Review

Evaluation of methods for the isolation, detection and quantification of cyanobacterial hepatotoxins

Titus A.M. Msagati [a,*], Bupe A. Siame [b], Deborah D. Shushu [b]

[a] *Department of Chemistry, University of Botswana, Private Bag, 00704 UB Gaborone, Botswana*
[b] *Department of Biological Sciences, University of Botswana, Private Bag, 00706 UB Gaborone, Botswana*

Received 2 April 2005; received in revised form 20 March 2006; accepted 27 March 2006

Abstract

Cyanobacterial hepatotoxins such as microcystins and nodularins have been responsible for the poisoning of both animals and humans who ingest or come into contact with toxic blooms. They are extremely stable in water due to their stable chemical structure and can tolerate radical changes in water chemistry, including pH and salinity. Different methods for the extraction and detection of these compounds have been reported. Extraction methods utilizing both aqueous and organic solvent systems have been reported. The detection methods ranging from immunological or biochemical assays such as enzyme linked immunosorbent assays (ELISA) and enzyme activity assays, to chemicals methods such as high performance liquid chromatography (HPLC) and more sophisticated liquid chromatography–mass spectrometry (LC–MS) have been documented as well. We review some important aspects of cyanobacterial hepatotoxins and methods of analysis for these toxins.

Keywords: Cyanobacteria; Hepatatoxins; Microcystins; Nodularins

Contents

* Corresponding author. Fax: +267 355 2836.
E-mail address: msagatit@mopipi.ub.bw (T.A.M. Msagati).

1. Introduction

Cyanobacteria, also known as blue-green algae, are Gram-negative photosynthetic prokaryotes which are found in a variety of habitats, colonizing both terrestrial and aquatic biotopes (Mankiewics et al., 2003; Briand et al., 2003). Their dominance over other species in the ecosystem is an indication that they possess some specific physiological capabilities which enable them to compete very efficiently.

2. Factors favouring bloom formation by cyanobacteria

2.1. Light intensity and buoyancy

Many species of planktonic algae and cyanobacteria have little means of active movement and are therefore, only photosynthetically active when the circulation maintains them in the euphotic zone (i.e. environment where light can penetrate). However, their biomass in euphotic waters can frequently become very high and cause substantial turbidity. Cyanobacterial movement within the water column is due to the presence of specialized gas-filled vesicles which gives them a lower density than that of water, making them buoyant (Walsby et al., 2006). These gas vesicles have hydrophilic outer surface and a hydrophobic inner surface. The structures have a density of about a 10th that of water and make cyanobacterial cells of lower density than that of water (Walsby, 1987). Cyanobacteria are therefore frequently exposed to light and this may account for a faster growth than in other microbes found in the same ecosystem (Abeliovich and Shilo, 1972; Eloff et al., 1976). High light intensities increase cellular iron intake, since Fe^{3+} seems to be converted to Fe^{2+} by light before it is transported into algal cells which may ultimately be responsible for higher growth rate (Utkilen and Gjølme, 1995; Sunda et al., 1991; Boyer, 1987; Finden et al., 1984). Iron is an essential component in a number of enzymes and protein complexes of respiratory and photosynthetic electron transport and nitrate assimilation (Michel et al., 1998).

2.2. Temperature

For optimal growth, cyanobacteria prefer warm conditions (temperatures between 20 and 25 °C), (Robarts and Zohary, 1987). These temperatures are higher than that for green algae and diatoms and therefore, cyanobacteria can out-compete the other species when subjected to extreme temperature conditions. This also explains why in temperate and boreal water bodies most cyanobacteria bloom during summer.

Temperature changes have also been found to induce variations in both the concentration and peptide composition of the toxins. Some studies have shown that different temperatures can be correlated with different chemical forms of toxin produced (Katircioğlu et al., 2004). At temperatures below 25 °C, *Anabaena* sp. produces microcystin-LR, instead of microcystin-RR which is preferentially synthesized at higher temperatures.

2.3. Nutrients

Nutrients, such as nitrogen and phosphorus are essential for cyanobacterial growth (Villareal and Carpenter, 2003; Metting and Pyne, 1986; Kaebernick et al., 2001). Several studies have shown that, cyanobacteria have higher affinity for nitrogen or phosphorus than many other photosynthetic organisms (Metting and Pyne, 1986; Kaebernick et al., 2001). This means that they thrive better than other phytoplanktonic organisms under conditions of phosphorus or nitrogen limitation. In addition to their high nutrient affinity, cyanobacteria can store substantial amounts of phosphorus (Metting and Pyne, 1986; Kaebernick et al., 2001) enough to perform two to four cell divisions, which correspond to a 4–32-fold increase in biomass. Low nitrogen to phosphorus ratio has also been observed to favour cyanobacteria blooms (Villareal and Carpenter, 2003; Metting and Pyne, 1986; Kaebernick et al., 2001).

3. Toxins produced by cyanobacteria

Toxins produced by cyanobacteria may be categorized according to their toxicological properties. Thus the cate-

gories are neurotoxins (anatoxin-a, anatoxin-a(s), saxitoxin and neosaxitoxin); the tumor promoters (microcystins and lipopolysaccharides); the dermatotoxins/irritant toxins (lyngbyatoxin A, apysiatoxins and lipopolysaccharides); hepatotoxins (microcystins, nodularins and cylindrospermopsin).

However, based on their chemical structure, cyanotoxins falls into three main groups: namely, cyclic peptides (microcystins and nodularins); alkaloids (neurotoxins and cylindrospermopsin); and lipopolysaccharides.

Most scientists have been more concerned about the cyclic peptide hepatotoxins than neurotoxic alkaloids or lipopolysaccharides, because the latter are not considered to be widespread, especially in water supplies (Falconer and Humpage, 2005). Among hepatotoxins, the most common are the microcystins and nodularins.

3.1. Microcystins and nodularins

Microcystins are cyclic heptapeptides cyanotoxins produced by members of several cyanobacterial genera including *Microcystis*, *Planktothrix* (*Oscillatoria*), *Anabaena*, *Nostoc*, *Anabaenopsis*, *Hapalosiphon* (Carmichael, 1997). Nodularins are cyclic pentapeptides produced by *Nodularia spumigena*, a brackish water cyanobacterium (Mankiewics et al., 2003).

Microcystins contain five invariant amino acids namely, D-alanine (position 1), D-methylaspartic acid (position 3), Adda (position 5), D-glutamic acid (position 6) and *N*-methyldehydroalanine (position 7) as well two variant amino acids at positions 2 and 4 which are normally L-amino acids (Fig. 1). One of the invariant amino acids is a unique β-amino acid abbreviated as Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-dienoic acid) (Falconer and Humpage, 2005). Microcystins are relatively polar molecules due to the presence of free carboxylic acids in their structures and the frequent presence of arginine with a free side chain in positions 2 and 4. The toxins are named according to the two variable L-amino acids at positions X and Z (Fig. 1). Microcystin-LR (Fig. 2) contains the amino acids leucine (L) and arginine (R) at these positions (McElhiney et al., 2002). Currently there are over 60 variants of microcystin, which have been characterized and which differ in their toxicities (Falconer, 2005a,b). However, microcystin-LR is the most common in cyanobacteria though it is common to find more than one microcystin in a particular strain of cyanobacterium (Falconer, 2005a,b).

The cyclic pentapeptide nodularin contains amino acids similar or identical to those found in microcystins, namely arginine, glutamic acid, β-methyl aspartic acid, *N*-methyldehydrobutyrine [2-(methyl-amino)-2-dehydrobutyric acid] and the Adda (Fig. 3). Nodularins are structurally similar to microcystins and exerts similar toxicities (Nicholson and Burch, 2001).

3.2. The chemistry of microcystins and nodularins

The toxicity of microcystins and nodularins is attributed to the presence of the unusual amino acid known as Adda. The compounds acts by inhibiting several eukaryotic protein phosphatases which are essential for many cell regulatory processes such as growth, protein synthesis, glycogen metabolism and muscle contraction (Falconer, 2005b; Edward et al., 1996; Ohta et al., 1994). The Adda moiety is also required in the binding of the toxin to protein phosphatases which is via covalent bonds and is highly specific.

The "Adda" amino acid also provides the microcystins and nodularins with a characteristic absorption wavelength at 238 nm due to the presence of a conjugated diene group in the long carbon chain. Absorption at 238 nm provides a means of analysis of microcystins and nodularins after separation by the reverse phase chromatography (Falconer, 2005b).

4. Physiological effects of hepatotoxins

Hepatotoxins induce massive haemorrhages and disruption in mammalian liver as well as some adverse kidney effects (Sandstrom et al., 1990; Eriksson et al., 1988). Microcystins and nodularins have also been observed to induce apoptotic changes in mammals when administered into mouse embryo fibroblast and rat promyeloctic leukemia cells (Fladmark et al., 1999; Mankiewicz et al., 2001). Morphological changes such as membrane budding, as well as cell shrinkage and organelle redistribution have also been observed in human fibroblasts, human endothelial cells, human epithelial cells, human lymphocytes and rat promyelocytes (McDermott et al., 1996; Mankiewicz et al., 2000, 2001). Microcystins have also been reported to have

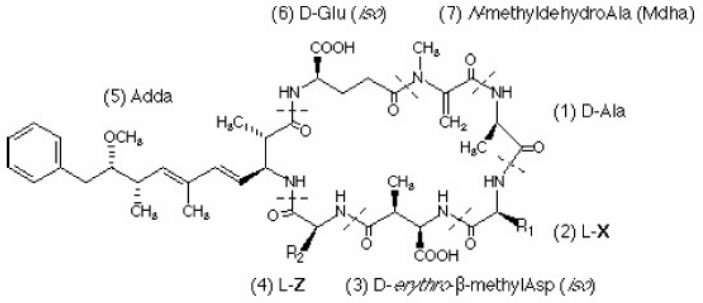


Fig. 1. General structure of microcystin.

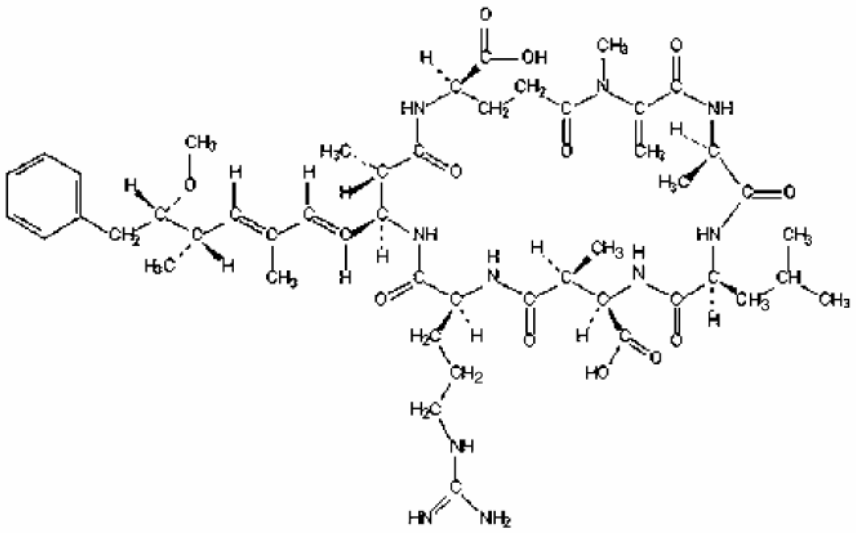


Fig. 2. Structure of microcystin-LR.

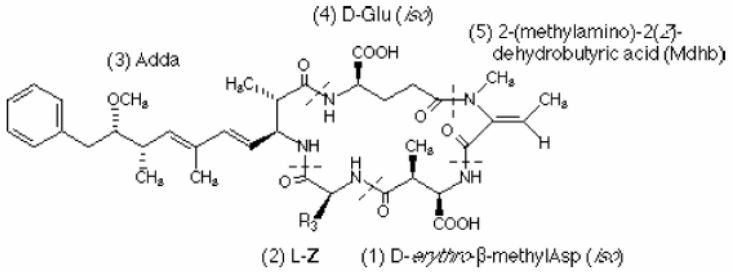


Fig. 3. Structure of nodularin.

a clastogenic effect in human lymphocytes connected with a dose-related increase of chromosomal breakage (Repavich et al., 1990; Premazzi and Volterra, 1993).

5. Human exposure to hepatotoxins

The exposure to cyanobacterial toxins is mainly through diet and/or direct contact with contaminated waters.

5.1. Possible exposure through consumption of animal meat and ingestion of water (or scum)

There are several reports on deaths of animals due to consumption of water contaminated with toxic blue-green algal blooms (Falconer, 2005a; Jochimsen et al., 1998; Elder et al., 1993; Carmichael, 1994; Codd et al., 1997; Mahmood et al., 1988; Carbis et al., 1995; Negri et al., 1995; Repavich et al., 1990; Sahin, 2000). There are also reports which associate poisoning of animal species to hepatotoxins from contaminated waters. Poisoning of animals such as ducks (Kalbe and Tiess, 1964), dogs (Edler et al., 1985; Nehring, 1993), young cattle (Gussmann et al., 1985) and sheep (Main et al., 1977) have been reported.

Ingestion of untreated natural waters, has also been reported to pose health risks or even death, not only to pets and livestocks but to human beings as well (Falconer et al., 1983; Oberholster et al., 2004). Poisoning of humans was reported from Brazil in 1996 where water containing high levels of cyanobacteial toxins was used for kidney dialysis (Jochimsen et al., 1998; Azevedo et al., 2002). The water responsible came from a reservoir with a major blue-green algal bloom.

5.2. Possible exposure through fish consumption and blue-green algal products used as food supplements

Microcystins can accumulate in the tissues of fish, particularly in the viscera (liver, kidney, etc.), and in shellfish (Prepas et al., 1997; Falconer et al., 1992; Chorus and Bartram, 1999;

Table 1
Summary table of WHO guidelines for cyanobacteria levels in drinking water (WHO Guidelines for drinking-water quality, 3rd ed., vol. 1, ©World Health Organization, 2004)

Risk category	Cell density (cells/ml)	Action recommended
Low risk	20000–100000	None
Moderate risk	>100000	Advisory and possible closure
High risk	Visible scum layer	Closure

Lehane, 2000). Levels in the tissues depend upon the severity of the bloom in the area where the fish or shellfish are caught or collected. In the case of fish caught from areas where major blue-green algal blooms occur, consumption of visceral parts of fishes may lead to cyanotoxin exposure.

Moreover, *Spirulina* and other blue-green algal species used as food supplements, though non-toxic, may lead to exposure of consumers to cyanotoxins if they have been harvested from wild pools where toxic and non-toxic species of cyanobacteria grow together (Falconer et al., 1992; Chorus and Bartram, 1999; Lehane, 2000).

5.3. Exposure through dermal contact

Swimming in waters containing toxic blooms of cyanobacteria may expose swimmers to cyanotoxins. Exposure through dermal contact may cause eye, ear and skin irritation, a condition known as dermatitis or swimmers' itch or seaweed dermatitis, and gastrointestinal symptoms such as vomiting and diarrhea in bathers (Falconer, 2005a).

In animals and humans, microcystins or nodularins are concentrated in the liver where they are transported to hepatocytes through an active bile acid transport system (Brooks and Codd, 1987a; Runnegar et al., 1991). These hepatotoxins are known to be resistant to digestion in the gastrointestinal tract of eukaryotes because the peptide bonds linking to the D-amino acids are not susceptible to normal hydrolytic enzymes (Falconer, 2005a). They are highly stable in water and are resistant to boiling even to irradiation; thereby presenting a high risk to consumers of animal products which have been contaminated.

For this reason, the WHO has set guidelines for water safety regarding the presence of cyanobacterial cells as shown in Tables 1 and 2. Table 1 gives a summary of WHO guidelines for the limit of cyanobacterial cells in drinking water suitable for human consumption, while Table 2 gives the toxicities of microcystins and nodularins by various administration routes.

Table 2
Toxicity of microcystins and nodularin by various administration routes (WHO Guidelines for drinking-water quality, 3rd ed., vol. 1, ©World Health Organization, 2004)

Cyanobacterial toxin	LD_{50} (μg/kg)				
	i.p.	i.n.	oral	LOAEL	NOAEL
Microcystins	25–150	36–122	5000–10900	100	40
Nodularin	50				

NB: i.p., intraperitoneal; i.n., intranasal; LOAEL, lowest observed adverse effect level; NOAEL, no observed adverse effect level.

Table 3
Conditions for culturing different strains of *Microcystis aeruginosa*

Strain	Species	Culturing medium	Culturing conditions	Comments	References
B.14.85	*M. aeruginosa*	ZEHNDER	Temp. 21–23 °C, light, Ca. 80 μmol m^{-2} s^{-1}, 16/8 L/D	Medium contains, $NaNO_3$, $Ca(NO_3)_2{\cdot}4H_2O$, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, Na_2CO_3, Fe–EDTA complex, micronutrient solution and distilled water	Pietsch et al. (2002)
ISACYA 2C, 6A, 9B, 10A, 12, 13, 14, 15 and 16A; other strains not from *M. aeruginosa* were present in the culture	*M. aeruginosa*	Z8	20 ± 1 °C, with 16/8 L/D and 30 μE m^{-2} s^{-1}		Moreno et al. (2004)
RST501	*M. aeruginosa*	BGN/2 (BG 11 with half the total N)	Salinity = 0; 25 °C; radiation, 63 μE m^{-2} s^{-1}; and pH 8.0	BG 11 Medium contains $NaNO_3$, $K_2HPO_4{\cdot}3H_2O$, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, citric acid, ferric ammonium citrate, EDTA (dinatrium-salt), Na_2CO_3 and micronutrient solution	Montagnoli et al. (2004)
NIVA-CYA 140	*M. aeruginosa*	WC	20 ± 1 °C, light intensities: 7 μmol photons m^{-2} s^{-1}	Medium contains, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, $NaHCO_3$, $K_2HPO_4{\cdot}3H_2O$, $NaNO_3$, $Na_2SiO_3{\cdot}5H_2O$, micronutrient solution, vitamin solution of thiamin HCl and biotin	Karlsson et al. (2004)
NPLJ-4	*M. aeruginosa*	ASM-1	pH 8.0, 23 ± 2 °C, 22 μE m^{-2} s^{-1} photoperiod = 12 h	Simple medium to prepare, it contains, $MgSO_4{\cdot}7H_2O$, KCl, $NaNO_3$, $CaCl_2{\cdot}2H_2O$, KH_2O_4, vitamins B1 and B12 and trace elements	Soares et al. (2004)

6. Isolation of cyanobacterial cells

Cyanobacterial cells may be obtained from laboratory cultures or from blooms in natural water bodies. However water blooms are excellent natural sources of cyanobacterial samples as these are usually dominated by one species of cyanobacteria.

6.1. Preparation of laboratory culture of Microcystis aeruginosa

Various conditions and different media formulations have been used for obtaining pure cultures of different strains of *M. aeruginosa* (Table 3). For instance, Lee and Chou (2000) reported the isolation of *M. aeruginosa* strain M.TN-2 from an eel pond. In another report by Pietsch et al. (2002) *M. aeruginosa* strain B 14.85 (Sammlung von Algenkulturen, University of Göttingen, Germany), was grown in ZEHNDER medium at 21–23 °C under continuous stirring and an illumination of 12 h/day (ca. 80 μmol s/m^2 photons, day–night change). Moreno et al. (2004) and Selheim et al. (2005) have reported the use of Z8 medium at 20 ± 1 °C, with 16/8 photoperiodic cycle and light intensity 30 μE m^{-2} s^{-1} for culturing *M. aeruginosa* strains ISACYA 2C, 6A, 9B, 10A, 12, 13, 14, 15 and 16A. Montagnolli et al. (2004) cultured the strain *M. aeruginosa* RST501 (UPC Culture Collection, Brazil) in 5 L batch cultures in BGN/2 medium (BG 11 with half the total N) under conditions of 0% salinity, temperature of 25 °C; radiation, 63 μE m^{-2} s^{-1}; and pH 8.0. Karlsson et al. (2004) cultured *M. aeruginosa* strain (NIVA-CYA 140) in WC medium in chemostat cultures (1 L, 20 ± 1 °C, light intensities: 7 μmol photons m^{-2} s^{-1}). Soares et al. (2004) have cultured strain NPLJ-4 of *M. aeruginosa* in ASM-1 medium, pH 8.0, temperature 23 ± 2 °C and 22 μE m^{-2} s^{-1} of light intensity and photoperiod of 12 h.

6.2. Sampling of cyanobacterial cells from natural waters

Waters suspected of containing toxic cyanobacteria are normally collected at points where the accumulation of cyanobacteria is likely to affect both humans and livestock, or at drinking water reservoirs. The spatial heterogeneity relating to the horizontal and vertical variations in cell densities, as well in the process of selecting the number and location of sampling sites and toxin content, are the other factors that may influence the decision of where samples should be collected.

The types of sample containers for sampling are normally chosen depending on the type of the analysis to be performed. Samples for microscopic identification and quantification of cyanobacterial cells are normally collected in brown glass bottles and preserved in Lugol's iodine solution or formaldehyde solution (Breitig and von Tümpling, 1982; Pietsch et al., 2002; Porfirio et al., 1999).

Collecting large samples of cyanobacterial cells for structural identification or toxicity analysis requires the use of plastic containers with wide necks for easier filling or when collecting samples for analysis of dissolved cyanobacterial toxins, 1 L minimum volume glass or plastic bottles have been reported to give good results (WHO, 2004; Breitig and von Tümpling, 1982; Pietsch et al., 2001).

When samples are to be freeze-dried, these samples have to be frozen in a layer of thickness of about 2 cm (Porfirio et al., 1999).

7. Sampling of test organisms from aquatic environments for hepatotoxin analysis

Test organisms in this case may include crustaceans, crabs, snails, bivalves, shrimps or fish. Normally organisms are collected and frozen at −20 °C, and for others like snails, crabs, bivalves, fish and crustaceans, they are first dissected into parts, e.g., stomach, intestine, hepatopancreas, gonad, muscle, eggs and gill (Chen and Xie, 2005a; Chen et al., 2005; Chen and Xie, 2005b; Lehtonen et al., 2003). The organs are then frozen in liquid nitrogen and stored at −80 °C prior to hepatotoxin extraction. In case the levels of hepatotoxins in various organs are low, all organs may be pooled together.

In some cases it has been necessary to adapt test organisms to particular conditions of salinity, temperature, light/dark cycle of 12:12 h and aeration (Montagnolli et al., 2004).

7.1. Vertebrate samples for hepatotoxin analysis

In the case of vertebrates, samples like hepatocytes are mostly preferred. De sousa et al. (1991), Batista et al. (2003), Dorko et al. (1994) and Isom et al. (1985) have reported the isolation of hepatocytes from humans and rats by a collagenase perfusion method (Desousa Get et al., 1991; Batista et al., 2003; Dorko et al., 1994; Isom et al., 1985). Large numbers of human hepatocytes are normally obtained from split and whole livers by using an adaptive form of the collagenase perfusion technique employed in rodent and human biopsies. In order to guarantee a homogenous distribution of the perfusate within the whole specimen, major hepatic veins are usually cannulated with large bore catheters. This technique allows for the isolation of human hepatocytes on a large scale (up to 18.5×10^9 in one case) from normal and diseased liver specimens (Dorko et al., 1994). After isolation, the rat hepatocytes are normally washed in L-15 medium supplemented with 5% fetal calf serum and cultured first in a chemically defined medium termed HCD, a serum-free medium supplemented with bovine serum albumin, insulin, dexamethasone, glucagon and transferrin then fed unsupplemented HCD medium or HCD medium supplemented with dimethyl sulphoxide (DMSO) or phorbol 12-myristate 13-acetate (PMA) and fed every 24 or 48 h. The hepatocytes are then seeded on round glass coverslips for about 40 h, placed in 24-well plates and then coated with type 1 collagen before incubation at 37 °C in humidified chambers with 95% air and 5% CO_2.

8. Extraction of cyanobacterial hepatotoxins

8.1. Extraction of hepatotoxins from cyanobacterial cells

After sampling, toxins are normally extracted by first disrupting or perforating the cell wall structure. Most procedures have used freeze-drying for this purpose (Li et al., 2005; Porfirio et al., 1999), but oven drying at 95 °C and freeze-thawing meth-

ods have also been used (Meriluoto, 1997). Freeze thawing has also been utilized in a number of analytical procedures for the peptide hepatotoxins analysis (Lawton et al., 1994). After lysing the cells, the hepatotoxins can then be extracted using several solvent systems. Acetic acid/water mixtures or alcohol/water mixtures were initially used as extractants (Harada et al., 1997); and a combination of one volume of acetic acid, two volumes of methanol:chloroform (1:1) solution has been used by Moreno et al. (2004). Water, in conjunction with sonication has also been shown to be applicable to live cells. With the more hydrophobic microcystins, methanol was found to be slightly more effective than methanol/water mixtures, and also effective with the more hydrophilic toxins (Vasa et al., 2004; Lawton et al., 1994). However methanol has been reported to be less effective than 5% acetic acid or 75% methanol for the extraction of the hydrophilic microcystin-RR (Fastner et al., 1998). However, methanol has been shown to be the most suitable solvent because it gives good extraction efficiency and has the added advantage of allowing rapid sample concentration through evaporation. It has since been suggested that 100% methanol may give poor recovery of more polar microcystins; but the addition of a small percentage of water overcomes this problem. Fastner et al. (1998) showed that, for lyophilised field samples dominated by *Microcystis* spp., extraction with a mixture of methanol and water (75:25, w/w) was most effective.

Simple lysis/extraction procedures such as heating in a boiling water bath or using a microwave oven employing water as solvent have also been evaluated and found effective for a range of microcystins including hydrophobic variants such as microcystins-LW and -LF (Metcalf et al., 2001). These procedures, including sonication, are preferable where solvents such as methanol interfere in the subsequent analytical determination. In terms of determining the intracellular component, the procedure of Lawton et al. (1994), i.e., filtration and freeze thawing, prior to solvent extraction, appear to be the most appropriate. Other extractants have employed supercritical carbon dioxide (Pyo and Shin, 1999). Such an approach may be useful for determining the intracellular content of microcystins in water samples, but requires evaluation with a wider range of toxins.

8.2. Extraction of hepatotoxins from aquatic organisms (shrimps, crabs, crustaceans, snails, bivalves and fish)

A number of studies have reported methods of analyzing the presence of hepatotoxins in fish (Magalhães et al., 2003; Xie et al., 2005; Magalhães et al., 2001; Xie et al., 2004; Li et al., 2005). Unlike mammalian cells where the transport of microcystins is via biliary acids, in fish species, the microcystin uptake is via the digestive tract (Tencalla et al., 1994). This makes digestive tracts of fish and other aquatic organisms, one of the target samples when extracting hepatotoxins. Krienitz et al. (2003) and Cazenave et al. (2005) reported the extraction of microcystin-RR from fish tissues (liver, gills, brain, intestine, gallbladder and blood) and fish muscles. In the method for the extraction of hepatotoxins from fish tissues by Cazenave et al. (2005) [based on method by Krienitz et al. (2003)], the fresh fish tissues were homogenized with 70% methanol containing 1% (v/v) trifluoracetic acid. After ultrasonication and centrifugation, the supernatants were separated and evaporated to dryness at 40 °C. The extraction of fish muscles were accomplished by using 5% acetic acid and the extracts were applied to solid phase extraction cartridges. Soares et al. (2004) have reported the extraction of microcystins from fish liver and muscles using of 100% methanol (Soares et al., 2004). Methanol extract was then mixed with an equal volume of hexane before discarding the hexane layer. The extract was then loaded onto C18 SPE cartridges for further purification.

Xie and coworkers have reported the extraction and analysis of hepatotoxins in the tissues and organs of aquatic organisms (Xie et al., 2004; Chen and Xie, 2005a; Chen et al., 2005; Li et al., 2005). The hepatotoxins in the lyophilized organ samples of the study organisms (shrimps, snails, etc.) were extracted by a mixture of solvents comprised of BuOH:MeOH:H_2O (1:4:15) for 24 h. The same extracting solvent and ratios was used by Lehtonen et al. (2003) in extracting nodularins in the clam *Macoma balthica* (Lehtonen et al., 2003). The extract was centrifuged and supernatant diluted with water. At this stage the extracts were ready for pre-concentration and further clean-up step using solid phase extraction (SPE).

8.3. Extraction of hepatotoxins from vertebrate and mammalian organ samples

Several reports are available which link hepatoxins to the health hazards of mammals and other vertebrates (Batista et al., 2003; Briand et al., 2003), but very few have reported on the extraction of hepatotoxins from vertebrate and mammalian tissues. Sahin (2000) developed a method to determine hepatotoxins in liver tissues of poisoned animals. Sahin's method involved a two step extraction steps, which involved the use of aqueous and organic solvents. In the aqueous extraction step, done at 4 °C, a buffer made up of 50 mM Tris–HCl, pH 7, 250 mM sucrose, 4 mM EDTA, 0.1% 2-mercaptoethanol was used. The aqueous extract which was obtained after centrifugation was freeze-dried and re-suspended in methanol. The methanol was evaporated and the organic extract was re-suspended in a sample buffer made up of 50 mM Tris–HCl, pH 7, 0.01% Brij-35, 0.1 mM EDTA, 0.1% 2-mercaptoethanol. The effectiveness of both the aqueous and organic extracts was checked for their ability to inhibit phosphatase activity in vitro. The phosphatase inhibitory potency was fully maintained when the aqueous samples were subjected to further extraction by methanol (Sahin, 2000).

8.4. Pre-concentration/clean-up procedures for peptide cyanotoxins

At low concentrations, direct determination of hepatotoxins may not be feasible due to the inadequate sensitivity of the detection procedures. This necessitates a sample pre-concentration step. This may also serve as a clean-up step by allowing removal of co-extracted material, which may interfere in the analysis. Moreover, cyanobacteria are considered protein-rich and the high protein content always poses serious problems during separation by high performance liquid chromatography. The proteins

denature on reversed-phase packing material resulting in distorted peak shapes or multiple peaks, hence the need for clean-up steps in order to eliminate interfering protein from the samples before analysis.

Pre-concentration can be performed using a number of methods, but the mostly used method has been by solid-phase extraction (SPE) (Cazenave et al., 2005; Soares et al., 2004; Xie et al., 2004). In SPE the sample is passed through a cartridge, which retains the toxins, by adsorption. Toxins can then be eluted from these cartridges using polar organic solvents such as methanol (Magalhães et al., 2003; Xie et al., 2005; Magalhães et al., 2001). Methanol extracts may be reduced in volume by evaporation resulting in increased toxin concentration. The choice of the SPE cartridges for use as sample cleanup and concentration is very critical. This is because some parameters, such as pore size, carbon load end-capping and functionality (mono- or tri-) may not be uniform for all cartridges. The pore size should be small enough to exclude protein (Edward et al., 1996; Meriluoto, 1997).

C18 cartridges also extract the naturally occurring organic matter (NOM) present in water. This co-extracted NOM can interfere in the chromatographic separation by producing peaks, which may obscure the toxin peaks. Masking of toxin peaks can therefore make quantification imprecise. Procedures involving selective elution of toxins versus co-extracted interferences such as NOM have been developed to minimize this problem. Such procedures include the use of immunoaffinity columns for concentration and cleanup of toxins (Nicholson and Burch, 2001).

Concentration by simple procedures such as rotary evaporation has been possible as the peptide hepatotoxins are heat stable (Meriluoto, 1997). However, this is a slow and laborious procedure, and may be impractical for determining toxins at low concentrations in water because of the high concentration factors required.

8.5. Sample preservation for cyanobacterial peptide toxins

Stability of the cyanobacterial peptide compounds after extraction especially where accurate determination is required is very critical. Degradation of samples may occur if not properly stored and this may lead to serious errors in the determination of the concentration in the original sample. Pure microcystins and nodularins have been found to be relatively stable in aqueous solutions of high purity or sterile water (Falconer and Humpage, 2005; Heresztyn and Nicholson, 1997). However, in the presence of pigments, photochemical degradation has been reported to occur relatively quickly (Tsuji et al., 1994). Therefore water samples that contain cellular material and dissolved microcystins or nodularins have always been kept in the dark. Microcystins and nodularins in solution may be stable to chemical breakdown by hydrolysis hence the pH of aqueous samples may as well not be important (Falconer and Humpage, 2005; Harada et al., 1997).

Toxins may also be susceptible to microbial degradation and this has been demonstrated for microcystins and nodularins in water bodies (Heresztyn and Nicholson, 1997). In all cases, degradation depends primarily on the level of degrading organisms and temperature. Microbial activity and hence degradation can be slowed by refrigeration (Heresztyn and Nicholson, 1997). The addition of preservatives such as copper to microorganisms may be useful. However, such preservative will kill cyanobacteria, thereby releasing the toxins, and will only be useful in the determination of total toxin content.

While refrigeration or freezing of sample extracts is generally accepted as preventing degradation, Gjolme and Utkilen (1996) showed that microcystin-RR had varying stability at 4 °C in various solvents. While the toxin was stable in methanol and 75% methanol for over 32 days, most of it was degraded in pure water and in 5% acetic acid after the same period of storage. These results indicated that methanol and/or aqueous methanol are preferred extraction solvents if there is likely to be delay between sample extraction and analysis.

Another factor that has been in consideration is the possible adsorption of peptide cyanotoxins on materials, which come into contact with extracts during analysis. Hyenstrand et al. (2001) investigated the adsorption of microcystin-LR on plastic (polypropylene) microcentrifuge tubes and pipette tips, and on glass. Significant losses by adsorption to plastics occurred with aqueous solutions or solutions containing less than 25% methanol. Losses by adsorption to glass also occurred with aqueous solutions. Therefore, solutions containing peptide cyanotoxins must also contain sufficient methanol in order to avoid these adsorptive losses.

9. Analysis of cyanobacterial hepatotoxic compounds

Two methods are generally employed in the detection and identification of cyanobacterial hepatotoxins; biological or biochemical screening assays and physicochemical methods. The methods differ in terms of principles of detection, information they provide and simplicity/complexity of the set-up. Selection of techniques depends on the availability of facilities and expertise as well as the type of information required. However, selectivity and sensitivity of these techniques are important criteria that can lead to the selection of the proper method, which will give more reliable information. Fig. 3 and Table 4 gives a comparison of several biological screening and physicochemical methods in terms of selectivity and sensitivity.

10. Biochemical screening methods of cyanobacterial hepatotoxins

Screening assays are diagnostic tests, which establish the presence or absence of a substance. Usually these tests provide quick, inexpensive and reproducible results. Among the biochemical screening methods commonly used for cyanobacterial peptide toxins are, the mouse assay, enzyme linked immunosorbent assay (ELISA) and phosphatase assays.

10.1. Mouse assays

In the mouse bioassay, toxin extracts are administered by intraperitoneal injection into the mouse (Nicholson and Burch, 2001). This has been used primarily to determine the toxicity

Table 4
Comparison of methods of analysis of cyanobacterial hepatotoxins

Method	Comments	References
Mouse assay	Qualitative Poor sensitivity and precision May need a number of mice May require license	Nicholson and Burch (2001), Nagata et al. (1997) and Falconer (1993)
Protein phosphatase inhibition assay	Very sensitive Colorimetric (requires purified enzyme) ^{32}P isotope has a short half-life Proteins are not commercially available Expensive due to the need of radioactive ATP and the commercial enzymes	Chorus (2001) An and Carmichael (1994)
ELISA	Very sensitive Variable cross-reactivities hence may underestimate concentration of cyanotoxins	McDermott et al. (1995) An and Carmichael (1994)
HPLC	Cannot differentiate between structural variants of cyanobacterial hepatotoxins, e.g., microcystins UV spectra may tentatively give the identity of sample and also with PDA characteristic UV spectra may be obtained Lack of available standards may hinder analysis since the method relies on retention time for identification Other components in the sample extract may have absorbance at the wavelengths where cyanotoxins absorb Problems of co-eluted extractives, e.g., plastic additives may contaminate sample	Lawton et al. (1994) Moollan et al. (1996) Ikawa et al. (1999)
LC–MS	Have a number of different interfaces: -ESI, MALDI-TOF, FAB, TSP Gives mass confirmation hence improved accuracy Sensitive and specific Equipment cost may be its limitation	Lawton et al. (1994) Robillot et al. (2000)
CE and CE-MS	Derivatization may be needed to give fluorescent products that can be detected using a laser-induced fluorescent detector Low sensitivity Method need to be further developed	Bateman et al. (1995) Li et al. (1999)
NMR	Enables structural determination of known and unknown cyanotoxin Its limitation is that it requires large amount of sample (mg quantities) Samples must be pure	Harada et al. (1995)

of bloom material, and, from the toxic response, the identity of the class of toxin can be determined. It has generally been used in a qualitative manner to determine a bloom as 'toxic' or 'non-toxic'.

The assay can potentially be calibrated against a specific toxin such as microcystin-LR, and therefore produce results in terms of microcystin-LR toxicity equivalents. However, the assay does not have the sensitivity or precision required to be applicable to water samples (Nagata et al., 1997). It is not practicable to use for water samples with concentrations around 1–2 μg/L, the approximate range of the guideline for microcystins with WHO (Nicholson and Burch, 2001). Another limitation of this assay is that, the number of mice required would be impracticable and unacceptable and moreover it is not permitted in some countries unless a license is issued (Falconer, 1993).

10.2. Enzyme linked immunosorbent assay (ELISA)

Screening methods such as enzyme linked immunosorbent assays (ELISAs), have been developed for peptide cyanotoxins and have proved to be sensitive (Pyo et al., 2005). Many researchers have generated antibodies against microcystins. For example, Kfir et al. (1986) generated monoclonal antibodies against microcystin-LA, which bound a wide range of other microcystins with equal efficiency. Brooks and Codd (1987b) generated antibodies against the toxin in one strain of *Microcystis*, which cross-reacted with other toxic *Microcystis* strains but not strains of other genera containing peptide toxins.

However, later studies have shown variable cross-reactivities, for example, Chu et al. (1989) generated antibodies against microcystin-LR and found cross-reactivities to vary between good (microcystin-RR) to poor (microcystin-LA). However, the procedure has been used successfully to determine the microcystin content of environmental samples (Chu et al., 1989; Nagata et al., 1995).

In another development, McDermott et al. (1995) generated antibodies against microcystin-LR which showed high cross-reactivity with microcystin-RR and there was good agreement between ELISA and HPLC results. An and Carmichael (1994) evaluated the cross-reactivity of a number of microcystins using ELISA techniques and found a poor correlation between reactivity and acute toxicity. This indicated that ELISA techniques can underestimate the concentration of some microcystin variants.

10.3. Phosphatase inhibition assays

Microcystins and nodularin, inhibit serine and threonine phosphatase enzymes responsible for the dephosphorylation of intracellular phosphoproteins (MacKintosh et al., 1990). The two enzymes are classified into two groups, i.e. Type 1 and Type 2. The types which are most inhibited by cyclic peptide hepatotoxins are Type 1 and a subset of Type 2 called protein phosphatase-2A. The extent of inhibition of these enzymes is related to the hepatotoxicity of these compounds and their tumor promotion properties as well.

The inhibition reaction by these toxins can be used as a measure of toxin concentrations. Phosphatase inhibition by microcystins and nodularin is determined using ^{32}P radiolabelled substrates (Chorus, 2001). Though the method is sensitive it suffers from one major drawback, that the ^{32}P isotope has a short half-life (approximately 14 days). This requires the labelled proteins for the assay to be prepared on a regular basis. Another limitation is that the proteins are not commercially available and require reasonably sophisticated procedures for their preparation. Furthermore, they use radioactive ATP and commercial enzymes in their preparation, most of which are expensive. In addition, many routine laboratories are not set-up to carry out radioactive determinations.

There are at least two variations of protein phosphatase inhibition assays, which are colorimetric and fluorescent assays. These assays use substrates such as *p*-nitrophenyl phosphate and determine microcystins and nodularins (An and Carmichael, 1994; Rivasseau et al., 1990; Heresztyn and Nicholson, 2001a,b). Heresztyn and Nicholson (2001b) have reported the use of phosvitin as substrate for measuring the phosphate released colorimetrically during the determination of peptide cyanotoxins. Metcalf et al. (2001) successfully combined the advantages of colorimetric assays with an immunoassay to produce a novel assay with high sensitivity and specificity for microcystins. Fluorescent assays on the other hand utilize highly fluorescent and luminescent products to give the inhibition properties related to toxicities (Heresztyn and Nicholson, 2001a,b).

11. Physicochemical methods of analysis of cyanobacterial hepatotoxins

These are analytical methods which use physicochemical properties of cyanobacterial hepatotoxins such the presence of UV chromophores within their structures, their reactivities are due to specific functional groups present in their structures and molecular weights. Among the methods employed are, high performance liquid chromatography (HPLC), capillary electrophoresis (CE), nuclear magnetic resonance (NMR) and mass spectrometry (MS).

11.1. HPLC separation and detection methods

The widely reported procedures for the separation of microcystins and nodularins employ the use of a high performance liquid chromatography (HPLC). Different stationary phases have been used, including reversed-phase C18 packed columns (Lawton et al., 1994), amide C16 columns (Spoof et al., 2001), internal surface reversed-phase columns (Meriluoto and Eriksson, 1988) or ion exchange columns (Gathercole and Thiel, 1987). Mobile phases have employed aqueous phases containing methanol or acetonitrile. The proper choice of the mobile phase can lead to a good resolution of the analytes and this is a prerequisite for accurate determination. For example, microcystins-LR and -YR co-elute with an acetonitrile/ammonium acetate mobile phase but can be resolved with methanol-based mobile phases (Lawton et al., 1994). For similar microcystins, a combination of mobile phases may be necessary to separate the compounds for unequivocal identification.

The means of detection of individual compounds is normally coupled within the HPLC system. Two mostly used detection techniques used with HPLC are UV/Vis absorbance and PDA.

UV/Vis absorbance is the most common technique of detecting peptide cyanotoxins after chromatographic separation. Most microcystins and nodularins have a UV absorption maximum at 238 nm (Lawton et al., 1994). However, those with aromatic amino acid constituents, such as microcystin-LW that contains tryptophan, have absorbance maxima at lower wavelengths, 222 nm (Lawton et al., 1994). The wavelength of the UV detector of the HPLC equipment can be set at these values to record the responses of peptide cyanotoxins in sample extracts separated on the HPLC column. However, other components in the sample extract can also have some absorbance at these wavelengths. The problems of co-eluted extractives and their effects on quantification, especially at low toxin concentrations, have been discussed by Moollan et al. (1996). In addition Ikawa et al. (1999) showed that common additives in plastics that contaminate water samples, co-elute with the toxins and have sufficient UV absorbance at 238 nm to produce erroneous results. Thus plastic materials should be avoided, or else their suitability checked, in the determination of peptide cyanotoxins using HPLC. Moreover HPLC cannot differentiate between structural variants of cyanobacterial hepatotoxins such as microcystins.

To determine peptide cyanotoxin concentrations in terms of toxicity equivalents, not only must the toxin be identified but the concentration must also be determined. The latter requirement necessitates that a quantitative standard be available which can also be used to indicate, to some extent, the identity of the toxin present, i.e., matching retention time of a peak in a sample chromatogram with that of an authentic standard. However, retention times cannot be used as unambiguous indicators of the presence of a particular toxin as toxins of similar structure can co-elute. In addition, co-extracted compounds may co-elute, e.g., the plastic additives mentioned above. It is, however, possible to estimate toxin concentrations, even when the toxin is not identified and standards are not available, as long as there is confidence that a microcystin or nodularin is being detected. Previous studies have indicated that microcystins and nodularins have similar absorption coefficients (Harada et al., 1997). Thus they have similar sensitivities when analyzed by HPLC with

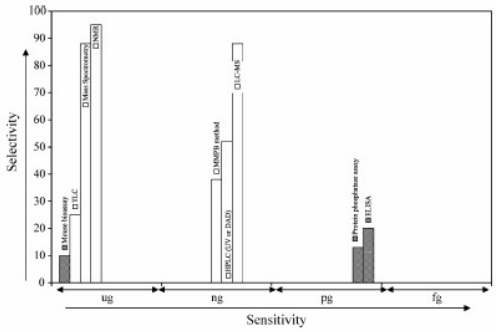


Fig. 4. Relationship between sensitivity and selectivity of biological screening assays and physicochemical methods of analysis in the determination of cyanobacterial hepatotoxins.

detection at 238 nm. Hence total concentrations have normally been estimated by reference to a known microcystin standard with a relatively high degree of accuracy. However, this result cannot be accurately converted to a concentration in terms of toxicity equivalents. Since toxicity data (LD50) are needed to calculate toxicity equivalents, the toxin must be unequivocally identified.

On the other hand, a photo-diode array (PDA) detector unlike UV/Vis, records the spectrum in addition to the UV response of the analyte. This provides much better evidence of the presence of a microcystin or nodularin than using single wavelength detection alone. The typical spectrum of a microcystin or nodularin with an absorbance maximum at 238 nm (or occasionally 222 nm in the case of microcystins containing tryptophan) provides a greater degree of confidence that a microcystin is present. As microcystins have similar sensitivities to UV detection, a concentration in terms of microcystin-LR equivalents can be readily determined by quantification of microcystin peaks, i.e., those with characteristic spectra, relative to a microcystin-LR standard. Detection limits considerably less than 1 μg/L for individual microcystins are normally attainable with appropriate concentration and clean-up procedures using this detection method.

Another approach to improving sensitivity and selectivity in the HPLC determination of microcystins and nodularins has involved the use of derivatizing reagents (Fig. 4). James and James (1991) reported a post-column system whereby the arginine residue of microcystin-LR was derivatized with a fluorescent reagent. This approach however, restricted the application to toxins containing arginine, e.g., microcystin-LR, while toxins such as microcystin-LA, could not be determined. Murata et al. (1995) showed that the dansyl-cysteine adducts of microcystins could be separated by HPLC and detected by chemiluminescence with high sensitivity. Harada et al. (1997) reported derivatization of microcystins with a fluorescent dienophile, DMEQ-TAD. These products were also separated by HPLC and detected by fluorescence with a high degree of sensitivity.

11.2. Liquid chromatography–mass spectrometric (LC–MS) detection of peptide cyanotoxins

When further confirmation and identification of cyanotoxins is required, more advanced and sophisticated method such as LC–MS may be employed. Mass spectrometry as a detection technique provides the best solution to the problem of peptide cyano peptide toxins since these toxins produce characteristic ions in their mass spectra (Lawton et al., 1994). The method enables the simultaneous separation and identification of cyanotoxins in a mixture. LC–MS with various interface and ionization configurations have been reported for the determination of peptide cyanotoxins with detection limits of ~0.02 μg/L. For example MALDI-TOF mass spectrometry has been successfully applied as a detection method following LC separation of microcystins (Robillot et al., 2000).

In the past, LC–MS systems were not common in the routine analytical laboratories, however, they are now increasingly become available and they give sufficient robust and cheap analysis for the routine determination of these toxins. Although work reported so far has shown that different peptide cyanotoxins have similar responses when determined with PDA detection, the same is not true of MS detection, i.e., the total ion current depends markedly on the particular cyanotoxin. Thus concentrations of unknown peptide cyanotoxins, for which standards are not available, cannot be reliably estimated with this method.

Pre-derivatization of peptide cyanotoxins for LC–MS analysis has also been reported as a technique to assist in identification (Nicholson and Burch, 2001). From the mass spectrum, which acts as a fingerprint, toxins could be identified as long as a mass spectrum of an authentic standard was available. A useful extension to this technique is MS/MS detection where the fragmentation pattern can be used to greatly assist in the identification of the unknown toxins and it has been reported by Edwards et al. (1993).

11.3. Electrochemical detection methods of peptide cyanotoxins

Other reported detection methods for peptide cyanotoxins include electrochemical detection but the sensitivity with peptide cyanotoxins such as microcystin-LA, which does not containing arginine, tryptophan or tyrosine, was very poor (Meriluoto et al., 1998).

Another limitation which may be applicable to all procedures that determine individual toxins is that, if several toxins are present, some may be present at concentrations below the detection limit. The total concentration may therefore be underestimated, thereby producing an erroneously low result. Standards must therefore be available for each toxin to determine individual concentrations, and toxicity data must be available to convert toxin concentrations to microcystin-LR toxicity equivalents. Although lack of both toxin standards and toxicity data may be a limitation in analytical approaches involving HPLC, assumptions can be made in order to produce a result in microcystin-LR toxicity equivalents. Taking these limitations into account, HPLC procedures are still the most appropriate for

monitoring for compliance with the guidelines as stipulated by WHO (Meriluoto et al., 1998).

11.4. Capillary electrophoresis (CE) detection of cyanobacterial peptide toxins

Other similar separation and detection techniques such as capillary electrophoresis and related techniques have also been considered for the separation and quantification of the peptide hepatotoxins (Bateman et al., 1995). But these have lower sensitivity when compared with HPLC procedures and are not suitable for routine monitoring of water without further development. CE is also not yet considered to be sufficiently robust for use in a routine analytical laboratory. Sensitivity can be increased in CE separation by derivatizing toxins to give fluorescent products that can be detected using a laser-induced fluorescent detector (Li et al., 1999). However, this method requires further evaluation in terms of technologies which may increase flow cell volume which will enhance the detection limits.

11.5. Gas chromatographic (GC) and GC–MS methods

Gas chromatographic (GC) methods based on oxidation of microcystins that splits the Adda side chain to produce 3-methoxy-2-methyl-4-phenylbutyric acid (MMPB) has been reported. Determination of the MMPB by either by GC or GC/MS (as its methyl ester) or by HPLC with fluorescence detection (after conversion to a fluorescent derivative) were reported with detection limits of 0.43 ng in water samples (Sano et al., 1992). The GC–MS approach was also used to monitor microcystins in Japanese lakes (Tanaka et al., 1993) and in sediments (Tsuji et al., 2001). Harada et al. (1997) reported a similar method whereby the MMPB was determined directly without derivatization using GC–MS or LC–MS. This approach results in a total toxin concentration, which can be expressed in terms of microcystin-LR. However individual toxins were not determined and consequently it was not possible to produce a result in terms of microcystin-LR toxicity equivalents. This procedure cannot therefore be used to monitor water samples in relation to the proposed WHO guidelines. The limitation of this method is that it requires tedious procedures during the extraction, cleanup, oxidation and post-treatment in order to eliminate the reagents used and derivatization for GC and HPLC analysis. However, Harada et al. (1995) have reported an improved method using ozonolysis which made it possible to reduce significantly the formation times of MMPB because the previously required extraction, clean-up and other procedures were eliminated. The resulting intact MMPB was directly analyzed by thermospray (TSP) LC–MS and EI-GC–MS using selected ion monitoring. This new procedure was short (took 30 min) and the analysis of MMPB was at picomol levels.

11.6. Spectroscopic characterization of cyanobacterial compounds

Two-dimensional NMR (2D NMR) measurements have been useful in elucidating chemical structures of cyanobacterial isomers (Harada et al., 1990). A general strategy for identification and assignment of all proton and carbon resonances of moderate complex metabolites (500–2000 Da) has been done by employing various correlation techniques of 2D NMR experiments (Harada et al., 1990). The chemical structures deduction has been performed by 2D H,H and H,C correlated NMR experiments, as well as selected one dimensional NOE difference spectra in combination with mass spectrometric data. NMR experiments for identification of isolated components are usually measured in deuterodimethylsulfoxide (DMSO-d_6) (Rowan, 1989). However, NMR usually requires relatively large amounts of sample (milligram quantities) and completely purified samples; and, moreover, the NMR instruments are very expensive.

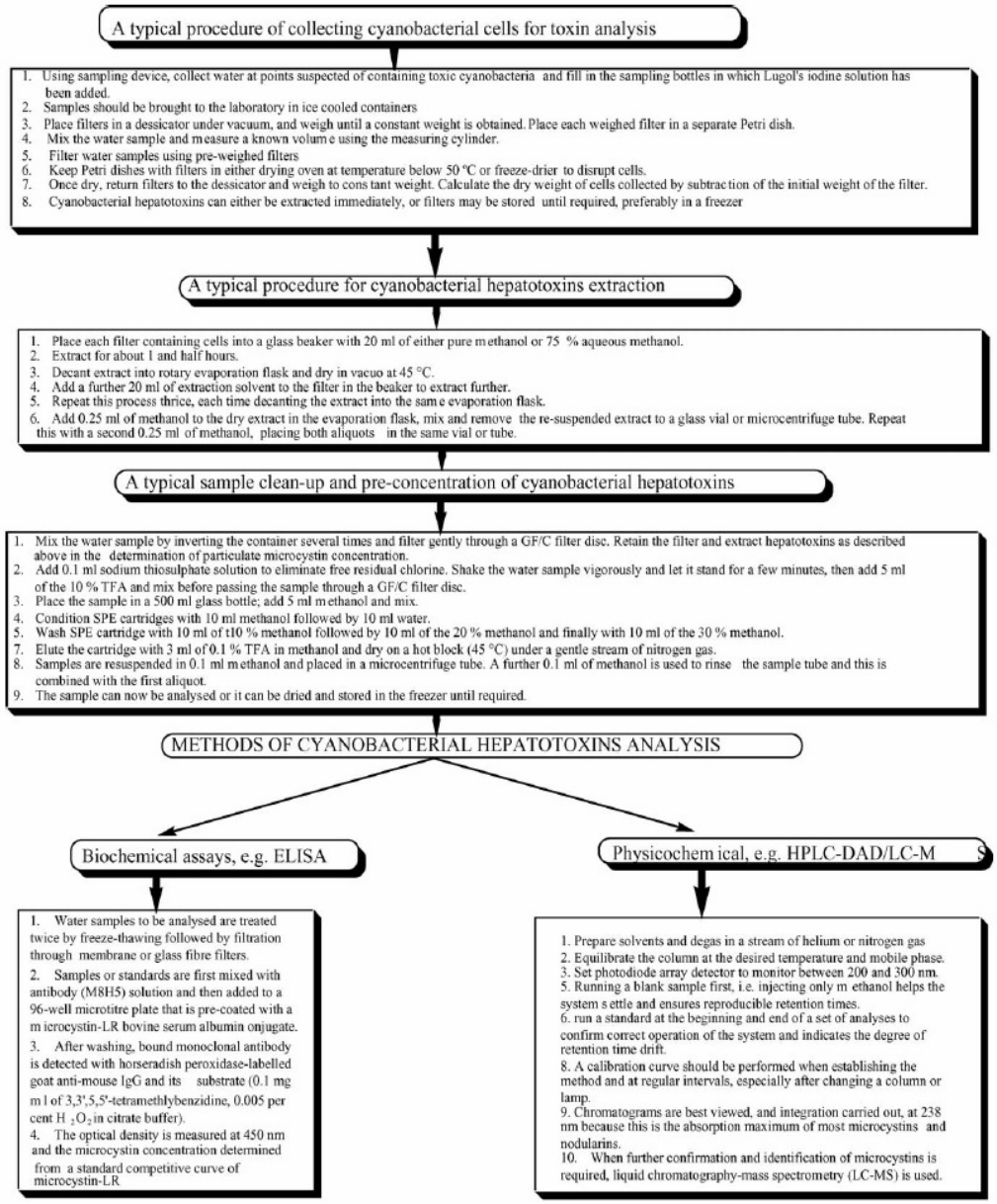
12. Conclusions

Due to the toxicity of cyanobacterial hepatotoxins to human beings and animals, it seems plausible that, the best response to safeguard the health and well being of humans and animals is the prevention of exposure to these toxins. Therefore, constant monitoring for cyanobacterial toxins in drinking water, recreational waters as well as other potential venues, is very important. Moreover, health surveillance and evaluation need to be introduced by authorities everywhere, where exposure may be suspected. Proper guidelines should be developed and put into effect for recreational and occupational use of potentially contaminated waters in line with the extent of potentiality of the toxin situation.

In cases where toxic blooms of cyanobacteria are present, public awareness should be made to the community and this may include avoidance of occupational and recreational exposure, warning the public of possible health consequences and that boiling water cannot destroy toxins. Also stricter control to reduce exposure of cyanobacterial blooms at critical points (e.g., drinking water sources) should be observed and put into immediate effect. The use of algicides such as copper sulphate to control toxic blooms should be avoided, as it leads to cell lysis hence releasing toxins into water. This makes removal of intact cells from water as the only recommended method which may be safer. Other methods such as the use of activated carbon may only decrease levels of toxicity. Also, since there is a possibility of exposure to hepatotoxins through consumption of contaminated sea foods (e.g., spirulina). It is important that such products should not be collected during the algal bloom season.

With regard to the techniques that have been reported for the determination of cyanobacterial hepatotoxins, the most reliable measurements which seem to comply with the international water guidelines standards of the World Health Organization (WHO) are HPLC (if standards for the toxins present are available) and liquid chromatography with mass spectral confirmation of toxin identity and quantification.

The ELISA technique has been useful for routine screening of water for cyanotoxin contamination. Mouse bioassays have been shown not to be suitable for determining microcystins at low concentration in water. These assays do not have sufficient sensitivity for application to water samples without impractical levels of pre-concentration. Mouse bioassays are useful for



Scheme 1. Example of procedures typically used in the analysis of cyanobacterial hepatotoxins.

initial screening of highly concentrated cyanobacterial samples (e.g., 'scums') of unknown toxicity. The results of a quantitative assay will indicate acute toxic effects and may also indicate the class of toxin from reactions and pathology in test animals. This can then allow for further quantitative testing by an alternative analytical method. Solid phase extraction has been shown to be the most reliable for sample cleanup and pre-concentration of cyanotoxins. Scheme 1 shows a typical example of the proce-

dures that may be followed to analyze peptide hepatoxins such as microcystins.

References

Abeliovich, A., Shilo, M., 1972. Photooxidative death in blue-green algae. J. Bacteriol. 110, 682–689.

An, J., Carmichael, W.W., 1994. Use of a colorimetric protein phosphatase inhibition assay and enzyme linked immunosorbent assay for the study of microcystins and nodularin. Toxicon 32, 1495–1507.

Azevedo, S.M.F.O., Carmichael, W.W., Jochimsen, E.M., Rinehart, K.L., Lau, S., Shaw, G.R., Eaglesham, G.K., 2002. Human intoxication by microcystins during renal dialysis treatment in Caruaru—Brazil. Toxicology 181, 441–446.

Bateman, K.P., Thibault, P., Douglas, D.J., White, R.L., 1995. Mass-spectral analyses of microcystins from toxic cyanobacteria using online chromatographic and electrophoretic separations. J. Chromatogr. A 712, 253–268.

Batista, T., de Sousa, G., Suput, J.S., Rahmani, R., Suput, D.A., 2003. Microcystin-LR causes the collapse of actin filaments in primary human hepatocytes. Aquat. Toxicol. 65, 85–91.

Boyer, K.W., 1987. Levels of Metals in Foods Packaged in Metal Cans—Recent Trends. Abstracts of Papers of the American Chemical Society. 193, 52-AGFD.

Breitig, G., von Tümpling, W. (Eds.), 1982. Ausgewählte methoden der wasseruntersuchung. Biologische, microbiologische und toxikologische methoden, vol. II, 2nd ed. Gustav Fischer Verlag, Jena.

Briand, J.-F., Jacquet, S., Bernard, C., Humbert, J.-F., 2003. Health hazards for terrestrial vertebrates from toxic cyanobacteria in surface water ecosystems. Vet. Res. 34, 361–377.

Brooks, W.P., Codd, G.A., 1987a. Distribution of *Microcystis aeruginosa* peptide toxin and interactions with hepatic microsomes in mice. Pharm. Toxicol. 60, 187–191.

Brooks, W.P., Codd, G.A., 1987b. Immunological and toxicological studies on *Microcystis aeruginosa* peptide toxin. Br. Phycol. J. 22, 301.

Carbis, C.R., Waldron, D.L., Mitchell, G.F., Anderson, J.W., McCauley, I., 1995. Recovery of hepatic function and latent mortalities in sheep exposed to the blue green alga *Microcystis aeruginosa*. Vet. Record. 137, 12–15.

Carmichael, W.W., 1994. The toxins of cyanobacteria. Sci. Am. Jan. 270, 78–86.

Carmichael, W.W., 1997. The cyanotoxins. In: Callow, J.A. (Ed.), Advances in Botanical Research, vol. 27. Academic Press Inc., San Diego, pp. 211–256.

Cazenave, J., Wunderlin, D.A., Bistoni, M.Á., Amé, M.V., Krause, E., Pflugmacer, S., Wiegand, C., 2005. Uptake, tissue distribution and accumulation of microcystin-RR in *Corydoras paleatus*, *Jenynsia multidentata* and *Odontesthes bonariensis*. A field and laboratory study. Aquat. Toxicol. 75, 178–190.

Chen, J., Xie, P., Guo, L., Zheng, L., Ni, L., 2005. Tissue distributions and seasonal dynamics of the hepatotoxic microcystins-LR and -RR in two freshwater snail (*Bellamya aeruginosa*) from a large shallow, eutrophic lake of the subtropical China. Environ. Pollut. 134, 423–430.

Chen, J., Xie, P., 2005a. Tissue distributions and seasonal dynamics of the hepatotoxic microcystins-LR and -RR in two freshwater shrimps, *Palaemon modestus* and *Macrobrachium nipponensis*, from a large shallow, eutrophic lake of the subtropical China. Toxicon 45, 615–625.

Chen, J., Xie, P., 2005b. Tissue distributions and seasonal dynamics of the hepatotoxic microcystins in various organs of four freshwater bivalves from the large eutrophic Lake Taihu of Subtropical China and the risk to human consumption. Environ. Toxicol. 20, 572–584.

Chorus, I., Bartram, J. (Eds.), 1999. Toxic Cyanobacteria in Water: a Guide to their Public Health Consequences Monitoring and Management. E & FN Spon, London.

Chorus, I. (Ed.), 2001. Cyanotoxins: Occurrence, Causes Consequences. Springer, Tokyo.

Chu, F.S., Huang, X., Wei, R.D., Carmichael, W.W., 1989. Production and characterization of antibodies against microcystins. Appl. Environ. Microbiol. 55, 1928–1933.

Codd, G.A., Ward, C.J., Bell, S.G., 1997. Cyanobacterial toxins: occurrence, modes of action, health effects and exposure routes. Arch. Toxicol. Suppl. 19, 3990–4410.

De sousa, G., Dou, M., Barbe, D., Lacarelle, B., Placidi, M., Rahmani, R., 1991. Freshly isolated or cryopreserved human hepatocytes in primary culture—influence of drug-metabolism on hepatotoxicity. Toxicol. In Vitro 5, 483–486.

Dorko, K., Freeswick, P.D., Bartoli, F., Cicalese, L., Bardsley, B.A., Tzakis, A., Nussler, A.K., 1994. A new technique for isolating and culturing human hepatocytes from whole or split livers not used for transplantation. Cell Transplant. 3, 387–395.

Edler, L., Ferno, S., Lind, M.G., Lundberg, R., Nilsson, P.O., 1985. Mortality of dogs associated with a bloom of the cyanobacterium *Nodularia spemigena* in the Baltic Sea. Ophelia 24, 103–107.

Edward, C., Lawton, L.A., Coyle, S.M., Ross, P., 1996. Laboratory-scale purification of microcystins using flash chromatography and reversed high performance liquid chromatography. J. Chromatogr. A 734, 163–173.

Edwards, C., Lawton, L.A., Beattie, K.A., Codd, G.A., Pleasance, S., Dear, G.J., 1993. Analysis of microcystins from cyanobacteria by liquid-chromatography with mass-spectrometry using atmospheric-pressure ionization. Rapid Commun. Mass Spectrom. 7, 714–721.

Elder, G.H., Hunter, P.R., Codd, G.A., 1993. Hazardous freshwater cyanobacteria (blue green algae). Lancet 341, 1519–1520.

Eloff, J.N., Steinitz, Y., Shilo, M., 1976. Photooxidation of cyanobacteria in natural conditions. Appl. Environ. Microbiol. 31, 119–126.

Eriksson, J.E., Meriluoto, J.A.O., Kujari, H.P., Osterlund, K., Fagerlund, K., Hallbom, L., 1988. Preliminary characterization of a toxin isolated from the cyanobacterium *Nodularia spemigena*. Toxicon 26, 161–166.

Falconer, I.R., Beresford, A.M., Runnegar, M.T., 1983. Evidence of liver damage by toxin from a bloom of the blue-green alga, *Microcystis aeruginosa*. Med. J. Aust. 1, 511–514.

Falconer, I.R., Choice, A., Hosja, W., 1992. Toxicity of mussel (*Mytilus edulis*) growing naturally in an estuary during a water bloom of the blue green algas *Nodularia spumigena*. Environ. Toxicol. Water Qual. 7, 119–123.

Falconer, I.R., Humpage, A.R., 2005. Health risk assessment of cyanobacterial (blue-green algal) toxins in drinking water. Int. J. Res. Public Health 2, 43–50.

Falconer, I.R., 1993. Measurement of toxins from blue-green algae in water and foodstuffs. In: Faconer, I.R. (Ed.), Algal Toxins in Seafood and Drinking Water. Academic Press, London, pp. 165–175.

Falconer, I.R., 2005a. Cyanobacterial toxins of drinking water supplies: cylindrospermopsins and microcystins. In: Chapter 5 Cyanobacterial Poisoning of Livestock and People. CRC Press, Boca Raton, FL.

Falconer, I.R., 2005b. Is there a human health hazard from microcystins in the drinking water supply? Acta Hydrochim. Hydrobiol. 33, 64–71.

Fastner, J., Flieger, I., Neumann, U., 1998. Optimised extraction of microcystins from field samples—a comparison of different solvents and procedures. Water Res. 32, 3177–3181.

Finden, D.A.S., Tipping, E., Jaworski, G.H.M., Reynolds, C.S., 1984. Light-induced reduction of natural iron (III) oxide and its relevance to phytoplankton. Nature (London) 309, 783–784.

Fladmark, K.E., Brustugun, O.T., Hovald, R., Boe, R., Gjersten, B.T., Zhivotovsky, B., Doskeland, S.O., 1999. Ultra rapid scapase-3 dependent apoptosis induction by serine/threonine phosphatase inhibitors. Cell Death Differ. 6, 1099–1108.

Gathercole, P.S., Thiel, P.G., 1987. Liquid chromatography detection of the cyanoginosins, toxins produced by the cyanobacterium *Microcystis aeruginosa*. J. Chromatogr. 408, 435–440.

Gussmann, H.J., Molzahn, J., Bicks, B., 1985. Poisoning of young cattle by *Nodularia spumigena*. Monatsh. Vet. 40, 76–80.

Harada, K., Ogawa, K., Matsuura, K., Murata, H., Suzuki, M., Watanabe, M.F., Itezono, Y., Nakayama, N., 1990. Structural determination of geometrical-isomers of microcystins LR and RR from cyanobacteria by 2-Dimensional NMR spectroscopic techniques. Chem. Res. Toxicol. 3, 473–481.

Harada, K., Fujii, K., Shimada, T., Suzuki, M., Sano, H., Adachi, K., Carmichael, W.W., 1995. 2 cyclic-peptides, anabaenopeptins, a 3rd group

of bioactive compounds from the cyanobacterium Anabaena-Flos-Aquae NRC-525-17. Tetrahedron Lett. 36, 1511–1514.
Harada, K.-I., Oshikata, M., Shimada, T., Nagata, A., Ishikawa, N., Suzuki, M., Kondo, F., Shimizu, M., Yamada, S., 1997. High performance liquid separation of microcystins derivatized with a highly fluorescent dienophile. Nat. Toxins 5, 201–207.
Heresztyn, T., Nicholson, B.C., 2001a. Determination of cyanobacterial hepatotoxins directly in water using a protein phosphatase inhibition assay. Water Res. 35, 3049–3056.
Heresztyn, T., Nicholson, B.C., 2001b. A colorimetric protein phosphatase inhibition assay for the determination of cyanobacterial peptide hepatotoxins based on the dephosphorylation of phosvitin by recombinant protein phosphatase 1. Environ. Toxicol. 16, 242–252.
Heresztyn, T., Nicholson, B.C., 1997. Nodularin concentrations in Lakes Alexandrina and Albert, South Australia, during a bloom of the cyanobacterium (blue-green alga) *Nodularia spumigena* and degradation of the toxin. Environ. Toxicol. Water Qual. 12, 273–282.
Hyenstrand, P., Metcalf, J.S., Beattie, K.A., Codd, G.A., 2001. Effects of adsorption to plastics and solvent conditions in the analysis of the cyanobacterial toxin microcystin-LR by high performance liquid chromatography. Water Res. 35, 3508–3511.
Ikawa, M., Phillips, N., Haney, J.F., Sasner, J.J., 1999. Interference by plastics additives in the HPLC determination of microcystin-LR and -YR. Toxicon 37, 923–929.
Isom, H.C., Secott, T., Georgoff, I., Woodworth, C., Mummaw, L., 1985. Maintenance of differentiated rat hepatocytes in primary culture, cell biology. Proc. Natl. Acad. Sci. 82, 3252–3256.
James, H.A., James, C.P., 1991. Development of an Analytical Method for Blue-green Algal Toxins (Report FR0224). Foundation for Water Research, Marlow, Buckinghamshire.
Jochimsen, E.M., Carmichael, W.W., An, J.S., Cardo, D.M., Cookson, S.T., Holmes, C.E.M., Antunes, M.B.D., de Melo, D.A., Lyra, T.M., Barreto, V.S.T., Azevedo, S.M.F.O., Jarvis, W.R., 1998. Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. N. Engl. J. Med. 338, 873–888.
Kaebernick, M., Rohrlack, T., Christoffersen, K., Neilan, B.A., 2001. A spontaneous mutant of microcystin biosynthesis: genetic characterization and effect on Daphnia. Environ. Microbiol. 3, 669–679.
Kalbe, L., Tiess, D., 1964. Entenmassessterben durch Nodularia-Wasserblute am kleiner Jasmunder Bodden auf Rugen. Arch. Exp. Vet. 18, 535–555.
Karlsson, K.M., Meriluoto, J.A.O., Kardinaal, E., Visser, P.M., Siewertsen, K., Van Donk, E., Ibelings, B.W., Pires, L.M.D., 2004. Assimilation and depuration of microcystin-LR by the zebra mussel, *Dreissena polymorpha*. Aquat. Toxicol. 69, 385–396.
Katircioğlu, H., Akin, B.S., Atici, T., 2004. Microalgal toxin(s): characteristics and importance. Afr. J. Biotech. 3, 667–674.
Kfir, R., Johannsen, E., Botes, D.P., 1986. Monoclonal antibody specific for cyanoginosin-LA: preparation and characterization. Toxicon 24, 543–552.
Krienitz, L., Ballot, A., Kotut, K., Wiegand, C., Pütz, S., Metcalf, J.S., Codd, G.A., Pflugmacher, S., 2003. Contribution of hot spring cyanobacteria to the mysterious deaths of Lesser Flamingos at Lake Bogoria, Kenya. FEMS Microbiol. Ecol. 43, 141–148.
Lawton, L.A., Edwards, C., Codd, G.A., 1994. Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. Analyst 119, 1525–1530.
Lee, T.-H., Chou, H.-N., 2000. Isolation and identification of seven microcystins from a cultured M.TN-2 Strain of *Microcystis aeruginosa*. Bot. Bull. Acad. Sinica 41, 197–202.
Lehane, L., 2000. Update on histamine fish poisoning. Med. J. Aust. 173, 149–152.
Lehtonen, K.K., Kankaanpää, H., Leiniö, S., Sipiä, V.O., Pflugmacher, S., Sandberg-Kilpi, E., 2003. Accumulation of nodularin-like compounds from the cyanobacterium *Nodularia spumigena* and changes in acetylcholinesterase activity in the clam *Macoma balthica* during short-term laboratory exposure. Aquat. Toxicol. 64, 461–476.
Li, P.C.H., Hu, S., Lam, P.K.S., 1999. Development of a capillary zone electrophoretic method for the rapid separation and detection of hepatotoxic microcystins. Mar. Pollut. Bull. 39, 250–254.
Li, L., Xie, P., Chen, J., 2005. In vivo studies on toxin accumulation in liver and ultrastructural changes of hepatocytes of the phytoplanktivorous bighead carp i.p-injected with extracted microcystins. Toxicon 46, 533–545.
MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P., Codd, G.A., 1990. Cyanobacterial microcystin-LR is a potent inhibitor of protein phosphatases 1 and 2A from both mammals and highest plants. FEBS Lett. 264, 187–192.
Magalhães, V.F., Marinho, M.M., Domingos, P., Oliveira, A.C., Costa, S.M., Azevedo, L.O., Azevedo, S.M.F.O., 2003. Microcystins (cyanobacteria hepatotoxins) bioaccumulation in fish and crustaceans from Sepetiba Bay (Brasil, RJ). Toxicon 42, 289–295.
Magalhães, V.F., Soares, R.M., Azevedo, S.M.F.O., 2001. Microcystin contamination in fish from the Jacarepaguá Lagoon (Rio de Janeiro, Brazil): ecological implication and human health risk. Toxicon 39, 1077–1085.
Mahmood, N.A., Carmichael, W.W., Pflahler, D., 1988. Anticholinesterase poisonings in dogs from a cyanobacterial (blue green algae) bloom dominated by *Anabaena flos-aquae*. Am. J. Vet. Res. 49, 500–503.
Main, D.C., Berry, P.H., Peet, R.L., Robertson, J.P., 1977. Sheep mortalities associated with the blue-green alga *Nodularia spumigena*. Aust. Vet. J. 53, 578–581.
Mankiewics, J., Tarczynska, M., Walter, Z., Zalewski, M., 2003. Natural toxins from cyanobacteria. Acta Biol. Cracov. Bot. 45, 9–20.
Mankiewicz, J., Walter, Z., Tarczynska, M., Zaleweski, M., Doskeland, S.O., Fladmark, K.E., 2001. Apoptotic effect of cyanobacterial extracts on rat hepatocytes and human lymphocytes. Environ. Toxicol. Water Qual. 3, 225–233.
Mankiewicz, J., Walter, Z., Tarczynska, M., Zaleweski, M., Fladmark, K.E., Doskeland, S.O., 2000. Apoptotic effect of cyanobacterial blooms collected from water reservoirs. Pol. J. Occup. Med. Environ. Health 13, 335–344.
McDermott, C.M., Nho, C.W., Howard, W., Holton, B., 1996. The cyanobacterial toxin, microcystin-LR, can induce apoptosis in a variety of cell types. Toxicon 36, 1981–1996.
McDermott, C.M., Feola, R., Plude, J., 1995. Detection of cyanobacterial toxins (microcystins) in waters of northeastern Wisconsin by a new immunoassay technique. Toxicon 33, 1433–1442.
McElhiney, J., Drever, M., Lawton, L.A., Porter, A., 2002. Rapid isolation of a single-chain antibody against the cyanobacterial toxin microcystin-LR by phage display and its use. Appl. Environ. Microbiol. 68, 5288–5295.
Meriluoto, J.A.O., Kincaid, B., Smyth, M.R., Wasberg, M., 1998. Electrochemical detection of microcystins, cyanobacterial peptide hepatotoxins, following high-performance liquid chromatography. J. Chromatogr. A 810, 226–230.
Meriluoto, J.A.O., 1997. Chromatography of microcystins. Anal. Chim. Acta 352, 277–298.
Meriluoto, J.A.O., Eriksson, J.E., 1988. Rapid analysis of peptide toxins in cyanobacteria. J. Chromatogr. 438, 93–99.
Metcalf, J.S., Bell, S.G., Codd, G.A., 2001. Colorimetric immune-protein phosphatase inhibition assay for specific detection of microcystins and nodularins of cyanobacteria. Appl. Environ. Microbiol. 67, 904–909.
Metting, B., Pyne, J.W., 1986. Biologically-active compounds from microalgae. Enzyme Microb. Technol. 8, 386–394.
Michel, K.-P., Exss-Sonne, P., Scholten-Beck, G., Kahman, U., Ruppel, H.G., Pistorius, E.K., 1998. Immunocytochemical localization of IdiA, a protein expressed under iron or manganese limitation in the mesophilic cyanobacterium Synechococcus PCC 6301 and the thermophilic cyanobacterium *Synechococcus elongates*. Planta 205, 73–81.
Montagnolli, W., Zamboni, A., Luvizotto-Santos, R., Yunes, J.S., 2004. Acute effects of *Microcystis aeruginosa* from the Patos Estuary, Southern Brazil, on the Microcrustacean *Kalliapseudes schubartii* (Crustacea: Tanaidacea). Arch. Environ. Contam. Toxicol. 46, 463–469.
Moollan, R.W., Rae, B., Verbeek, A., 1996. Some comments on the determination of microcystin toxins in waters by high-performance liquid chromatography. Analyst 121, 233–238.
Moreno, I.M., Pereira, P., Franca, S., Cameán, A., 2004. Toxic cyanobacteria strains isolated from blooms in the Guadiana River (Southwestern Spain). Biol. Res. 37, 405–417.

Murata, H., Shoji, H., Oshikata, M., Harada, K.I., Suzuki, M., Kondo, F., Goto, H., 1995. High-performance liquid chromatography with chemiluminescence detection of derivatized microcystins. J. Chromatogr. A 693, 263–270.

Nagata, S., Soutome, H., Tsutsumi, T., Hasegawa, A., Sekijima, M., Sugamata, M., Harada, K.-I., Suganuma, M., Ueno, Y., 1995. Novel monoclonal antibodies against microcystin and their protective activity for hepatotoxicity. Nat. Toxins 3, 78–86.

Nagata, S., Tsutsumi, T., Ueno, Y., Watanabe, W.F., 1997. Enzyme-immunoassay for direct determination of microcystins in environmental water. JAOAC 80, 408–417.

Negri, A.P., Jones, G.J., Hindmarsh, M., 1995. Sheep mortality associated with paralytic shellfish poisons from the cyanobacterium *Anabaena circinalis*. Toxicon 33, 1321–1329.

Nehring, S., 1993. Mortality of dogs associated with a mass development of Nodularia spemigena (Cyanophycea) in a brackish lake at the German North Sea coast. J. Plankton Res. 15, 867–872.

Nicholson, B.C., Burch, M.D., 2001. Evolution of analytical methods for determination and quantification of cyanotoxins in relation to Australian drinking water guidelines, a report to the National Health Authority.

Oberholster, P.J., Botha, A.-M., Grobberlaar, J.U., 2004. *Microcystis aeruginosa*; source of toxic microcystins in drinking water. Afr. J. Biotechnol. 3, 159–168.

Ohta, T., Sueoka, E., Lida, N., Komori, A., Suganuma, M., Nishinaki, R., Tatematsu, M., Kim, S.-J., Carmichael, W.W., Fujik, H., 1994. Nodularin, a potent inhibitor of protein phosphatase-1 and phosphatase-2A, is a new environmental carcinogen in male F344 rat-liver. Cancer Res. 54, 6402–6405.

Pietsch, J., Bornmann, K., Schmidt, W., 2002. Relevance of intra- and extracellular cyanotoxins for drinking water treatment. Acta Hydrochim. Hydrobiol. 30, 7–15.

Porfirio, Z., Ribeiro, M.P., Estevam, C.S., Houly, R.L.S., Sant'Ana, A.E.G., 1999. Hepatosplenomegally caused by an extract of cyanobacterium *Microcystis aeruginosa* bloom collected in the Manguaba Lagoon, Alagoas—Brazil. Rev. Microbiola. 30, 278–285.

Premazzi, G., Volterra, L., 1993. Microphyte Toxins. A Manual for Toxin Detection Environmental Monitoring and Therapies to Counteract Intoxications. JRC CEC, Luxembourg.

Prepas, E.E., Kotak, B.G., Campell, L.N., Evans, J.C., Hrudey, S.E., Holmes, C.F.B., 1997. Accumulation and elimination of cyanobacterial hepatotoxins by the freshwater clam *Anodonta grandis simpsoniana*. Can. J. Fish Aquat. Sci. 54, 41–46.

Pyo, D., Lee, J., Choi, E., 2005. Trace analysis of microcystins in water using enzyme-linked immunosorbent assay. Microchem. J. 80, 165–169.

Pyo, D., Shin, H., 1999. Supercritical fluid extraction of microcystins from cyanobacteria. Anal. Chem. 71, 4772–4775.

Repavich, W., Sonzogni, W.C., Standridge, J.H., Wedepohl, R.E., Meisner, L.E., 1990. Cyanobacteria (blue green algae) in Wisconsin waters: acute and chronic toxicity. Water Res. 24, 225–231.

Rivasseau, C., Racaud, P., Deguin, A., Hennion, M.C., 1990. Development of a bioanalytical phosphatase inhibition test for the monitoring of microcystins in environmental water samples. Anal. Chim. Acta 394, 243–257.

Robarts, R.D., Zohary, T., 1987. Temperature effects on photosynthetic capacity, respiration, and growth-rates of bloom-forming cyanobacteria. Z. J. Mar. Freshw. Res. 21, 391–399.

Robillot, C., Vinh, J., Puiseux-Dao, S., Hennion, M.C., 2000. Hepatotoxin production kinetics of the cyanobacterium *Microcystis aeruginosa* PCC 7820, as determined by HPLC-mass spectrometry and protein phosphatase bioassay. Environ. Sci. Technol. 34, 3372–3378.

Rowan, K.S., 1989. Photosynthetic Pigments of Algae. Cambridge University Press, NY.

Runnegar, M.T.C., Gerdes, R.G., Falconer, I.R., 1991. The uptake of cyanobacterial hepatotoxin microcystin by isolated rat hepatocytes. Toxicon 29, 43–51.

Sahin, A., 2000. Enzymatic detection of minute amounts of cyanobacteria hepatotoxins in liver tissue. Eastern J. Med. 5, 52–54.

Sandstrom, A., Gelmarec, C., Meriluoto, J.A.O., Eriksson, J.E., Chattopadhyaya, J., 1990. Structure of hepatotoxic pentapeptide from cyanobacterium *Nodularia spumigena*. Toxicon 28, 535–540.

Sano, T., Nohara, K., Shiraishi, F., Kaya, K., 1992. A method for microdetermination of total microcystin content in waterblooms of cyanobacteria (blue-green algae). Int. J. Environ. Anal. Chem. 49, 163–170.

Selheim, F., Herfindal, L., Martins, R., Vasconcelos, V., Døskeland, S.O., 2005. Neuro-apoptogenic and blood platelet targeting toxins in benthic marine cyanobacteria from the Portuguese coast. Aquat. Toxicol. 74, 294–306.

Soares, R.A., Magalhaes, V.F., Azevedo, S.M.F.O., 2004. Accumulation and depuration of microcystins (cyanobacteria hepatotoxins) in *Tilapia rendalli* (Cichlidae) under laboratory conditions. Aquat. Toxicol. 70, 1–10.

Spoof, L., Karlsson, K., Meriluoto, J., 2001. High-performance liquid chromatographic separation of microcystins and nodularin, cyanobacterial peptide toxins, on C-18 and amide C-16 sorbents. J. Chromatogr. A 909, 225–236.

Sunda, W.G., Swift, D.G., Huntsman, S.A., 1991. Low iron requirement for growth in oceanic phytoplankton. Nature 351, 55–57.

Tanaka, Y., Takenaka, S., Matsuo, H., Kitamori, S., Tokiwa, H., 1993. Levels of microcystins in Japanese Lakes. Toxicol. Environ. Chem. 39, 21–27.

Tencalla, F.G., Dietrich, D.R., Schlatter, C., 1994. Toxicity of *Microcystis aeruginosa* peptide toxin to yearling rainbow trout (*Oncorhyunchus mykiss*). Aquat. Toxicol. 30, 215–224.

Tsuji, K., Masui, H., Uemura, H., Mori, Y., Harada, K., 2001. Analysis of microcystins in sediments using MMPB method. Toxicon 39, 687–692.

Tsuji, K., Naito, S., Kondo, F., Watanabe, M.F., Suzuki, M., Harada, M., 1994. Stability of microcystins from cyanobacteria—effect of light on decomposition and isomerization. Environ. Sci. Technol. 28, 173–177.

Utkilen, H., Gjølme, N., 1995. Iron-stimulated toxin production in *Microcystis aeruginosa*. Appl. Environ. Microbiol. 61, 797–800.

Vasa, G., Gápár, A., Páger, C., Surányi, M., Hamvas, M.M., Borbely, G., 2004. Analysis of cyanobacterial toxins (anatoxin-a, cylindrospermopsin, microcystin-LR) by capillary electrophoresis. Electrophoresis 25, 108–115.

Villareal, T.A., Carpenter, E.J., 2003. Buoyancy regulation and the potential for vertical migration in the oceanic cyanobacterium *Trichodesmium*. Microb. Ecol. 45, 1–10.

Walsby, A.E., 1987. Mechanisms of buoyancy regulation by planktonic cyanobacteria with gas vesicles. In: Fay, P., Van Baalen, C. (Eds.), The Cyanobacteria. Elsevier, Amsterdam, pp. 377–414.

Walsby, A.E., Schanz, F., Schmid, M., 2006. The Burgundy-blood phenomenon: a model of buoyancy change explains autumnal waterblooms by *Planktothrix rubescens* in Lake Zürich. New Phytol. 169, 109–122.

WHO, 2004. Guidelines for Drinking-water Quality, vol. 1., 3rd ed. ©World Health Organization.

Xie, L., Xie, P., Guo, L., Li, L., Miyabara, Y., Park, H.-D., 2005. Organ distribution and bioaccumulation of microcystins in freshwater fish at different trophic levels from the eutrophic Lake Chaohu, China. Environ. Toxicol. 20, 293–300.

Xie, L.Q., Xie, P., Ozawa, K., Homma, T., Yokoyama, A., Park, H.D., 2004. Dynamics of microcystins-LR and -RR in the phytoplanktivorous sliver carp in a sub-chronic toxicity experiment. Environ. Pollut. 127, 431–439.