Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management
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Chapter 13. LABORATORY ANALYSIS OF CYANOTOXINS

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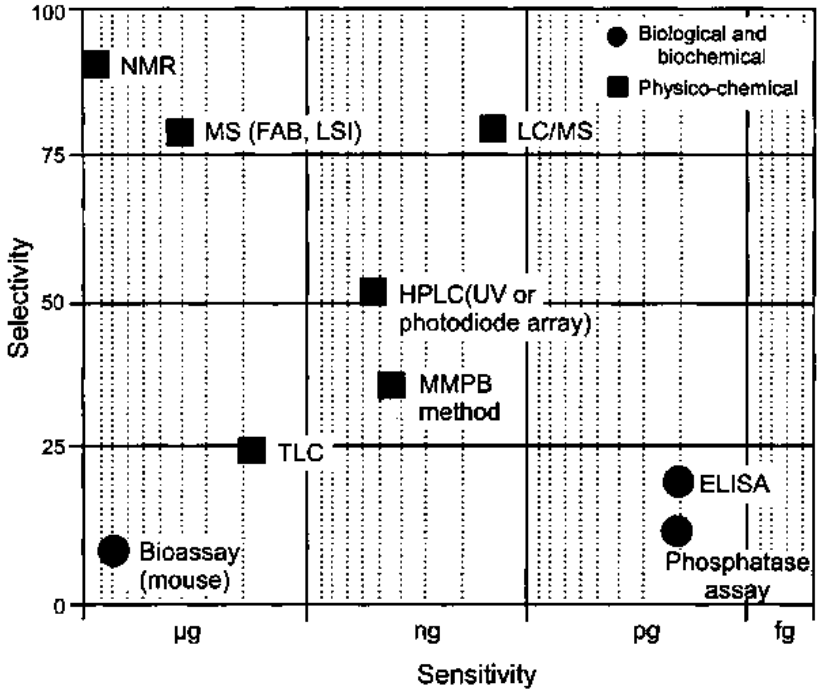
There is a diverse range of laboratory methods used to detect and identify cyanotoxins in water and cyanobacterial cells. These methods can vary greatly in their degree of sophistication and the information they provide. Relatively simple low cost methods can be employed which evaluate rapidly the potential hazard and allow management decisions to be taken. In contrast, highly sophisticated analytical techniques can be employed which determine precisely the identity and quantity of cyanotoxins. Techniques can be selected depending on the facilities and expertise available, coupled with the type of information required. For example, analysis of water for recreational purposes may only require detection of potentially harmful cyanobacteria, whereas ensuring potable water does not exceed guideline levels for cyanotoxins may require highly specialised equipment and expertise. Information obtained from simple, rapid screening methods such as microscopic examination can be used to make an informed decision on the type of bioassay or physicochemical technique which should be employed. It is important to remember that, currently, there is no single method which can be adopted which will provide adequate monitoring for all cyanotoxins in the increasing range of sample types which have to be evaluated. Selectivity and sensitivity are important criteria for the selection of methods. Figure 13.1 compares three biological and six chemical methods with respect to these criteria.

13.1 Sample handling and storage

When samples arrive in the laboratory, the type of analysis that will be carried out should have been anticipated previously. Full consideration should be given to the type of information required prior to sample collection (see Chapters 10 and 11). However, this is not always possible, particularly when a routine monitoring programme is not in place. Information obtained from the microscopic examination of the cyanobacterial taxa (Chapter 12) may also influence the choice of analysis but this information is not usually available until samples have reached the laboratory. Many samples will therefore require immediate evaluation on arrival in the laboratory to determine if any pre-treatment is needed prior to appropriate sample storage.

Figure 13.1 Relationship between sensitivity and selectivity of analytical methods for microcystins (see text for explanation of methods)



Samples for cyanotoxin analysis should be refrigerated in the dark to prevent toxin degradation but it is essential that storage be kept to a minimum (preferably less than 24 hours). Where prolonged storage is required, samples can be frozen, although this will release toxins from the cells and only the total amount of toxin in the sample can then be determined. It is often desirable, especially for water treatment purposes, to estimate the amount of toxin contained within cells as well as that which is dissolved in the water. Where this information is required, samples should be gently filtered as soon as practically possible and if necessary the filters and filtrate can be refrigerated or frozen.

A concentration step for cyanobacterial cells can be useful especially for less sensitive cyanotoxin assay methods. This can be achieved at the sampling location with the use of a plankton net or by using lakeside filtration apparatus (Chapter 12). In the laboratory, concentration can be achieved by allowing buoyant cells to accumulate in the upper part of a separating funnel which enables excess water to be removed. This method can concentrate cells by at least ten fold but is dependent on cells being vacuolate (i.e. floating). It may also lead to a bias if most of the cells do not accumulate at the surface because floating cells may have different toxin quotas than cells which do not. Centrifugation is useful but is often limited by the relatively small volume of laboratory centrifuge tubes and, furthermore, problems can be encountered when trying to pellet vacuolate cells. Filtration is increasingly popular because it can allow concentration of cells by several orders of magnitude and enables the weight of the cell mass to be determined. The following filtration method can be employed (Figure 13.2).

Apparatus

- Oven/incubator set at 45 °C or freeze-drier

- Vacuum dessicator

- Balance accurate to 0.0001 g

- Glass fibre filters 70 mm, GF/C, 1.2 µm will retain most cyanobacteria but the smaller pore size of GF/F (0.7 µm) is required for picoplankton cyanobacterial cells.

- Filtration cup and vacuum pump

- Measuring cylinder

- Plastic Petri dishes

Procedure
1. Place filters in dessicator under vacuum, then remove at intervals and weigh. Filters are ready to use once a constant weight is obtained. It is useful to place each weighed filter in a separate Petri dish with the weight recorded on the dish. This makes samples easy to handle and prevents confusion.

2. Mix the water sample by inverting the bottle several times, then measure a known volume using the measuring cylinder. This volume will differ depending on the concentration of cells present, because only small volumes of water with a high concentration of cells can be filtered before the filter becomes blocked.

3. Using one of the pre-weighed filters, filter the water sample and then return the filter to the labelled Petri dish.

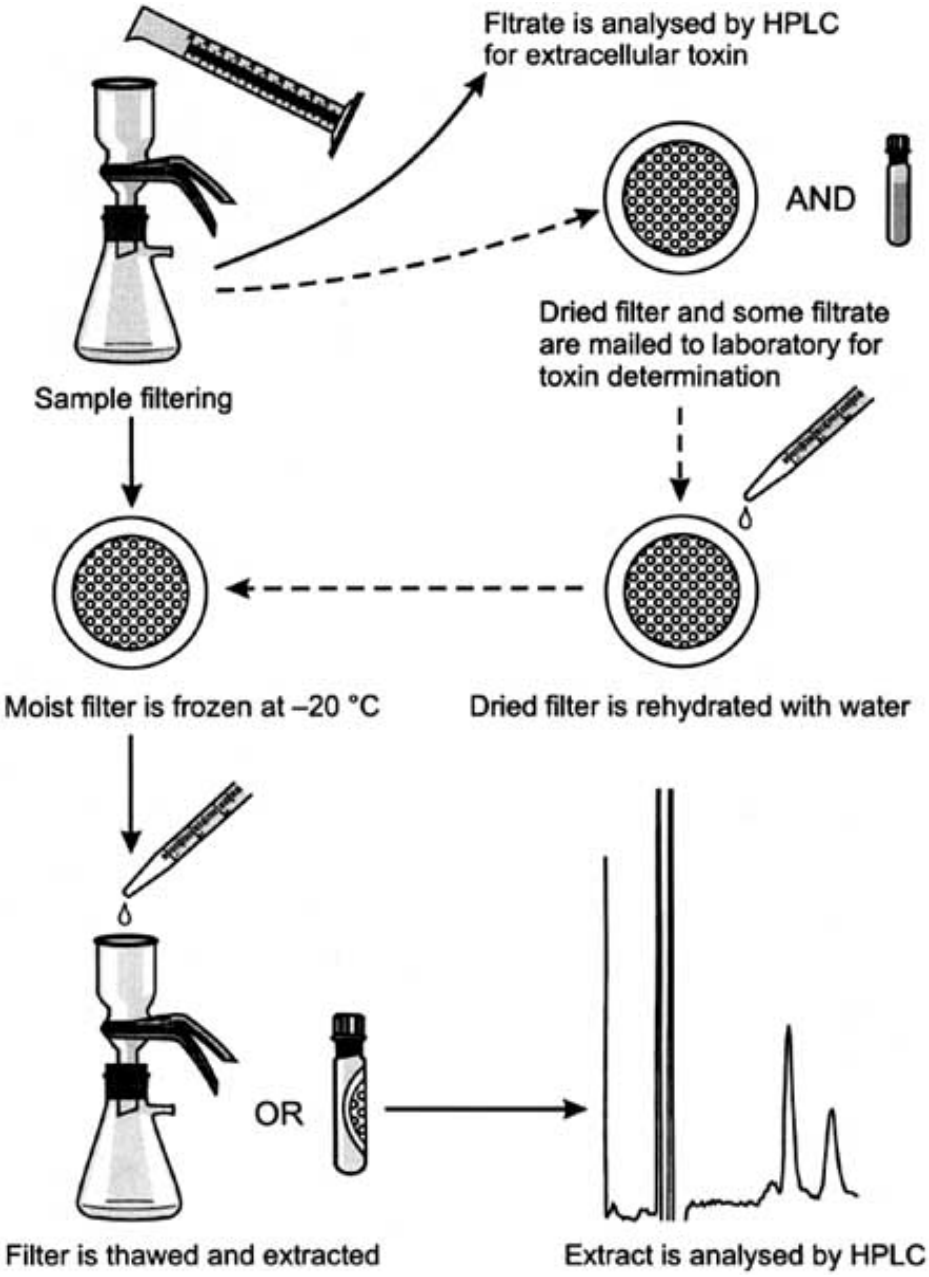
4. Keeping the filter in the Petri dish place it either in the drying oven or freeze-drier. If using on oven, the temperature must be kept below 50 °C.

5. Once dry, return filters to the dessicator and weigh to constant weight. Calculate the dry weight of cells collected by subtraction of the initial weight of the filter.

6. Cyanotoxins can either be extracted immediately, or filters may be stored until required, preferably in a freezer.

By following this procedure it is possible to relate cyanotoxin content to the dry weight of particulate matter extracted as well as to the volume of water filtered. Sometimes dry weights are affected by the presence of large particles in the sample (e.g. zooplankton or feathers from waterfowl). This can easily be overcome by carrying out pre-screening using a 1-2 mm sieve if necessary. Furthermore, particulate matter (i.e. seston) may consist of plankton organisms in addition to cyanobacteria (e.g. algae, rotifers, bacteria, detritus). A brief microscopic check of the fresh sample or a preserved sub-sample (see Chapter 11) will reveal whether or not the dry weight can be attributed mainly to cyanobacteria. In dense blooms and scums this is usually the case but, in samples from more homogenously dispersed situations, other components may dominate and the relating of the toxins to dry weight will therefore underestimate the toxin content of the cells. If an accurate balance is not available, the filtration method can be used but the cyanotoxin content can be related only to the volume of water filtered.

Figure 13.2 Toxin extraction by filtration



Dense cyanobacterial scums or samples concentrated using a plankton net are often freeze-dried. This provides a dry powder which can easily be weighed prior to extraction. However, great care must be taken with such dry powders because they can easily become airborne and may present a health hazard through inhalation.

Most sample handling and storage methods for cyanotoxin analysis have been evaluated primarily for microcystins, hence the stability of other cyano-toxins may not be fully understood. It is therefore important to evaluate the chosen method if other cyanotoxins are being monitored.

13.2 Sample preparation for cyanotoxin determination and bioassays

13.2.1 Extraction

For cyanotoxin detection, samples may be extracted from cells or biological matrices such as animal tissue, or water samples. Cells have successfully been extracted in a number of different liquid phases, and some of the most popular are 5 per cent acetic acid, methanol, acidified methanol (Trifluoroacetic acid (TFA) added), aqueous methanol, and butanol:methanol:water (1:4:15) (Harada, 1996). The efficiency of these methods depends on the sample and the cyanotoxins present. It has been found that the more polar extraction media, such as 5 per cent acetic acid, provide reasonable extraction efficiency of the more polar microcystins but give very poor recovery of hydrophobic microcystins (Lawton *et al.,* 1994a). Methanol has been advocated as 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 per cent methanol may give poor recovery of more polar microcystins; but the addition of a small percentage of water overcomes this. Fastner *et al.* (1998) showed that, especially for lyophilised field samples dominated by *Microcystis* spp., extraction with 75 per cent methanol and 25 per cent water (by weight) was most effective. One approach which has been used routinely, with success, for microcystins is as follows:

Apparatus

- Rotary evaporation equipment
- Glass beakers, 50 ml
- Measuring cylinder, 20 ml
- Rotary evaporation flasks, 50 ml, pear-shaped
- Pipette, 0.25 ml
- Glass vials or microcentrifuge tubes, ~1 ml.

Reagents

- Methanol, high purity if possible.
- Aqueous methanol (75 per cent, v/v)

Procedure

1. Place each filter containing cells into a glass beaker with 20 ml of either pure methanol or 75 per cent aqueous methanol. Filters may be cut into small pieces with scissors but gloves must be worn and care must be taken not to cross contaminate samples or to lose material sticking to the scissors.

2. Allow filters to extract for 1 hour. If time is limiting, extract for 30 minutes because the shorter time has been found to reduce only slightly the recovery of microcystins.

3. Decant extract into rotary evaporation flask and dry *in vacuo* at 45 °C.

4. Add a further 20 ml of extraction solvent to the filter in the beaker and allow to extract as before.

5. Repeat this process a total of three times, each time decanting the extract into the same rotary evaporation flask.

6. Add 0.25 ml of methanol to the dry extract in the rotary evaporation flask, mix and remove the resuspended extract to a glass vial or microcentrifuge tube. Repeat this with a second 0.25 ml of methanol, placing both aliquots in the same vial or tube.

7. Consideration must be given to the type of analysis which is going to follow extraction because organic solvents are toxic to bioassay organisms. This can often be overcome by using a volatile medium, followed by evaporation and resuspension in a medium which is compatible with the assay method. With very toxic samples and/or sensitive bioassays, dilution with the assay medium (using a control containing the same per cent of solvent) is sufficient to obtain non-toxic solvent concentrations.

Two other rapid extraction procedures have also been successfully applied:

- After freezing and thawing to disrupt cells, toxins may be extracted in the filtration device by passing aqueous methanol or water, followed by methanol, through the filters by suction (use a total of 5-25 ml of solvent). Extracts can be used directly for analysis or further concentrated if enhanced sensitivity is needed (Figure 13.2) (Utkilen and Gjølme, 1994). This method requires little equipment other than a filtration device and deep-freezer.

- Membrane filters with approximately 20 mg of freeze-dried material may be extracted in 2 ml microcentrifuge tubes by adding 1.5 ml solvent (preferably 75 per cent aqueous methanol), sonicating, shaking for 30 minutes and centrifuging. This step extracts a large share of the microcystins, and extraction is more complete if the pellet is re-extracted twice and the supernatants are pooled (Fastner *et al*., 1998).

Saxitoxins are often extracted in acidified media including acetic acid, hydrochloric acid (HCl) and acidified methanol (Fernandez and Cembella, 1995). A recent study found methanol acidified with TFA was the most efficient solvent when extracting saxitoxin and neosaxitoxin from cyanobacterial cells (McElhiney *et al.,* 1998). Anatoxin-a has been successfully extracted with water, acidified water, acidified methanol (Edwards *et al.,* 1992), chloroform followed by hydrochloric acid (Harada *et al.,* 1989) or by dichloromethane after an acidification and neutralisation step (Bumke-Vogt *et al.,* 1996).

Box 13.1 When is sample cleanup needed?

Try analysis or bioassays without performing cleanup if:

- Samples are dominated by cyanobacteria; or

- Precise quantification of toxins and complete identification of minor toxin components is less important than rapid analysis of a larger number of samples.

Introduce cleanup if:

- Cyanobacteria are a minor component of the sample.

- Low concentrations of cyanotoxins are anticipated.

- Identification and quantification is important.

- Chromatograms obtained without cleanup show considerable baseline problems or poor peak separation.

- Bioassay results suggest influence from further substances.

To store extracts prior to analysis or bioassays, blow to dryness and deepfreeze (-18 °C). Samples may then be re-dissolved in the solvent at a concentration adequate for the subsequent chemical analysis or bioassay.

13.2.2 Sample cleanup

The purpose of cleanup is to eliminate impurities by a simple operation without loss of analyte and where the concentration of cyanotoxins is low, it also enables enrichment of the analyte. Whether or not cleanup is necessary depends largely on the precision of the toxin determination and quantification required. Without cleanup, small toxin peaks in chromatograms may be missed due to masking by other matrix substances eluting simultaneously, and toxin concentrations may be overestimated if peaks are not clearly separated from matrix substances. However, for many screening and monitoring purposes, particularly of bloom samples containing little material other than cyanobacteria, the carrying out of bioassays or toxin analysis without sample cleanup has given satisfactory results (see Box 13.1 for criteria when to perform cleanup).

The establishment of a versatile cleanup method would give an additional advantage to the analysis of microcystins in other matrixes such as biological samples, fish and shellfish. Furthermore, it would give useful information for preparative separation of microcystins and their degradation products. Octadecyl silanised (ODS) silica gel has been employed extensively to facilitate sample cleanup and trace enrichment of microcystins and nodularin because it retains the toxins and allows interference compounds to pass through (Lawton *et al*., 1994a; Harada, 1996).

Concentration and cleanup method for microcystins and nodularins in water samples

Apparatus

- Porcelain filter funnel, 110 mm diameter and Buchner flask, 1 litre or similar
- Filter disks, GP/C, 110 mm
- Measuring cylinder, 500 ml
- Pipettes, various
- Water vacuum pump
- Glass bottles, 500 ml
- Vacuum manifold
- Solid phase extraction cartridges, 1 g trifunctional, end-capped C18
- PTFE tubing and cartridge adapters
- Glass sample tubes
- Drying hot block (45 °C) with blow-down nitrogen
- Microcentrifuge tube (1.5 ml)

Reagents

- Sodium thiosulphate solution, 1 g $Na_2O_3.5H_2O$ made up to 100 ml with distilled water
- Aqueous methanol, solutions containing 10, 20 and 30 per cent methanol in water
- Trifluoroacetic acid solution, 10 per cent TFA v/v in water
- Trifluoroacetic acid solution, 0.1 per cent TFA v/v in methanol
- Methanol
- Water

Note: all reagents should be analytical quality

Procedure

1. Mix the water sample by inverting the container several times, then measure a 500 ml portion of the sample and filter it gently through a GF/C filter disc. The filter can be retained and extracted as described above to determine the particulate microcystin concentration.

2. Add 0.1 ml sodium thiosulphate solution to eliminate free residual chlorine. Shake the water sample vigorously and let it stand for a few minutes, then add 5 ml of the 10 per cent TFA and mix before passing the sample through a GF/C filter disc.

3. Place the sample in a 500 ml glass bottle, add 5 ml methanol and mix. The sample is now ready for solid phase extraction (SPE).

4. Solid phase extraction cartridges are prepared by attaching them to the vacuum manifold system and then conditioning them with 10 ml methanol followed by 10 ml water, ensuring that the cartridge does not become dry at any time. The methanol and water eluates are discarded.

5. Using PTFE tubing and adapter, attach a tube from the bottle containing the water sample to the top of the SPE cartridge. The vacuum draws the sample through the tubing and through the cartridge. The water is not collected but allowed to run to waste through the water pump.

6. Once all of the water sample has passed through it, the cartridge is washed with 10 ml of the 10 per cent methanol followed by 10 ml of the 20 per cent methanol and then

finally washed with 10 ml of the 30 per cent methanol. The eluate from the three washes is discarded.

7. The cartridge is eluted with 3 ml of 0.1 per cent TFA in methanol. This is collected in a sample tube and dried on a hot block (45 °C) under a gentle stream of nitrogen gas.

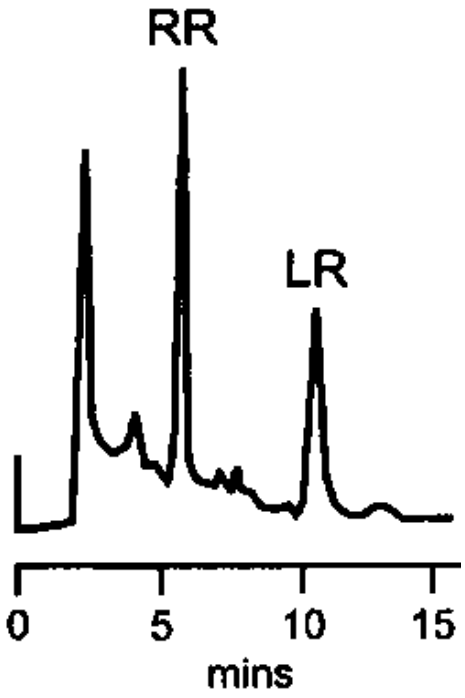
8. Samples are resuspended in 0.1 ml methanol and placed in a microcentrifuge tube. A further 0.1 ml of methanol is used to rinse the sample tube and this is combined with the first aliquot.

9. This sample can now be analysed or it can be dried and stored in the freezer until required.

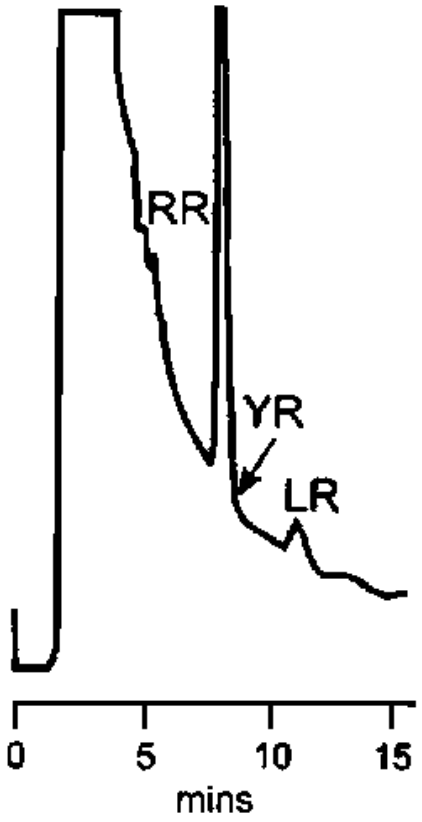
10. It is recommended that this procedure is carried out in duplicate for each water sample analysed.

Figure 13.3 HPLC profiles of water samples containing microcystins.

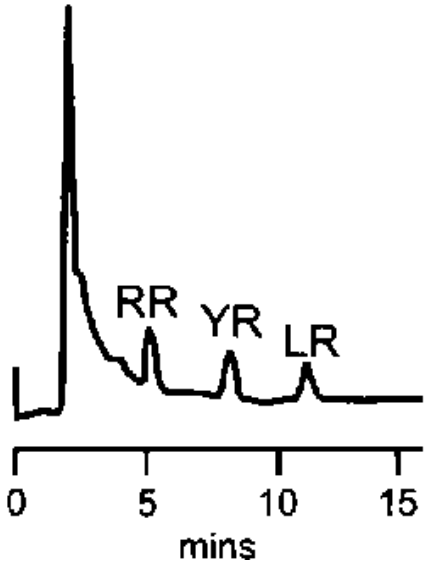
A. A toxic fraction from a bloom sample and of fractions from lake water (1 µg each of microcystin-RR, -YR, -LR added);



B. Before cleanup with silica gel cartridge;



C. After cleanup with silica gel cartridge



Notes

- Different systems can be used to pass the water sample through the SPE cartridge but a specially designed vacuum manifold apparatus is available for use with SPE cartridges. Most of these systems are designed so that a number of cartridges can be attached at one time (e.g. 10 or 20). Automated systems are also available, and peristaltic pumps have also been used successfully, but are not as practical.

- It is vitally important that all apparatus used is thoroughly cleaned to ensure no external contamination. Equipment used for trace enrichment should be dedicated to this procedure only, in order to reduce the opportunities for contamination.

Some modifications to the single SPE method have been proposed with promising results. Figure 13.3A shows the High Performance Liquid Chromatography (HPLC) chromatogram of a toxic fraction from a cyanobacterial bloom sample; the microcystins have been detected with limited interference. However, this method is not always effective for analysis of the toxins in raw water samples because of the occurrence of serious background peaks as shown in Figure 13.3B. To eliminate effectively the impurities, Tsuji *et al.* (1994) established a tandem cleanup method using ODS silica gel and silica gel cartridges as follows. The water sample was first applied to an ODS silica gel cartridge and the desired fraction was eluted with 90 per cent aqueous methanol. Next, the resultant eluate, in 100 per cent methanol, was applied to a silica gel cartridge. After washing with methanol, the toxin-containing fraction was obtained by elution with 10 per cent water-0.1 per cent TFA in methanol. Figure 13.3C shows the chromatogram after the silica gel cleanup, indicating that most of the impurities can be removed. Tsuji *et al.* (1996) used HPLC with ultra violet (UV) detection and Liquid Chromatography/Mass Spectrometry (LC/MS) combined with this tandem cleanup system to report the intracellular and extracellular microcystin levels between 1992 and 1995 for Japanese lakes. Microcystins-LR, -YR and -RR were detected at 0.02-2.64 μg l^{-1} in cell-free water and at 0.02-378 μg l^{-1} in the cells during this period.

Recently, an immunoaffinity purification method has been developed using an anti-microcystin-LR monoclonal antibody (Kondo *et al.,* 1996). This cleanup method was found to be remarkably effective in the removal of contaminants in the hepatic cytosol and enabled the analysis of microcystins and their metabolites, formed *in vivo* in mouse and rat livers, by HPLC and Frit-FAB LC/MS. Figure 13.4 shows the HPLC profiles of a cytosolic extract from mouse liver spiked with 5 μg each of microcystins-RR and -LR. Before the immunoaffinity purification, the spiked microcystins could not be accurately quantified due to the many impurities (Figure 13.4A), whereas after the immunoaffinity purification, the impurities were effectively eliminated and the peaks of the microcystins were clearly detected (Figure 13.4B). Although this immunoaffinity column is not commercially available, it has the potential to enable significant progress in a number of important areas of research; for example in metabolism studies and in elucidation of the fate of microcystins in the environment.

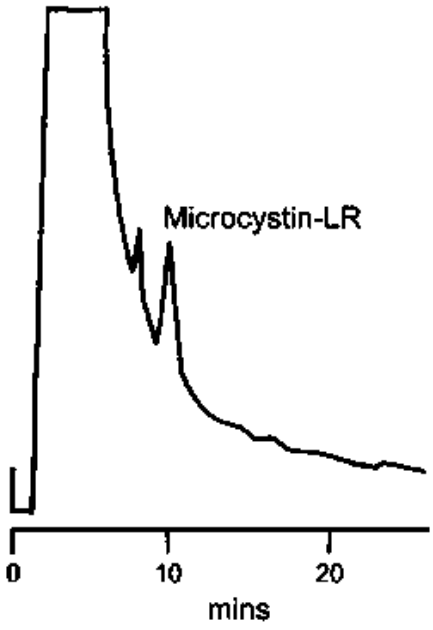
Research into appropriate cleanup methods for other cyanotoxins has still to be carried out, although a similar approach to that which has been developed for microcystins may prove useful.

13.3 Toxicity tests and bioassays

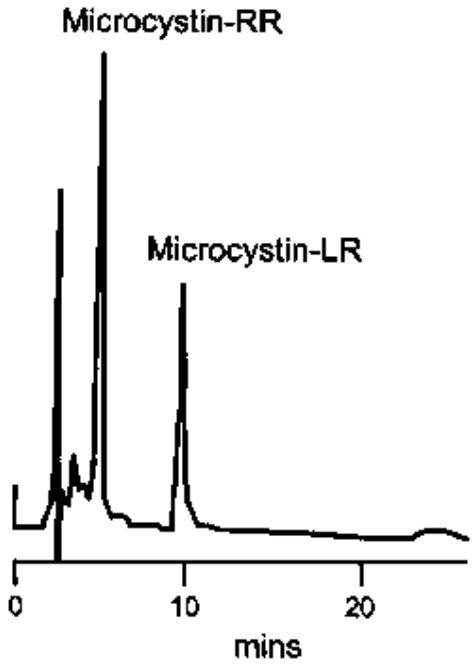
There have been many biological detection methods developed for cyanotoxins that use the bioactivity of the toxins (Table 13.1), such as potent hepatotoxicity, neurotoxicity, cytotoxicity, enzymatic activity and immunological interactions. However, for many years, the mouse bioassay alone has been used to determine bloom toxicity. Although this bioassay provides a measure of the total toxicity (response) within a few hours, it is generally not very sensitive or specific. Considerable research efforts have been made to find suitable alternative methods to the mouse bioassay as a routine monitoring assay for cyanotoxins and many novel and sensitive methods have become available in recent years. However, no single method is currently available to replace the mouse for the detection of all cyanotoxins using a single assay, and further validation and comparison of methods is needed before general recommendations on their application can be given (see also Box 13.2).

Figure 13.4 HPLC profiles of a cytosolic extract from mouse liver spiked with 5 µg each of microcystin-RR and -LR.

A. Extract after heat-denaturation, pronase digestion, and ODS silica gel cleanup;



B. Extract after further immunoaffinity purification



Different bioassays are described below. Users are advised to test their suitability for locally-prevalent cyanotoxins with other methods, such as chemical toxin analysis (see section 13.4). Sensitivity and selectivity are important criteria for the selection of methods. Figure 13.1 shows the sensitivity of three different biological methods in relation to six methods of chemical analysis.

13.3.1 Mouse bioassay

Male Swiss Albino mice are the most used animals for toxicity testing for cyanotoxins. Toxicity is tested by intraperitoneal injection (i.p.) of 0.1-1.0 ml of a lysate of cyanobacteria prepared either by sonication or by freeze-thawing of a cell suspension which has been sterilized by membrane ultra-filtration. Samples can be suspended in water or physiological saline solution which is preferred if the volume to be injected is 0.5 ml or greater.

Table 13.1 Bioassays for the detection of cyanotoxins

Method	Toxins[1]	Cost[2]			Comments	Reference(s)
		Cap.	Con.	Pers.		
Vertebrate						
Mouse	M,N,A, A(s),C,S				Requires licence, not permitted in some countries	Falconer, 1993
Invertebrate						
Brine shrimp	M,N	L	L(H)	M	Commercial kit available but increases cost	Kiviranta *et al*., 1991; Campbell *et al.,* 1994
Daphnia sp.	M,N	L	L	H	Culturing is labour intensive	Lawton *et al.*, 1994b
Thamnotox	M,A,C(?)	L	H	M	Commercial kit available but requires full evaluation for cyanotoxins	Kozma, 1997
Mosquito	M	L	L	M	Difficult to handle	Kiviranta *et al*., 1993
Fruitfly	M,N	L	L	L	Easy to culture	Swoboda *et al*., 1994
Locust	S	L	M	L	Easy to handle	McElhiney *et al*., 1998
House fly	S	L	L	L	Difficult to administer toxin	Ross *et al*., 1985
Bacterial						
Microtox	M,N	H	H	L	No correlation	Lawton *et al*., 1994b
Serratia sp.	M,S	L	L	M	Poor correlation	Lawton *et al*., 1994b
Biochemical						
PPase inhibition	M,N				Very sensitive	
Radioactive		M	M	L	Requires special facilities	Holmes, 1991
Colorimetric		L/M	M	L	Requires purified enzyme	An and Carmichael, 1994
AChE	A(s)	L/M	L	L	Only alternative bioassay for A(s) may react with OP[3] pesticides	Mahmood and Carmichael, 1987
ELISA					Very sensitive	
Polyclonal	M,N	M	H	L	Reactivity for variants may vary	Chu *et al*., 1989
Monoclonal	M,N	M	H	L	Reactivity for variants may vary	Ueno *et al*., 1996
Polyclonal	S	M	H	L	Variable cross-reactivity, does not detect C-toxins	Cembella *et al*., 1995
Mammalian cells						
Hepatocytes	M,N	M	M	M	Sensitive and rapid bioassay	Heinze, 1996

V79 fibroblasts	M	H	H	H	Some false negatives observed	Lawton *et al.,* 1994b
Neuroblastoma	S	H	H	H	Requires careful standardisation	Cembella *et al*., 1995; Gallacher and Birkbeck, 1992

ELISA Enzyme linked immuno sorbent assay

AChE Acetylcholinesterase

[1] M Microcystins; N Nodularins; A Anatoxin-a A(s) Anatoxin-a(S); C Cylindrospermosin; S Saxitoxin

[2] Cap. Capital; Con. Consumable; Pers. Personnel; L Low; M Medium; H High

[3] OP Organophosphorus

Box 13.2 When are bioassays needed?

Bioassays are recommended if any of these conditions are fulfilled:

- A laboratory can easily establish them, but has little or no access to adequate equipment and expertise for establishing physicochemical analysis, or little means of subcontracting analysis.
- There is indication of cyanotoxins other than (or in addition to) the known toxicants.
- Cyanobacterial taxa dominate, the toxins of which have not yet been well studied and which may therefore contain unknown toxic metabolites.
- Confirmation of results from physicochemical analysis is required, especially to confirm bioactivity.
- Validation of physicochemical methods by an alternative method is desired.

Mice should be observed for 24 h and then killed by an approved method (Falconer, 1993). The observation period must be extended to seven days where cylindrospermopsin is suspected and animals should be injected with sterile samples. This toxin demonstrates protracted symptoms which result from progressive organ failure, specifically liver and kidneys. At the end of the observation period post-mortem examination of tissue injury is performed. The observed symptoms and the results of the post-mortem are used to determine which cyanotoxin is present (see Chapter 3 and 4 for toxicology). However, where more than one type of cyanotoxin is present, the more rapid-acting toxin may mask other symptoms.

Toxicity is expressed as LD_{50} mg cell dry weight per kg mouse body weight. Values are classified as follows (Lawton *et al.* 1994b):

> 1,000	non toxic
500-1,000	low toxicity
100-500	medium toxicity
< 100	high toxicity

Note that in some countries non-toxic limits may be at least 2,000 mg cell dry weight per kg mouse body weight.

13.3.2 Invertebrate bioassays

A number of invertebrates have been investigated for use in routine bioassays for cyanotoxins. Of these, the brine shrimp (*Artemia salina*) has been the most popular, because no culture maintenance or specialist equipment is required. Brine shrimps have been exploited for many years for the detection of toxic secondary metabolites and are now commercially available as standardised test kits (although use of the kits increases the cost of performing the assay). Brine shrimp eggs are readily available from biological supply companies and can be stored for several years at -20 °C without loss of viability.

Brine shrimp bioassay

Apparatus

- Conical flasks, glass, 250 ml
- Microtitre plates, 96-well
- Incubator, 25 °C
- Desk lamp
- Pasteur pipette and bulb
- Pipette, 0.1 ml
- Universal bottle, 25 ml
- Dissecting microscope with low power objective

Reagents

- Brine shrimp eggs, stored in freezer
- Brine shrimp medium (BSM) stock solution, composed of:

Sodium chloride (NaCl) 300 g
Calcium chloride dihydrate ($CaCl.2H_2O$) 3 g
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$) 15 g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) 5 g
Potassium chloride (KCl) 8 g
Glycine 60 g
Disodium glycerophosphate 30 g

- Methanol or formalin

The stock solution chemicals are dissolved in 1.25 litres of distilled water, dissolving each chemical separately in the order shown. It is important to add the disodium glycerophosphate last to prevent an insoluble precipitate occurring. The stock solution should be stored in a brown glass bottle in the refrigerator (~4 °C).

Procedure

1. Mix 20 ml of BSM stock solution with 140 ml distilled water in a 250 ml conical flask.

2. Add 100 mg of brine shrimp eggs and incubate at 25 °C until they hatch (usually 36-48 hours). Once a regular hatching time has been established try to keep it constant.

3. Separate the hatched larvae from unhatched eggs and egg cases by transferring them into a shallow dish, e.g. Petri dish or slightly deeper.

4. Position a desk lamp to one side of the dish and allow the larvae to concentrate towards the light.

5. Using a Pasteur pipette carefully, using slow steady suction on the pipette bulb, collect the hatched larvae.

6. Place larvae in a universal bottle and repeat the collection process until most of the hatched larvae have been collected.

7. If a substantial number of unhatched eggs have been collected in error, the separation can be repeated by placing the collected larvae in a clean dish and illuminating.

8. Using a mechanical pipette with plastic tips (first use a sharp blade to remove the first few mm of the tip) pipette 0.1 ml of the larvae suspension into a microtitre well. Make sure the larvae suspension is regularly mixed because they tend to cluster rapidly in the bottle. Mixing is easier if the bottle is only half full.

9. Examine the well containing larvae under the microscope to determine the approximate number of larvae in 0.1 ml. Ideally this should be between 15 and 25 larvae. Dilute the sample with fresh media if the larvae are too concentrated. Repeat the pipetting a few times to ensure reasonably reproducible numbers of larvae are being deposited in each well and that they are alive and look healthy.

10. Fill as many wells with larvae suspension as will be required to carry out the bioassay.

11. To determine the toxicity of cyanobacterial cells, the sample must first be extracted. This can be done as described above (section 13.2.1) from filter discs, but if the final extract is in methanol it must be diluted in BSM so that the final concentration of methanol does not exceed 5 per cent in the test well.

12. A dilution series of each sample should be prepared and 0.1 ml of the test solution added to the larvae in the wells in triplicate. The microtitre plate is then incubated for 18 hours at 25 °C after which the percentage mortality is calculated.

13. First, with the help of a microscope, count the number of dead or immobilised larvae in each well, then add a few drops of formalin or methanol and wait until all the larvae are dead. Now count the total number of larvae in each well and calculate the percentage mortality using a mean of the three replicates.

14. By plotting the concentration of cyanobacteria against the percentage mortality the LC_{50} value (i.e. the concentration of cell extract which caused 50 per cent mortality) for each sample can be determined.

Notes

- Care must be taken to exclude particulate material from the assay and therefore extracts must be filtered or centrifuged.

- High concentrations of cell extract cause false positives, probably due to oxygen depletion caused by the activity of bacteria, therefore sample cleanup (see section 13.2.2) is necessary to detect low concentrations of toxin successfully. Sample cleanup, e.g. the use of SPE, can also enhance the specificity of the assay because it selectively concentrates microcystins and nodularins, reducing interference by other compounds.

When 21 hepatotoxic bloom samples were assessed by this assay the results compared very favourably with both mouse bioassay and HPLC (Lawton *et al.,* 1994b). This assay has not been fully evaluated for toxins other than microcystins, although there does appear to be a correlation between anatoxin-a content and toxicity.

Daphnia bioassays can successfully detect microcystins, although standardised culturing is extremely labour intensive (Baird *et al.,* 1989). A commercially prepared test kit, similar to the brine shrimp assay in that it uses the resting stage of an aquatic invertebrate, *Thamnocephalus platyurus* has been found to be sensitive to a number of cyanotoxins (Kozma, 1997). These kits are relatively expensive and have a limited shelf-life (6 months), although the standardised format of this bioassay leads to highly reproducible results and low inter-laboratory variability (Kozma, 1997). The use of mosquito adults and larvae have both been investigated as potential bioassays (Turell and Middlebrook, 1988; Kiviranta *et al.,* 1993). Adults were injected and larvae immersed in aqueous extracts. Both methods were relatively sensitive but have not been widely adopted due to the difficulties of handling this organism. The other insect which has been shown to detect microcystins successfully in bloom samples is the fruit fly (*Drosophila melanogaster*) (Swoboda *et al.,* 1994). These organisms are easy to maintain in the laboratory, with no special equipment required. Toxin is administered orally by adding filter discs spotted with sample plus sucrose to tubes containing pre-starved (24 h) flies. The flies were not, however, sensitive to neurotoxic *Aphanizomenon* (Swoboda *et al.,* 1994).

Two invertebrate assays have been investigated for the detection of saxitoxins. Firstly, adult house flies injected with purified toxins and natural samples (shellfish extracts) gave results which compared well with toxicity determined by mouse. However, the flies are difficult to handle and require microinjection (1.5 µl) which is difficult to administer (Ross *et al.,* 1985). A locust bioassay has recently been found to detect saxitoxins successfully in a range of samples, namely cyanobacteria and shellfish (McElhiney *et al.,* 1998). Locusts are easy to handle because they can be readily immobilised by holding their rear legs. Samples are administered by injection (10 µl) and results are obtained within 90 minutes (McElhiney *et al.,* 1998). The LD_{50} for pure saxitoxin was 8 µg g^{-1} but the bioassay was not sensitive to microcystin-LR or anatoxin-a.

Locust bioassay for saxitoxins

Apparatus

- 10-25 pi syringe, e.g. type used for GC analysis
- Desert locust (*Schistocerca gregaria*), male early fifth instar
- Plastic container, 500 ml, foil covered with a number of small air holes

Reagents

- Simple saline, 7.5 g sodium chloride (NaCl) and 0.37 g potassium chloride (KCl) dissolved in 1 litre distilled water

Procedure

1. Prepare a dilution series in simple saline of the test sample. It has been found that saxitoxins can be extracted well from cyanobacterial cells with acidified methanol (TFA added). Samples can be dried then resuspended in saline for use in the bioassay.

2. Pick up locust by hind legs and inject 10 pi along the abdomen, (parallel to the body) between the second and third segment. Inject three locusts for each concentration and inject controls with saline only.

3. Place each locust in a clear plastic container and observe for 90 minutes.

4. Death or the inability to self-right when placed on their back is recorded as a positive result.

5. The time of death is an indication of saxitoxin concentration.

13.3.3 Bacterial bioassays

Bacterial bioassays have been investigated to determine if they can provide simple routine methods for cyanotoxin detection. The one that has received the most attention is the Microtox bioluminescence assay which indicates toxicity by a reduction in the light emitted by the test bacterium (*Photobacterium phosphoreum).* Initial investigations suggested that this system may be suitable for the rapid detection of microcystins in bloom samples (Lawton *et al.,* 1990) although more detailed analysis revealed that the assay responded to unknown components of cyanobacterial extracts other than microcystins (Campbell *et al.,* 1994). Several studies have now been published that clearly indicate there is no correlation between response in the Microtox assay and cellular content of the known cyanotoxins (Lawton *et al.,* 1994b; Vezie et al., 1996).

A second bacterial bioassay which used the inhibition of pigment (prodigiosin) formation in *Serratia marcescens* as an indication of toxicity has been proposed by Dierstein *et al.* (1989). This bioassay was thought to be useful for saxitoxins and microcystins. However, like the Microtox system, little correlation was found between actual content of known cyanotoxins and inhibition of pigment formation (Lawton *et al.,* 1994b).

13.3.4 Biochemical assays

The protein phosphatase inhibition assay is a sensitive screening method for microcystins and nodularins which uses the biochemical activity of these toxins. One

version is based on the quantitation of ^{32}P-phosphate released from a radiolabelled substrate (Holmes, 1991; Lambert *et al.,* 1994) by the activity of the protein phosphatase enzyme (PP1 and PP2A). It is sensitive to sub-nanogram levels of microcystin and is a rapid assay allowing the analysis of many samples in a few hours. The method has been applied to detect microcystin class compounds in the marine environment, to extracts of liver tissue taken from Atlantic salmon afflicted with netpen liver disease (Andersen *et al.,* 1993) and to hydrophobic microcystins from freshwater cyanobacteria (Craig *et al.,* 1993). The method has also been successfully used for quantitation of microcystins in drinking water before and after water treatment (Lambert *et al.,* 1994). The detected amounts in raw and treated waters were estimated to be 0.12-0.87 and 0.09-0.18 µg l^{-1}, respectively. Although this method has been widely used in research, there is a reluctance in adopting it for the routine monitoring of microcystins because of the requirement to use radioactivity which necessitates specialised laboratory equipment and regulations.

An and Carmichael (1994) have used a colorimetric protein phosphatase inhibition assay which avoids the complications of using radioactive materials. Isobe *et al.* (1995) reported a firefly bioluminescence system for the detection of protein phosphatase 2A inhibitors, in which luciferin phosphate is hydrolysed to luciferin and inorganic phosphate by protein phosphatase 2A. The use of the protein phosphatase inhibition assay is extremely helpful to confirm biological activity, and hence toxicity, of microcystins in environmental samples. The non-radioactive bioassay may therefore be Used increasingly for the routine screening of water samples, as shown recently by Ward *et al.* (1997).

The biochemical activity of anatoxin-a(S) can be exploited in an enzyme-based assay to detect the inhibition of acetylcholinesterase (AChE), thereby providing an indication of the presence of this toxin (Mahmood and Carmichael, 1987). This is a sensitive method and is the only alternative to the mouse bioassay currently available for this toxin. The assay is not selective because it will also detect other toxicants, such as organophosphorus-based pesticides.

13.3.5 Immunological detection

The Enzyme-Linked Immuno Sorbent Assay (ELISA) technique is currently the most promising method for rapid sample screening for microcystins because of its sensitivity, specificity and ease of operation. Monoclonal antibodies raised against microcystin-LA were initially developed by Kfir *et al.* (1986) and offered a simple approach to a general immunoassay for microcystins. An ELISA technique was subsequently developed by Chu *et al.* (1989) as a practical method. This assay is based on polyclonal antisera raised in rabbits against bovine serum albumin conjugated to microcystin-LR. The antisera showed good cross-reactivity with microcystins-LR, -RR, -YR and nodularin, but less with -LY and -LA. The sensitivity of the assay showed approximately 50 per cent binding at a toxin concentration of 1 ng ml^{-1} which is appropriate for normal water quality testing. In fact, this method has been successfully employed for quantitation of cyanobacterial hepatotoxins in domestic water supplies and biomass extracts with detection limits of 0.2 µg l^{-1} and 0.25 µg g^{-1} for water and biomass samples, respectively (Chu *et al.,* 1990).

Recently, Nagata *et al.* (1995) produced six monoclonal antibodies against microcystin-LR. Among them, M8H5 antibody showed cross-reactivity with microcystin-RR (106 per cent), microcystin-YR (44 per cent), microcystin-LA (26 per cent), [D-Asp^3]microcystin-LR (51 per cent), [Dha^7] microcystin-LR (48 per cent), glutathione conjugate of microcystin-LR (47 per cent), monomethyl ester of microcystin-LR (30 per cent), nodularin (46 per cent) and 6(Z)-ADDA microcystin-LR (< 4 per cent). Although the epitope of this antibody is not clear, the importance of the Adda moiety for antibody binding has been indicated. It should be noted that this antibody also reacts to the non-toxic monomethyl ester of microcystin-LR giving a false positive from the toxicological point of view. Using this monoclonal antibody, a more sensitive competitive ELISA method has been developed by Ueno *et al.* (1996) with detection limits of 0.05 µg l^{-1} for water samples. This method has been successfully applied to detect microcystins in drinking water in China (Box 13.3).

Box 13.3 Application of the ELISA method to the detection of microcystins in drinking water in China

Using an ELISA method, Ueno *et al.* (1996) analysed microcystin concentrations in drinking water collected in Haimen and Fusui in China, where the rates of primary liver cancer (PLC) do not correlate with PLC-causing agents such as aflatoxin and hepatitis-B virus. It had also been observed that people who drank pond and ditch water had a higher risk of PLC than people who drank well water. The authors investigated the levels of microcystins in four types of water (pond/ditch, river, shallow well and deep well) collected from 989 different sampling sites in Haimen. The results showed a positive detection of microcystin for 17,32,4 and 0 per cent of the total samples of pond/ditch, river, shallow well and deep well water, respectively. The average microcystin concentration in the pond/ditch and river water were 101 and 160 pg ml^{-1}, respectively, which were significantly higher than those of the shallow and deep well water. Among the samples examined, two samples from the river showed microcystin levels over 1,000 pg ml^{-1}. These data suggested that microcystin in drinking water from ponds/ditches and rivers, or both, is one of the risk factors for the high incidence of PLC in China. Furthermore, the results indicate that ELISA can be applied successfully to the monitoring of microcystins in environmental samples.

Commercially, a polyclonal ELISA kit is available for microcystins. The antibodies are fixed to the walls of the wells of a microtitre plate. The first step involves binding of the calibrators (a non-toxic microcystin-LR surrogate at 0.1, 0.4 and 1.6 µg l^{-1}) a negative control and the samples to the antibodies in the wells. This is followed by addition of a microcystin-enzyme conjugate which binds to the remaining antibodies. After thorough rinsing, the concentration of bound enzyme is measured colourimetrically in an ELISA plate reader. The microcystin concentration is inversely proportional to the colour intensity.

ELISA method for microcystins

Apparatus

- Filtration equipment for samples containing particles
- Automatic 100 µl pipette
- Timer
- Multichannel pipette for washing the microtitre plate

- Apparatus for shaking the microtitre plate
- ELISA reader with filter at 450 nm

Reagents

- ELISA test kit
- Distilled water

Procedure

1. Water samples to be analysed are treated twice by freeze-thawing followed by filtration through membrane or glass fibre filters.

2. Samples or standards are first mixed with antibody (M8H5) solution and then added to a 96-well microtitre plate that is pre-coated with a microcystin-LR bovine serum albumin conjugate.

3. After washing, bound monoclonal antibody is detected with horseradish peroxidase-labelled goat anti-mouse IgG and its substrate (0.1 mg ml^{-1} of 3,3',5,5'-tetramethlybenzidine, 0.005 per cent H_2O_2 in citrate buffer).

4. The optical density is measured at 450 nm and the microcystin concentration determined from a standard competitive curve of microcystin-LR.

Development of immunodiagnostic systems for the detection of saxitoxins have primarily been aimed at replacing the mouse bioassay for the routine monitoring of shellfish from the marine environment and are discussed in more detail elsewhere (Cembella *et al.,* 1995). Both polyclonal and monoclonal antibodies have been produced, although none have shown cross-reactivity with all the known variants. Antibodies tend to be raised to saxitoxin mainly because it has been the most extensively studied and because it is also the most readily available; hence methods reliably detect this variant but most notably fail to cross-react with neosaxitoxin which is of similar toxicity. Methods continue to be developed (e.g. Kralovec *et al.,* 1996) and may provide a suitable routine monitoring system in the future.

13.3.6 Mammalian cells

Bioassays using mammalian cells have received attention as suitable replacements for mouse toxicity tests. The well documented fact that microcystins have caused acute liver damage has prompted studies using hepatocytes (liver cells). Freshly isolated rat hepatocytes were first investigated by Aune and Berg (1986) who reported good correlation between toxicity measured by leakage of the enzyme lactate dehydrogenase (LDH) from hepatocytes and results from mouse bioassay. More recently this method has received renewed interest due to legislative restrictions in the use of the mouse LD_{50} (Heinze, 1996). Isolated rat hepatocytes have been incubated with pure toxin or bloom extracts for 4 h and 20 h and then the viability has been assessed using the MTT ((3,4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) test. Toxicity was found to be time-dependent with the LC_{50} for microcystin-LR being 0.20 µg ml^{-1} at 4 h reduced to 0.05 µg ml^{-1} after 20 h. Different LC_{50} values were found for microcystin variants, most notably microcystin-RR was found to be at least an order of magnitude less toxic in this assay. This is consistent with *in vivo* toxicity data.

An *in vitro* fibroblast cytotoxicity assay for microcystins has been investigated by Codd *et al.* (1989) as a replacement for the mouse bioassay. The assay, using V79 hamster

fibroblast cells, responded to microcystins with the results correlating reasonably well with those of the mouse bioassay (Lawton *et al.,* 1994b). However, the assay demonstrated a number of false positives and, of more concern, false negatives were also recorded.

Box 13.4 When is physicochemical analysis needed?

Physicochemical analysis of cyanotoxins is recommended if:

- Cyanobacterial species composition or bioassay results indicate which toxins to look for.
- Unambiguous identification of toxins is required.
- Quantification of toxins is required.
- Rapid screening of a large number of samples is required, especially for regular monitoring of sites where the toxin patterns are well established.
- Low toxin concentrations which may not be detected by a bioassay are expected (e.g. in drinking water).
- New toxic cyanobacterial metabolites are to be identified.

Another cell-based assay proposed in the past has used blood cells, with agglutination of the cells being reported as an indicator of microcystin level (Carmichael and Bent, 1981). Although this bioassay did appear promising, it has since been found to be a poor indicator for microcystins.

Two *in vitro* cell bioassays have been found to be successful in detecting saxitoxins and, like the immunoassay, they were developed primarily for monitoring toxins in shellfish. First, a neuroreceptor binding assay was developed that uses radiolabelled saxitoxin and works on the basis of competitive displacement (Davio and Fontelo, 1984). The initial protocol has subsequently been refined (Doucette *et al.,* 1994) and the data obtained correlate well with the mouse bioassay (Cembella *et al.,* 1995). A neuroblastoma cell line technique for sodium channel blocking activity has also been developed (Gallacher and Birkbeck, 1992; Jellett et al., 1992). This assay, which is currently undergoing evaluation for its suitability as a replacement for the mouse in shellfish monitoring, is now available as a commercial test kit.

13.4 Analytical methods for cyanotoxins

Analytical methods use the physicochemical properties of cyanotoxins such as molecular weight, chromophores and reactivities due to the functional groups in the molecules. Physicochemical methods used for cyanotoxin detection are summarised in Table 13.2, which also indicates that the initial capital expenditure to establish most of these methods is high. Appropriate use of such methods is discussed in Box 13.4.

Table 13.2 Physicochemical methods for the detection of cyanotoxins

Method[1]	Cost[2]			Comments	Reference(s)
	Cap.	Con.	Pers.		
Microcystins and nodularins					
HPLC-PDA	H	M	L	UV spectra can give tentative id	Lawton *et al.,* 1994b
LC/MS	VH	M	M/L	A number of different interfaces; mass confirmation; can have PDA	Kondo *et al*., 1992; Edwards *et al.,* 1992
TLC	L	L	M	Qualitative; requires standards and further confirmation of toxins	Harada, 1996
MMPB	H/VH	M	M	Detection by GC-MS or LC-MS detects total microcystin/nodularin	Sano *et al*., 1992; Harada *et al*., 1996
MALDI	VH	L	M/L	Initially poor but recent developments have improved accuracy	Erhardt *et al*, 1997
CE-MS	H	L	M	Requires further development but has future promise	
NMR	VH	M	M/H	Can characterise cyanotoxins; needs mg quantities and expert interpretation	Botes *et al*., 1984; Harada, 1996
Anatoxin-a and homoanatoxin-a					
HPLC-PDA	H	M	L	Characteristic UV spectra	Edwards *et al.,* 1992
GC-MS	H	M	L	Characteristic ion spectra	Smith *et al*., 1987
GC-ECD	H	M	L	Requires sample cleanup	Stevens *et al*., 1988
LC/MS	VH	M	M/L	Sensitive and specific	Harada *et al*., 1993
Anatoxin-a(S)					
HPLC	H	M	L	Very poor chromophore, not suitable for routine detection	Matsunaga *et al*., 1989
Cylindrospermopsin					
HPLC-PDA	H	M	L	Lack of available standards; give characteristic UV spectra	Harada *et al*., 1994; Hawkins *et al*., 1997
Saxitoxins					
HPLC-pre	H	M	H	Precolumn derivatisation; poor stability of derivative	Lawrence *et al*., 1995
HPLC-post	H	M	M	Three solvent systems required to analyse for all variants	Oshima *et al*., 1995
LC/MS	VH	M	M/L	Best method for all variants but equipment cost can be prohibitive	Quilliam *et al*., 1989; Hines *et al*., 1993

CE-MS	H	L	M	Poor detection limits; needs further development	Pleasance *et al*., 1992; Lock *et al*., 1994

[1] See text for an explanation of the different methods listed
[2] Cap. Capital; Con. Consumables; Pers. Personnel; L Low; M Medium; H High; VH Very high

Another approach for further confirmation and identification of microcystins has been proposed using a LC-linked protein phosphatase assay (Boland *et al*., 1993; Chen *et al*., 1993). Essentially, this method makes use of HPLC to separate and identify tentatively the cyanotoxins present, and then monitors the bioactivity of the detected peaks using the protein phosphatase inhibition assay. This provides excellent confirmation of protein phosphatase inhibiting cyanotoxins, especially in complex samples.

13.4.1 Detection methods for microcystins and nodularins

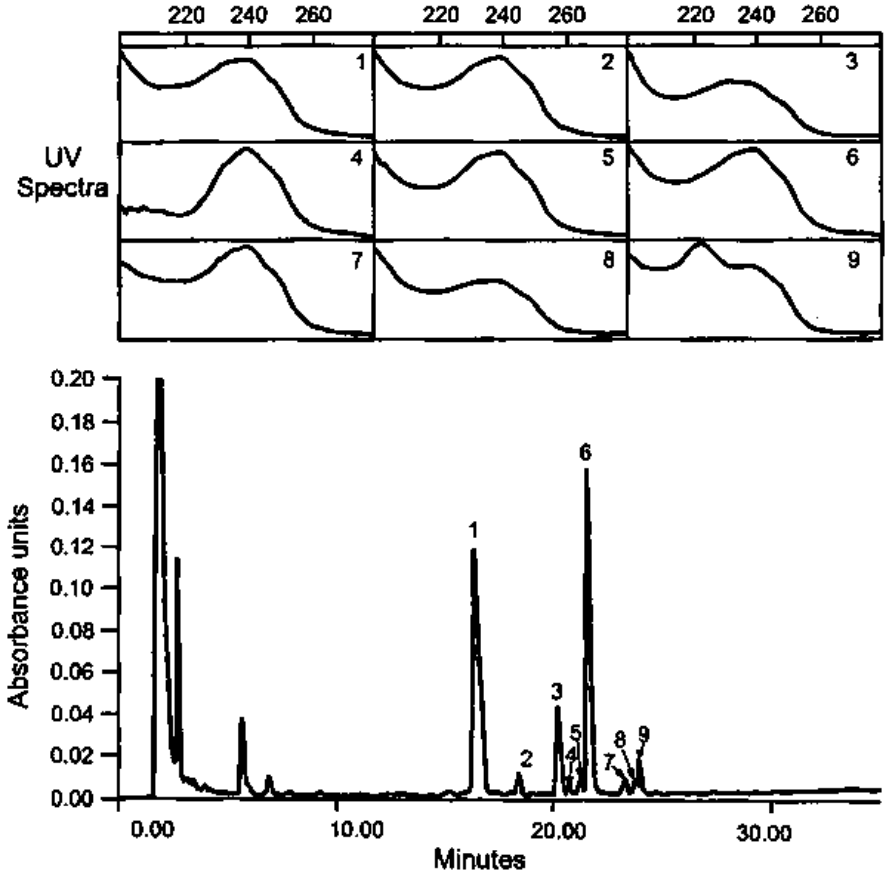
The majority of analytical methods have been developed primarily for microcystins although because both nodularins and microcystins show similar physicochemical properties, nodularins can easily be analysed by the same methods. The most commonly-used analytical system for this class of cyanotoxins is HPLC (see Box 13.5). Combined with UV detection, HPLC has been used extensively for the detection of microcystins, but because this method relies on retention time for identification, microcystin standards are required (Harada, 1996). Detection by UV can be made more specific by using a photodiode array (PDA) UV detector (Lawton *et al.,* 1994a) but it has very limited ability to identify individual microcystins because almost all microcystins show a similar UV spectrum. Recent advances in detector hardware can now provide high resolution spectra that detect very slight variations in chemical composition and can be used in conjunction with advanced spectral matching software. These developments may assist in the identification of microcystins by spectral match data in conjunction with retention times. However, a fundamental problem still exists in the availability of standards. With over 60 microcystins known, it is currently impossible to create a definitive spectral library, which is a limiting factor when using this method to identify unknown microcystins.

Typical HPLC analysis uses a reverse-phase C18 silica column with separation achieved over a gradient of water and acetonitrile, both containing 0.05 per cent triflouroacetic acid (TFA). The gradient has to cover a sufficient range of polarities (e.g. 30-70 per cent acetonitrile) to allow the analysis of all microcystins which are known to vary considerably in their polarities. Data is gathered at 238 nm and where PDA is used spectral information is collected between 200 and 300 nm. Use of HPLC-PDA can allow tentative identification of microcystins and this method was found to perform very well when over 20 samples were assessed by HPLC and compared with mouse bioassay data. No false negatives were observed and only one false positive was reported, the latter being attributed to a relatively low level of microcystin which failed to cause death in the mouse bioassay (Lawton *et al.,* 1994b). Other solvent systems have also been successfully used including methanol/water and ammonium acetate/acetonitrile.

Box 13.5 Possibilities and limitations of HPLC with UV spectra for microcystin analysis

High pressure liquid chromatography can be used routinely to identify and quantify microcystins, but not to differentiate between structural variants of most microcystins. For example, in a sample from Radeburg Reservoir (1 July 1996, monospecific population of *Microcystis* spp.), UV-spectra indicated nine microcystins, three of which could be identified with commercially available standards by their retention times to be microcystin-RR (peak 1), microcystin-YR (peak 3), and microcystin LR (peak 6). However, six other minor microcystins could not be further specified by this method alone. For a tentative assessment of the toxicity of this sample, a "worst case" approach was chosen by calculating the sum of all of the nine microcystins and assuming them to be as toxic as -LR and -YR, the most toxic variants currently known.

For preliminary microcystin screening and for routine monitoring, HPLC with photodiode array detection is an excellent approach because it efficiently provides an overview of toxin content, and a worst-case toxicity estimate can be derived. Therefore, if a local or regional authority must deal regularly with microcystin monitoring, establishment of HPLC techniques with photodiode array detection of UV spectra is recommended. Further identification of microcystins can then be performed with selected samples. In many cases, it will be advisable to subcontract this to laboratories with specialised expertise.



Results of HPLC analysis of a sample from Radeburg Reservoir (1 July 1996, monospecific population of *Microcystis* spp.) showing nine microcystins identified by their characteristic UV-spectra (Fastner, unpublished data)

Analysis of microcystins and nodularins by HPLC-PDA

Apparatus

- Gradient HPLC system with photodiode array detection
- Data acquisition system
- Auto-sampler, recommended for high sample throughput
- C18 column e.g. 4.6 × 250 mm Symmetry (Waters)
- Column oven, 40 °C
- Pipette, 0.1-0.5 ml

Laboratory conditions

Effective ventilation, taking into account that acetonitrile is heavier than air and accumulates at ground level.

Reagents

- Eluent A, water plus TFA, 0.5 ml TFA added to 1,000 ml water
- Eluent B, acetonitrile plus TFA, 0.5 ml TFA added to 1,000 ml water
- Helium gas
- Methanol

Note: all reagents must be high purity, HPLC grade.

Procedure

1. Prepare solvents and degas in a stream of helium gas

2. Program a linear gradient (1 ml min^{-1}) as follows:

Time (mins)	0	10	40	42	44	46	55
Eluent A%	70	65	30	0	0	70	70
Eluent B%	30	35	70	100	100	30	30

3. Equilibrate the column at the desired temperature and gradient starting conditions.

4. Set photodiode array detector to monitor between 200 and 300 nm.

5. Running a blank sample first, i.e. injecting only methanol, helps the system settle and ensures reproducible retention times.

6. Samples and standards are usually prepared in methanol. However, some microcystins are less soluble in methanol (e.g. microcystin-YR). Check product information where available. Samples should be centrifuged or filtered to remove particulates before carrying out HPLC analysis.

7. It is advisable always to run a standard at the beginning and end of a set of analyses, because this helps to confirm correct operation of the system and indicates the degree of retention time drift.

8. A calibration curve should be performed when establishing the method and at regular intervals, especially after changing a column or lamp.

9. Chromatograms are best viewed, and integration carried out, at 238 nm because this is the absorption maximum of most microcystins and nodularins.

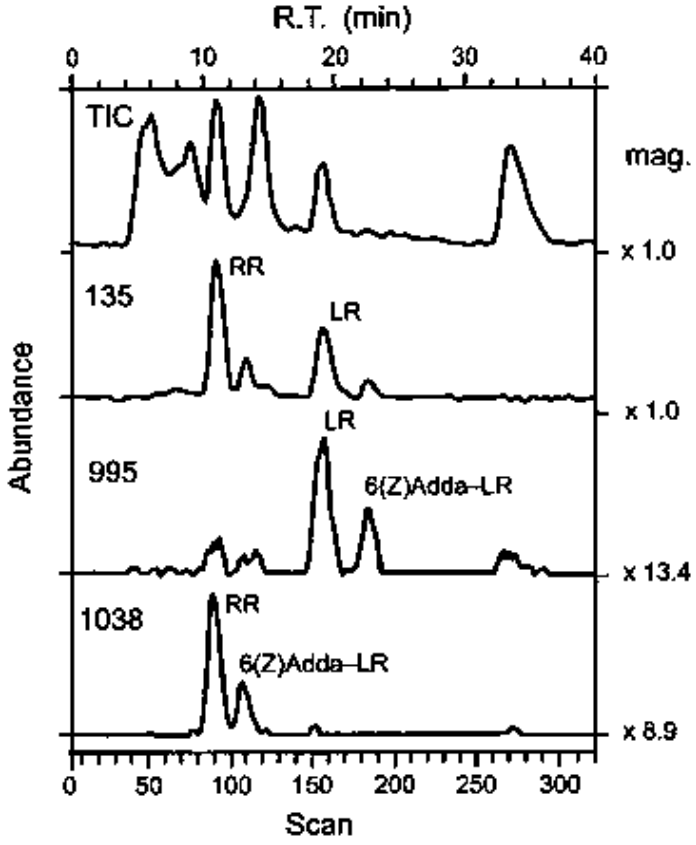
10. Microcystin congeners can be identified where they have the same retention time and spectrum as a standard, but for many microcystins no standards exist. These can be identified as microcystins, but the respective congener may only be tentatively inferred from published retention times in relation to identified congeners. However, advances in spectral matching software provide increased confidence in microcystin identification by providing a numeric indication of how similar an unknown is to a range of microcystins in a spectral library.

When further confirmation and identification of microcystins is required, more advanced methodology must be used. Liquid chromatography/mass spectrometry (LC/MS) is a very promising method because it enables the simultaneous separation and identification of microcystins in a mixture (Kondo *et al.,* 1992; Edwards *et al.,* 1993; Poon *et al.,* 1993). Figure 13.6 shows the Frit-FAB (fast atom bombardment) LC/MS analysis data of a toxin from a bloom sample collected in Japan. The toxic fraction contains mainly two microcystins as shown by the mass chromatogram monitored at the characteristic ion m/z 135 derived from Adda, which has proved to be useful for the discrimination of microcystins from other types of compounds (Kondo *et al.,* 1992). The two peaks were readily identified as microcystins-RR and -LR according to the mass spectra and mass chromatograms at their $[M+H]^+$. An advanced Frit-FAB LC/MS method using a microbore column (0.3 mm internal diameter) enabled the identification of nanogram levels of microcystins in water and biological samples (Kondo *et al.,* 1995, 1996). This increase in sensitivity was achieved by splitting the mobile phase between the pump and the injector so that the total effluent could be introduced into the mass spectrometer.

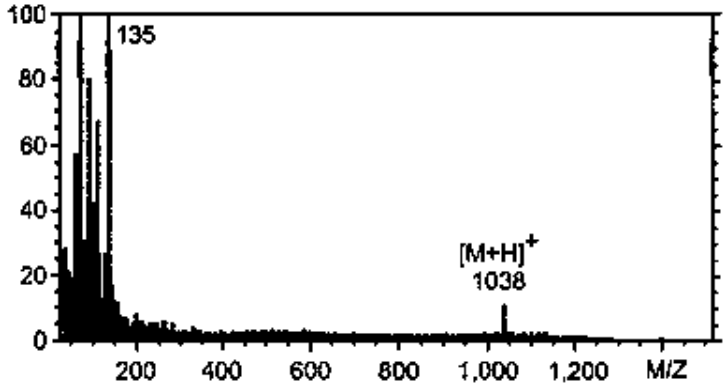
A physicochemical screening method that is based on the detection of 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) as an oxidation product of microcystins has been reported. The MMPB was initially prepared by Lemieux oxidation, followed by analysis by gas chromatography (GC) with a flame ionisation detector or HPLC with fluorescence detection (Sano *et al.,* 1992). However, it required tedious procedures such as extraction, cleanup, oxidation and post-treatment in order to eliminate the reagents used, and derivatisation for GC and HPLC analysis. An improved method using ozonolysis made it possible to reduce significantly the formation times of MMPB because the previously required extraction, cleanup and other procedures could be entirely eliminated (Harada *et al.,* 1996). The resulting intact MMPB was directly analysed by thermospray (TSP) interface LC/MS and EI-GC/MS using selected ion monitoring. This new procedure, from the ozonolysis of the microcystins to analysis of MMPB at picomol levels, took only 30 minutes to perform. The quantification of bloom samples achieved by this method were consistent with those obtained by HPLC analysis, showing that the method provided a means of screening for microcystins, as well as for their accurate quantification. Additionally, the most remarkable feature of this method is the applicability to complex sample matrixes, including solid material such as animal tissue, without the requirement for any complicated processing.

The structural determination of microcystins and nodularins has been carried out by nuclear magnetic resonance (NMR) spectroscopy and recent advances in 2D NMR techniques have proved to be essential for the structural determination of known and unknown microcystins. Fast atom bombardment MS and liquid secondary ion (LSI) MS give a protonated molecule $[M+H]^+$, providing information about molecular weight with further structural information obtained by Tandem FAB MS (FAB-MS/MS) as used in recent studies (Namikoshi *et al.,* 1995). However, NMR and MS (except LC/MS) usually require relatively large amounts of sample (milligram quantities) and completely purified microcystins, therefore they are not used in routine monitoring (Botes *et al.,* 1984; Namikoshi *et al.,* 1995; Harada, 1996).

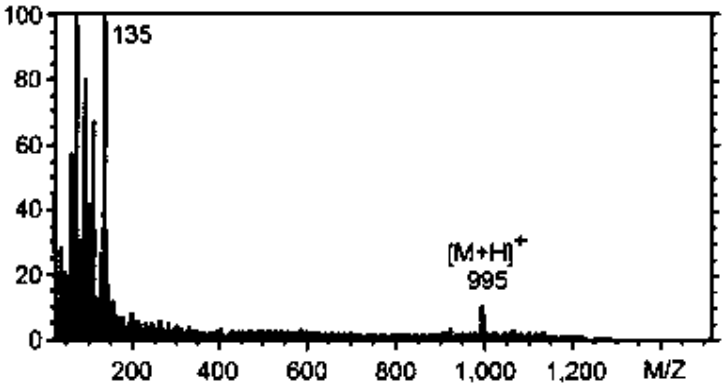
Figure 13.6 Frit-FAB LC/MS analysis of a toxic fraction from a bloom sample collected in Japan (Data supplied by Ken-ichi Harada and Fumio Kondo)



Microcystin-RR (MW 1037)



Microcystin-LR (MW 994)



For identification of microcystins with very small sample volumes (less than 1 mm^3 freeze-dried material). Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-TOF) has recently been developed (Erhardt *et al.,* 1997). This method provides the molecular mass of all of the peptides in a sample and thus gives strong indications of the microcystin variants present. Post Source Decay (PDS) spectra may be obtained, which are characteristic for different microcystins. A library is currently being established. For rapid qualitative assessment of microcystins and other cyanobacterial peptides, this method is highly promising. Quantitative assessment is not yet possible.

Different methods provide different and often complementary information, therefore combined use of suitable methods is recommended according to the purpose and type of data required. This is necessary because none of the methods currently available provides all the information which may be required. Furthermore, individual laboratories must identify the techniques that are both suitable for their analytical requirements and use their own expertise and available technology (see Meriluoto (1997) for a recent review of chromatographic methods for microcystins). Initial screening of samples can check rapidly for the presence of microcystins in a small amount of sample using sensitive and simple methods. Figure 13.1 summarised the relationship between sensitivity and selectivity of analytical methods for microcystins, showing that different methods provide different and complementary information.

For studies requiring enhanced precision, accuracy and sensitivity in detection of individual toxins, a multistage procedure is required. This may comprise initial screening of samples to check for the presence of microcystins in a small amount of sample using sensitive and simple methods, such as bioassays (e.g. ELISA, protein phosphatase inhibition assay) and MMPB method. The use of screening helps to reduce the number of samples which require full analytical investigation and thus reduces the laboratory commitment. Furthermore, rapid results from an initial screen may aid prompt regulatory responses regarding the suitability of water for human use. If a sample is positive in a screening test, it will be necessary to follow through with identification and quantitative analysis. Prior to this, cleanup and sample concentration is often very important for many samples because relatively low levels of microcystins, around 1-2 µg l^{-1}, are usually present in water samples. A tandem cleanup system using ODS silica gel and silica gel cartridges, facilitates the accurate analysis of trace amounts of microcystins in water. Finally, quantitation of microcystins is usually essential, and although the screening methods can provide an indication of total microcystin concentrations in samples, a separation and spectroscopic method such as HPLC with UV detection should be applied at this final step. Availability of authentic standards would strengthen the capability of HPLC with UV detection (preferably with photodiode array detection) but otherwise LC/MS should be the method of choice despite the requirement of a more specialised technique.

13.4.2 Anatoxin-a

Three methods for analysis of anatoxin-a have been reported to date: GC/MS, GC with electron capture detection (ECD) and HPLC. One GC/MS method has been published as a confirmation of anatoxin-a in material taken from a toxic bloom that resulted in the deaths of 16 cows (Smith and Lewis, 1987). In the procedure, following liquid-liquid extraction with *N*-acetylation, the resulting acetylated toxin was analysed by a capillary GC/MS. Although confirmation was based on interpretation of the mass spectrum, the detection limit was unclear. Another GC/MS method has also been established for *N*-acetylated anatoxin-a (Himberg, 1989) and it was successfully employed for preliminary characterisation of neurotoxic cyanobacteria from Finland (Sivonen *et al.,* 1989). The GC/ECD method provided a higher sensitivity than other analytical methods, it used an internal standard for accurate quantification, and it could be applied to analysis of field samples (Stevens and Krieger, 1988). Although GC/ECD requires a considerably more complicated cleanup operation and derivatisation prior to analysis, it has been used successfully for stability studies on anatoxin-a (Stevens and Krieger, 1991). Derivatisation with N-pentafluorobenzylbromide was shown to achieve a sensitivity of 2.5 pg (Bumke-Vogt *et al.,* 1996). It is possible to use HPLC with UV detection to analyse intact anatoxin-a which has a strong absorption at 227 nm. After extraction of a cyanobacterial suspension with chloroform, followed by re-extraction with 0.01N hydrochloric acid, the resulting extract is separated under the following conditions: column, ODS silica gel; mobile phase, methanol-0.01M perchloric acid (7:3); detection, UV (227 nm). Harada *et al.* (1989) reported an alternative approach using a reversed phase HPLC method with a methanol-10.01M ammonium chloride (1:9) solvent system.

Mass spectrometry is a very effective method for identification of anatoxin-a and its derivatives. Conventional electron ionisation (EI) and chemical ionisation (CI) can yield molecular ions and protonated molecules, respectively. Ross *et al.* (1989) evaluated secondary ion mass spectrometry (SIMS), GC/MS, desorption CI and TSP-MS for

detection of the toxin and its derivatives. The use of HPLC coupled with mass spectrometry using thermospray interface (TSP-LC/MS) has also been investigated (Harada *et al.,* 1993). The latter method made possible a sensitive, specific and reproducible analysis of anatoxin-a and its non-toxic oxidation product when used in combination with a cleanup method including a solid phase extraction with a reversed phase carboxylic acid cartridge. Using this method, trace amounts of anatoxin-a were detected in three strains and two bloom samples collected in Japan.

13.4.3 Anatoxin-a(S)

Matsunaga *et al.* (1989) purified anatoxin-a(s) and determined its structure, although no analytical method has been developed. Traditional HPLC plus UV detection is not suitable because this cyanotoxin lacks a strong chromophore. Liquid chromatography/mass spectrometry may prove useful, but this analytical technique has not been evaluated yet for anatoxin-a(S).

13.4.4 Cylindrospermopsin

The first analytical method reported for this toxin consisted of a combination of a cleanup step using HP-20 and ODS silica gel cartridges followed by HPLC with photodiode array detection. This method was applied to a bloom sample collected in Japan which was thought to contain Cylindrospermopsin (Harada *et al.,* 1994). However, the authors suggested that the mobile phase used in this study was not satisfactory because Cylindrospermopsin showed poor retention power on the ODS column and slight tailing, and their method requires further development to allow precise analysis.

A recently published method (Hawkins *et al.,* 1997) describes the extraction of cells in 5 per cent aqueous acetic acid and analysis using Spherisorb ODS-2 with a 10 minute linear gradient from 0 to 5 per cent methanol followed by a further 10 minutes at 5 per cent methanol. This gave good retention, separation and peak shape. Cylindrospermopsin was found to have a characteristic UV absorbance spectrum between 200 and 300 nm, with a maximum absorbance at 262 nm.

13.4.5 Saxitoxins

The methods mentioned here have been developed primarily for the analysis of saxitoxins in the marine environment, particularly in shellfish. However, they have been found to be equally suitable for cyanobacterial samples. The most commonly used analytical method for the saxitoxins is HPLC with on-line post-column oxidation and fluorescence detection (Oshima *et al.,* 1995). Although this method has been found to be the most satisfactory to-date, it requires three different mobile phase systems to allow analysis of all the saxitoxins. Furthermore, there is limited availability of analytical standards for all saxitoxin variants, which are necessary for peak confirmation. Mass spectrometry with FAB (Mirocha *et al.,* 1992), TSP (Wils and Hulst, 1993), electrospray (Hines *et al.,* 1993) and ion-spray ionisation (Quilliam *et al.,* 1989) has been reported for the paralytic shellfish poison (PSP) toxins. The application of an LC/MS method for saxitoxins has been reported, although it appears that it will be difficult to achieve analysis of all PSP toxins within one LC/MS run. The combination of capillary electrophoresis (CE) with ion-spray ionisation has proved to be successful for the saxitoxins (Pleasance *et al.,* 1992; Locke and Thibault, 1994). Analysis using CE was

found to give poor detection limits due to the very small injection volumes (< 10 nl), but it may be possible to overcome this problem with an on-column sample preconcentration system. Furthermore, developments in CE technology are anticipated in the near future which will increase flow cell volume, hence enhancing detection limits.

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