LR, MC-RR, 3-dmMC-LR, dhMC-LR, MC-LR-Cys, NOD	[67,83]
<i>Sphingomonas</i> sp. CBA4	MC-RR	[57]
<i>Sphingomonas</i> sp. MD-1	MC-LR, MC-RR, MC-YR	[56]
<i>Sphingomonas</i> sp. MDB2	MCs	[70]
<i>Sphingomonas</i> sp. MDB3	MCs	[70]
<i>Sphingomonas</i> sp. MJ-PV	MC-LR	[49]
<i>Sphingomonas</i> sp. Y2	MC-LR, MC-RR, MC-YR, 6(Z)-Adda-MC-LR	[64,70,84]
<i>Sphingopyxis</i> sp. C-1	MC-LR	[58]
<i>Sphingopyxis</i> sp. LH21	MC-LR, MC-LA	[52]
<i>Sphingopyxis</i> sp. USTB-05	MC-RR	[85]
<i>Stenotrophomonas</i> sp. strain EMS	MC-LR, MC-RR	[76]
17 different strains (Gram-negative, <i>Proteobacteria</i>)	MCs	[47]

Table 1. Reported microcystin-degrading bacteria

Recently, Gram-positive bacteria isolated from freshwater, belonging to *Actinobacteria* and identified as *Arthrobacter* sp., *Brevibacterium* sp. and *Rhodococcus* sp., were shown to remove MC-LR [72]. The mechanism of MC-LR removal for *Rhodococcus* sp. C1 [73] was shown to be similar to the previously reported degradation pathway for *Sphingomonas* by Bourne *et al.* [63]. A new strain AMRI-03 with close relationship to the genus *Bacillus* was isolated from a Saudi freshwater lake [74]. Another strain J10 isolated from Lake Taihu in China was identified as *Methylobacillus* sp. [75]. An EMS strain similar to *Stenotrophomonas maltophilia* was described by Chen *et al.* and was the first report of microcystin-degrading bacteria carrying the *mlrA* gene in the genus of the gamma division of proteobacteria [76]. Other reported examples of bacteria with such ability are *Morganella morganii* and *Pseudomonas* sp. [77]. Further recent findings include two isolates from Lake Okeechobee, Florida, capable of microcystin-degradation and classified as *Rhizobium gallicum* and *Microbacterium* sp. [78].

6.2. Enzymatic mechanisms of microcystin-biodegradation

The first proposal of microcystin-biodegradation suggested a proteolytic mechanism [63]. Within the genome of the first isolated microcystin-degrading bacterium, *Sphingomonas* sp. ACM-3962, Bourne *et al.* identified a gene cluster, *mlrA*, *mlrB*, *mlrC* and *mlrD*, responsible for the degradation of MC-LR [63,82]. Based on MS-analysis a linear MC-LR (protonated molecular ion at m/z 1013) and a tetrapeptide (protonated molecular ion at m/z 615) were recognised as the degradation products. The microcystin-degradation pathway was described as a linear, three-step process. It was suggested that the *mlrA* gene encoded an enzyme responsible for the hydrolytic cleaving of the cyclic structure of MC-LR (ring-opening at the Adda-Arg peptide bond). The resulting linear MC-LR molecule was then sequentially hydrolysed by peptidases encoded by the *mlrB* and *mlrC* genes to a tetrapeptide, and further to smaller peptides and amino acids (Figure 2). The final gene, *mlrD*, encoded for a possible transporter protein that may have allowed for active transport of microcystin or its degradation products. The genes *mlrA*, *mlrB* and *mlrC* encode a 336-residue metalloendopeptidase (responsible for linearization of microcystins), a serine protease and a metalloprotease, respectively. Further studies have confirmed the existence of the *mlr* cluster components also in other microcystin-degrading bacteria; Ho *et al.* identified homologues of four *mlr* genes in *Sphingopyxis* sp. LH21 [52]. Similarly, a homologous gene cluster was also detected in *Sphingopyxis* sp. C-1 [58].

However, as has been recently indicated, *mlrC* acts not only on the tetrapeptide but is also able to hydrolyze linear microcystin without earlier processing by *mlrB* [86]. Other products of microcystin-degradation have consequently been documented, but the complete fate of microcystin-derivatives is still unknown [83,87]. Additionally, enzymes other than proteases have been suggested to be involved in microcystin-utilisation, and besides typical proteolytic activity, also decarboxylation and demethylation have been proposed as alternative mechanisms [87].

Various studies have designed qualitative polymerase chain reaction assays for detection of *mlrA* [51,52,56]. Saito *et al.* reported gene homologues of *mlrA* in two microcystin-degrading bacteria, *Sphingomonas* sp. MD-1 and *Sphingomonas* sp. Y2, both of which were previously

isolated from Japanese lakes [56]. More recently, Hoefel *et al.* designed and optimised a quantitative real-time polymerase chain reaction assay for the detection of the *mlrA* gene [88].

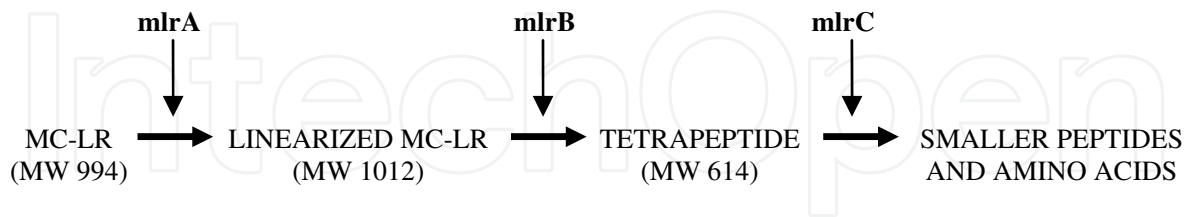


Figure 2. MC-LR degradation pathway by *Sphingomonas* sp. ACM-3962; *mlrA-C*: microcystinases A-C (modified from [63]).

6.3. Further aspects of microcystin-biodegradation

Before biological treatment can be considered a feasible option for effective removal of microcystins, there is a need to determine if any toxic biodegradation by-products are generated. Different studies have demonstrated that the biodegradation of microcystins does not yield toxic by-products. Bourne *et al.* [63] and Harada *et al.* [67] identified two intermediate products from the bacterial degradation of MC-LR by *Sphingomonas* sp. ACM-3962 and *Sphingomonas* sp. B9, respectively. Both studies identified linearized MC-LR and a tetrapeptide as the intermediate products, and isolated Adda as one of the final degradation products (Figure 2). Both these intermediate products were less active than the parent MC-LR. Studies with *Sphingopyxis* sp. LH21 in treated reservoir water concluded that the decrease in cytotoxicity indicated that no cytotoxic by-products of microcystins were being generated [52].

Different factors may influence the biodegradation efficiency, such as water temperature. Published results suggest that the temperature range for the effective biodegradation of microcystins is between 11 and 37 °C, with more rapid degradation at the higher temperatures in most cases [52,64,79,88]. In addition, the bacterial composition and cell density within the water body also affects degradation; both the types of organisms present and their concentration.

Only few studies with respect to the biodegradation of a range of cyanobacterial metabolites in water bodies have been performed. This is relevant since multiple classes of cyanobacterial metabolites are often simultaneously present in water bodies. The following sections regarding the removal of cyanotoxins by probiotic bacteria will assess this issue, and results regarding the removal of a range of cyanotoxins are presented. The results on a range of bacterial species demonstrate the feasibility of biodegradation as a possible removal option for microcystins. The most important practical use of microbial aggregates, such as biological filters and biofilm, is in biological wastewater treatment, and some new technologies already utilize bacterial aggregates for degradation [89].

7. Probiotic bacteria involved in cyanotoxin-removal

Probiotics were earlier defined as “live microbial food supplements which beneficially affect the host either directly or indirectly by improving its intestinal microbial balance” [90]. Today, the most commonly accepted definition by WHO states that probiotics are “live microbial food supplements which, when given in adequate amounts have a demonstrated beneficial effect on human health” [91]. In order to be effective the probiotic micro-organisms must be able to survive the digestive conditions, including bile acids, and they must be able to colonise the gastrointestinal tract at least temporarily without any harm to the host [92]. Only certain strains of micro-organisms have these properties. Most probiotic micro-organisms are grouped in two bacterial genera, *Lactobacillus* (L.) and *Bifidobacterium* (B.).

The main site of action for the health benefits of probiotic bacteria is the gut. The intestinal mucosa forms a barrier between the external and internal environment of the human body. There are several important modes of action for probiotic bacteria, including modification of gut pH, colonisation ability, inhibition of the colonisation, adhesion and invasion of pathogens, direct antimicrobial effect, replacement of already adhered pathogens, competing for available nutrients and growth factors, regulation of the immune system of the host, normalisation of the gut microbiota, and different metabolic effects (reviewed in [93,94]). It is therefore believed that by adding these bacteria as probiotics to the diet, the normal microbiota can be altered. Many probiotic organisms originate in fermented foods, and they have a long history of safe use in human consumption. Lactobacilli and bifidobacteria common in the food industry belong to the European Qualified Presumption of Safety (QPS) status organisms, which can be used in foods and feeds [95].

7.1. Efficiency of probiotic strains in microcystin-removal

Recently published studies have reported efficient cyanotoxin-removal by several strains of probiotic bacteria [79,80,96,97]. The aim of these studies was to characterise the potential of probiotic lactic acid bacteria and bifidobacteria in removal of microcystins and cylindrospermopsin from aqueous solutions. Different physiological conditions possibly affecting the removal efficiency were studied and the mechanism of toxin removal was investigated.

In an initial screening study, 15 different strains of probiotic lactic acid bacteria and bifidobacteria were tested for their MC-LR removal capacities and evaluated for their potential in water decontamination [79]. The results showed a reproducible reduction of MC-LR in solution by the majority of the tested bacterial strains; the most efficient removal was achieved with *L. rhamnosus* strains GG and LC-705, *B. lactis* strains 420 and Bb12 and *B. longum* 46 [79]. The removal of MC-LR continued during the entire 24-hour incubation, which indicates that the removal process is quite slow. The effect of pH during incubation was also studied. pH was found to have an influence on toxin removal, with a higher removal percentage observed at neutral pH than at pH 3 [79]. It was also shown that viable bacteria were more efficient in microcystin-removal than non-viable bacteria [80]. Further studies showed that several strains were efficient in microcystin-removal and that different physiological conditions, including

the effect of pH, temperature, toxin concentration, bacterial cell density and cell viability, had an effect on the removal efficiency [80].

The removal of MC-LR was shown to be temperature dependent, with the highest removal observed at 37 °C for all studied strains. At 4 °C, practically no removal of MC-LR could be observed and the removal percentages increased with increasing temperature [79]. This can be explained by the fact that at 4 °C, the bacterial cells are metabolically inactive, but at 22 and 37 °C, the bacteria become metabolically active, which is required for enzymatic activity. In addition, the role of glucose in activating the metabolism of the probiotic bacteria was assessed [96]. Since it was shown that viability is a requirement for efficient toxin removal, glucose was added as a source of nutrient to the bacterial solutions to enhance the bacterial viability. Glucose addition improved the removal efficiencies of all tested strains by enhancing both the removal rate and the amount of MC-LR removed after 24 hours of incubation [96]. Supplementation of glucose provides energy to the bacteria, and thereby, the microcystin-removal efficiencies also increase.

To investigate the role of the probiotic bacterial cell density, a range of bacterial cell densities were screened and tested for their microcystin-removal efficiencies [79]. The removal of MC-LR was shown to be dependent on the bacterial cell density, with a minimum of approximately 10^9 CFU/mL required for significant MC-LR removal [79]. The removal of MC-LR was further enhanced with increasing bacterial cell density. To assess whether a combination of several probiotic strains could enhance their microcystin-removal efficiencies the microcystin-removal of three probiotic strains (*L. rhamnosus* GG, *L. rhamnosus* LC-705 and *B. longum* 46) separately and in combination was studied [80]. With the probiotic mixture, microcystin-removal percentages of up to 90% could be observed and the results showed that the removal efficiency was improved with a mixture of the strains and compared to the individual strains [80].

In addition to MC-LR, probiotic bacterial strains were also incubated with other microcystins, including MC-RR, -YR, -LY, -LW and -LF. The results of the study show that probiotic strains were effective in the elimination of several different microcystins from solution [79]. Simultaneous removal of several toxins present in cyanobacterial extracts was also investigated. The time course for the removal of microcystins present in the cyanobacterial extracts *Microcystis* NIES-107 and *Microcystis* PCC 7820 by the probiotic strain *L. rhamnosus* GG is shown in Figures 3 a and b, respectively. The removal of all studied microcystins increased over time. The removal of the microcystins present in *Microcystis* NIES-107 after 24 hours of incubation was around 65–85% of total microcystin and for microcystins present in *Microcystis* PCC 7820 around 60–80%. The toxin-removal was thus shown to be efficient also when several different microcystins were present in the solution. This indicates that there is no competition taking place among the toxins during incubation with probiotic bacteria. In addition, the strains were shown to remove the cytotoxin cylindrospermopsin from aqueous solutions; the removal was somewhat less efficient, around 30% for all tested strains [80].

Probiotic bacteria have several advantages in comparison with the previously reported microcystin-degrading bacteria, as they have been classified as food grade, safe bacteria by the European Food Safety Authority (EFSA) [95]. Therefore probiotic bacteria can safely be

included in both food and water. Previous studies have also shown the effect of probiotic bacteria in the removal of other environmental contaminants, such as heavy metals [98] and mycotoxins including aflatoxins and ochratoxins [99].

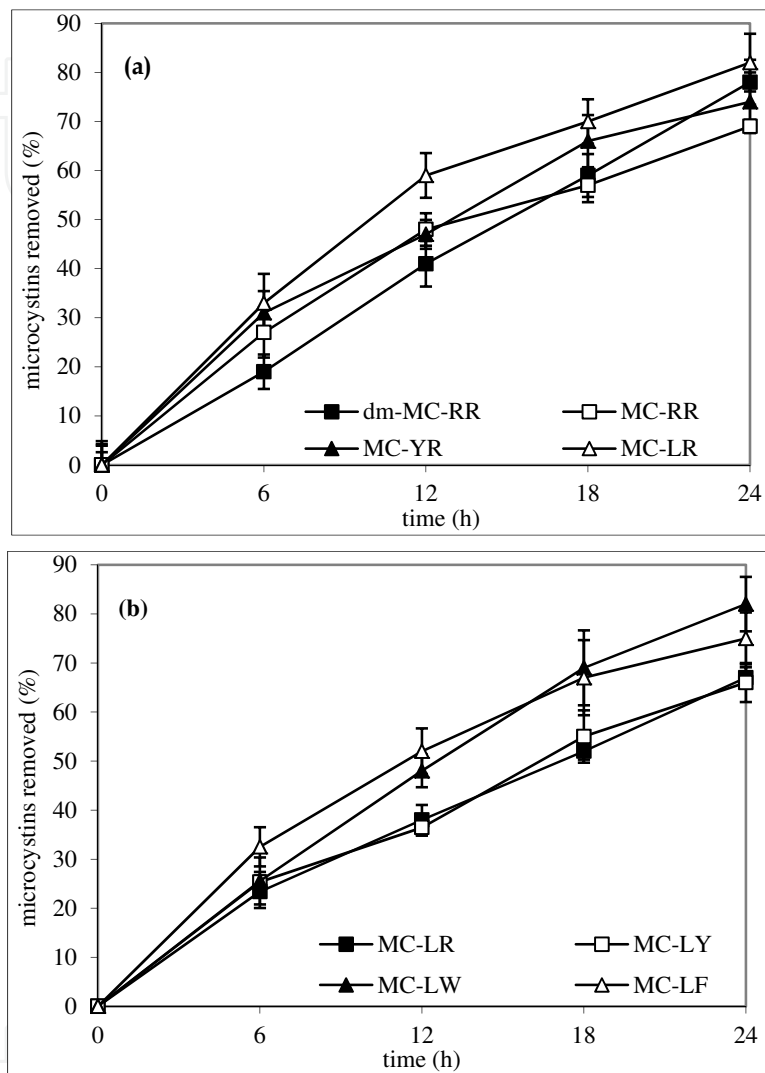


Figure 3. Removal of microcystins in cyanobacterial extracts by probiotic strain *Lactobacillus rhamnosus* GG (a) *Microcystis* NIES-107 and (b) *Microcystis* PCC 7820. Initial concentration of microcystins in extracts: 20-100 $\mu\text{g/L}$, bacterial concentration 10^{10} CFU/mL, temperature 37 $^{\circ}\text{C}$, average \pm SD, $n = 3$ (modified from [80]).

7.2. Mechanisms of microcystin-degradation by probiotic bacteria

As specific probiotic bacterial strains were shown to be efficient in microcystin-removal, the subsequent aim was to identify and specify the removal mechanisms. The location and mechanism of microcystin-removal were investigated by studying a possible extracellular enzymatic degradation of microcystins [97]. Furthermore, a comparison of the degradation pathways of previously identified microcystin-degrading bacteria with probiotic bacteria was performed.

The participation of cell-envelope proteinases in microcystin-removal was investigated. Following standard peptidase assay no proteolytic activity was found in the supernatants of the bacterial cell cultures of the investigated strains; enzymatic activity was found only in the cell suspensions. The activity of cell-associated proteinases of probiotic strain *L. rhamnosus* GG was measured after incubation with protease inhibitors. The protein inhibitor EDTA was shown to inhibit MC-LR removal [97]. The results suggest that the main proteolytic activity observed for the strain was due to metallo-enzymes. A possible extracellular enzymatic degradation of microcystins by probiotic bacteria was therefore investigated and it was suggested that extracellularly located cell-envelope proteinases appear to be involved in the decomposition of MC-LR [97]. A correlation between proteinase activity and MC-LR removal was also found when these parameters were simultaneously measured. The correlation between the activity of cell-envelope proteinases and the decrease of MC-LR concentration suggests that enzymes are involved in microcystin-removal [97]. The findings support the theory that enzymatic degradation of microcystins occurs when the toxin is incubated with probiotic bacteria, but the exact mechanism still remains unidentified.

Bacterial degradation of microcystins has previously been reported for strains of *Sphingomonas* and the degradation products and patterns have been determined for strains ACM-3962 and B9 [63,66,67,82]. For possible identification of toxin removal by probiotic bacteria, the removal process of MC-LR for *L. rhamnosus* GG was compared with the two *Sphingomonas* strains, and the degradation products were identified [97]. Linearized MC-LR and the tetrapeptide were observed for the two *Sphingomonas* strains, but these degradation products were not obtained using the probiotic strain, suggesting that the removal mechanisms between the strains differ [97]. Furthermore, no additional degradation products could be identified from samples incubated with the probiotic strain, which suggested that microcystin is rapidly degraded to smaller peptides and amino acids. Further studies are needed to identify possible degradation products and the precise steps of the degradation mechanism by probiotic bacteria.

8. Discussion and conclusions

The majority of cyanotoxin-biodegradation studies have focused on bacteria isolated from water sources exposed to microcystin-containing blooms. As described in this review, it is clear that many of the cyanobacterial metabolites are susceptible to biodegradation in water supplies. Currently an increasing focus on bacterial degradation of hepatotoxic cyanobacterial peptides is being observed [56,59,64,66,76]. Previous studies have demonstrated that the ability of bacteria to degrade microcystins is related to the presence of the gene *mlrA* that encodes a hydrolytic enzyme with specificity to the toxins. The potency to utilize these bacteria in microcystin-degradation has also been demonstrated in laboratory scale [51,54, 100]. Recently, a new type of bacteria, specific probiotic bacterial strains, was presented to be efficient in cyanotoxin-removal. Probiotic bacteria have several advantages in comparison with the previously reported microcystin-degrading bacteria, as they have been classified as food grade, safe bacteria by the EFSA [95]. Therefore probiotic bacteria can

safely be included in both food and water, and can also safely be used in food technology. Furthermore, the beneficial health effects of probiotic bacteria give them an advantage for the use in different applications. A potential area of use could be probiotic dietary supplements used as a personal defense mechanism against cyanotoxins in the gastrointestinal tract when ingested through contaminated drinking water and to reduce the health risks caused by microcystins, as well as applications in biological decontamination of microcystin-containing water.

Several reports have showed that biological degradation of cyanotoxins may be a feasible method of water treatment. The bacterial strains or possible enzymes identified in the removal process could be used in a degradation process to remove toxins from drinking water. Technologies using potential purified enzymes identified in the removal process of bacteria could be a future approach for efficient cyanotoxin-removal. Today, the best way for cyanotoxin biodegradation is the use of biofilters with immobilised micro-organisms, as most water treatment processes already employ a filtration step. Also the removal of other cyanobacterial toxins, such as anatoxins, saxitoxins, and cylindrospermopsin, should be taken into account. In conclusion, the development of new water treatment technologies using efficient bacteria that would be able to remove or inactivate cyanotoxins, as well as other types of environmental contaminants, such as heavy metals, viruses and pathogenic bacteria found in drinking water, is an important aspect to consider in the future.

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