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European Journal of Experimental Biology, 2012, 2 (2):321-336

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Bioassay methods to identify the presence of cyanotoxins in drinking water supplies and their removal strategies

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ABSTRACT

A diversified group of toxins produced by freshwater cyanobacteria pose threat to human health as they frequently occur in drinking water sources. Though numerous qualitative as well as quantitative chemical analytical methods are now available, relatively simple low cost methods that are able to evaluate the potential health hazard and allow management decisions to be taken, are more useful to agencies that monitor drinking water supplies. Given that there is no single method that can provide adequate monitoring for all freshwater cyanotoxins in the increasing range of sample types, bioassays that can detect the toxic effects and safe levels of cyanobacterial toxins in drinking water supplies are discussed. Methods for removal of cyanobacterial cells as well as dissolved toxins in drinking waters prior to supply are also discussed.

Key Words: Freshwater cyanobacteria, toxins, microcystins, bioassay, removal of toxins, waterworks.

INTRODUCTION

Most, though not all, cyanobacterial blooms and scums produce secondary metabolites that are toxic to aquatic animals, fishes, cattle and even human [1, 2, 3]. The most frequently found toxin producing cyanobacterial species in freshwaters are *Microcystis, Anabaena, Nodularia, Planktothrix, Aphanizomenon, Cylindrospermopsin* and *Lyngbya* etc. These colonial or filamentous cyanobacteria produce a variety of chemically and biologically different toxic products such as hepatotoxins (microcystin and nodularin), neurotoxins (anatoxins and saxitoxins), paralytic shellfish toxins (as produced by *Aphanizomenon*), cytotoxins (cylindrospermopsin) and dermatotoxins (lipopolysaccharides) [2]. Awareness of toxic cyanobacterial blooms and scums in freshwaters, and of health hazards which they can present, is long established and a pronounced history of animals and fish deaths as well as outbreaks of human illness and poisonings are present. The extreme cases of human poisonings were manifested in the death of more than 60 hemodialysis patients in Caruaru, Brazil in 1996 [4] and in the incidences of primary liver cancer in China [5]. Freshwater cyanobacteria are reported to produce hepatotoxins, microcystins and nodularins more potently than other toxins [6] and, that is why, most research on cyanobacterial toxins has been centered on these toxins and their producer cells in freshwaters. Microcystins and nodularins cause severe disruption of liver architecture and function and induce clinical signs such as weakness, recumbency, pallor, vomiting and diarrhea and death occurs due to pooling of blood in the liver [2]. These hepatotoxins irreversibly inhibit protein phosphatase PP1 and PP2 A and can have diverse inhibitory effects at genetic, developmental, metabolic, and physiological levels,

including and beyond liver function [7]. The high susceptibility of liver cells to damage by microcystins *in vitro* and *in vivo* is accounted for by the active uptake of the toxins by bile acid transport system.

During summers, cyanobacterial blooms tend to flourish due to the nutrient enrichment of the water and a thick oily layer is filmed on the water surface. These algal blooms add bad taste and odor to the water. When this bloom degenerates, the toxins are released to the water, and the concentration of toxin may exceed the safe level for human consumption. The toxic level as high as 2.8 mg toxin per liter of lake water (~3000 times more then safe level) has been reported in Central Indian waters during summers [3]. Similar studies in Bangladesh showed presence of toxic level of more then 10 µg L^{-1} in almost twenty drinking water resources [8]. Among microcystins, microcystin-LR (Leucine-arginine variant) is most abundant as well as most toxic among more then 70 variants [1]. Based on such reports, the World Health Organization (WHO) has considered 1 µg toxin (equivalent to microcystin-LR) per liter of water (1 ppb) to be safe for human consumption [9]. The WHO guidelines are more suitable to the temperate and sub temperate countries, where the prevalence of the MC-LR has been shown widely, but in tropical countries where MC-RR dominates the blooms in most of the time [3, 10], this guideline proves to be a little fragile. Given that the structure-function relationship is present amongst MCs, the toxicity of over 70 structural variants differs to a great extent. For instance MC-RR is at least 20 times less toxic than MC-LR. In the light that more than one microcystin often contributes to the total microcystin content, and hence may contribute towards total toxicity of the cyanobacterial bloom, analytical methods that identify and quantify the microcystins in water samples may not be adequate while making predictions on total toxicity. Such toxin variants may mask or enhance the toxicity when act together. That is why; bioassays provide definitive support towards making water management strategies, as they provide the total and the actual toxicity of the given sample.

Bioassays present a direct toxicity impact in lesser time and help waterworks to maintain safe toxin level in drinking water supplies. There have been enormous number of bioassay methods developed so far for cyanotoxins that use the bioactivity of toxins, such as potent hepatotoxicity, cytotoxicity, enzymatic activity and immunological interactions, though till date, no single bioassay is available that can safely detect all structural variants of toxins (i.e. microcystins) produced by cyanobacteria. Considerable research efforts are being made since last two decades to find out alternative methods to the mouse bioassay as a routine monitoring assay for cyanotoxins. A number of alternative bioassays have become available in recent years. A comprehension of bioassay methods is however required, so that a suitable bioassay can be employed based on the initial information on type of cyanobacteria and their toxins prevailing in the water reservoir at a given time and space. However, further validation and comparison of methods are needed prior to their application and data prediction. The bioassays methods including microbes, animals, plants and their extracts along with sophisticated biochemical bioassays for detection of cyanotoxins are reviewed critically.

Besides posing health hazards, these toxins and the cells containing them, add bad tastes and odors, which significantly impair drinking water quality [11]. Removal of intact cyanobacterial cells with their intracellular compounds during water treatment would potentially reduce the concentration of taste, odor and toxic cell metabolites present in the treated water. Removal of toxins dissolved in water due to the decomposition of cells is equally important (at least below the safe level as per the WHO guidelines) before the water is safely used for drinking purposes.

The MCs are stable compounds and do not degrade fast in natural waters. Jones and Orr [12] showed that MCs persisted for nine weeks before degradation after an algicide treatment in a recreational lake. Lahti et al. [13] demonstrated that MC-LR was detectable in lake water during decomposition of a *Microcystis* bloom and was present in detectable amounts even weeks after the bloom disappeared. Tsuji et al. [14] showed that MC-LR was very stable because of limited decomposition by exposure with sunlight as compared to MC-RR and MC-YR. However, the addition of pigments extracted from cyanobacteria accelerated the decompositions and converted the microcystins into its inactive geometrical isomer 6(Z)-Adda microcystin. Though, the converted MC-LR from its inactive geometrical isomer showed essentially the same toxicity as that of intact microcystin LR. This clearly indicates that MCs are not degraded fast under natural conditions and hence need removal strategies that employ traditional as well as modern methods of water treatment. The toxin removal strategy should be planned based on the microcystin or other cyanotoxin composition in the given water bloom.

Treatment of drinking water traditionally employs screening, settling, filtration and disinfection steps, although not all steps are used in every case, and the arrangement and variations on each differ from facility to facility, and

around the world. Since, in recent past, so many methods evaluated safe and easy removal of toxic cells and toxins by applying new methods, or by alteration of traditional methods and their sequence, it is worth reviewing these methods.

A. Bioassay methods-

Bioassays using microbes-

Use of microbes in biotoxicity analysis helps in detecting low amounts with in short time, though this idea might not be very suitable for detecting toxicities presented by cyanobacteria. Use of viruses does not provide good support for identification of toxins, though methanolic and aqueous extracts from selected cyanobacteria were found toxic against adenovirus, herpesvirus Type 1 and influenza A virus [15]. The methanolic extracts of *Nodularia spumigena* HÜ 280 and *Synechocystis aquatilis* 428 were found to be weakly effective against herpesvirus Type 1 while the aqueous extracts of the *Calothrix gracilis* 96 and *Oscillatoria* species 234 inhibited the replication of Influenza A virus in MDCK cells. In all the above cases, experiments were performed with crude extracts and no correlation of antiviral activity to pure toxins has been established. In other study, the antiviral property of cyanobacteria against HIV 1 virus has been linked to some sulphoglycolipids from cyanobacteria [16] and not to microcystins or other toxic metabolites. Though the other possibilities are yet to be explored, it seems that the use of viruses may not provide good bioassay system for detection of cyanotoxins at present.

Bioassays using bacterial community for detection of cyanobacterial toxins have been confined only to the detection of toxic extracts from cyanobacteria and not to pure toxins such as microcystin-LR. Bioassays based on *Aeromonas hydrophila*, *Bacillus cereus* and *Bacillus subtilis* were found to be sensitive and suitable when aqueous and methanolic extracts from five cyanobacteria were examined [17]. The n-hexane extracts of *Oscillatoria*, *Nostoc*, *Cylindrospermum majus*, *Calothrix gracilis* and *Limnothrix redekei* and the methanol extracts of *Anabaena variabilis*, *Gloeocapsa caldariorum*, *Pseudanabaena catenata* and *Limnothrix redekei* inhibited the growth of *Bacillus subtilis* SBUG 14. The n-hexane extract of *Limnothrix redekei* was also active against *Staphylococcus aureus* SBUG 11 and *Micrococcus flavus* SBUG 16 [15]. In no case, the toxicity to bacterial strains was correlated with microcystin or any other cyanotoxin. The use of bacterial strains, however, needs further investigations. At the contrary, some studies show that natural heterotrophic bacterial population are not reduced by ambient toxin concentrations [18, 19] and the some strains of bacteria are actually stimulated by cyanobacterial blooms and extracts [12, 18]. Jones et al. [20] found that *Pseudomonads* were possibly involved in degradation of cyanobacterial blooms.

Other bacteria based bioassay is the Microtox bioluminescence assay that was based on toxicity indication by the reduction in the light emitted by the test bacterium *Photobacterium phosphoreum.* Initially this test was suggested suitable for the detection of microcystins in bloom samples [21]. However, detailed analysis suggested otherwise. The bioassay responded to unknown components of cyanobacterial extracts that were free from microcystins [22] and no correlation between response in the Microtox assay and cellular content of the known cyanotoxins could be found [23]. Pomati et al. [24] show Microtox assay for saxitoxins using three gram negative bacteria where saxitoxins have been shown to cause decrease in total cellular levels of Na^+ and K^+, as demonstrated by flame photometry. Dierstein et al. [25] proposed another bacteria-based bioassay based on the inhibition of prodigiosin pigment formation in *Serratia marcescens* due to the toxicity. Though, little correlation was found between cyanotoxin concentration and its pigment formation inhibitory activity. It can be assumed that some strains of bacteria might be used in bioassay systems as far as detection of toxic cyanobacterial blooms is concerned. Moreover, the choice of bacteria is highly important in Microtox assays and a variety of bacteria should be checked for lethality prior to the final experimentation.

Bioassay using invertebrate animals

For analysis of toxins and other secondary metabolites from cyanobacteria, invertebrates, especially, zooplankton has been used extensively [26, 27]. Zooplankta are small animals that feed on other plankton. Freshwater zooplankta are dominated by four major groups of animals: protozoa, rotifers, and two subclasses of the Crustacea, the cladocerans and copepods. Some of the neonates and larvae of larger animals, such as fish, crustaceans, and annelids, are also included here [28]. Since, these filter feeders graze directly on cyanobacterial blooms, and are affected directly by cyanotoxins; they offer a good use of them in a bioassay system.

Cyanobacterial blooms, isolated cells, cultured cells, extracts and purified substances; affect a variety of zooplankton negatively. Among the affected animals are protozoa; *Heteromita globosa* and *Spumella sp.* [19],

Rotifera; *Brachionus* rubens [29], Crustacea; Copepoda; *Diaptomus bergii* [30], Cladocera*; Daphnia* [31, 32, 33], *Moina* [26, 33], *Ceriodaphnia* [35], Insect larvae; *Aedes egypti* [36] and *Culex pipens* [37] and shrimp larvae; *Artemia salina* [19, 38]. During cyanobacterial blooms, the smaller cladoceran and rotifers dominate the zooplankton community [39]. Several studies indicate that cladocerans and copepods are more affected by cyanobacteria and the rotifers are least [40, 41]. This is the reason why most of workers preferred a test cladoceran *Daphnia sp.* as a bioassay system for detection of negative effects posed by cyanobacteria. *Daphnia* is prevalent in freshwaters of temperate countries, while another cladoceran, *Moina* prevails in tropical freshwaters. Both these water flea (commonly called 'daphnids') have been used extensively for cyanobacterial toxicity bioassay.

The literature dealing with effects of toxic cyanobacteria on growth, reproduction, survival and feeding of daphnids is extensive, but conflicting (see Agrawal & Agrawal [42] for a review). It is well documented that most species of *Daphnia* are sensitive to toxic cyanobacteria at high toxin concentrations i. e. >10 µg toxin ml^{-1} [19], and other non-toxic substances like protease inhibitors may also affect the daphnids [26, 27, 43, 44]. This makes the use of daphnids as a bioassay system for detection of toxic cyanobacterial blooms a little fragile. However, using different experimentation techniques such as survival, feeding inhibition, population growth rate etc. the toxicity of cyanobacteria can be assigned. It is very important to break colonies or large filaments of cyanobacteria before using in daphnids based bioassay, as large colonies and filaments can present mechanical interference and feeding inadequacy to daphnids, and the mortality may not reflect the toxicity of cyanobacteria [26, 45]

Shrimps, especially larvae of brine shrimp; *Artemia salina* has been found to be affected by toxic cyanobacteria and has been used extensively to detect the cyanobacterial toxicity in the recent past [22, 38, 46]. The toxic levels ranging 1-10 µg toxin ml^{-1} [19] can be identified using this test system. Though the larvae of *Artemia salina* are found sensitive to microcystins and nodularin, a further research is needed that includes use of other cyanobacterial toxins as well as cyanobacterial protease inhibitors in *Artemia* lethality assay, before this bioassay can be accepted universally.

Daphnid bioassays are not well suited for detecting microcystins and nodularins in lower concentrations, and their standardized culturing is time consuming and labour intensive [47]. In contrast, the eggs of *Artemia salina* are commercially available, and are viable for years under subzero temperatures. However, the toxicity towards all variants of microcystins, nodularins, anatoxins and saxitoxins as well as protease inhibitors has not been shown towards *Artemia salina* and that limits the use of this bioassay. Another bioassay that uses another aquatic invertebrate, fairy shrimp (*Thamnocephalus platyurus*) has been found to be sensitive to a number of, though not all, cyanotoxins [48]. This bioassay generated highly reproducible results by using commercially prepared test kit, as that of brine shrimp assay, but these kits are relatively expensive and have a limited shelf life (6 months) [49]. Moreover, when six microcystin congeners (including MC-LR) were tested for acute toxicity and protein phosphatase inhibition with *Thamnocephalus platyurus*, no correlation was found between the two activities. The toxicity was highest for [D-Asp^3, (E)-Dhb^7] MC-RR but the protein phosphatase activity was much weaker [50]. The study indicates that mechanisms other than the inhibition of protein phosphatase play in MC induced toxicity to *Thamnocephalus platyurus*.

The mosquito adults and larvae have also been investigated as potential bioassay systems against cyanobacterial toxins [37, 51]. Larvae of *Aedes aegyptii* have been found to be affected by neurotoxins and hepatotoxins from cyanobacteria. Adults of *Culex pipens* were found to be sensitive towards MC-LR [37] when injected. Both mosquitoes were relatively sensitive but have not been widely adopted due to the difficulties of handling this organism [49]. Similarly, adult houseflies (*Musca sp*), diamond-backed moth (*Plutella* sp.) and cotton leaf worm (*Spodoptera* sp.) were found sensitive towards MC-LR when injected with purified toxins and natural samples gave positive results that were comparable with mouse toxicity results and various insecticides [52]. However, the flies are difficult to handle and require microinjection, which is difficult to administer [49].

The other insect, fruit fly (*Drosophila melanogaster*) can detect microcystins successfully in bloom samples [53]. Fruit flies are easy to maintain in the laboratory, with no special equipment required. Toxin can be administered orally by adding filter discs spotted with sample plus sucrose to tubes containing pre-starved (24 h) flies [49]. The flies were not, however, sensitive to neurotoxic *Aphanizomenon* extract [53] and hence of limited use.

Bioassay using vertebrate animals

Mouse bioassay has been intensively used during last two decades, and still is most preferred bioassay as far as tests for microcystins are concerned. Male Swiss albino mouse are the mostly used strains for toxicity testing for cyanotoxins [49]. Toxicity is tested by intra peritoneal injection of cell lysate of cyanobacteria. Samples prepared in physiological saline solution are preferred if the volume to be injected is 0.5 ml or greater [54]. Mice are observed for 24 h and then sacrificed by cervical dislocation. A postmortem of liver tissue at the end of the observation period is necessary as hepatotoxins show characteristic symptoms of liver damage [3]. These hepatotoxins are known to induce signs of hepatotoxicity characterized by degeneration and vacuolation of the hepatic parenchyma, congestion and hemorrhaging, and hepatic vacuolation, etc. [3, 54] (Fig. 1). Additionally, the leakage of key hepatic enzymes i.e. glutamate pyruvate transaminase (GPT), glutamate oxalloacetate transaminase (GOT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in serum can be investigated in cases of lack of symptoms, or if mouse survives even after the observational period. For this, blood is collected from retro orbital plexus before sacrificing the mouse and the hepatic enzymes can be investigated in serum using commercially available diagnostic kits [3].

Fig. 2 shows leakage of hepatic enzymes after administration of an LD_{50} dose of MC-RR purified from a water bloom in India. Cylindrospermopsin shows protracted symptoms resulted from progressive organ failure, specifically liver and kidneys, which necessitates longer observation period [55]. Bernard et al. [56] showed acute hepatotoxicity with severe liver, kidney, and thymus damage with the Australian cylindrospermopsin-producing strain. Histological examination of the liver revealed only moderate and multifocal necrosis.

The mouse toxicity is expressed as LD_{50} mg dry weight of toxin or cyanobacteria per kg mouse body weight [57] and a LD_{50} of <1000 mg dry weight is considered the cyanobacteria as non-toxic. The first major drawback in using mouse assay is the need of an animal house facility for rearing the animals for routine experiments. Secondly, the use of animals in toxicity studies is against scientific ethics and is actually banned in most of the countries. Moreover, where more than one type of cyanotoxin is present, the more rapid-acting toxin (i.e. microcystin-LR) may mask other symptoms [58]. But the overall toxicity due to cyanobacteria can be estimated in drinking water supplies using mouse bioassays.

Fishes are also affected by cyanobacterial toxins in the ways of liver damage, disturbed ionic regulation, behavioral changes and mortality [59, 60]. Young brown trout [60], Tilapia and Carp [61] are the fishes reported to be most sensitive, and can be used as a test system against cyanobacteria. Unlike mouse bioassay, fish bioassays may not prove to be easy and sensitive. Injecting cyanobacterial extracts to fishes is a difficult task, and immersion in media containing cyanobacterial extracts might need more amounts of cyanobacterial extracts in order to get lethal effects, and the oral toxicity can be subsided by the detoxification of toxin in various ways.

A desert locust (*Schistocerca gregaria*) based bioassay has been applied for detecting saxitoxins successfully in cyanobacteria and shellfish [62, 63]. Similar to mouse bioassay, locusts are easy to handle and samples can be administered by injecting low volumes (10 µl). The results, characterized by the paralytic stroke, are obtained within 90 minutes [62]. The LD_{50} for pure saxitoxin was found to be 8 µg g^{-1} locust body weight, but the bioassay was not found sensitive to microcystin-LR or anatoxin-a. Moreover, relative toxicities of selected saxitoxin analogues differed from those reported in mammalian systems [63]. Authors discussed the use of locusts as simple, ethically acceptable, broad-specificity functional bioassay, for the monitoring of saxitoxins and other paralytic shellfish toxins.

Bioassay using cell cultures

Since most of the vertebrate animals including mammals are affected by toxic cyanobacteria in various ways, bioassays using cultured mammalian cells instead of using animals have emerged as suitable replacements for animal bioassays. The well-documented fact that microcystins cause acute liver damage has prompted studies using hepatocytes (liver cells). Aune and Berg [64] used freshly isolated rat hepatocytes for the first time. The toxicity was measured by leakage of a key hepatic enzyme, lactate dehydrogenase (LDH) from hepatocytes. Typically, isolated rat hepatocytes are incubated with pure toxin or bloom extracts for a specified time and then the viability of the cells is assessed using the (3,4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) test [65]. The bioassay provided the first authenticated report for comparison of toxicities with the change in structure of microcystin and showed that MC-LR is most toxic and MC-RR is at least 100 times less toxic as compared to MC-LR [58]. As a replacement of rat hepatocytes, a liver slice culture was introduced for assessing hepatotoxicity of freshwater cyanobacteria [66]. This method was also based upon the leakage of liver enzyme (LDH) from freshly prepared liver

slice culture due to the toxicity of microcystins, and has an advantage over hepatocytes in its costing and non-requirement of sophisticated instruments and culture conditions

Apart from hepatic cells, other cells have also been used an indicator of cyanobacterial toxicity. A blood cell-based assay was proposed as early as 1981 by Carmichael and Bent [67] that showed the agglutination of the red blood cells as an indicator of microcystin level. However, this test failed in further experiments and found to be a poor indicator for microcystins. Codd et al. [68] were the first to use *in vitro* fibroblast cytotoxicity assay for microcystins. Lawton et al. [57] used the assay using V79 hamster fibroblast cells, which correlated well with the mouse bioassay. However, the assay demonstrated a number of false positives and false negative results, which make this bioassay a little fragile. Gonçalves et al. [69] showed that in addition to its hepatotoxicity, microcystin might have an immunomodulatory effect. Authors discussed the use of leukocytes as a bioassay parameter for monitoring microcystin. The leukocytes of healthy volunteers presented an increase in apoptosis rates while leukocytes from hemodialysis patients exhibited a lower production of oxygen-reactive species when incubated with microcystin-LR

Apart from mammalian cell lines, two fish cells lines PLHC-1 (derived from a hepatocellular carcinoma of the topminnow *Poeciliopsis lucida*) and RTG-2 fibroblast-like cells (derived from the gonads of rainbow trout *Oncorhynchus mykiss*) were found to be sensitive enough to several concentrations of extracts from a natural cyanobacterial bloom and a *Microcystis aeruginosa*–isolated strain as elucidated by succinyl dehydrogenase (SDH) activity. The similar EC_{50} values were recorded for natural and isolated cyanobacteria strains. Increased secretion vesicles, rounding effects, decreased cell numbers and size, hydropic degeneration, esteatosis, and apoptosis were observed in the morphologic studies of both cell lines. However, cyanobacterial bloom was more toxic to the PLHC-1 cell line [70].

For neurotoxins assays, a neuroreceptor-binding assay was developed earlier for saxitoxins, which works on the basis of competitive displacement and used radio-labeled saxitoxin [71]. A neuroblastoma cell line technique for sodium channel blocking activity has also been developed for the analysis of neurotoxins [72, 73]. Again, the use of cell cultures for toxicity needs further experiments before a universal cell line can be adopted for all known cyanotoxins in freshwaters.

Bioassay using plants and plant extracts

Secondary metabolites including microcystins produced by cyanobacteria are known to have algicidal or herbicidal properties [74, 75]. Bioassay using *Anacystis, Phormidium, Plectonema* and *Chlorella* has been used to investigate algicidal effects posed by *Oscillatoria* [76]. Little work has, however, been done on establishing a simple, cost-effective and sensitive plant based bioassay for the detection of cyanotoxins in drinking water. Gehringer et al. [77] investigated the effect of a microcystin-LR extract on the growth of *Lepidium sativum* over 6 days. Exposure to 10 μg L^{-1} microcystin-LR concentration resulted in a significant decrease in root and leaf lengths as well as fresh weights of seedlings when compared to the controls. Glutathione S-transferase and glutathione peroxidase activities were also significantly raised in plants at investigated toxin levels. Authors discussed the use of *Lepidium sativum* as a bioassay against microcystins, though the effect of microcystins other then MC-LR and other cyanobacterial toxins have not been included in the study. The use of this bioassay needs vast exploration.

Cylindrospermopsin was shown to pose negative effects on the germination of pollen from tobacco (*Nicotiana tabacum* cv Samsun NN). Pollen germination was inhibited by cylindrospermopsin between 5 and 1000 μg ml^{-1} [78]. The inhibition of tobacco pollen germination may be amenable for development as a bioassay for cylindrospermopsin, although this would require a pre-concentration step for the monitoring of environmental samples.

Enzyme Bioassay

Microcystins and nodularins are reported to inhibit protein phosphates (PP) 1 and 2A [79]. In this way, the protein phosphatase inhibition assay has proved to be a sensitive screening method for microcystins and nodularins. Microcystins bind equally well with PP1 and 2A. Earlier versions of PP1 and 2A bioassay were based on the quantitation of ^{32}P-phosphate released from a radiolabelled substrate [80, 81]. This bioassay was sensitive to sub-nanogram levels of microcystin and nodularin. The method has also been used successfully for quantitation of microcystins in environmental samples such as drinking water before and after water treatment [81]. Being sensitive enough, this method was not used widely because of the use of radioactive substrate, which necessitates specialized

laboratory equipment and regulations. The refinement of this method was introduced by An and Carmichael [82] who used a colorimetric protein phosphatase inhibition assay which avoids the complications of using radioactive materials. The use of the protein phosphatase inhibition assay is extremely helpful to confirm toxicity of microcystins in environmental samples. The non-radioactive bioassay may therefore be used increasingly for the routine screening of water samples, as shown by Ward et al. [83]. It is however, noteworthy, that false positive and negative results can be obtained using PP1 and PP2A assays, and expert staff should perform the use of these enzyme assays before making any general statement. Moreover, this bioassay can be applied to microcystins, nodularins and their variants only.

Bioassays for neurotoxins use inhibition of acetylcholin esterase (ACE) enzyme, which is a neuro receptor [84]. This is a sensitive method and is the only alternative to the mouse bioassay currently available for anatoxins. However, the assay is not selective as it can also detect other toxicants, such as organophosphorus-based pesticides in the environmental samples such as surface waters [1].

Enzyme Linked Immuno Sorbant Assay (ELISA)

The Enzyme-Linked Immuno Sorbant Assay (ELISA) technique is currently the most promising method for rapid sample screening for microcystins and nodularins because of its sensitivity, specificity and ease of operation. A large number of environmental samples can be screened within no time. This assay is based on monoclonal or polyclonal antisera raised in rabbits against bovine serum albumin (BSA) conjugated to microcystins or other toxins. Sheng et al. [85] showed that polyclonal antibodies generated by immunization with MC-LR-BSA showed good cross-reactivity with microcystins-LR, -RR, -YR, -LF, -LW and nodularin. The detection limit of 0.12 µg L^{-1} corresponding to MC-LR was achieved. Earlier, Nagata et al. [86] produced six monoclonal antibodies against microcystin-LR. Among them, M8H5 antibody showed cross-reactivity with microcystin-RR, -YR, -LA, [D-Asp_3] microcystin-LR, [Dha_7] microcystin-LR, glutathione conjugate of microcystin-LR, mono methyl ester of microcystin-LR, nodularin and 6(Z)-ADDA microcystin-LR. It should be noted that this antibody also reacts to the non-toxic mono methyl ester of microcystin-LR giving a false positive result. Using a commercially available monoclonal antibody, MC10E7, a more sensitive competitive ELISA method has been developed by Lindner et al. [87] with detection limits of 4 ng L^{-1} for water samples. In a typical ELISA bioassay, the antibodies are fixed to the walls of the wells of a microtiter plate. The first step involves binding of the calibrators (a non-toxic microcystin-LR surrogate at different concentrations), a negative control and the samples to the antibodies in the wells. The addition of a microcystin-enzyme conjugate binds to the remaining antibodies. After thorough rinsing, the concentration of bound enzyme is measured colorimetrically in an ELISA microplate reader. The microcystin concentration is inversely proportional to the color intensity [49].

Development of immuno-diagnostic systems for the detection of PSPs such as saxitoxins has been aimed for the routine monitoring of shellfish from the marine environment though can be applied in freshwaters also [88]. Both polyclonal and monoclonal antibodies have been produced, although none have shown cross-reactivity with all the known variants. Kralovec et al. [89] developed a novel ELISA technology for detecting saxitoxin, which was based on non-covalent immobilization of free saxitoxin. The efficacy of this technology was demonstrated on a polyclonal rabbit anti-saxitoxin antibody and compared with a conventional ELISA of saxitoxin using saxitoxin-bovine serum albumin conjugate as the coating antigen. A detection limit of 35 *p*M ml^{-1} was found by this method. Micheli et al. [90] produced polyclonal antibodies from rabbits immunized with saxitoxin-keyhole limpet hemocyanin. Results showed the saxitoxin detection limit to be 3 and 10 pg ml^{-1} for direct and indirect ELISA formats, respectively. Though, ELISA techniques look promising for detection of cyanotoxins in drinking waters, they are of restricted use. Antibodies against all possible variants of hepatotoxins and anatoxins are still not available. Moreover, the ELISA kits and consumables are far more expensive then any other bioassay system.

B. Methods of removal of toxins from drinking water supplies

Using surface water contaminated with cyanobacterial cells and/or dissolved toxins, as drinking or recreational purposes, different approaches of water treatment are needed. First, intact cyanobacterial cells or colonies are to be removed from water without causing damage to the cells, so the toxin contained within the cells is not released to the water. A typical full-scale water treatment plant uses co-agulation-sedimentation, duel media filtration and chlorination [91]. Processes such as settling, coagulation, filtration and flocculation can achieve safe removal of algal cells from drinking water, and these methods may be used in combination to remove the algal bloom cells. However, there seems to be some disagreement in the literature regarding the efficiency of conventional treatment (C/F/S, filtration, chlorination) for cyanobacterial cells removal. Some papers report the occurrence of cell lysis,

release of intracellular toxins and taste and odor compounds [18, 92], while others refer no release of such compounds to the water [93, 94]. However, the removal efficiencies of *Microcystis* cells have been shown from 58% to 90% by the conventional treatments, and showed that such procedures are not effective for extra-cellular toxin removal [95]. Fig. 4 shows the schematic of water treatment with reference to cyanotoxins.

Coagulation/flocculation with alum is being used worldwide. Coagulants such as aluminum sulphate, ferric sulphate, ferric chloride and polymerized coagulants as poly aluminum chloride have been successfully used for treating algal-rich waters. Studies suggest that pre-polymerized coagulants have some advantages over metal salt coagulants: better overall treatment efficiency, better flock separation, wider working pH range, lower sensitivity to low temperatures and lower residual metal-ion concentration [96, 97]. Chow et al [95] showed removal of *M. aeruginosa* by alum flocculation method without damaging the cultured cells. Ribau-Teixeira and Rosa [98] showed that an aluminum pre-polymerized coagulant of high basicity has the potential of removing single cells of *Microcystis aeruginosa* yielding very high chlorophyll *a* removal (93-98%), with very less toxin release to water (8–15%) when coupled with dissolved air flotation. Sedimentation for removal of cyanobacterial bloom cells might not be a good idea, as the buoyant cells and colonies tend to float on the surface. The dissolved air flotation (DAF) is widely considered more effective than sedimentation in the treatment of algal-rich water. According to Lam et al. [18]), flock blanket clarification had shown approximately 80% removal of *Microcystis* cells while DAF removed 98% in the presence of other algae. The same high DAF removal efficiencies for *Microcystis aeruginosa* and *Anabaena circinalis* [99] and for *Chlorella* and *Cyclotella* [100] have been reported. C/F/DAF process showed the best cyanobacterial removal efficiencies, higher than 92%, as well as the lowest residuals for lower optimal coagulant dose. Extra cellular microcystins were practically not removed from the water by both processes, but no release of toxins from *Microcystis* cells during treatment was found [98]. These experiments were performed with cultured single celled *Microcystis aeruginosa* cultures, and it will be worth investigating if natural populations of *Microcystis* would also be removed effectively by this process, given that high amount of mucous is associated with their buoyant colonies.

Another method for removal of *Microcystis aeruginosa* cells from raw water is by ultrasonic removal. Zhang et al, [11] showed that algal cells could be effectively removed by sonication and gas vesicle collapse was the main mechanism. However, higher ultrasonic power and long irradiation caused cell lysis and increased the amount of microcystins in water. Ultra-filtration is able to remove microorganisms (>99.99%), but a point of concern is the possible release of cell-bound cyanotoxins due to the shear of the feed pump. Ultra-filtration experiments carried out with a dense cell-culture of *Planktothrix agardhii* and with a bloom of *P. rubescens* in Germany showed release of a maximum of 2% of the cell-bound microcystin, from the cells into the medium. Thus, the removal of the cells resulted in a removal of cell-bound microcystin of over 98% [101].

The second step needed for water treatment is to effectively remove dissolved toxins i.e. microcystins. Removal of microcystin through the sand filters was shown to be primarily through biological degradation processes. Using polymerase chain reaction (PCR), biofilm, extracted from one of the sand filters that had effectively removed the microcystins, was shown to contain bacteria with the *mlrA* gene. Detection of this gene provided additional evidence that biological degradation of microcystin was the primary removal mechanism [102].

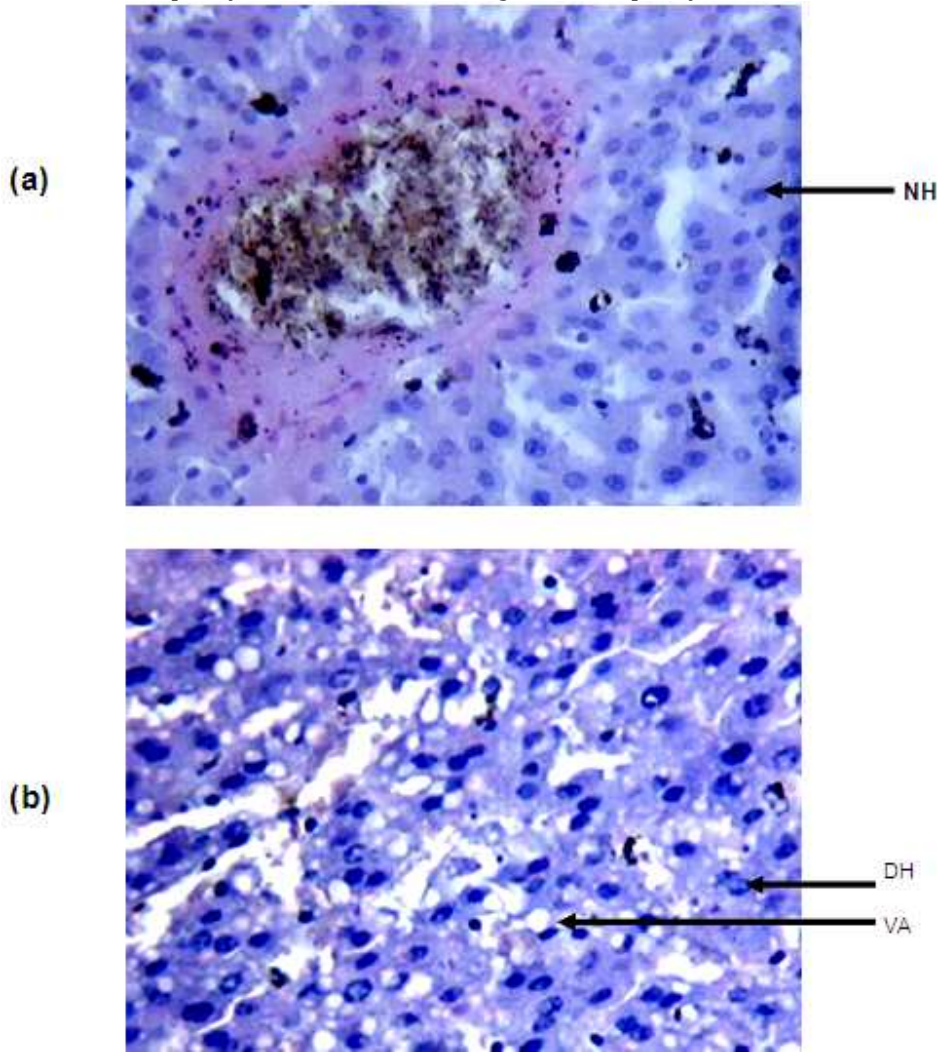
Chlorination of water is a long established method for disinfection of drinking water. According to Shi et al. [103] microcystins (MCs) in water can be directly and effectively removed by active chlorine transformed *in situ* from the naturally existing Cl^- in water resource using electrochemical method. Titanium coated with RuO_2 and TiO_2 was used as the anode. The results suggested that high concentrations of MC-RR and MC-LR in aqueous solution could be synchronously decomposed within 15 min of electrolysis. The qualitative analysis showed that the heptapeptide ring and the Adda group of both treated MCs were changed. Almost complete removals could be obtained in the case of indirect electro-oxidation with *in situ* electro-generated active chlorine from Cl^- in water [103].

The oxidation of the microcystins was related to the chlorine exposure of the sample waters following the trend: microcystin-YR>microcystin-RR>microcystin-LR>microcystin-LA [104]. Though results suggested that chlorination at an adequate chlorine dose is very effective for the removal of microcystin in raw water, pre-oxidation of the cell itself with chlorine must be avoided, because it frequently causes toxin release from algae and produce tri halomethanes during water treatment [105]. The chlorine treated water was not toxic to mouse as shown by the histological examinations except the cases when cylindrospermopsin was present in the water samples [106]. It is recommended that further toxicological studies should be performed with chlorinated cyanobacterial solutions.

Holst et al. [107] demonstrated that indigenous microorganisms in the sediment of a water recharge facility were capable of degrading microcystin. At oxic or micro aerophilic (<2% O_2) conditions, microcystin added to sediment slurries was reduced to 70% of its original concentration in 1–2 weeks, and to 96% after 7 weeks. At anoxic conditions and with addition of nitrate, the degradation was significantly stimulated, reducing microcystin to 80% within a day. The simultaneous production of N_2O in the samples suggests that the microcystin degradation was coupled to dissimilative nitrate reduction. Since aquifers and sediments beneath drinking water reservoirs often are anoxic, nitrate respiration may be an important process in removal and detoxification of microcystins.

Figure 1- Histology of mouse liver architecture (400 X magnification) after i.p injection of *Microcystis* bloom extract (a) control liver injected with phosphate buffered saline and (b) liver of mouse injected with bloom extract equivalent to 10 mg cell dry weight collected from Lake Kundam, India.

NH, normal hepatocytes with nucleus; DH, degenerated hepatocytes; VA, vacuolation.



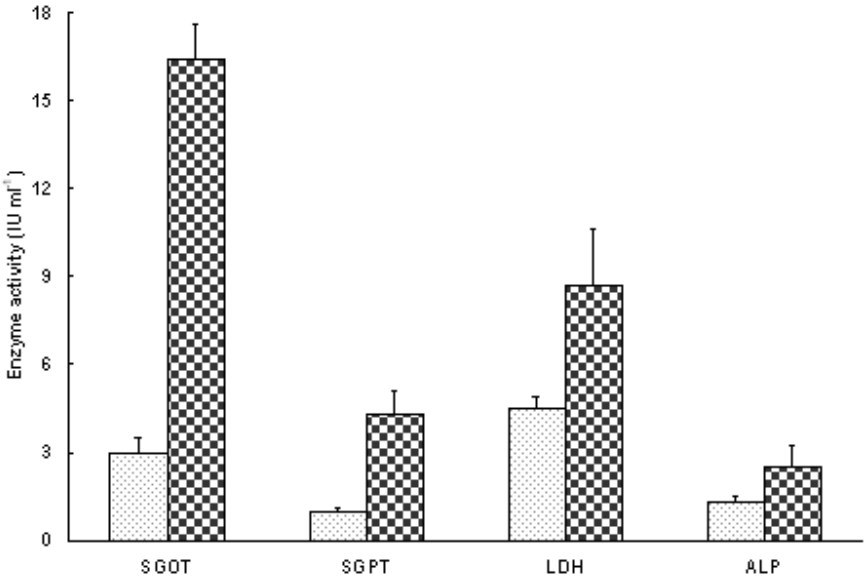
Three human probiotics, *Lactobacillus rhamnosus* strains GG and LC-705, and *Bifidobacterium lactis* strain Bb12, were also found to bind the cyanobacterial peptide toxin microcystin-LR from water solutions [108]. The highest removal percentage was observed with heat-treated *L. rhamnosus* strain GG for microcystin-LR.

Addition of catalysts to water treatment is also a good choice for the removal of toxins, though most of the work dealt with removal of microcystins only. Some treatment methods used the combined UV/ hydrogen peroxide (H_2O_2) system. The combined UV/H_2O_2 process proves to be an effective technology for the removal of microcystins. The ultra violet radiations lead to photocatalysis of the microcystins and the reaction is enhanced by

electron acceptor (H_2O_2) [109, 110]. Similarly, UV induced photocatalysis in combination with TiO_2 effectively destroys microcystin-LR in aqueous solutions, however non-toxic by-products were detected. Yuan et al. [111] showed degradation of MC-LR following the addition of ferrate as the indirect electron acceptors to the photocatalytic process. Low ferrate dose added to the photocatalytic process, yielded a significant enhancement in the photocatalytic rate and the efficiency could be increased to 100%. Ferrate treatment may be an effective and practical method for the removal of cyanobacterial peptide toxins from eutrophic waters, especially, which hold high total organic carbon [112]. Compared the effect of Fe(III) and Fe(VI) on the photocatalysis rate, the process of Fe(VI)-assisted photocatalytic degradation of MC-LR apparently existed the synergistic effect and the photocatalysis rate constant of Fe(VI)/UV/TiO_2 process was higher than Fe(III)/UV/TiO_2 by 2.5 times and than UV/TiO_2 by 4.4 times, but an overdose of ferrate will retard the rate due to the short circuiting reactions between Fe3+ and Fe2+ and the lower absorption of UV light. The pH had a remarkable influence on the reaction rate of detoxification of microcystin-LR and pH 6.0 was beneficial to the photocatalytic process [111].

Figure 2- Release of hepatic enzymes after administration of a LD_{50} dose of MC-RR purified from a *Microcystis* bloom ( ) against saline control ( ) in India

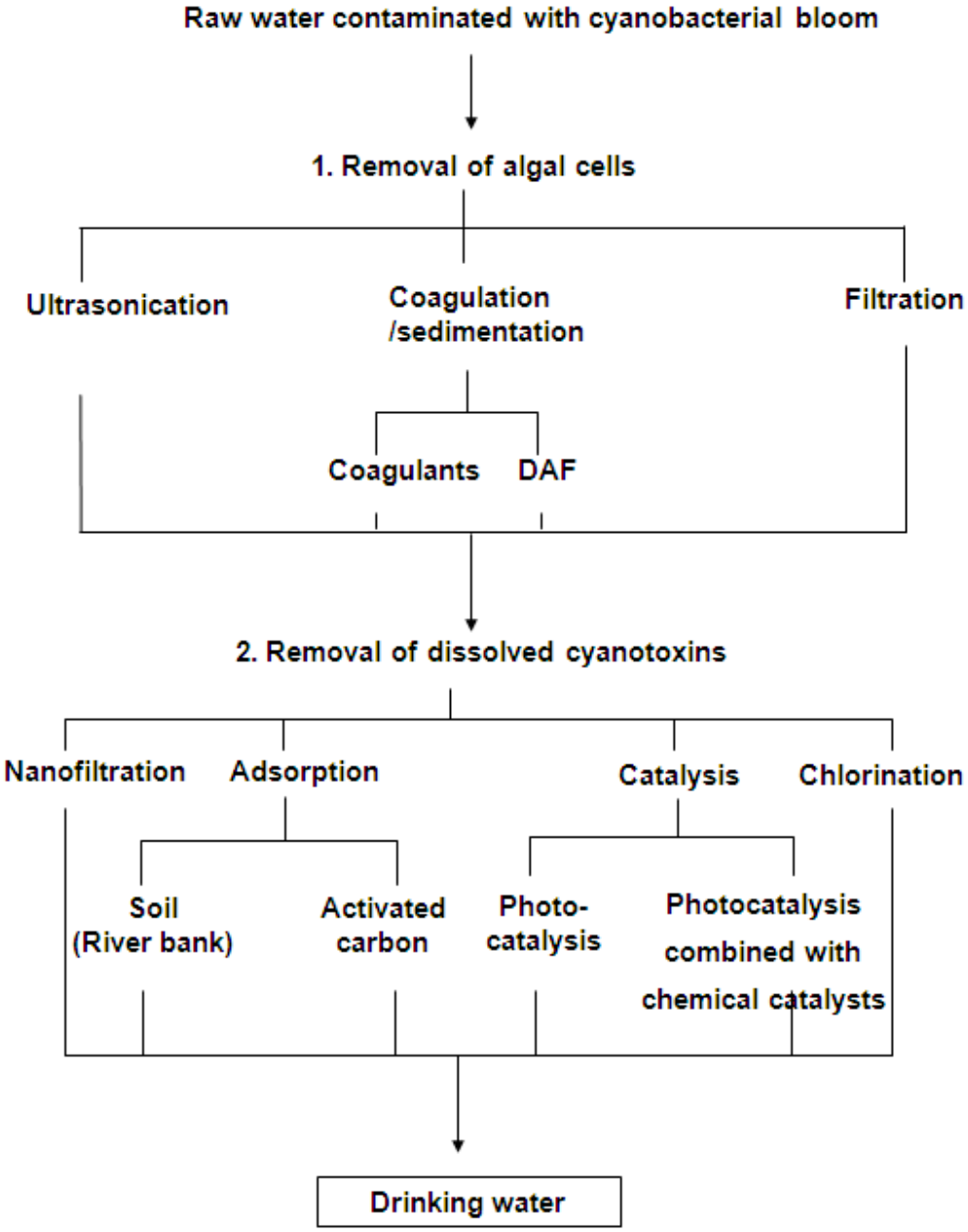
The enzymes were assayed using commercially available kits (Merck, India) and enzyme activity is presented as mean ±SE international unit per ml of blood serum. SGOT=serum glutamate oxalloacetate transaminase, SGPT= serum glutamate pyruvate transaminase, LDH= lactate dehydrogenase and ALT=alkaline phosphatase



The use of activated carbon has been increased as a potential remover of toxins from water by adsorption. Maatouk et al. [113] showed total elimination of cyanobacterial cells and the low concentration of hepatotoxins through the combined action of pre-ozonation and adsorption on powdered activated carbon. However, pre-chlorination followed by powdered activated carbon removed only 45% of hepatotoxins. Warhurst et al. [114] showed that a low-cost activated carbon from the pan-tropical multipurpose drumstick tree (*Moringa oleifera*) removes the cyanobacterial hepatotoxin microcystin-LR in quantitative amounts from water in batch adsorption trials. Pendleton et al. [115] compared activated carbon of two types; the wood carbon and the coconut carbon and found that the wood-based carbons adsorb more microcystin than the coconut-based carbons. The simple reason behind that is that the wood carbons contain both micropores and mesopores while the coconut carbons contain micropores only. Falconer et al. [116] showed that the toxicity could be removed by both powdered and granular activated carbon, with and without chlorination, alum flocculation, and polyelectrolyte addition. Bhaskar et al. [117] evaluated three grades of active carbon namely 40, 60 and 80 CTC for their removal efficiency of MC-LR from contaminated water and found 80 CTC carbon to be most efficient in removing MC-LR from contaminated water. Lee et al. [118] employed TiO_2-coated granular activated carbon for the removal of MC-LR from water. The granular activated carbon provided high surface area for rapid adsorption of MC-LR and TiO_2 particles degraded the toxin showing a synergistic effect. Adsorption of toxins by soil (river bank filtration) has been proposed by Pendleton et al. [115] as a low cost yet effective method for degradation of microcystins and nodularins in water. The soils with the high clay

and/or organic carbon contents had the higher nodularin adsorption coefficients. The implications for bank filtration are that higher water pH values and lower salinities will enhance the *in situ* mobility of the toxins, resulting in an increased distance of filtration through the river bank before toxin free water could be abstracted for human consumption [115]. Another method shows mixing of surface water and ground water reduces the hazards caused by toxic cyanobacterial blooms in the reservoir [119].

Figure 3- A schematic overview of water treatment strategy to remove algal cells and toxins from freshwater sources



Ribau-Teixeira and Rosa [120] suggested the removal of *Microcystis aeruginosa* cells as well as associated microcystins by a dissolved air flotation (preceded by coagulation/flocculation)—nano-filtration (NF) sequence. DAF–NF sequence is found to be a safe barrier against *M. aeruginosa* and microcystins in drinking water. In addition, it ensures an excellent control of particles, disinfection, by-products formation, and other micropollutants that may be present in raw water. Pawlowicz et al. [121] investigated various filters for effective removal of

microcystin-LR from the water and found carbon filters to be most effective. Ribau-Teixeira and Rosa [122] investigated the influence of nano-filtration for cyanotoxins removal, namely the neurotoxic anatoxin-*a* and the hepatotoxic microcystins and found that NF membranes are an effective barrier against anatoxin-a and microcystins in drinking water. Anatoxin-*a* and especially microcystins were almost completely removed. Anatoxin-*a* removal was governed by electrostatic interactions and steric hindrance, whereas for microcystins the latter was the main mechanism.

Recently, a biological removal method for cyanobacterial toxins has been proposed by Tsuji et al. [123]. The method used the bacterial strains of *Sphingomonas* sp. and show that MC-LR and MC-RR were completely degraded with in a day, when these bacterial strains were immobilized with a polyester resin. Though the method seems promising, need further investigations for removal of cyanobacterial cells as well as non-microcystin types of cyanotoxins. Since most of the methods claim effective removal of cyanotoxins from water, the analytical check of water for safe drinking level is however required to ascertain the removal of toxins and nontoxicity of by-products generated due to the degradation of toxins.

CONCLUSION

Bioassay methods are particularly relevant to waterworks agencies, as their prime mandate is to keep the toxin level below the safe level guidelines proposed by WHO. However, analytical techniques such as reverse phase HPLC and MALDI TOF are required in order to identify and quantify the cyanotoxins in the source water. Once, the toxin is identified, appropriate bioassay can be chosen based on the biological activity of toxin as well as the facilities available. Detection of toxin is equally important in treated waters for monitoring purposes. It should be noted that, those bioassays, which provide results faster are more appropriate, then the assays, which last for more then one day. Daphnids and shrimps are excellent organisms to use in bioassays because they are sensitive to changes in water chemistry and are simple and inexpensive to grow in an aquarium. They are parthenogenic and mature in just a few days and because daphnids are transparent, it is possible to conduct bioassays using endpoints other than death. For example, through a microscope their somatic growth, heart rate or feeding behavior etc. can be observed [49]. Biochemical tests and ELISA methods are more precise and useful for waterworks and most of the toxin forms can be identified in raw as well as treated water in very short time. However, such methods have limited implications when the compounds other then toxins (microcystins) are present such as protease inhibitors along with the toxins. Though, every method has its own limitation, a combination of bioassays can be adopted in cases where more then one type of toxin is suspected, or where one technique is not sufficient to identify all the variants.

So many methods are adopted worldwide for the removal of toxins in raw water. Since most the methods discussed effectively remove toxins from the raw water, techniques in which no external chemical is added to the water, should be adopted. Biological sand filtration and river bank filtration are some of the methods which not only effectively remove cyanotoxins and other toxic substances from water, are cheap as well as environment friendly methods. It should be however, noted that some biological control program should be introduced to the water reservoir, so that toxic cyanobacterial blooms can be controlled and the aquatic ecology can be maintained. Various studies showed that some species of aquatic grazers consume toxic cyanobacteria without getting affected by it. A mixotrophic flagellate *Ochromonas danica* is able to ingest and digest *Microcystis aeruginosa* single cells [124]. Fulton and Pearl [125] found a rotifer *Brachionus calyciflorus* Pallas to be able to ingest and survive on a diet of toxic *Microcystis aeruginosa* though on the contrary the same species were found to be most sensitive towards a toxic strain of *Anabaena flos-aquae* [126]. Some rotifers can graze on cyanobacteria without getting affected by it [41]. The second approach for biological control of toxic cyanobacteria may be the application of allelopathic interactions between a toxic and non-toxic cyanobacteria [12]. Algicidal compounds from cyanobacteria such as one from *Oscillatoria laete-virens,* whose algicide effectively eliminates and detoxicify *Microcystis* blooms; yet lack presence of any type of toxic metabolite [74, S. N. Bagchi, Pers. Comm.] can be introduced to the water reservoir. However, strains should be introduced only after proper screening for non-production of other toxic metabolites. Genetically modified strains, which lack toxin-producing genes, may provide a better solution in this regard. The biological control of toxic cyanobacterial bloom will not only provide support to the waterworks, but will help in protecting the environment too.

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