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REVIEW

Recent developments in the methods of quantitative analysis of microcystins

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Abstract

Cyanotoxins are produced by the toxic cyanobacterial species present in algal blooms formed in water bodies due to nutrient over-enrichment by human influences and natural environmental conditions. Extensive studies are available on the most widely encountered cyanotoxins, microcystins (MCs) in fresh and brackish water bodies. MC contaminated water poses severe risks to human health, environmental sustainability, and aquatic life. Therefore, commonly occurring MCs should be monitored. Occasionally, detection and quantification of these toxins are difficult due to the unavailability of pure standards. Enzymatic, immunological assays, and analytical techniques like protein phosphatase inhibition assay, enzyme-linked immunosorbent assay, high-performance liquid chromatography, liquid chromatography-mass spectrometry, and biosensors are used for their detection and quantification. There is no single method for the detection of all the different types of MCs; therefore, various techniques are often combined to yield reliable results. Biosensor development offered a problem-solving approach in the detection of MCs due to their high accuracy, sensitivity, rapid response, and portability. In this review, an endeavor has been made to uncover emerging techniques used for the detection and quantification of the MCs.

KEYWORDS
biosensor, ELISA, HPLC, LC-MS, microcystins, PPIA

1 | BACKGROUND

The population explosion, industrialization, and climate change have led to nutrient over-enrichment (eutrophication) of water bodies around the globe.[1] Increase in the temperature, light attenuation, pH, concentration of phosphate, nitrogen, and other natural organic matter due to disposal of untreated domestic, sewage and industrial wastes, and inflow of agricultural runoff polluted by livestock feces are considered to be the main reason for the development of harmful algal or cyanobacterial blooms (HABs), both in fresh and marine water bodies, and this could encourage cyanotoxin production.[2] Apart from these reasons, natural environmental conditions like leaching of nutrients and minerals from the soil, rocks, and mountains are also causing HABs.[3] This build-up of algal or cyanobacterial bloom renders water undesirable for drinking, recreational activities, and agricultural usage.[4] The formation of cyanobacterial blooms are severe in tropical regions, which tends to favor the growth of cyanobacteria. Factors like light intensity and increased incidence of rainfall promote the growth of blooms. The cyanotoxins produced by cyanobacteria includes microcystins (MCs, cyclic heptapeptides), nodularin (NOD, cyclic pentapeptides), cylindrospermopsins (tricyclic guanidine alkaloids), anatoxins (ANA, cyclic alkaloids), saxitoxins (alkaloids), lyngbyatoxins or aplysiatoxins (indole alkaloids), β-methylamino-L-alanine (BMAA) or 2,4-diaminobutyric acid (DAB) and lipopolysaccharides.

MCs are the most commonly and widely studied group of cyanotoxins. The generalized structure of MC and its important variants are presented in Figures 1A and 1B, respectively. MCs are commonly produced by cyanobacterial genera *Microcystis*, *Anabena*, *Aphanizomenon*, *Planktothrix*, and *Anabaenopsis*.[5] Furthermore, some species of cyanobacteria can produce multiple types and variants of MCs. The conditions of MCs production by cyanobacteria are not well understood, and they can only be identified by molecular tests. Even small cell densities are even sufficient to attain hazardous toxicity levels of MCs. MCs are released into the water bodies when an algal bloom dies off. However, sometimes MCs can also be released into the water by live cells.[6] Taking into account the challenges in the unavailability of safe water for daily livelihood, due to global occurrences of MCs in water bodies, demands more effective solutions to remove these toxins in water bodies. MCs cannot be completely removed or reduced by conventional methods, particularly in developing countries.[7–9] Additionally, water or sea-food contaminated with MCs is odorless, tasteless, and cannot be destroyed by cooking.[10] Therefore, there is a need to develop efficient, cost-effective, and highly sensitive methods for estimation of MCs at low levels so that it can be suitable for use as a routine, high-throughput, and fast regulatory water quality monitoring tool. Figure 2 addresses the increase in the number of research papers and scientific reports published strictly related to MC detection and indexed in the Web of Science categorized by continents in the last 10 years (2009-2019) in relation to the development of techniques for the detection and quantification of MCs concentration in water bodies. The data presented in Figure 2 has been collected from Web of Science through screening papers by using the keyword "microcystin detection" and "microcystins" and then categorizing the resulting articles by continents using the filters "year" and "region." The country of the corresponding author has been used to define the

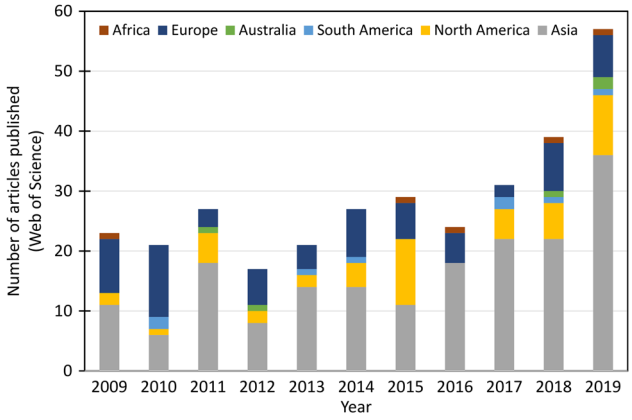
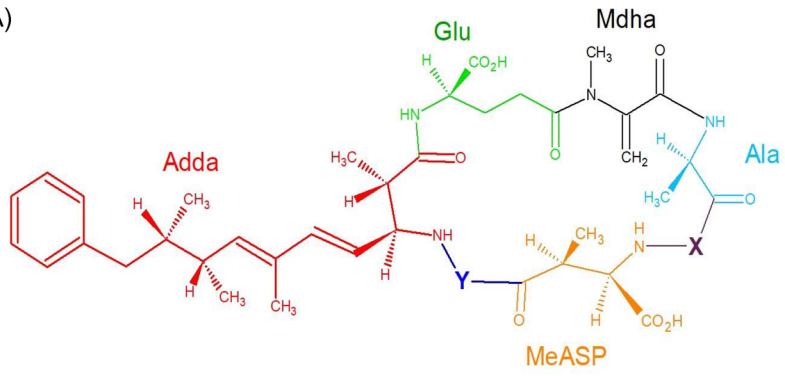


FIGURE 2 Research publication on microcystin detection in last decade (2009-2019). Source: Web of Science (http://www.webofknowledge.com)

(A)



(B)

Variant	X	Y
MC-LR	Leucine	Argenine
MC-RR	Argenine	Argenine
MC-YR	Tyrosine	Argenine
MC-LW	Leucine	Tryptophan
MC-LA	Leucine	Alanine
MC-LF	Leucine	Phenylalanine
MC-WR	Tryptophan	Argenine
MC-VF	Valine	phenylalanine
MC-LY	Leucine	Tyrosine
MC-YA	Tyrosine	Alanine
MC-LL	Leucine	Leucine
MC-FR	Phenylalanine	Argenine

FIGURE 1 Structures of cyanotoxins (A) general structure of MC (B) important variants of MC. MC, microcystin

continent from which the research article has been published. There have been several studies regarding the comparison of different methods for the detection of MCs.[11–16]

Recent advancements include the efforts by researchers like Turner et al,[17] who have been able to develop and optimize the novel ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for the quantification of MCs and NOD in shellfish, algal supplement tablet powder, water, and cyanobacteria. Zervou et al[18] have developed a fast, simple, and sensitive analytical method for the simultaneous detection of multi-class cyanobacterial and algal toxins in the water. Zhang et al[19] used the fluorescence polarization immunoassay (FPIA) for the detection of MCs and NOD in water. Triantis et al[20] were able to achieve a limit of detection (LOD) of 0.02 μg/L by combining protein phosphatase inhibition assay (PPIA), high-performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC-MS) for MCs. These techniques have certain disadvantages and cannot be used in routine and on-site monitoring. Thus, there is an earnest need for the development of a technique which is fast, portable, easy to handle, and also reduces the time for detection. This has led to the development of biosensor technique, which consists of a bioreceptor with direct contact to transducer.[21]

The efforts in the studies mentioned above can help to realize the urgent need to establish a fast, accurate, and reliable method. Therefore, this review discusses the summary of techniques and strategies developed so far for the detection and quantification of commonly encountered MCs.

2 | HARMFUL EFFECTS OF MCs AND OTHER CYANOTOXINS

Cyanotoxins produced by cyanobacteria have varying toxicological effects (hepatotoxic, neurotoxic, and dermatotoxic).[22] Cyanotoxins in drinking and recreational water have a serious deleterious health effect worldwide.[23,24] This phenomenon has been recognized by environmental quality regulatory authorities in many countries around the world,[24] such as by European Committee and WHO.[23] The most widespread cyanotoxins in the environment are MCs, the nonribosomal cyclic heptapeptide hepatotoxins (100 MC variants) and sometimes anatoxin (neurotoxins) or cylindrospermopsin[25] and BMAA that has been linked to neurodegenerative diseases.[26] Mouse assays indicated that the MC-LR and MC-LA variants were equally toxic, but were 12 times more toxic than another common variant MC-RR. Among cyanotoxins, MCs are considered as most threatening to humans and wildlife.[27,28] This may be because of their greater persistence compared with anatoxins, which are more rapidly degraded in surface waters. Besides this, cyanotoxins can accumulate in organisms and are transferred via aquatic food webs, presenting potential risks to human health. Cyanotoxins may enter the human body through different routes like oral, inhalation, or dermal passages. The most common modes of exposure are through recreational activities and drinking water.[29] Exposure to MCs by eukaryotic organisms affects not only the liver but also causes oxidative stress, cytoskeletal disruption, renal, pulmonary, cardiac toxicity, and reproductive toxicity.[30–32] An extensive review on cyanotoxins and their effects on human health has been provided by Buratti et al.[33] Exposure to these toxins results in common acute symptoms, which usually include nausea, headache, fever, pneumonia, respiratory problems, blistering in mouth, and liver damage.[34] Lone et al[35] have provided a detailed review on the immunotoxicity of MC-LR in mammals. There have been reports that the MCs promote the formation of reactive oxygen species (ROS), which leads to apoptotic processes and cause tissue damage. They have also discussed the activation of neutrophils and macrophages on exposure to MCs and the effect of MCs on the DNA damage in human lymphocytes. It has been observed that MCs are also responsible for the mitochondrial disruption due to the formation of ROS, which in turn, leads to the onset of mitochondrial permeability transition and loss of mitochondrial membrane potential.[36] There has been an enhancement in understanding the mechanisms of MCs toxicity in animal cells and how they are transferred into the cell. It is known that MCs interacts with a number of eukaryotic proteins like PP1, PP2A, P53, NADPH oxidase, etc. to bring about changes in their activities. Hepatocytes have been shown to have specificity for the uptake of MCs. The uptake of MCs occurs via transmembrane organic anion transporter peptides.[37] The MCs are bio-transformed into a relatively less toxic form by the conjugation of glutathione (GSH) and cysteine conjugates.[38] The conjugation of GSH to MC-LR is mediated by soluble glutathione *S*-transferase in various aquatic organisms. The presence of conjugates in cells has been confirmed in various studies by the use of fluorogenic bimane probe (monochlorobimane), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and Nuclear magnetic resonance (NMR).[38] Also, the effects on the reproductivity of different organisms have been summarized by Chen and Xie.[36] MCs can either have a direct effect on the testis and ovaries or can indirectly affect sex hormones by damaging the hypothalamic-pituitary-gonadal axis and liver.[36] Due to these harmful effects of MCs, WHO has laid down a guideline for its permissible limit in drinking water as 1 μg/L. Many countries have set guidelines for their countries based on the guidelines provided by WHO. Therefore, the development of reliable approaches for detection and analysis of MCs is highly demanding in the present scenario and should be practiced.

3 | APPROACHES FOR DETECTION OF MCs

3.1 | HPLC and capillary electrophoresis

For analysis and detection of MCs, in general, HPLC is coupled with an ultra-violet/visible (UV/Vis) detector for UV absorbance. The separation of cyanotoxins can be done either by normal-phase HPLC or by reverse-phase HPLC. The reverse-phase HPLC using

the acidic mobile phase is more popular due to its ability to resolve a large range of MCs.

Spoof et al[39] have tested short, narrow-bore (50 mm × 2.1 mm *i.d.*) reversed-phase BEH (bridged ethylsiloxane/silica 1.7 mm hybrid particles) shield columns to separate MCs and NODs. The use of such columns can shorten time and save the solvent required for the run. They also recommend the use of columns with sub 3 μm particle size for routine analysis at low pressures and a faster pace. HPLC has been employed in combination with other methods like enzyme-linked immunosorbent assay (ELISA), where ELISA is used for the screening of the presence of MCs and other cyanotoxins, and then HPLC is used for their quantifications, respectively.[40] The combination of ELISA and HPLC has also been used for MC and NOD detection and quantification in crops.[41] Shamsollahi et al[42] have achieved an LOD of 20 μg/L for MC-LR without the use of trifluoroacetic acid and high silanol activity of the HPLC column to get a short retention time of 5.33 minutes.

The HPLC method requires the availability of standard of cyanotoxins for correct identification and precise estimation but poses a problem for certain variants of MCs as standard of many variants are not available.[43] Sometimes, the amount of cyanotoxins in a given sample is low. Therefore, there is a need to concentrate the sample for its analysis with HPLC. To achieve this, there have been studies on different methods of sample preparation. Some researchers have used solid-phase extraction (SPE) to concentrate MC-LR followed by its detection with HPLC-DAD (diode array detector).[44] Using SPE requires a high amount of organic solvent during pre-treatment and desorption stages; this in turn, makes the process expensive. To prevent these demerits of SPE, Lian et al[45] have used magnetic solid-phase extraction (MSPE) combined with HPLC-UV to achieve LOD of 0.75×10^{-3} μg/L for detection of MC-LR. It involves the use of magnetic nanoparticles. MSPE has been used to extract the MC-LR out of the sample using a magnetic molecularly imprinted polymer on a magnetic graphene surface, which resulted in an LOD of 0.08 μg/L.[46] The HPLC can under-report total MC concentration due to method specificity and the high number of MC variants. An alternate MMPB (3-methoxy-2-methyl-4-phenylbutyric acid) technique has been followed to mitigate this problem. This approach quantifies the MMPB molecule, which is common to all the variants of MC and is formed by cleaving the ADDA moiety by oxidation.[47] A similar approach has been followed by Foss and Aubel,[48] who compared the total MC concentration from MMPB with ADDA ELISA and individual variants (MC-RR, MC-YR, MC-LR, MC-WR, MC-LA, MC-LY) and showed that the individual variant analysis does not account for the total MCs in the sample. They achieved an LOD of 0.05 μg/L for the total MCs. The HPLC has also been used for the determination of MCs (MC-LR, MC-RR, MC-YR, MC-LW, MC-LF) in vegetables after subjecting the sample to matrix solid-phase dispersion (MSPD) and coupling it with mass spectrometry.[49] They have reported an LOD and limit of quantification (LOQ) of 13 μg/kg of dry weight (DW) and 43 μg/kg DW, respectively. Various matrices like natural water and supplement tablet powders of cyanobacterial, shellfish, and algal origin have been studied for assessment of MCs and NODs extraction by UHPLC-MS/MS.[17]

3.2 | Liquid chromatography-mass spectrometry

The approach for LC-MS is similar to HPLC; but it couples the liquid chromatography column with the mass spectrometer. This technique enables the simultaneous separation and identification of toxins. The analytes eluted from the column are analyzed with MS. It is used as a confirmatory test for the presence of a particular cyanotoxin. Table 1 summarizes the modifications made with the LC-MS method for the analysis of MCs and some of the other cyanotoxins. Ortea et al[56] have used mass spectrometry in combination with PPIA. The general protocol follows the initial screening with PPIA or ELISA, followed by the concentration of the sample with SPE, and then mass spectrometry is done.

Pekar et al[57] have used this technique for the analysis of 22 different toxins simultaneously. They have reported the limit of quantification as 0.1 μg/L for 75% cyanotoxins (anatoxins, cylindrospermopsins, NOD, and MCs) in drinking water. As in HPLC, SPE can also be coupled with LC-MS. Zervou et al[18] have demonstrated the use of SPE for water samples of the lake. The technique was able to detect a range of MCs (MC-RR, MC-YR, MC-HtyR, MC-LR, MC-HilR, MC-WR, MC-LA, MC-LY, MC-LW and MC-LF) in the concentration range of 0.034-63 μg/L. Fayad et al[58] have developed a rapid method using SPE coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS) for MC detection in bloom samples, which yielded an LOD of less than microgram per litre level. The optimization of the technique is critical to establish a robust method capable of identifying and quantifying cyanotoxins even after matrix influences. SPE has also been coupled to liquid chromatography-quadrupole time-of-flight high-resolution mass spectrometry for the detection of MCs and anatoxins and was able to reach detection limits between 0.004 and 0.01 μg/L.[59] In the SPE online coupled to UHPLC-MS/MS method, desalting of the sample has shown a critical role to increase the robustness of the method.[60] Oehrle et al[50] have used UHPLC using particles smaller than 2 μm in combination with tandem mass spectrometry to improve sensitivity, speed, and resolution. The technique has been used for the detection of nine cyanotoxins (including six MCs) with a LOD of 0.002-0.1 μg/L, and the method has shown good applicability for water samples as well.[61] UHPLC-MS/MS with double SPE techniques has been implemented for the simultaneous extraction and detection of MC-LR, MC-RR, MC-YR, and cylindrospermopsin in vegetable samples.[51] LC-MS has been used for MC quantification in muscle and liver tissues of freshwater fishes with a LOD and LOQ of 0.06 and 0.21 μg/L, respectively, and 0.04 and 0.13 μg/L, for MC-LR and MC-RR, respectively.[53] UPLC-MS has been used with other multi-detection methods like microsphere-based method coupled with flow cytometry to monitor different toxins at the same time.[62]

LC-MS has been used for the quantification of cyanotoxins as well as for their derivatives formed after the biotransformation in plants and animals. Guo et al[54] have used LC-MS for the analysis of MC-LR, MC-LR-GSH, and MC-LR-Cys in rat liver. They used SPE and LC-electrospray ionization mass spectroscopy (LC-ESI-MS/MS) to achieve an LOD of 0.005, 0.007 and 0.006 μg/g DW and LOQ

TABLE 1 Modifications in LC-MS for the analysis of MCs and other cyanotoxins

Modification	Cyanotoxin	LOQ (μg/L)		LOD (μg/L)	Reference
		Lower limit	Upper limit		
Solid-phase extraction	MCs, NOD, CYN, ANA	0.034	63	NA	[18]
UPLC-MS/MS	MCs, ANA, and CYN	2	100	NA	[50]
LC/MS/MS	MCs	NA	NA	0.02	[20]
SPE-LC-MS/MS	MCs and NODs	NA	NA	<1	[19]
SPE-LC-quadrupole time-of-flight high-resolution mass spectrometry	MCs and ANA	0.004	0.01	NA	[51]
LC-MS/MS	MCs and NOD-R	0.018[a]	0.084[a]	0.006-0.028[a]	[52]
LC-MS	MC-LR	NA	NA	0.06	[53]
SPE and LC-ESI-MS/MS	MC-LR	NA	NA	0.005[b]	[54]
Mixed-mode cation-exchange SPE and LC-ESI-ITMS	MC-RR	NA	NA	NA	[55]

Abbreviations: ANA, anatoxin; CYN, cylindrospermopsin; LC-MS, liquid chromatography-mass spectrometry; LC-MS-MS, liquid chromatography with tandem mass spectrometry; LC-ESI-ITMS, liquid chromatography electrospray ionization ion trap mass spectrometry; LC-ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; LOD, limit of detection (the minimal amount of toxin whose presence or absence can be detected); LOQ, limit of quantification (upper and lower limits of the method are extremes of the linear range of the method); MCs, microcystins; NA, not available; NOD, nodularin; NOD-R, nodularin-R; SPE, solid-phase extraction; SPE-LC-MS/MS, solid-phase extraction and liquid chromatography-tandem mass spectrometry; UPLC-MS/MS, ultraperformance liquid chromatography-tandem mass spectrometry.
[a]μg/g—microgram of cyanotoxin per gram of algal dietary supplement.
[b]μg/g—microgram of cyanotoxin per gram of rat liver.

as 0.017, 0.023 and 0.020 μg/g DW for MC-LR, MC-LR-GSH, and MC-LR-Cys, respectively. A rapid and sensitive LC-MS has been used for simultaneous determination of MC-RR and its GSH and cysteine conjugates in fish plasma and bile. LOD for MC-RR, MC-RR-GSH, and MC-RR-Cys of 6, 12, and 9 μg/L have been reported.[63] However, Wu et al[55] have quantified MC-RR and its metabolites through mixed-mode cation-exchange SPE, then quantifying them through liquid chromatography electrospray ionization ion trap mass spectrometry (LC-ESI-ITMS) in the fish liver. The MMPB technique has also been used in combination with LC/MS to determine the total MC content in the sample.[64] LC-MS precursor ion screening has been employed for MC detection in the polar region, and this method could be useful for cyanotoxin detection in unusual matrices.[65] Bogialli et al[66] have used LC-QTOF for the screening of cyanotoxins in water samples. Also, they have developed a database of cyanobacterial metabolites using high-resolution mass spectrometry (HRMS). LC-MS has been used as a method to detect the presence of MCs on Koka reservoir (Euthopia). They have been able to identify five variants of MCs, namely MC-LR, MC-YR, MC-RR, MC-LA, and MC-dmLR.[67]

Liquid chromatography with electrospray ionization triple quadrupole mass spectrometry (LC-ESI-MS/MS) after SPE has been used to determine NOD in water. The detection limits were 0.002 μg/L for all hepatotoxins.[68] A LC-MS/MS method was developed for the simultaneous detection and quantitation of seven MC congeners and nodularin-R in blue-green algal dietary supplements with limits of detection of 0.006-0.028 μg/g dry supplement and quantitation of 0.018-0.084 μg/g dry supplement.[69]

3.3 | Biochemical methods

3.3.1 | Protein phosphate inhibition assay

It is an enzymatic assay for the detection of MCs and NODs. MCs and NODs are known to bind specifically to and inhibit the activity of serine and threonine phosphatase enzymes like PP1 and PP2A. Inhibition of protein phosphatase by these cyanotoxins is the basis for the detection of the heptapeptides. It measures the change in the amount of phosphate released from phosphorylated protein substrates.[70] The test typically requires about 1 hour on a 96-well microtitre plate, which makes it a simple, economical, and rapid method for MC detection. Due to its tendency to overestimate the toxin concentration, this method is used only as a screening method.[71] All the approaches used to assay require the exposure of enzyme by samples to be tested. The decrease in the activity of enzymes linearly correlated with the concentration of cyanotoxins present in the sample. Based on the method applied to detect the released phosphorus, assay can be of two types: where, (a) a radio-labeled substrate is used or (b) a chromogenic substrate is used.

In the early phases of the assay, researchers have employed the use of radio-labeled phosphorus. In this case, phosphatase inhibition is measured by measuring the release of ^{32}P isotope of phosphorus from a radio-labeled substrate. Yoshizawa et al[72] have used this approach by measuring the ^{32}P released from the phosphorylated histone H1. However, MacKintosh et al[73] have described the use of [^{32}P] glycogen phosphorylase to measure the quantity of MC. The drawbacks of this method are short life of ^{32}P and use of

sophisticated procedures as well as instruments which are not possible to be used by every lab.

In recent times, the colorimetric analysis has been in practice to overcome the drawbacks of the radio-labeled method. It uses the activity of protein phosphatase-1 toward a chromogenic substrate *p*-nitrophenyl phosphate (*p*NPP) (Eq. 1).[74] The rate of color produced due to liberation of *p*NP from *p*NPP is measured at 405 nm at 37°C.[75] The detection limit reported has been as low as 0.01 and 10-20 μg/L by Ward et al[76] and Almeida et al,[52] respectively.

$$\text{PP1} + p\text{NPP} \rightarrow p\text{NP(yellow)} + \text{PP1}. \quad (1)$$

The assay is sensitive to other inhibitors of protein phosphatase as well, such as okadaic acid, tautomycin, and calyculin A.[77] Rivasseau et al[78] have developed this test for a wide working range of MC concentration for screening purposes, and it has a detection limit of 0.4-10 μg/L, having a narrow working range of 0.2-0.8 μg/L for monitoring of the MC present. They attained this difference in sensitivity by adding nickel chloride and dithiothreitol (DTT) to wide and narrow reaction mixtures, respectively. Garibo et al[79] developed PPIA to evaluate the MC equivalent in the natural cyanobacterial bloom sample using recombinant and wild-type PP1 and PP2A. The method has been used for the evaluation of the toxicity of MC-LR and its derivatives after transformation.[80] Moore et al[81] have compared the PPIA and LC-MS for the quantification of MC congeners in veterinary samples. The variations in enzyme purity and instability of enzyme dimers in solution are the significant limitations of PPIA.[82]

3.3.2 | Enzyme-linked immunosorbent assay

This method offers advantages such as simplicity and cost-effectiveness. It is a commercially viable technique; at present, there are many kits available for MC and other cyanotoxin detection in the market (Table 2). ELISA is based on the interaction of MC-LR and NODs with monoclonal antibodies (mAbs) or polyclonal antibodies (pAbs) generated against them. Antibodies (Abs) having specificity and cross-reactivity against a wide range of variants of MCs and NODs are desirable, and achieved by increasing the Abs specific for a similar structural feature among different cyanotoxins. Generally, the unusual amino acid ADDA serves as a common recognition site for such Abs. The ability of an antibody (Ab) to have high cross-reactivity, makes this method cost-effective and applicable to a wide range of cyanotoxins. The Abs specific for ADDA also detects the free ADDA present in water bodies after the degradation of toxins, and this is not toxic and may fail to recognize modified ADDA containing toxins.

Abs specific to a particular toxin raised by injecting a small size toxin conjugated with a more prominent immunogenic carrier protein into mice has been shown by Yang et al[90] Kfir et al[91] have produced mAbs against MC-LR by the hybridoma technique. This has been done by using polylysine and muramyl dipeptides to make a high molecular weight complex. The Abs generated by this method had shown equal efficiency against different types of MCs. The use of mAbs has also been reported by Nagata et al,[92] and they also reported the protective effect of these Abs on the toxicity of MC-LR for the first time. Detection of MC-LR in trace amounts in the range of 0.002-0.1 μg/L has been possible by mAbs.[93] Long et al[94] have reported a LOD of 0.032 μg/L for MC-LR using the mAbs. Yu et al[95] have generated pAbs against MC-LR by conjugating MC-LR with γ-globulin. The generation of pAbs by conjugation of hapten and MC-LR has been reported by Metcalf et al.[96] To get Abs having high cross-reactivity, use of multihapten has also been made.[97] For this, a mixture of haptens conjugated to different variants of MC have used, which resulted in the generation of pAbs.

By ELISA, various other techniques for the detection of MC-LR have been developed. One such method is been called the chemiluminescence enzyme immunoassay. An enzyme immunoassay (EIA) has been developed by Khreich et al[98] with an LOD of 99.5×10^{-4} μg/L. They have reported cross-reactivity for MC-LR, MC-YR, and MC-LA. This method has the ability to detect extracellular as well as intracellular MC; which often goes undetected by other methods. Yang et al[90] developed a multi-analyte immunoassay with Abs having broad specificity and high affinity. The Abs had high sensitivity and high affinity for MC-LR, MC-LA, MC-YR, MC-LW, MC-RR, MC-LY, MC-WR, and MC-LF. Using these Abs, an indirect competitive ELISA (icELISA) has been developed, having a LOD of 0.16 μg/L for MC-LR.

The Abs directed against toxins are not only used for their detection but also used for their removal as well. Tsutsumi et al[99] have used an immunoaffinity column (IAC) for the concentration and detection of MC in tap water. It has also been shown that ELISA can be used to detect the presence of MCs in human serum. Although there are limitations associated with this, such as time requirement for sample preparation and overestimation of certain MC congeners need to be further pursued.[100]

A broad-spectrum non-competitive immunoassay has been used for NOD as well. The assay is based on the unique combination of a generic anti-immunocomplex (anti-IC) single-chain fragment of the antibody variable domain (scFv) and a mAb capable of binding to an ADDA-group present in all MCs/NOD. The anti-IC scFv was isolated from a large synthetic Ab library with phage display and used to develop a single-step sandwich-type non-competitive immunocomplex assay. The assay is capable of detecting NOD at a concentration below 0.1 μg/L in a 1 hour assay. The detection limits are ~0.026 μg/L in 1 hour and ~0.1 μg/L in 10 minutes assay time.[101] Table 3 presents biochemical (PPIA and ELISA) estimation methods for MCs detection.

3.3.3 | Biosensors

Biosensors offer a portable, simple, fast, sensitive, and handy way for the quantification of MCs. They could allow the development of cost-effective and easy techniques for the detection and enable the in situ analysis of MCs. Table 4 summarizes the increased focus on MCs detection by the biosensor method. Biosensors use the basic

TABLE 2 Commercial kits available in the market for the detection of microsystins and other cyanotoxins in freshwater

Kit name	Target cyanotoxin	Principle	LOD	Reference
Abraxis microcystin test strip	Microcystin	Direct competitive ELISA	0.1[a]	[83]
Abraxis microcystin tube kit (PN 520012A)	Microcystin	Direct competitive ELISA	0.09[a]	
Abraxis Microcystin/Nodularin PP2A microtiter plate test kits (PN 520032)[c]	Microcystin	PPIA	0.25[b]	
Abraxis (PN 520011 SAES)	ADDA in microcystin and nodularins	Indirect competitive ELISA	0.016[a]	
Envirologix Qualitube (PN ET 022)	Microcystin	Competitive ELISA	0.3[a]	[84]
ZEU-INMUNOTEC MicroCystest Plate Kit	Microcystin	PPIA	0.25[a]	[85]
Abnova Microcystin-LR ELISA kit (KA1496)	Microcystin	ELISA	0.1	[86]
Biorbyt Microcystin ELISA kit (orb59527)	Microcystin	ELISA	0.01	···
Enzo Life Sciences Microcystins- ADDA ELISA (microtitre plate) (ALX-850-319)	Microsystin and nodularin (ADDA specific)	ELISA	0.1	[87]
Aviva Microcystin-LR ELISA Kit (Water) (OKVA00009)	Microcystin-LR	ELISA	0.1	[88]
Creative Diagnostics Microcystin-LR ELISA kit (DEIA3935)	Microcystin-LR	ELISA	0.1	···

Abbreviations: ELISA, enzyme-linked immunosorbent assay; PN, product number; PPIA, protein phosphatase inhibition assay; NA, not available.
[a]μg/L—microgram of cyanotoxin per liter of sample.
[b]μg/g—microgram of cyanotoxin per gram of food sample.
[c]This kit was used by Marsan et al.[89]

TABLE 3 Biochemical estimation methods used for the detection and analysis of microsystins

Method	Modification	LOQ (μg/L)		LOD (μg/L)	Reference
		Lower limit	Upper limit		
PPIA	Colorimetric analysis using *p*NPP	0.01	NA	0.01	[76]
	Addition of nickel chloride	0.4	10	0.4	[78]
	Addition of DTT	0.2	0.8	0.2	[78]
	Colorimetric analysis using *p*NPP	10	20	10	[52]
	No modification	0.2	1.0	0.2	[20]
ELISA	Monoclonal antibodies	0.002	0.1	NA	[91]
	Competitive ELISA	NA	NA	0.1	[20]
	Indirect competitive ELISA	0.16	NA	NA	[90]
	Chemiluminescence enzyme immunoassay	NA	NA	0.032	[94]
	Enzyme immunoassay	NA	NA	0.00995	[98]
	Non-competitive immunoassay	0.1	NA	NA	[101]

Abbreviations: DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; LOD, limit of detection (the minimal amount of toxin whose presence or absence can be detected); LOQ, limit of quantification (upper and lower limits of the method are extremes of the linear range of the method); NA, not available; PPIA, protein phosphatase inhibition assay.

detection techniques of MCs, such as their reaction with specific Abs and binding to aptamers, etc. as their driving principle. For example, there is a biosensor based on the inhibition of PP1 by MC-LR. The approach followed to develop this biosensor required the use of a recombinant PP1 and a substrate, which is electrochemically active after its dephosphorylation.[102] One such substrate is phosphoparacetamol. The biosensor showed an LOD of 0.93 μg/L and has a working range of 0.93-40 μg/L. Herranz et al[103] presented a surface plasmon resonance (SPR)-based biosensor for MCs detection. The authors compared four mAbs in different formats, from which a amino-terminated self-assembled monolayer (SAM) with covalently immobilized mAbs was found to be the best to show a sufficient and selective signal for MC-LR detection. An SPR optical biosensor based on immunoassay has been developed, Abs produced for the biosensor has shown cross-reactivity for MC-LR, MC-RR, MC-LA, MC-LW, MC-LF, and NOD. The assay developed could detect an extracellular concentration of 0.5×10^{-3} μg/mL and intracellular concentration of 0.05×10^{-3} μg/mL.[124] An electrocemical non-enzymatic immunosensor has been developed using carbon nanofibers (CNFs) as an immobilization matrix and gold nanoparticles (AuNPs) as an electrochemical label. The assay was based on a competitive immunoassay format utilizing CNFs bound to MC-LR antigen, which competes with MC-LR to form antigen-antibody immunocomplex with MC-LR Abs. Then, this immunocomplex is reacted with AuNPs labeled with secondary Abs. The detection of MC-LR was performed after electro-oxidizing with HCl.[104] A nanoprobe based on quantum dots (QDs) is another means to quantify MC-LR. The MC-LR was immobilized on the QD through alkyl spacers to avoid hindrance during immunoreaction. The QD conjugated with MC-LR and free MC-LR are mixed with anti-MC-LR Abs, where they compete with each other to bind with Abs. The quantity of MC-LR present in a given sample is inversely proportional to fluorescence intensity.[125] A three-dimensional graphene-based biosensor for MC-LR detection and quantification has been developed. It utilizes the electrical properties of graphene. A detection limit of 0.05 μg/L was achieved with a linear correlation over a range of 0.05-20 μg/L MC-LR.[110] Zhang et al[111] have developed an electrochemical biosensor using the effect of MC-LR on DNA. They have immobilized calf thymus DNA (ctDNA) on a gold electrode. The presence of MC-LR changes the conformation of the immobilized ctDNA, which further decreases the electron transfer impedance.

Immunosensors have been around in the market for some time. Recently, there has been a boom in the field of electrochemical immunosensors. Pang et al[126] have developed an immunosensor using molybdenum disulfide/gold nanoclusters nanocomposites as a platform. Based on ELISA, many immunological biosensors has been developed. Liu et al[105] have used Abs developed against MC-LR to form immunochromatographic strips with a cut-off value of 0.001 μg/L. Lotierzo et al[106] used affinity membrane-bound Abs with a disposable amperometric immunosensor. Like electrodes, paper and fabric have also been used to make biosensors by coating it with the mixture of an Ab and single-walled carbon nanotubes (SWNTs). The nanotubes make the paper conductive and the change in the conductivity of the paper has been used to detect MC-LR.[107] Immunosensors, which utilize the competitive nature of Abs, have also been developed. This is an impedimetric immunosensor, which is based on decline in the conductivity of the electrode due to the formation of a precipitate on the surface of the electrode.[108] An SPR-based immunosensor from avian-Abs having cross-reactivity for multiple MC variants with a LOD of

TABLE 4 Estimation of MCs using various types of biosensors

Biosensor	Bioreceptor	LOQ (μg/L)		LOD (μg/L)	Reference
		Lower limit	Upper limit		
Electrochemical biosensor	Recombinant PP1	0.93	40	0.93	[102]
SPR biosensor	Monoclonal Ab of MCs	NA	NA	0.073	[103]
Non-enzymatic immunosensor	MC-LR antigen	0.0025	5	0.0016	[104]
Immunochromatographic biosensor	Monoclonal Abs for MC-LR	0.001	NA	NA	[105]
Amperometric immunosensor	MC-LR Ab	NA	NA	0.5	[106]
SWNT-paper biosensor	MC-LR Ab	NA	NA	0.6	[107]
Impedimetric immunosensor	MC-LR-bovine serum albumin	0.01	100	0.004	[108]
SPR immunosensor	Recombinant anti-microcystin avian Ab	NA	NA	1.9×10^{-4}	[109]
3D graphene-based biosensor	MC-LR Abs	0.05	20	0.05	[110]
Electrochemical biosensor	Calf thymus DNA	0.004	0.512	0.0014	[111]
Electrochemical immunoassay sensor	Alkyne end-terminated Ab	0.01	200	0.004	[112]
Electrochemical immunosensor	Antigen of MC-LR	0.005	50	0.004	[113]
Photoelectrochemical immunoassay sensor	Ab of MC-LR	0.005	500	0.001	[114]
Electrochemical immunosensor	Ab of MC-LR	0.01	20	0.005	[115]
Electrochemical impedance biosensor	MC-LR aptamer	0.049	99.5	0.017	[116]
Photoelectrochemical aptasensor	MC-LR aptamer	NA	NA	1.691×10^{-5}	[117]
Colorimetric sensor	MC-LR aptamer	0.4	7462.5	0.368	[118]
Fluorescence aptasensor	MC-LR aptamer	0.01	50	0.002	[119]
Fluorescence sensor	MC-LR aptamer	0.398	1194	0.137	[119]
Colorimetric sensor	MC-LR aptamer	NA	NA	0.497	[120]
Photoelectrochemical aptasensor	Single-stranded DNA aptamer of MC-LR	0.01	5	5×10^{-9}	[122]
Electrochemical aptasensor	Thiolated MC-LR aptamers	5×10^{-6}	3×10^{-2}	2×10^{-6}	[123]

Abbreviations: 3D, three-dimensional; Ab, antibody; MC, microcystin; NA, not available; LOD, limit of detection (the minimal amount of toxin whose presence or absence can be detected); LOQ, limit of quantification (upper and lower limits of the method are extremes of the linear range of the method); SPR, surface plasmon resonance; SWNT, single wall nanotubes.

1.9×10^{-4} μg/L has been developed.[109] A porous Au-paper working electrode (Au-PWE) has also been used in an electrochemical immunosensor to develop point-of-care testing. The procedure included the azide functionalization of Au-PWE combining functionalized Au-PWE with alkyne end-terminated Ab.[112] Gan et al[113] used a carbon nanotubebased immunosensor for MC-LR detection in the range of 0.005-50 μg/L and achieved a detection limit of 0.004 μg/L. Wei et al[114] have developed an ultrasensitive photoelectrochemical (PEC) immunoassay, which has a detection range of 0.005-500 μg/L with a detection limit of 0.001 μg/L. An electrochemical immunosensor based on molybdenum disulfide (MoS_2) and gold nanorod composite has been developed for the detection of MC-LR.[115] Zhang et al have reported a grapheme film/polyethylene terephthalate (GF/PET) composite-based electrochemical biosensor for the online detection of MC-LR in water bodies. The surface (GF/PET) of the developed biosensor was functionalized with ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS). The developed biosensor has detected a LOD of 2.3×10^{-3} μg/L and MC-LR detection in a wide range of 0.005-10 μg/L.[127]

In comparison to Abs, better specificity and stability with MC-LR can be achieved with aptamers.[128] Aptamer-based biosensors have also been developed, a brief summary has been presented in the work of Zhang et al.[129] These types of biosensors utilize the change in electrochemical properties of the electrodes to measure the concentration of MC-LR. This property has been utilized by Lin et al[116] to develop a label-free aptamer-based electrochemical impedance biosensor. The aptamer immobilized on the gold electrode would bind to MC-LR to form a complex, hence reducing the impedance of the electrode. The decrease in impedance could be linearly related to the concentration of MC-LR. A PEC aptasensor utilizing AgI nitrogen-doped graphene (AgI-NG) composites as photocathode and aptamer as recognition element has been developed for ultrasensitive detection of MC-LR. It has been found that AgI-NG has more photocurrent than AgI. It can be used for the detection of MC-LR in fish samples with a detection limit of 1.7×10^{-5} μg/L.[117] An aptamer

based colorimetric sensor has also developed using AuNPs.[118] Besides this, an aptamer based fluorescent sensor based on SWNTs with high selectivity toward MC-LR has been developed.[120] A portable analyzer has been developed using an aptamer-antibody immunoassay (AAIA) in which the horseradish peroxidase (HRP)-labeled Ab produces chemiluminescence to detect MC-LR. It has been tested for a range of 0.5-4.0 μg/L MC-LR and LOD of 0.3 μg/L and was found to be highly specific for MC-LR.[130] An electrochemical aptatoxisensor capable of measuring MC-LR in the range of 0.1-1.1 μg/L with a LOD of 0.04 μg/L has been developed on a glassy carbon electrode (GCE) by using cobalt(II) salicyladiimine metallodendrimer (SDD-Co(II)) doped with silver nanoparticles (AgNPs). The electrode GCE|SDD-Co(II)|AgNPs uses a 5′-thiolated aptamer.[130] Lv et al[119] have used a fluorescence aptasensor for the specific determination of MC-LR in the linear range of 0.01-50 μg/L with a LOD of 0.002 μg/L. Wang et al[121] have used an aptamer to form AuNP dimers. In the presence of the target molecule, the aptamer binds, which leads to disruption of the dimer and change in the color of the solution from blue to red. It has yielded an LOD of 0.497 μg/L. A photoelectrochemical aptasensor for MC-LR has been fabricated on a Ti-Fe plate after its electrochemical anodic oxidation. Then, Ti-Fe-O nanotubes were functionalized with reduced graphene oxide (rGO) upon the immobilized aptamer. The sensing platform operates under visible light conditions. The platform could give a linear range of $0.01–5.0 \times 10^{-6}$ μg/L with a detection limit of 5×10^{-9} μg/L.[122]

Liu et al[123] have used a dual signal amplification strategy in an electrochemical aptasensor for the quantification of MC-LR. They used TiO_2 nanobeads (TiONBs) coated with MoS_2 as a substrate to deposit AuNPs. Thiolated MC-LR aptamers were then immobilized on AuNP@MoS_2-TiONBs. The assay involves the competitive binding of MC-LR and biotin-cDNA complementary to MC-LR aptamer to the immobilized aptamer. MC-LR has been quantified by the extent of binding of avidin conjugated HRP to biotin-cDNA. The reduction of current is inversely proportional to the concentration of MC-LR in the sample. The amplification of the signal is first driven by the AuNP@MoS_2-TiONB ternary composite itself. The initial amplification is due to the large surface area provided by TiONB to MoS_2 nanosheets, which leads to a higher number of aptamers being immobilized on the surface. The second amplification is by the action of HRP. The sensor has a linear range of 5×10^{-6} to 3×10^{-2} μg/L with a LOD of 2×10^{-6} μg/L.[123] It is important to evaluate the toxin concentration in the water (extracellular) and within the cell (intracellular). Considering the detection of toxin at early stages, it becomes important to know the intracellular concentration as well. Based on this strategy, a portable biosensor involving an integrated approach to analyze total (intracellular and extracellular) as well as free (extracellular) MC-LR content has been developed.[131] The integrated system consists of sample processing and detection modules. The sample processing modules involve operations like lysing, filtration, and mixing. The detection modules work on the principle of electrochemical impedance spectroscopy (EIS) using electrochemical cell chips. The developed platform has an LOD of 5.7×10^{-4} μg/L with a linear dynamic range between 330 and 0.1 μg/L.

An aptasensor has been developed for MC-LR using fluorescence resonance energy transfer (FRET) on a QD. FRET involves the nonradiative transfer of energy from the excited donor fluorophore to

TABLE 5 A general comparison of different methods used for MC analysis

Method	Advantage	Disadvantage	Reference
PPIA	High selectivity and sensitivity	Unable to differentiate among MCs and NODs congeners	[15]
	Fast and easy to use		[20]
ELISA	High specificity	Expensive method due to specific antibody required against a cyanotoxins	[133]
	Quick to perform	Requires sophisticated laboratory equipments and skilled personnel for analysis	[134]
		Some Abs may have low cross-reactivity toward different cyanotoxins	
HPLC	Can be used routinely to identify and quantify cyanotoxins	Unable to differentiate structural variants of most microcystins	[14]
		Matrix effect can give false results	[43]
		Non availability of standards may hinder analysis since the method depends on retention time for detection	
Biosensor	Fast	Consistent reproducibility	[15]
	Portable		
	Reduction in time delay	Initial development cost is high	
	Easy to handle		
LC/MS	Enhanced sensitivity and selectivity	Requires availability of standards for analysis	[68]
		Expensive and requires skilled personnel for operation and data analysis	

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; MC, microcystin; NOD, nodularin; PPIA, protein phosphatase inhibition assay.

the acceptor fluorophore. The QD nanoparticles have been coated on a magnetic bead core, then the aptamer was immobilized on the QD surface. In the QD-aptasensor, QD transfers its energy to PoPo-3 dye. The QD gets excited at a wavelength of 360 nm and emits at 530 nm, this 530 nm wavelength then excites PoPo-3 dye to emit at 561 nm. The PoPo-3 molecules remain intercalated in the aptamer and give fluorescence in the absence of MC-LR. The QD-aptasensor had an LOD of 10^{-4} μg/L and a detection range of 12.7-15.8 μg/L in cyanobacterial culture and 1.0 and 7.2 μg/L in environmental samples.[132] The methods discussed above have their own advantages and disadvantages, which have been summarized in Table 5.

4 | CONCLUSION

Cyanotoxins are a modern-day threat to aquatic ecosystem and drinking water quality. They are known to be ubiquitous and pollute most water bodies. The need for the detection and quantification of the most common cyanotoxins, MCs, is paramount. Over the last few years, researchers have been regularly working on development of methods for routine analysis. There has been the development of various methods like PPIA, ELISA, HPLC, LC-MS, and biosensors. The methods discussed in this review make use of the diverse physical and chemical properties of MCs for their detection, as well as each method having different basic principles; therefore, no single approach seems to be sufficient to detect all types of MCs accurately. However, newly emerged approaches such as biosensors, in particular, have provided more significant advances for the detection and monitoring of MCs in water bodies. The speed, portability, and sensitivity of this method make it an effective approach in the area of water quality management in the field conditions. This review focuses on information about advanced approaches used for MC detection. This should provide assistance for future research directions and contribute toward a better understanding in selecting a suitable approach by microbiologists, ecologists, and water managing authorities for appropriate environmental assessment, as this is of concern as a public health risk. Concerning the emerging diversity of MCs variants with time, there is an urgent need for the development of a total analysis method, which is capable of detecting and analyzing all MCs rapidly with good sensitivity.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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