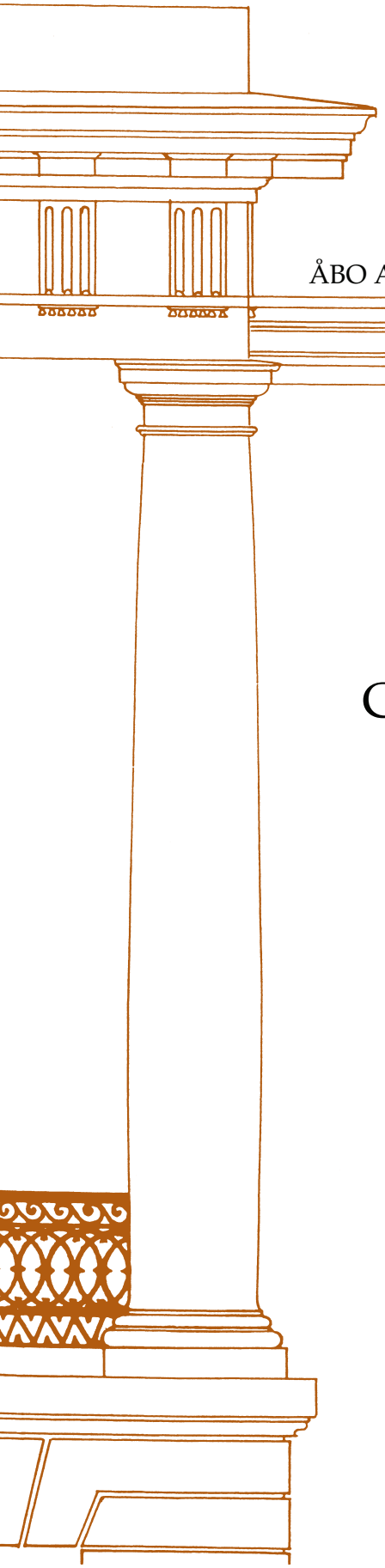

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Cyanobacterial Monitoring and Cyanotoxin Analysis

Jussi Meriluoto and Geoffrey A. Codd
Editors

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Preface

This manual serves as a practical guide for the monitoring of harmful cyanobacteria (blue-green algae) and their toxins. It is one of the deliverables of the research project "TOXIC - Barriers against Cyanotoxins in Drinking Water". The TOXIC project was funded by the European Commission under the Fifth Framework Programme (contract number EVK1-CT-2002-00107) in 2002-2005.

The TOXIC project involved ten European research groups in nine countries and comprised four programmes focussing on Raw Water Quality, Analysis, Treatment and Exploitation. This manual presents the core methods which have been developed, standardised and used within the Raw Water Quality and Analysis programmes. The research on raw water quality was coordinated by Prof. Geoffrey A. Codd at the University of Dundee, UK, while the analytical work was coordinated by Dr. Jussi Meriluoto at Åbo Akademi University, Turku, Finland.

The book addresses an increasing worldwide need for standard protocols for cyanotoxin monitoring in lakes, reservoirs and treated waters. Toxic cyanobacteria are encountered around the world, and problems related to safe drinking water production are common. The presence of cyanobacterial toxins in drinking and bathing waters has been recognised as a human health hazard by the World Health Organization, and a provisional guideline value for the common hepatotoxin, microcystin-LR, in drinking water has been established. National legislation has been recently introduced in some European countries and elsewhere to control microcystins.

Chronic exposure to low levels of cyanotoxins in drinking water is a health risk which is still only partially understood. Sample preparation protocols and analytical methods for microcystins, anatoxin-a and cylindrospermopsin have been developed and refined in the TOXIC project. These results, together with field data generated in the raw water quality subproject and effective water treatment options developed in the treatment-related workpackages of TOXIC, contribute to consumer health risk evaluation and minimisation.

The coordinator of the TOXIC project was Dr. Wido Schmidt, DVGW Technologiezentrum Wasser, Branch Office Dresden. The authors of this book are indebted to Dr. Schmidt for his enthusiasm and project coordination done in a proficient and constructive manner. We would also like to thank all project partners for fruitful collaboration in the laboratory and field, and for their written contributions to this manual.

This book is dedicated to the memory of Dr. Malgorzata Tarczynska.

Contents

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Abbreviations

AAU	Åbo Akademi University
ACN	Acetonitrile
Adda	3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid
Antx-a	Anatoxin-a
CE	Capillary electrophoresis
Chl-a	Chlorophyll-a
CID	Collision-induced dissociation
CZE	Capillary zone electrophoresis
Cyn	Cylindrospermopsin
DAD	Diode-array detector
Dm (dm)	Demethyl
DW	Dry weight
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
FAB	Fast atom bombardment
FID	Flame ionisation detector
FISH	Fluorescent *in situ* hybridisation
Glu	Glutamic acid
GV	Guideline value
HPLC	High-performance liquid chromatography
i.p.	Intraperitoneal
IT	Ion-trap
LC-MS	Liquid chromatography-mass spectrometry
LC-MS-MS	Liquid chromatography-tandem mass spectrometry
LD_{50}	Lethal dose that kills 50% of the test organisms
LLE	Liquid-liquid extraction
LOAEL	Lowest observed adverse effect level
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time-of-flight
MC, MCYST	Microcystin
Mdha	*N*-methyldehydroalanine
Mdhb	2-(Methylamino)-2(*Z*)-dehydrobutyric acid
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
MIP	Molecular imprinted polymer
MMPB	3-Methoxy-2-methyl-4-phenylbutyric acid
MRM	Multiple reaction monitoring
MS	Mass spectrometry
m/z	Mass-to-charge ratio
N-tot	Total nitrogen
P-tot	Total phosphorus
PDA	Photodiode-array
PPIA	Protein phosphatase inhibition assay
Q	Single-quadrupole
QQQ	Triple-quadrupole
RP	Reversed-phase

S/N	Signal-to-noise
SELDI-TOF	Surface-enhanced laser desorption/ionisation-time-of-flight
SIR	Single ion recording (selected ion recording)
SOP	Standard operating procedure
SPE	Solid-phase extraction
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
TOF	Time-of-flight
TZW	DVGW Technologiezentrum Wasser
UDU	University of Dundee
ULO	University of Lodz
UV	Ultraviolet
WHO	World Health Organization

1 Basic guide to detection and monitoring of potentially toxic cyanobacteria

Tomas Kull and Tore Lindholm, Åbo Akademi University, Turku, Finland

1.1 Introduction

Aquatic cyanobacteria may live as phytoplankton dispersed in the water or attached to surfaces of e.g. plants, rocks and sediments, sometimes as thick mats. Attached cyanobacteria may appear among phytoplankton if their habitat is disturbed. Most harmful cyanobacteria are, however, planktonic and possess gas vesicles which give them capacity to regulate buoyancy. Cyanobacteria tend to increase in warm and nutrient-enriched waters, but some species occur also in clear, nutrient-poor and cold waters and even under ice. Planktonic cyanobacteria exploit and affect large water volumes. They may live quite dispersed, or form surface or subsurface maxima. Scums or surface blooms of cyanobacteria together with associated other microbes may become redispersed, accumulate in embayments, sedimentate, or be washed ashore. Accumulation also occur e.g. in sand filters. The risks for water users increase markedly in connection with periods of fast growth of cyanobacteria or physical biomass accumulation which may be favoured by periods of stable weather.

Knowledge about cyanobacterial properties and likely distribution patterns of important species is useful for those involved in drinking water production and reservoir monitoring. In North European countries most cyanobacterial problems arise during the period May-October. Blooms may form or disappear rapidly due to (un)favourable weather conditions. Despite the dynamic nature of most cyanobacterial blooms, some problems are predictable. Predictability is improved if monitoring data are available for some years. Also simple measurements and notes may be useful.

1.2 Examples of cyanobacterial occurrence and distribution

In the following text common cyanobacterial distribution patterns and some bloom cases are illustrated with real examples from the Åland Islands, SW Finland. Hints are also given about how to observe cyanobacteria.

1.2.1 *Anabaena* blooms

Species of the filamentous genus *Anabaena* frequently form slimy summer blooms on the surface of eutrophic lakes and reservoirs. *Anabaena* blooms may develop rather fast, and they may look like green paint (Fig. 1.1). In less eutrophic waters some species also form colonies, which are seen as large dark dots in water samples and on filters after filtration. Not infrequently, *Anabaena* filaments and cells may disintegrate soon when they are covered with a cover slip. One such fragile species is *A. solitaria*, which forms non-toxic blooms in Lake Markusbölefjärden (Fig. 1.2). During calm and sunny weather conditions the cyanobacterial biomass may be restricted to the topmost few meters, while the underlying water may be little affected (Fig. 1.3). However, if the wind speed increases, the bloom material will be dispersed into deeper layers. Both toxic and non-toxic *Anabaena* species occur, and some species produce both neurotoxins and hepatotoxins. Quite often, several *Anabaena* species occur simultaneously in a bloom.

1.2.2 *Microcystis* blooms

Many different species of the colony-forming unicellular genus *Microcystis* cause dense blooms in eutrophic lakes and reservoirs, particularly in the summer and autumn. Thick, paint-like (sometimes granular or cheese-like) bloom material may accumulate along the shores. In contrast to *Anabaena* blooms, *Microcystis* blooms often remain fresh for weeks. In less eutrophic basins, or early in the season, scattered colonies are often found only with a plankton net. Although *Microcystis* is typical in nitrogen-rich waters, single colonies or small bloom patches may occur in rather clear lakes. At low concentrations of a few colonies per litre, *Microcystis* is difficult to detect in water samples, but can be seen on filters (Fig. 1.4).

Usually, *Microcystis* colonies rise to the surface in fresh samples, and thus, under the microscope, they may have to be looked for at the top of counting chambers. Several *Microcystis* species often co-occur and some of them can be identified on the basis of cell size, cell aggregation patterns and colony margins. Not infrequently, smaller cyanobacteria and various protists grow in or on the colonies. *Microcystis* overwinter on the lake bottom. Several species produce potent hepatotoxins. Rather similar, often markedly buoyant colonies, are produced by the common cyanobacterium *Woronichinia naegeliana*, but its cells are ovoid.

1.2.3 *Planktothrix agardhii*

Planktothrix agardhii (previously named *Oscillatoria agardhii*) forms long, slender, straight filaments that usually remain separate. The end cells are short. A somewhat similar species, *Aphanizomenon flos-aquae*, usually forms bundles and has long end cells. *P. agardhii* seldom forms dense surface scums. Its presence may be revealed by a strong earthy odour. The filaments are easily detected visually in a water sample in a clear glass beaker placed in strong perpendicular light against a dark background. The filaments show gliding movement when studied on an object glass in a microscope. *Planktothrix* filaments are motile and may pass through sand filters and plankton nets.

Planktothrix agardhii is common in nutrient-rich basins in most seasons. In shallow basins it occurs in the whole water mass. In stratified basins like Östra Kyrksundet it may be dispersed in the epilimnion or have mass occurrence at intermediate depths (*1*), but other vertical distribution patterns also occur. As with microcystin-producing *Anabaena* and *Microcystis* blooms, the microcystin content may approximately equal the chlorophyll-a concentration. Thus, chlorophyll-a values give a rough estimate of the (maximum) microcystin concentration of the water.

Figure 1.1: Collecting a surface bloom sample of cyanobacteria.

Figure 1.2: *Anabaena* bloom in Lake Markusbölefjärden in August 2002.

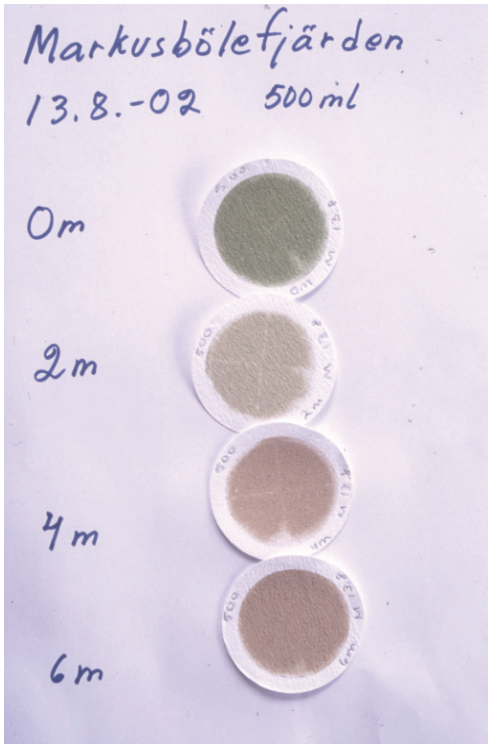


Figure 1.3: Water collected at various depths from Lake Markusbölefjärden in August 2002 and filtered onto GF/C filters. The cyanobacterial biomass was restricted to the surface layer, while other phytoplankton dominated at deeper levels. Note the different colours of the filters.

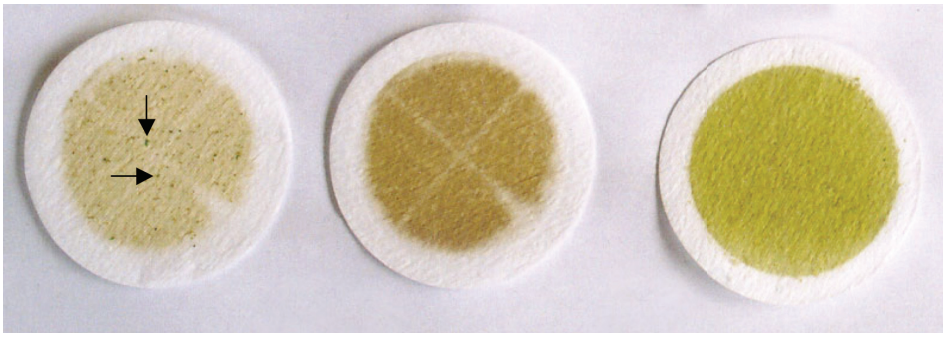


Figure 1.4: Water collected from three lakes with increasing nutrient status (left-right) and filtered onto GF/C filters. The first lake was rich in chrysophytes (left filter), the second in cryptophytes and diatoms (middle filter), and the third in cyanobacteria and green algae (right filter). Single colonies of *Microcystis* (marked with arrows) can be seen as green spots on the filter to the left.

Table 1.1: Suggested workflow for detection and monitoring of potentially toxic cyanobacteria

Basic level	**Site**	**Frequency**	**Water quality**	**Proceed to:**
A. Visual inspection	Intake at water works	Daily	Clear water Turbid water Visible flocks or filaments	*No action* *B* *C*
	Lake or reservoir (shore) Lake or reservoir (water column)	Daily-weekly Weekly-monthly	Clear water Turbid water Visible flocks or filaments Surface scum	*No action* *B* *C* *C and D*
Basic level	**Volume**	**Filters**	**Filter quality**	**Proceed to:**
B. Technical test (filtration)	500 ml	GF/C glass fibre filters, 47 mm diameter	No odour, filter not green or red, whole volume easily passing the filter Earthy smell, filter green or red, reduced volume passing the filter	*No action* *C*
Basic level	**Instruments**	**Samples**	**Water quality**	**Proceed to:**
C. Microscopic investigation and chlorophyll-a analysis	Inverted microscope (phase contrast preferred), 40×-400× magnification Spectrophotometer	Preserved Lugol's samples in 2.5-10 ml sedimentation chambers Use filters from level B for chlorophyll-a analysis	Low amount of cyanobacteria present (< 2000 cells ml^{-1}), or chl-a < 1 μg l^{-1} Moderate - high amount of cyanobacteria present (< 100000 cells ml^{-1}), or chl-a < 50 μg l^{-1} Very high amount of cyanobacteria present (>100000 cells ml^{-1}), or chl-a >50 μg l^{-1}	*No action* *D* *D, consider other measures*
Advanced level	**Toxins to be analysed**	**Methods**	**SOPs**	
D. Toxin analysis	Analysis based on dominating cyanobacterial genera present	HPLC analysis and PPIA according to SOPs, ELISA according to the manufacturer or supplier; test both raw and treated water for toxins	SOP_TOXIC_AAU_06F SOP_TOXIC_UDU_08F SOP_TOXIC_UDU_09F SOP_TOXIC_UDU_10F SOP_TOXIC_EXT_01F	

1.3 Instructions to Table 1.1

1.3.1 Basic level A. Visual inspection

- **Frequency:** The incoming raw water at the water works should be inspected visually, and if possible microscopically, daily for the occurrence of cyanobacterial colonies or filaments, while, depending on the distance to the raw water source and available resources, inspection of the lake or reservoir from the shore can be performed on a daily-weekly basis. The water column in the raw water source should be checked on a weekly-monthly basis, with more frequent, e.g. weekly, inspections during periods of bloom development and persistence.
- **Water quality:** Check the water quality in a clear glass bottle or beaker, preferably in strong perpendicular light against a dark background. Flocks and filaments are then easily spotted. If flocks or filaments are observed in the incoming water at the water works, inspection of the shore and the water column in the raw water source should be performed.
- **Proceed to:** *No action* means follow normal procedures, *B* means perform filtration test, *C* means perform microscopic investigation and chlorophyll-a analysis, *D* means perform toxin analysis.
- **Additional information:** In the field, use a water sampler with an integrated thermometer (preferred) or a 1 l glass bottle attached to a stick when collecting water samples from discrete depths. A plastic tube (2 m long, ~2.5 cm inner diameter) with rubber plugs can be used when collecting an integrated sample of the surface water (0-2 m). If microscopic examination is to be performed, we recommend that the sample is preserved with Lugol's Iodine. Add 50-100 ml of water to a sealable glass bottle and preserve the sample with 0.5-1 ml of Lugol's Iodine solution (acidified Lugol's Iodine solution is preferred if the samples will be stored for a longer time). The bottle should be filled to the neck and stored in darkness, preferably in a cool place, until analysis. When inspecting the water column, the Secchi depth and a depth profile of the temperature, pH, conductivity, O_2 concentration and saturation should be measured. Samples from the surface water, thermocline (if present) and the bottom water for analysis of total phosphorus (P-tot) and total nitrogen (N-tot) can also be collected at the same time. Anoxia in the bottom water is connected with the release of phosphorus from the sediment, and this phosphorus may trigger a cyanobacterial bloom when the water column is mixed. In temperate regions, there is an increased risk of cyanobacterial blooms when the temperature of the surface water peaks and thereafter. Increase in surface water pH and O_2 saturation above 100% indicate high photosynthetic activity and may be related to high cyanobacterial numbers.

1.3.2 Basic level B. Technical test (filtration)

- **Filter quality:** Filter the water at low vacuum pressure (max. 100 mm Hg or 13.3 kPa). Brown filters indicate that the phytoplankton assemblage is dominated by diatoms and/or cryptophytes, light brown-yellow filters indicate that the phytoplankton assemblage may be dominated by chrysophytes, while green filters indicate that the water is rich in green algae or cyanobacteria (Fig. 1.4). A reddish filter might be due to high abundances of *Planktothrix rubescens*.

1.3.3 Basic level C. Microscopic investigation and chlorophyll-a analysis

- **Chlorophyll-a analysis:** Follow a standardised method or SOP_ULO_01F. Filters from the filtration test can be used for chlorophyll-a analysis.

- **Microscopic investigation of Lugol's Iodine-preserved material:** Allow the Lugol's preserved sample to equilibrate to room temperature. Invert the sample bottle gently 50-100 times before filling the sedimentation chamber (an inverted microscope is necessary if a sedimentation chamber is used). Dilution of the sample might be necessary if the phytoplankton abundance is high. Typically, samples with a chlorophyll-a concentration up to 10 μg l^{-1} can be counted in a 10 ml sedimentation chamber without dilution. Allow the suspended particles to settle by placing the sedimentation chamber on a horizontal surface shielded from light. A minimum of 8 h sedimentation time is required for a 10 ml chamber. Begin the microscopic investigation by scanning the whole bottom area at low, e.g. 40×, magnification. Large *Microcystis* colonies, *Anabaena* filaments and *Aphanizomenon* bundles are easily spotted. If their abundances are not too high, count the colonies, filaments and bundles. Switch to higher magnification, e.g. 400×, and scan 1-2 transects for qualitative analysis. Identify the cyanobacterial genera present with the help of the identification key provided in this chapter or with a cyanobacteria/phytoplankton flora. If several species of the same genera are present, denote these with sp_1, sp_2, etc.

  For quantitative analysis, scan 2-4 transects and count the number of cells, colonies or filaments of respective cyanobacterial genera or species. Calculate the average cell number in the 30 first filaments of each filamentous cyanobacterial genera or species present. Use this value later on when calculating the abundances. For *Microcystis* colonies of irregular shape and tangled *Anabaena* filaments it is, however, difficult to estimate the cell number and approximate values must be utilized. A small *Microcystis* colony, e.g. 40 μm in diameter, may contain less than 100 cells, while a large *Microcystis* colony, e.g. 400 μm in diameter, may contain more than 10000 cells. Calculate the cyanobacterial abundance, C_{ab}, by multiplying the total number of cyanobacterial cells, N_{cf}, with the total bottom area, A_{tot}, (in mm^2) and divide this value with the scanned area, A_{scan}, (in mm^2) multiplied with the volume of the sedimentation chamber, V_{sc}, (in ml). Correct for possible dilution of the sample by multiplying with the dilution factor, df. The formula is: $C_{ab} = ((N_{cf} \times A_{tot}) / (A_{scan} \times V_{sc})) \times df$. The unit of the cyanobacterial abundance will then be in cells per ml.

- **Additional information:** The threshold values for cyanobacterial abundance and chlorophyll-a concentrations given in the workflow are the same as in *Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management* (*2*), based on the WHO guidelines. This requires that all cyanobacteria are quantified, not only the potentially toxic ones. However, since microscopic quantification may be very time consuming, and there is usually a need for a rapid answer, we recommend toxin analysis to be performed if there are more than 1 colony or 5 filaments per ml of those cyanobacterial genera related to a specific toxin (microcystin, anatoxin-a or cylindrospermopsin) listed in this manual. For more detailed information about how to quantify cyanobacteria and other phytoplankton we refer to e.g. (*2, 3*).

1.4 References

1. *Lindholm, T., Meriluoto, J. A. O.*: Recurrent depth maxima of the hepatotoxic cyanobacterium *Oscillatoria agardhii*. Can. J. Fish. Aquat. Sci. **48**, 1629-1634 (1991).
2. *Chorus, I., Bartram, J.* (Eds.): Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management. E & FN Spon, London (1999).
3. *Olrik, K., Blomqvist, P., Brettum, P., Cronberg, G., Eloranta, P.:* Methods for Quantitative Assessment of Phytoplankton in Freshwaters, part I. Swedish Environmental Protection Agency Report 4860. Naturvårdsverkets förlag, Stockholm (1998).

1.5 Identification key

1. Main cyanobacterial genera that produce microcystins

   1.1. *Microcystis* characters: cells round, typically 4-6 μm in diameter, cells loosely bound into gelatinous, spherical-irregular colonies of varying size, typically 40-400 μm in diameter.....**1**

   1.2. *Anabaena* characters: cells round or oval, typically 4-8 μm in diameter, cells form straight, curled or spiral formed trichomes (filaments) of varying length with nitrogen fixing heterocysts and resting cells (akinetes) often present.....**2**

   1.3. *Planktothrix* characters: disk-shaped cells, typically 4-8 μm wide, cells form straight trichomes in which single cells are somewhat difficult to distinguish, end cell of different shape, planktonic, has gas vesicles, some reddish species, e.g. *P. rubescens*, are usually found in the metalimnion in thermally stratified lakes.....**3**

   1.4. *Oscillatoria* characters: disk-shaped cells that are much wider than long, form straight trichomes, end cell rounded or distinctive, benthic, does not have gas vesicles.....**4**

   1.5. *Nostoc* characters: cells round, form bent or kinked trichomes with heterocysts, akinetes found in some species, trichomes encapsulated in a firm mucilaginous matrix, colonies often macroscopic.....**5**

2. Main cyanobacterial genera that produce anatoxin-a

   2.1. *Anabaena* characters: see above.....**2**

   2.2. *Planktothrix* characters: see above.....**3**

   2.3. *Oscillatoria* characters: see above.....**4**

   2.4. *Aphanizomenon* characters: cells elongated, typically 2-4 times longer than wide, cells form straight trichomes which tend to aggregate into bundles, if present, heterocysts and akinetes are found in the central part of the trichome, always separated from each other, cell length increases towards the ends of the trichome.....**6**

3. Main cyanobacterial genera that produce cylindrospermopsin

   3.1. *Anabaena* characters: see above.....**2**

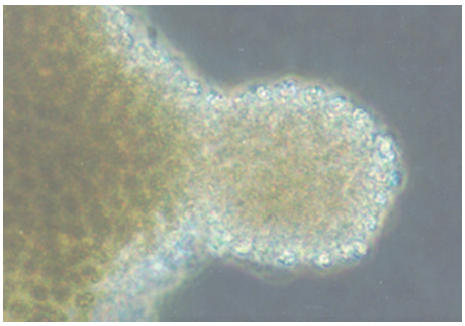
   3.1. *Aphanizomenon* characters: see above.....**6**

   3.2. *Cylindrospermopsis* characters: cells rectangular, form linear or coiled trichomes with a basal heterocyst, akinetes basally, but not immediately adjacent to the heterocyst.....**7**

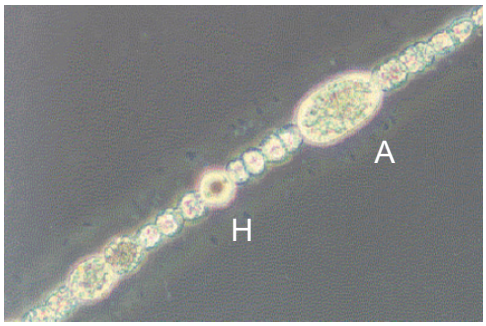
The following books contain detailed instructions for the identification of cyanobacteria and algae:

John, D.M., Whitton, B.A., Brook, A.J. (Eds.): The Freshwater Algal Flora of the British Isles. Cambridge University Press, Cambridge (2002).

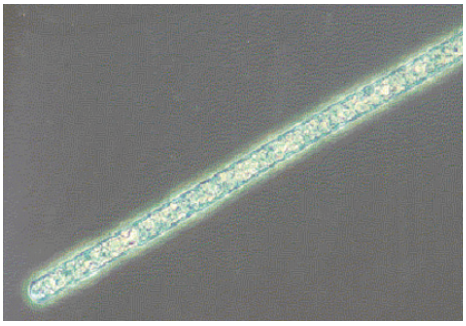
Canter-Lund, H., Lund, J.W.G.: Freshwater Algae. Biopress, Bristol (1995).



1. *Microcystis* sp.

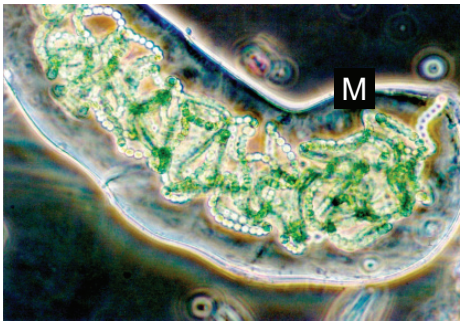


2. *Anabaena* sp. (H=heterocyst, A=akinete)

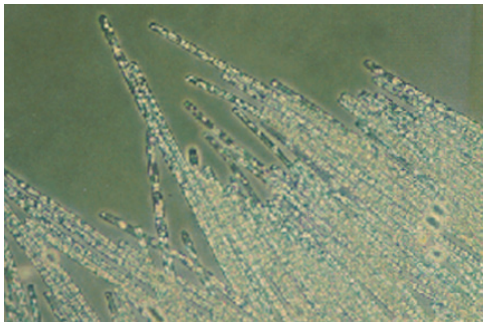


3. *Planktothrix* sp.

4. *Oscillatoria* sp.



5. *Nostoc* sp. (M=mucilaginous layer)



6. *Aphanizomenon* sp.

7. *Cylindrospermopsis* sp.
(H=terminal heterocyst)

Acidified Lugol's Iodine solution

- 20 g potassium iodide (KI)
- 200 ml distilled water
- 10 g resublimated iodine (I_2)
- 20 g glacial acetic acid (CH_3COOH)

Store in a dark glass bottle, well sealed, and protected from light.

2 Practical guidance for environmental sampling of cyanobacteria and cyanobacterial toxins

James S. Metcalf, Marianne Reilly and Geoffrey A. Codd, University of Dundee, UK

2.1 Introduction

Much of the current information and knowledge on the occurrence of cyanobacteria and their toxins in the natural environment has been made possible through the monitoring of natural waterbodies. Although this has proved useful, in order to obtain the most appropriate and accurate information, and to permit safe sampling of cyanobacteria and waterbodies, various strategies can be employed so that the subsequent information obtained during the analytical phase is meaningful and appropriate.

2.2 Prior to commencement of environmental sampling

As environmental sampling of waterbodies is potentially dangerous, careful planning is essential. Local rules and regulations should be adhered to and a formal risk assessment of the sampling sites and equipment to be used should be performed. Furthermore, training of personnel in the proper use of equipment and sampling procedures should be routinely updated and recorded. This includes proper recording facilities to account for scientists in the field including the route taken, waterbodies visited, approximate expected return times and designated responsible persons. Safety equipment to be used including disposable rubber gloves, emergency throw ropes, life preservers and fully charged mobile phones should all be checked for condition. In addition, when environmental sampling takes place a minimum of two persons is required.

Before environmental field work, sampling equipment to be used should be checked and cleaned. This is particularly important when samples are required for molecular biological techniques such as the Polymerase Chain Reaction (PCR; e.g. *1*) or fluorescence *in situ* hybridisation (*2*) as very small amounts of cyanobacterial DNA containing cyanotoxin genes can potentially interfere with subsequent analysis and produce false positive results. In addition to making sure that the equipment is clean, this will also give users the opportunity to make sure that everything is working correctly, aiding the subsequent field work.

With respect to the types of equipment available, these range from the use of plankton nets to dipping equipment and bottles and are dependent on the types of samples and information that are required (*3*). For representative water samples, dipping equipment consisting of beakers attached to poles offer safe means of sample isolation for collection in containers. Glass containers are potentially better than plastic as the latter have been shown to adsorb microcystins (*4, 5*) although plastic containers are more amenable to field work due to their strength and durability. For concentrated cyanobacterial samples from water, plankton nets either towed behind boats or along shorelines result in the accumulation of cyanobacterial filaments and colonies for isolation and enumeration. Much of the research on cyanobacteria has involved naturally accumulated scum samples and these permit the collection of large quantities of natural aggregations in small volumes. However, knowing the kinds of

samples required will permit the proper choice of sampling equipment (Fig. 2.1). More detailed information on the kinds of sampling equipment available is given by Franks (*6*).

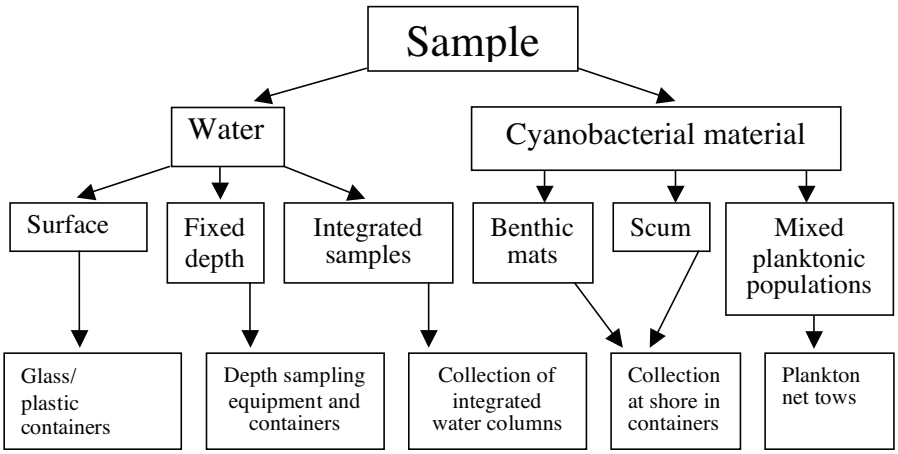


Figure 2.1: Water and cyanobacterial sampling locations and procedures.

2.3 On-site considerations for environmental sampling

For effective sampling of cyanobacteria, their toxins and water there are various options for sites where samples can be collected (*7*). Easy to use sites such as jettys and recreational entry points allow simplified collection of samples and can provide data on the likelihood of recreational exposure to cyanobacteria. Cyanobacterial scums are often found on leeward shores or in sheltered bays and offer the best chance for collection. Boats can be used for the safe collection of samples in open waters. Although the majority of sampling occurs in recreational waterbodies, if information is required by drinking water suppliers, samples can also be obtained at drinking water off-take points or entry points into the drinking water plant.

The majority of cyanobacterial blooms in European waters are seasonal and the duration of the bloom can be highly variable. This has implications for the frequency of sampling for effective risk assessment and the collection of appropriate data. The frequency of sampling should increase relative to the importance of the waterbody in terms of recreation, agriculture (e.g. crop-spray irrigation) and drinking water (human and animal) use. For low priority waterbodies, one to two samples per season may be sufficient. This should be increased as the importance of the waterbody increases, for example when the waterbody is used as a drinking water source, or for recreation, livestock watering or irrigation. Furthermore, sampling should also be increased in line with alert levels on the occurrence of cyanobacteria and toxins once they have been detected in a waterbody (e.g. *8*). Once the bloom has subsided and toxin concentrations (if measured) have decreased, the frequency of sampling can then be reduced.

During field sampling, in addition to samples for cyanobacteria and their toxins, various other physicochemical parameters such as temperature, pH, dissolved oxygen,

water transparency (Secchi depth) can also be taken to provide supplemental information which may be of use during the assessment of cyanobacterial toxins in the waterbody. However, if any environmental sampling involving water is performed, safety considerations should always be the primary concern. Further information on environmental sampling of cyanobacteria is given by Utkilen et al. (*3*) and Codd et al. (*7*).

2.4 Transportation and storage of samples prior to laboratory processing

After representative samples of cyanobacteria and water containing cyanobacteria have been obtained, these may require further processing in the laboratory and should be transported there within 24 hours, in a cool box, if possible. During transit, samples should be stored correctly so as to minimise movement and breakage of containers and cyanobacterial aggregations contained within. After arrival in the laboratory samples can either be processed immediately or stored for later analysis. Short-term storage of water samples should be performed at 4 °C. Scum and concentrated cyanobacterial samples can also be stored at 4 °C short-term or frozen and/or lyophilised for later analysis and long-term storage.

2.5 Processing of cyanobacterial samples prior to analysis

After receipt in the laboratory, various options for analysis exist and each requires varying amounts of material depending on the sensitivity required and the analytical method used. For microscopy and molecular methods such as enzyme-linked immunosorbent assay and PCR, small (approx. 1 ml) volumes can be sufficient. When physicochemical methods such as high-performance liquid chromatography (HPLC) and solid phase extractions are used, larger volumes are potentially required (up to 1-2 l) and consequently the sampling regime used in the field should take this into account.

In terms of the processing of the samples this should be performed as soon as possible to prevent biological and physical degradation of samples. Water samples can be filtered to separate the particulate from the cell-free phase and to permit analysis of the partitioning of cyanobacterial toxins in environmental waterbodies. Carefully designed sampling programmes before, during and after fieldwork can result in the production of sound data which can meaningfully be used by all persons interested in the occurrence and persistence of cyanobacterial toxins.

2.6 References

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3 Microcystins and nodularins

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3.1 Producers and structures

The most frequently reported cyanobacterial toxins are cyclic heptapeptide hepatotoxins, microcystins, found primarily in some species of the freshwater genera *Microcystis* (*1*), *Planktothrix* (primarily *P. agardhii*, called *Oscillatoria agardhii* in earlier literature) (*2*), *Anabaena* (*3*) and *Nostoc* (*4*). *Anabaenopsis* (*5*), *Oscillatoria* (*tenuis*) (*6*), picoplanktonic cyanobacteria (*7, 8*), *Radiocystis* (*9*), *Arthrospira* (*10*) and the terrestrial cyanobacterium *Hapalosiphon* (*11*) have also been identified as producers of microcystins. At least 76 different analogues of microcystins have been found in natural blooms and laboratory cultures of cyanobacteria (60 microcystins were listed by Sivonen & Jones in 1999 (*12*); at least 17 additional microcystins have been listed elsewhere.

The general structure of microcystins (Fig. 3.1) is cyclo(-D-Ala-L-**X**-D-*erythro*-β-methylAsp(iso-linkage)-L-**Z**-Adda-D-Glu(iso-linkage)-*N*-methyldehydro-Ala) where Adda stands for the unique β-amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid (*1, 13-15*). The main structural variation in microcystins is in the L-amino acid residues 2 (**X**) and 4 (**Z**), which are indicated by a two-letter suffix. For instance, the commonly occurring microcystin-LR contains leucine (L) at position 2 and arginine (R) at position 4. The Adda stereochemistry was assigned to 4*E*, 6*E* (*1*) and 2*S*, 3*S*, 8*S*, 9*S* (*14*).

Variability has been observed in all residues of microcystins (*12*). Changes in residues other than 2 and 4 are described by a prefix. For instance, [D-Asp^3]microcystin-LR, also known as 3-demethylmicrocystin-LR, contains D-aspartic acid at position 3, and [6(*Z*)-Adda^5]-microcystin-LR has an Adda residue with the biologically inactive 6(*Z*) configuration (*16, 17*). Microcystins are relatively polar molecules due to carboxylic acids at positions 3 and 6 and the frequent occurrence of arginine at positions 2 and 4. The Adda residue and variable hydrophobic amino acid residues give a partially hydrophobic character to the microcystins.

Nodularins (Fig. 3.1) have been found in the brackish water cyanobacterium *Nodularia spumigena* (*14*) and in the marine sponge *Theonella swinhoei* (*18*). Fewer than 10 analogues of nodularins are known (*12*). Nodularins are cyclic pentapeptides with the general structure cyclo(-D-*erythro*-β-methylAsp(iso-linkage)-L-**Z**-Adda-D-Glu(iso-linkage)-2-(methylamino)-2(*Z*)-dehydrobutyric acid). The second residue (designated as **Z** above) is L-Arg in the common nodularin-R (often called simply nodularin) (*14*) and L-Val in nodularin-V (also known as motuporin) isolated from *Theonella*.

This chapter originates from Lisa Spoof's PhD thesis examined at Åbo Akademi University in June 2004. New information from 2004-2005 has been added.

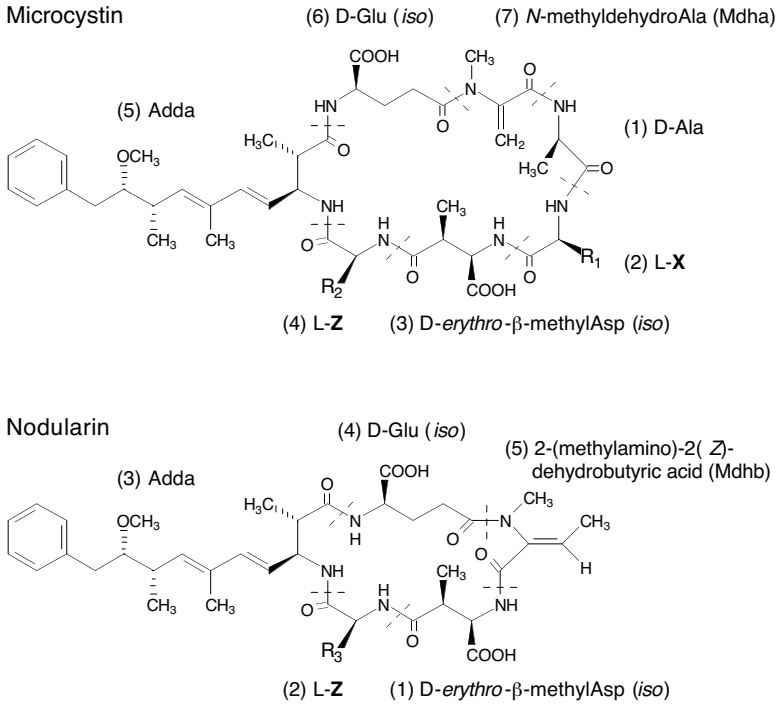

Figure 3.1: General structures of microcystins and nodularins.

3.2 Toxin concentrations in environmental waters

Cyanobacterial peptide toxins have been found both in planktonic species and in mats of benthic species. The peptide toxins are not actively secreted to the surrounding water. Studies with laboratory cultures of cyanobacterial strains have shown that most (>80%) of the toxin is intracellular in healthy growing cells, and that the release of toxin occurs during senescence of the cultures and the shift from growth to stationary phase and cell death (*19-22*). Furthermore, during the active growth of cyanobacteria under field conditions, the majority of microcystin is intracellular (*23, 24*). The ambient concentration of dissolved microcystin is a function of several factors (*25*) such as dilution, chemical degradation accelerated by temperature and pH extremes (*26*), photodegradation (*27*), adsorption to particles (*28*) and biodegradation (*28, 29*).

The quantitative determination of toxins has been traditionally performed with freeze-dried samples of culture or seston material (particulate suspended material, which contains not only cyanobacterial cells but usually other algae, some zooplankton, and inorganic material such as clay particles). Results are then expressed as micrograms of toxin per gram (dry weight, DW). The highest published microcystin and nodularin concentrations in bloom samples have been 7300 μg g^{-1} microcystin in a *Microcystis* sample from China (*30*) and 18000 μg g^{-1} nodularin in *Nodularia* from the Baltic Sea (*31*).

Toxin levels expressed in volumetric units are often more suitable for the estimation of risk for aquatic organisms, wildlife and humans. A provisional guideline value for microcystin-LR of 1 μg per litre in drinking water has been derived by the World Health Organization (*32*). In pioneering work from Finland reported by Lindholm et al., concentrations of roughly 20-40 μg l^{-1} of demethylmicrocystin-RR were recorded in the eutrophic Lake Östra Kyrksundet in Åland during a persistent metalimnetic maximum of toxic *Oscillatoria* (*Planktothrix*) *agardhii* (*33*). Typical microcystin concentrations in (German) fresh water bodies were published by Fastner et al. (*34*). In over 70% of the samples from pelagic (open) water, microcystin concentrations in the intracellular fraction were below 10 μg l^{-1}. In *Microcystis* blooms the concentrations of microcystins varied between 2-25000 μg l^{-1}. The microcystin to chlorophyll-a ratio in the German study was usually between 0.1-0.5 with maxima of 1-2. Chlorophyll-a, which is a basic parameter measured in routine water monitoring, can be useful as a first estimation of intracellular microcystin concentration (*33, 34*). Recorded concentrations of released microcystins in water vary from trace concentrations up to at least 1800 μg l^{-1} (*12*). This high concentration was found after algicide treatment of a water bloom (*23*).

3.3 Toxicity and organotropism

The LD_{50} values (mouse, i.p.) of the different microcystins and nodularins vary from about 50 μg kg^{-1} to over 1 mg kg^{-1}. Minor differences in structure may lead to pronounced differences in toxicity. For instance, the reported LD_{50} values (mouse, i.p.) are 180-250 μg kg^{-1} for demethylmicrocystin-RR variants but 600 μg kg^{-1} for microcystin-RR (*12*). In contrast, demethylmicrocystin-LR variants are less potent toxins (LD_{50} 90-300 μg kg^{-1}) than microcystin-LR (50 μg kg^{-1}). The structure-activity relationships of microcystins and nodularins have been studied by using naturally occurring toxin variants or by introducing modifications at different sites of the toxins. Dahlem demonstrated that the removal or saturation of the Adda structure in microcystin-LR resulted in a loss of toxicity of the derivative (*35*). He also selectively reduced the α,β-unsaturated carbonyl group (dehydro moiety) in the Mdha residue of microcystin-LR and the Mdhb residue of nodularin with sodium borohydride. The reduction of the dehydro moiety and the Michael type addition of glutathione to Mdha did not significantly weaken the toxicity. Besides Adda, another structural feature necessary for toxicity is the free carboxylic acid group in the D-Glu unit as demonstrated by Stotts et al. (*36*). The cyclic structure of the toxins has also been shown to be crucial for toxicity. Linear microcystins are more than 100 times less toxic than the cyclic compounds (*15, 37*). However, the linear microcystin, *seco*[D-Asp^3]microcystin-RR, was shown to inhibit recombinant protein phosphatase 1 in a preliminary examination (*38*).

Microcystins and nodularins enter hepatocytes through the bile acid transport mechanism (*39, 40*). The molecular basis of microcystin/nodularin toxicity is by the inhibition of protein phosphatases 1 and 2A (*41, 42*). The intoxication routes of concern for human health include intraperitoneal, intravenous and oral exposure as well as the intranasal/inhalation route (*43*). The intravenous route has relevance during dialysis treatment (*44, 45*). The most important exposure route is the oral route (*46*). This route involves not only the drinking of water containing cyanobacterial toxins but also the consumption of toxin-containing animal or plant tissues. Dietary supplements made from cyanobacteria comprise one possible source for the toxins (*47, 48*). Low-level chronic exposure is probably of greater human health significance than some rare acute lethal poisonings.

The drinking water guideline value for microcystin-LR relies heavily on the data from Falconer et al. who studied the health effects of microcystin-containing extracts in pigs receiving microcystins in their drinking water over a 44 day period (*49*). The lowest observed adverse effect level (LOAEL) was 100 µg microcystin-LR equivalents per kg bodyweight per day (*49*). The tolerable daily intake of microcystins was established at 0.04 µg kg^{-1} of bodyweight per day (*46, 50*). From these studies the provisional guideline value for microcystin-LR in drinking water was derived and adopted by the WHO at 1.0 µg l^{-1} (*32*).

3.4 Extraction

3.4.1 Intracellular toxins

Effective toxin recovery is difficult without disrupting the strong cyanobacterial cell wall structure. Most procedures for cyanotoxins use freeze-drying or freeze-thawing for cell wall disruption. Toxins can be extracted from filter discs containing harvested algal cells or from weighed lyophilized bloom material. The disruption of cells is enhanced by ultrasonication in a bath or probe-type sonicator. The advantages of probe sonication have been underlined by Rapala et al. since intact cells were present in their samples even after freeze-thawing and bath sonication (*51*). Microcystins have been extracted with a number of solvents, some of the most popular being 5% acetic acid (*52*), methanol (*53*), aqueous methanol (most often 75% methanol) (*54*) and water:methanol:n-butanol (75:20:5) (*55*).

The differences in extraction efficiency are not solely dependent on the solvent used but also on the morphological differences of cyanobacteria and on the microcystin variants present in the cells (*54*). Recently an international intercomparison exercise was conducted between 31 laboratories (*56*). All laboratories received the same set of samples of lyophilised cyanobacterial material. Substantial differences were observed in the extraction efficiency between the in-house methods of the laboratories (using different solvents and apparatus). The intercomparison organisers, Fastner et al., concluded that pure microcystin-LR standard could be measured with adequate precision by all participating laboratories but a standardisation of field sample analysis was necessary. In addition, Fastner et al. emphasised the need for certified reference materials.

3.4.2 Extracellular toxins

The concentrations of extracellular toxins are usually too low for direct analysis by HPLC-UV. Solid-phase extraction (SPE) is the most widely used technique in sample concentration and clean-up for HPLC of cyanobacterial toxins. It has almost completely replaced the old liquid-liquid extraction (LLE) method. Alkyl-bonded silicas are most often used but polymeric materials are becoming increasingly popular.

Some comparisons between different SPE C_{18} cartridges from different manufacturers were made by Harada et al. (*52*). Cyano cartridges were also demonstrated to show retention of microcystins (*57*). Tsuji et al. studied different types of SPE chemistries and developed a two-step clean-up method, with solid phase extraction on a C_{18} cartridge followed by a silica gel cartridge (*58*). Kondo et al. introduced immunoaffinity purification as an alternative to silica in the two-step purification protocol (*59*) and finally replaced the C_{18} cartridge with a polymeric sorbent (*60, 61*). The nonpolar poly(styrene–divinylbenzene) copolymer sorbent has been shown to concentrate microcystins from water samples effectively (*60*) and the

poly(divinylbenzene-co-*N*-vinylpyrrolidone) material (Oasis HLB cartridges from Waters) has also been shown to function well in this respect (*51, 62*).

Validation of the chosen SPE method is necessary. This can be done by spiking a blank water sample with a known amount of microcystin and determining the recovery after SPE. The recovery of microcystins in SPE has been evaluated in raw water, tap water and ultra-pure water (*63, 64*). In practice, the recovery varies not only due to the cartridge type used but also due to the sample matrix and the microcystin analogues present in the sample.

Immunoaffinity columns utilising antibodies against microcystins (antibodies covalently bound onto an appropriate sorbent such as Sepharose or silica) have been shown to effectively eliminate co-eluting substances in extracted water samples (*62, 65-67*). The shortcomings of the immunoaffinity columns are the variable cross-reactivity/recovery of the different microcystins, difficulties in the production of large amounts of antibodies required and the limited stability of the antibodies.

Molecular imprinted polymers (MIPs) allow selective molecular recognition for a certain compound and have, instead of conventional SPE, been used for pre-treatment and concentration of microcystin-LR. The possibility of using MIPs in a piezoelectric sensor was also explored (*68, 69*). Fragment imprinting technique enabled the preparation of microcystin group-specific adsorption media (*70*).

3.5 Screening

As it is not possible to determine whether a cyanobacterial bloom is toxic by its appearance or species composition, a number of biological, biochemical and physicochemical methods have been developed for the assessment of toxin content. Cyanobacterial blooms can produce complex microcystin mixtures and sometimes both hepatotoxins and neurotoxins. For example, 15 and 19 microcystin variants were reported in *Microcystis* blooms in the UK and USA, respectively (*71, 72*). Physicochemical methods should ideally separate and quantify individual microcystins which have different toxicities.

A multi-step procedure may comprise initial screening of samples by sensitive and simple methods, such as the enzyme-linked immunoassays and protein phosphatase inhibition assays for microcystins/nodularins. These methods have now almost completely replaced the mouse bioassay but they are, of course, selective for a single class of cyanobacterial toxins, microcystins and nodularins. The use of screening helps to reduce the number of samples which require full analytical investigation and thus reduces the work burden in a laboratory. Furthermore, rapid results may speed up regulatory measures needed to protect water users.

The mouse bioassay has been the recognised standard method for establishing the LD_{50} values of and symptoms/effects caused by cyanobacterial toxins (*73*). Adult mice are injected intraperitoneally with the sample dissolved in physiological saline and observed for specific symptoms of intoxication. Due to ethical problems and an increasing opposition to the use of higher animals for any form of toxicity testing, other types of bioassays have been developed. The most popular of these has been the brine shrimp (*Artemia salina*) assay (*74*). It is, however, not always clear whether the invertebrates react to microcystins or to other compounds present in the samples (*75*). Marsálek and Bláha compared 17 biotests (using crustaceans, protozoans, insects, rotifers, cnidarians, nematodes, oligochaetes and plants) for detection of cyanobacterial toxicity (*76*). They found the crustacean *Thamnocephalus platyurus* to

be the most suitable assay organism for routine assessment of cyanobacterial toxicity even though mortality was also observed with extracts containing no microcystins.

The protein phosphatase inhibition assay (PPIA) is based on the biochemical activity of microcystins and nodularins. Many kinds of substrates can be used to perform this assay. The first developed method determined the release of ^{32}P-phosphate from the radiolabelled substrate phosphorylase *a*, a reaction inhibited by the hepatotoxins (*41, 77, 78*). The method is sensitive (detects 50 ng microcystin per litre) but suffers from the extensive preparation of reagents and the use of a radioisotope. Methods based on the use of *p*-nitrophenyl phosphate as substrate for recombinant protein phosphatase 1 and measuring the release of the coloured *p*-nitrophenol have been reported by An & Carmichael, Ward et al. and Wirsing et al. (*79-81*). Heresztyn and Nicholson investigated the use of protein phosphatase 2A and *p*-nitrophenyl phosphate to determine cyanobacterial toxins directly in water. They found that this method responded well to a range of microcystins, as well as nodularin, at concentrations of 0.2-1 μg l^{-1} (*82*). IC_{50} values of seven microcystin analogues in protein phosphatase 2A inhibition assay were compared and found to range between 0.29-0.84 nM (*83*). Endogenous protein phosphatase activity could mask the presence of microcystins in cyanobacterial samples and lead to an underestimation of the toxin content (*84*). This problem can be dealt with by heating the samples to destroy the endogenous phosphatase activity. Quantitation problems of microcystins related to the presence of iron Fe^{3+} and aluminium Al^{3+} ions in the sample water matrix were reported by Oliveira et al. (*85*). A significant reduction of microcystin detection in HPLC, PPIA and ELISA assays, either because of toxin decomposition or through microcystin complexation with the metal ions, was observed.

The most popular screening method for microcystins and nodularins is based on an immunological method, the enzyme-linked immunosorbent assay (ELISA). ELISA kits for the cyanobacterial peptide hepatotoxins are available from several manufacturers, for example Strategic Diagnostics and Envirologix. ELISA can only determine the total microcystin/nodularin content, and although many major microcystins give a strong response in the assays, the exact epitope and specificity of the used antibodies are somewhat uncertain. The quantitation ranges of the commercial ELISA kits are typically about 0.1-2 μg l^{-1}, thus covering the provisional drinking water guideline for microcystin-LR, 1 μg l^{-1}, without concentration. Earlier work in the preparation of antibodies against microcystins has been reported by Brooks and Codd, Chu et al., and An et al. (polyclonal antibodies) (*79, 86, 87*) and by Nagata et al. (monoclonal antibodies) (*88, 89*). Recent work include polyclonal antibodies by Metcalf et al. (*90*), a range of poly- and monoclonal antibodies by Mikhailov et al. (*91*), recombinant antibodies derived from a phage display library (*92*) and antibodies against the Adda residue (*93, 94*). Kim et al. developed a rapid and sensitive fluorescence immunochromatographic assay for the on-site quantification of microcystins (*95*). Practical applications relating to the monitoring of environmental and process waters for microcystins have been reported e.g. by Ueno et al. (epidemiological studies of microcystins vs. primary liver cancer in China) (*96*), Rapala et al. (comparison between ELISA, PPIA and HPLC-UV) (*51*), Spoof et al. (comparison between ELISA and chromatographic methods in lake monitoring) (*97*), Hoeger et al. (comparison between Adda-ELISA and PPIA methods in the assessment of water treatment efficacy) (*98*) and Vieira et al. (screening for microcystins in raw and treated waters) (*99*). Immunological methods for cyanobacterial toxins were reviewed by Metcalf and Codd (*100*).

Detection of 3-methoxy-2-methyl-4-phenylbutyric acid (MMPB) by gas chromatography using a flame ionisation detector (FID) or HPLC with a fluorescence detector have also been used for the purposes of screening for microcystins. MMPB is an oxidation product of Adda in microcystins and nodularins (*101*). The original method by Sano et al. utilised permanganate/periodate oxidative cleavage but an alternative method of MMPB release relying on ozonation was suggested by Harada et al. (*102*). Kaya and Sano later advocated the use of deuterium-labelled MMPB as an internal standard and concluded that MMPB detection is the most sensitive method for determination of total microcystin (*103*). One strength of the MMPB method is that no microcystin standards are necessary. The weakness of the MMPB method is that different microcystins give an equal signal with MMPB detection, although their toxicities may vary. Also, non-toxic microcystin/ nodularin precursors and still unidentified degradation products containing Adda may lead to an overestimation of the toxin content of a sample.

3.6 Trace analysis

3.6.1 HPLC coupled with diode-array UV detection

Microcystins and nodularins comprise an interesting family of closely related peptides for separation and detection studies. The number of toxin analogues is high and therefore the separations require high resolution and good selectivity. The molar absorbance of microcystins and nodularins is strong at 238 nm enabling sensitive UV detection but the derivatisation of the toxins e.g. for fluorescence detection is challenging. The main chromophore of the toxins absorbing at 238 nm is the conjugated diene in the Adda residue (*104*) with additional absorbance from the α,β-unsaturated carbonyl group in Mdha/Mdhb residue. The UV spectra of microcystins were divided by Lawton into two categories (*53*): the usual microcystin spectra, e.g. that of microcystin-LR, with a local maximum at 238 nm and the spectra of tryptophan-containing microcystins with an additional maximum at 222 nm. Additionally, the UV spectra of tyrosine-containing microcystins, e.g. that of microcystin-YR, are flatter in the region of 230-240 nm. The reported molar absorbances of microcystins at 238 nm (in methanol) were 39800 for microcystin-LR, -RR (*17*) and -YR (*105*), and 31600 for 3-demethylmicrocystin-LR and 46800 for 7-demethylmicrocystin-LR (*106*). The application of known molar absorbances has constituted the basis of toxin quantitation in many papers.

The chromatographic systems used in microcystin and nodularin separations fall largely into five categories: a) neutral mobile phases with ammonium acetate and acetonitrile, RP chromatography, b) acidic mobile phases with TFA and acetonitrile, RP chromatography, c) methanol-containing mobile phases with different buffers and pHs, RP chromatography, d) other RP chromatographic systems and e) other mobile and stationary phases. Examples of separations in these categories are given below. Acidic mobile phases have been shown to be able to resolve more microcystins and nodularins than the neutral ones. Examples of HPLC analyses can be found in Table 3.1 (isocratic separations) and Table 3.2 (gradient separations).

a) *Neutral mobile phases with ammonium acetate and acetonitrile, RP chromatography.* The first successful analytical procedure for the extraction and quantification of "microcystins" from a cyanobacterial bloom by HPLC was introduced in 1984 by Siegelman et al. (*55*). The Hypersil column (50 mm × 4.6 mm I.D.), a modern choice, containing C_{18} silica with 3 μm particles was eluted with aqueous 500 mM ammonium acetate and 26% acetonitrile. Siegelman et al. detected

and quantitated one toxin (toxin-LR) in cultured *M. aeruginosa* as well as in a natural bloom sample. Jones et al. chromatographed 24 compounds with a microcystin-like UV spectrum on a C_{18} column with a gradient of aqueous 8 mM ammonium acetate and acetonitrile (*107*).

b) *Acidic mobile phases with TFA and acetonitrile, RP chromatography.* Among microcystin chromatographers Krogman et al. were the first to use a gradient of acetonitrile in water containing 0.1% TFA (*108*). Wicks and Thiel (*109*) chromatographed microcystin-YR, -LR, -FR, -YA, -LA and -LAba (in this elution order, Aba = aminoisobutyric acid) on a C_{18} column. They used an isocratic mobile phase of 38% aqueous acetonitrile and 0.1% TFA. The elution order of microcystins in the acidic TFA - acetonitrile mobile phase is thought to be the genuine reversed-phase elution order according to increasing hydrophobicity. Guo et al. had developed retention coefficients for amino acid residues in peptides and they could predict the retention times of small linear peptides (not microcystins) on reversed-phase HPLC with water - acetonitrile - 0.1% TFA gradients (*110, 111*). Retention coefficients at pH values 2.0 and 7.0 were published. These coefficients are thought to give an indication of the elution order of microcystins with different variable residues in aqueous TFA - acetonitrile eluents. Lawton et al. developed further the acidic chromatography system (*53*). They used a gradient of 0.05% TFA in water and 0.05% TFA in acetonitrile, starting at 30% acetonitrile, with separation on a C_{18} column. The detection was performed with a diode-array UV detector collecting spectral information between 200 and 300 nm. The observed elution order was 3-demethylmicrocystin-RR, microcystin-RR, nodularin-R, microcystin-YR, -LR, -FR, -LA, -LY, -LW and -LF. This matched the predicted order (*110*) for the microcystin-**X**R and microcystin-L**Z** series with one exception: the elution order of microcystin-LW and -LF was reversed. As expected, the arginine-containing microcystins and nodularin eluted earlier than the uncharged and more hydrophobic microcystins -LA, -LY, -LW and -LF. The arginine-containing toxins also had somewhat broader peaks which is typical for basic analytes on certain older C_{18}-silica columns (secondary interactions with residual silanols on the silica surface). Spoof et al. assessed the performance of several reversed-phase sorbents in the separation of eight microcystins and nodularin-R (*134*). The best overall performance was delivered by a polar-embedded reversed-phase column, the amide C_{16} column. Spoof and Meriluoto also explored rapid chromatography of microcystins and nodularin-R using a monolith C_{18} column (*135*).

c) *Methanol-containing mobile phases with different buffers and pHs, RP chromatography.* In 1988 Harada et al compared different HPLC solvent systems in the C_{18} separation of microcystins-LR, -RR and -YR and suggested three methanol-containing mobile phases (*52*). In 1991 Harada et al. published a chromatographic method using normal-phase TLC and reversed-phase HPLC for the separation of 3-demethylmicrocystin-LR and 7-demethylmicrocystin-LR (*106*). The HPLC separation was conducted on a C_{18} column with methanol - 0.05 M sodium sulphate as the mobile phase. Watanabe et al. (*112*) demonstrated that a chemical analysis method gave comparable results to the mouse bioassay. They used Harada's methods for the detection and quantification of microcystins in different *Microcystis* species and strains. The method was applicable to the determination of the toxins at a concentration of 50 $\mu g\ g^{-1}$ freeze-dried cells.

d) *Other RP chromatographic systems.* In 1988 Meriluoto et al. introduced the use of internal surface reversed-phase (ISRP) HPLC for microcystin and nodularin

separations (*113*). The general idea was to minimise the clean-up work prior to HPLC as these multifunctional columns combine size-exclusion chromatography with a reversed-phase and cation-exchange mechanism. Large protein molecules pass unretained through the column while the small analytes, e.g. cyanobacterial peptide toxins, with a molecular weight of about 1000 Da, penetrate the pores of the packing, interact with the internal reversed phase and are separated. The partitioning phase of the ISRP column was a hydrophobic peptide, glycine-L-phenylalanine-L-phenylalanine. The free carboxyl group in the terminal phenylalanine functioned as a weak cation exchanger. The ISRP column could resolve at least five microcystins during an isocratic run of less than 10 minutes (*114*). Later the capacity factors of five major microcystins were measured at different pH values and in eluents with different organic modifiers (*115*). Meriluoto et al. concluded that for the reversed-phase mode, the best separation was achieved at acidic pH values. At pH 2 the elution order of toxins was microcystin-RR, nodularin, microcystin-LR, -YR and -LA and it was also possible to separate demethylated microcystin-RR analogues from microcystin-RR. Alkaline mobile phases with base-stable C_{18} columns have not been fully explored but they can offer alternative selectivity for microcystins (*116*).

e) *Other mobile and stationary phases.* Gathercole and Thiel compared diethylaminoethyl (DEAE) anion-exchange chromatography with the reversed-phase method and succeeded in the separation of microcystin-LR, -FR, -YR, -LR and -LAba, and the quantitation of microcystin-LR, -YR and -LA on the anion-exchange column (*117*).

Methodological comparisons of microcystin analysis on different HPLC stationary and mobile phases have been quite rare. Spoof et al. assessed five reversed-phase stationary phases and four mobile phases in microcystin and nodularin separation (*134*). Rapala et al. studied the chromatography of microcystins, with emphasis on the demethylated variants, on three analytical HPLC C_{18} columns from different manufacturers (*51*). Both ammonium acetate based and TFA-based mobile phase systems were used. It was concluded that the chromatographic performance in both mobile phases was strongly dependent on the analytical column. In their view, the ammonium acetate - acetonitrile mobile phase showed potential for better separation of demethylmicrocystins. In addition to the chromatography comparison, the paper of Rapala et al. included useful information of the performance of HPLC, PPIA and ELISA for the analysis of cyanobacterial toxins from laboratory strains and natural waters.

Much of the early work in microcystin analysis has been done with isocratic mobile phases and with single-wavelength UV detection. This was feasible when the number of known microcystins was less than ten. The primary choice in modern HPLC work with optical detection is to use aqueous TFA-acetonitrile gradients and a diode-array detector. These gradients should span ca 25-70% acetonitrile to cover both hydrophilic and hydrophobic microcystins. The use of the diode-array detector enables the tentative identification of microcystins and nodularins for which there are no standards.

3.6.2 LC-MS

Before liquid chromatography-mass spectrometry was used for the quantitative analyses of cyanobacterial toxins, fast atom bombardment (FAB) mass spectrometry had become an important tool for the determination of molecular weights and amino acid sequences of microcystins and nodularins, e.g. (*1-3, 13-15, 72, 118-121*). Later,

electrospray ionisation MS collision-induced dissociation (CID) mass spectra have been used for the structural determination of the toxins (*122, 123*). Matrix-assisted laser desorption/ionisation time-of-flight MS (MALDI-TOF-MS, an off-line technique) is also a useful technique for a tentative structural characterisation of cyanobacterial metabolites, including microcystins (*124, 125*), when high-resolution reflector instruments and a post-source decay mode are used. Another TOF technique is the surface-enhanced laser desorption ionisation mass spectrometry or SELDI-TOF-MS described for microcystins and nodularin in 2004 (*126*).

Kondo et al. applied LC-frit-FAB-MS to the analysis of microcystins (*127*). In the early 1990s the electrospray ionisation (ESI) technique made LC-MS much more convenient to use and gains in sensitivity were achieved. Poon et al. pioneered the use of LC-ESI-MS in the quantitative analyses of microcystins, nodularin, anatoxin-a and saxitoxin (*128*). Edwards et al. improved LC-ESI-MS for the identification of microcystins from cyanobacteria and water samples (*129*). They described the use of a parent scan of *m/z* 135, a fragment ion formed from the side chain of Adda, in an LC-MS-MS screening method for microcystins. The same Adda cleavage had been observed earlier by Krishnamurthy et al. (*3*), Namikoshi et al. (*130*) and Kondo et al. (*127*) with FAB-MS. Bateman et al. utilised both LC and capillary electrophoresis (CE) combined with electrospray mass spectrometry for the determination and quantitation of microcystins (*131*).

Recent advances in LC-MS (better electronics and ion transfer optics in the MS instruments etc) have resulted in lowered detection limits, typically in the low picogram range injected on column. Zweigenbaum et al. applied microbore LC coupled to an ion-trap mass spectrometer for the analysis of microcystins from environmental samples (*136*). Pietsch et al. designed a single LC-MS-MS method for cyanobacterial hepato- and neurotoxins (*137*). Lawrence et al. analysed several cyanobacterial health food products for microcystins by ELISA, protein phosphatase inhibition assay, LC-MS and LC-MS-MS, at concentrations down to 0.1 μg g^{-1} (*138*). MRM (LC-MS-MS) was the preferred mode of toxin identification and quantification for regulatory purposes. Hummert et al. demonstrated the usefulness of LC-MS-MS in identifying microcystin variants in environmental cyanobacterial samples (*139*). Barco et al. used narrow-bore columns for the analysis of hepatotoxins by electrospray LC-MS (*140*). Dahlmann et al. analysed a wide range of algal toxins, i.e. microcystins, nodularin, anatoxin-a, domoic acid, okadaic acid and dinophysistoxin-1 in a single LC-MS run (*141*). Meriluoto et al. introduced an LC-MS method for high-throughput screening of microcystins and nodularins. A sample throughput of over 500 samples a day was achieved by using a short C_{18} column, rapid aqueous formic acid - acetonitrile gradient and MS detection (*156*). A summary of LC-MS conditions for microcystins is given in Table 3.3.

Table 3.1: Detection and quantification of microcystins with isocratic HPLC-UV

Stationary phase	Column dimensions and particle size	Solvent A and B	% B	Toxins detected	Authors
Hypersil C_{18}	50 mm × 4.6 mm I.D., 3 µm	0.5 M ammonium acetate, pH 6 - ACN	26	toxin-LR (microcystin-LR)	Siegelman et al. (*55*)
Spherogel-TSK DEAE 2-SW	250 mm × 4.6 mm I.D., 5 µm	0.04 M sodium phosphate, pH 7 + 0.2 M NaCl - MeOH	3	microcystin-LR, -FR, -YR, -LA, -LAba	Gathercole & Thiel (*117*)
Novapak C_{18}	150 mm × 3.9 mm I.D., 5 µm	0.01 M ammonium acetate - ACN	26	microcystin-LR/YR, -LA	
Nucleosil C_{18}	75 mm × 4.6 mm I.D., 3 µm	0.05% TFA - MeOH	60	microcystin-YR, -LR	Harada et al. (*52*)
		0.05 M phosphate, pH 3.0 - MeOH	60	microcystin-YR, -LR	
		0.05 M $NaSO_4$ - MeOH	50	microcystin-YR, -LR	
	150 mm × 4.6 mm I.D., 5 µm	0.05 M phosphate, pH 3.0 - MeOH	58	microcystin-RR, -YR, -LR	
GFF ISRP	250 mm × 4.6 mm I.D., 5 µm	0.1 M potassium dihydrogenphosphate, pH 6.8 - ACN	15	microcystin-LA, nodularin, microcystin-LR, -YR, -RR	Meriluoto et al. (*114*)
Ultrasphere ODS	150 mm × 4.6 mm I.D., 5 µm	0.1% TFA -ACN	38	microcystin-YR, -LR, -FR, -YA, -LA, -LAba	Wicks & Thiel (*109*)
ODS Hypersil	100 mm × 4.6 mm I.D., 5 µm	0.01 M ammonium acetate - ACN	23	several microcystins including de- and didemethylated variants	Rapala et al. (*51*)
Eclipse XDB-C_{18}	250 mm × 3.0 mm I.D.				

Table 3.2: Detection and quantification of microcystins with gradient HPLC-UV

Stationary phase	Column dimensions and particle size	Solvent A and B	Gradient % B	Toxins detected	Authors
Ultrasphere C_{18}	250 mm × 4.6 mm I.D., 5 µm	0.1% TFA - ACN	46-55	Two toxins	Dierstein et al. (*132*)
Partisil-5		A: acetic acid in chloroform (1:99) B: acetic acid in MeOH (1:99)	30-80	Two toxins	Birk et al. (*133*)
µBondapak C_{18}	300 mm × 3.9 mm I.D.	0.05% TFA - 0.05% TFA in ACN	30-100	dm-microcystin-RR, microcystin-RR, nodularin, microcystin -YR, -LR , -FR, -LA, -LY, -LW, -LF	Lawton et al. (*53*)
µBondapak C_{18}	150 mm × 6 mm I.D.	0.008 M ammonium acetate - ACN	15-35	24 compounds with microcystin UV spectra	Jones et al. (*107*)
Nucleosil C_{18} Discovery C_{18} Discovery AmideC_{16} Purospher RP-18e LiChrospher RP-18e	250 mm × 4.6 mm I.D., 5 µm 250 mm × 4.6 mm I.D., 4 µm 250 mm × 4.6 mm I.D., 4 µm 250 mm × 4.0 mm I.D., 5 µm 250 mm × 4.0 mm I.D., 5 µm	I: 0.05% TFA - ACN II: 0.05% TFA - 0.05% TFA in ACN III: 0.5% formic acid - 0.2% formic acid in ACN IV: (A) 0.0105 M ammonium acetate-ACN (95:5) (B) 0.05 M ammonium acetate-ACN (20:80)	I, II,III: 25-70 IV: 15-60	dm-microcystin-RR, microcystin-RR, nodularin, microcystin-YR, dm-microcystin-LR, microcystin-LR, -LY, -LW, -LF	Spoof et al. (*134*)
Eclipse XDB-C18 LiChrospher PAH ODS Hypersil ODS	250 mm × 3.0 mm I.D., 5 µm 250 mm × 3.0 mm I.D., 5 µm 100 mm × 4.6 mm I.D., 5 µm	0.05% TFA - ACN 0.01 M ammonium acetate - ACN	30-64 23-40	several microcystins including de- and didemethylated variants	Rapala et al. (*51*)
Chromolith Performance RP-18e	100 mm × 4.6 mm I.D.	0.05% TFA - 0.05% TFA in ACN	25-70	dm-microcystin-RR, microcystin-RR, nodularin microcystin-YR, dm-microcystin-LR, microcystin-LR, -LY, -LW, -LF	Spoof & Meriluoto (*135*)

Table 3.3: Examples of LC-MS of microcystins

Chromatographic conditions	Ionisation and mass analyser	Acquisition mode	Analytes besides microcystins	Authors
C_{18}, 0.01% TFA - MeOH, containing 0.8% glycerol	Frit-FAB, positive	scan, SIR		Kondo et al. (*127*)
C_{18}, water - ACN, both containing 0.1% acetic acid	ESI, positive, QQQ	scan	nodularin, anatoxin-a, saxitoxin	Poon et al. (*128*)
C_8, water - ACN, both containing 0.1% TFA	ESI (ionspray), positive, QQQ	scan, parent scan, SIR		Edwards et al. (*129*)
C_{18}, water - ACN, containing 0.1% TFA	ESI (ionspray), positive, QQQ	scan, SIR, MRM		Bateman et al. (*131*)
C_{18}, 0.5% formic acid - ACN - MeOH	ESI, positive, IT	scan, MS^n		Zweigenbaum et al. (*136*)
C_{18}, aqueous TFA (pH 2.5) - ACN	ESI, positive, Q	scan		Robillot et al. (*142*)
C_{18}, water - ACN, both containing 0.05 M formic acid and 0.002 M ammonium formate	ESI (turbo ionspray), positive, Q or QQQ	scan, SIR, daughter scan		Hummert et al. (*139*)
C_8, 0.08% formic acid - ACN	ESI, positive, QQQ	SIR, MRM	nodularin	Lawrence et al. (*138*)
C_{18}, three-component gradient of A: 0.002 M heptafluorobutyric acid, pH 3.5; B: 0.002 M ammonium acetate - 0.1% acetic acid in ACN; C: 0.002 M ammonium acetate - 0.1% acetic acid in water	ESI, positive, QQQ	scan, daughter scan, SIR, MRM	nodularin, anatoxin-a, saxitoxin	Pietsch et al. (*137*)
C_{18}, water - ACN, both containing 0.08% formic acid	ESI, positive, Q	scan, SIR	nodularin	Barco et al. (*140*)
C_{18}, 0.01M TFA containing 0.01% heptafluorobutyric acid - ACN	ESI (turbo ionspray), positive, Q (LC-MS) or Q-TOF (by flow injection)	scan, SIR	nodularin, anatoxin-a, domoic acid, okadaic acid, dinophysistoxin-1	Dahlmann et al. (*141*)
C_{18}, 0.5% formic acid - ACN	ESI, positive, QQQ	scan, SIR, MRM	nodularin	Spoof et al. (*97*)
C_{18}/phenyl-hexyl, water - MeOH, both containing 0.006% acetic acid	ESi, positive, TOF	scan	nodularin	Maizels and Budde (*143*)
C_{18}, water - ACN, containing 0.01% TFA	ESI, positive, IT	scan, MS^n		Zhang et al. (*144*)
C_{18}, water - ACN, both containing 0.05% TFA	ESI, positive, IT	scan, MS^n	nodularin	Ortea et al. (*145*)
C_{18}, 0.5% formic acid - ACN	ESI, positive, Q	SIR	2 nodularins	Meriluoto et al. (*156*)

3.6.3 Comparison of HPLC-DAD and LC-MS methods for microcystins

HPLC-DAD. This instrumentation is available in most advanced laboratories and the operation of the HPLC-DAD instrument is easily automated and relatively cheap. The HPLC-DAD instrument is also a good quantitative tool which has a fairly uniform response factor for most microcystins as the toxins have the same principal chromophore, the conjugated diene of the Adda moiety. There are two main problems with HPLC-DAD: a) authentic standards which are difficult to obtain are required and b) the sample matrix affects the spectral-based identification of the toxins. The limit-of-detection in HPLC-DAD analyses of microcystins and nodularins is usually less than 1 ng (of each toxin analogue) per injection for pure samples but several times higher for many field samples. Consequently, small peaks of uncommon microcystins in complex samples are easily overlooked. The usefulness of the HPLC-DAD technique is dependent on the sample pre-concentration and elimination of coeluting impurities. HPLC-DAD is the preferred method of individual quantitation of microcystins at higher toxin levels (giving good UV spectra, ca 20 ng/injection or more) but the use of LC-MS is necessary for the trace analyses of individual microcystins. Samples can be concentrated using SPE techniques but the clean-up effect of traditional sorbents is limited as they usually rely on reversed-phase separation, the same mechanism which is used in the analytical separation. The recovery of the different microcystins in SPE varies. Immunoaffinity purification cartridges to microcystin clean-up and concentration are available. These may simplify the HPLC-DAD detection as the very selective immunoaffinity step eliminates most of the co-eluting impurities and thus gives a better S/N ratio.

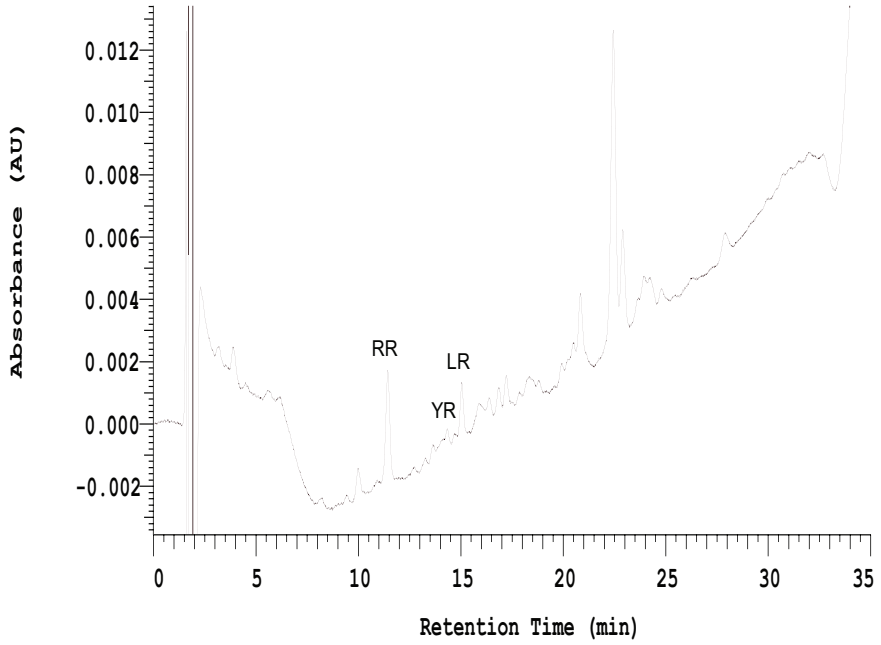

Figure 3.2: HPLC-UV trace at 238 nm of a water sample from a Finnish lake, total microcystin concentration in the lake water was 3.1 µg per litre. Supelco Discovery Amide C_{16} column, 150 mm × 2.1 mm I.D. Mobile phase: 0.05% aqueous TFA (A) and 0.05% TFA in acetonitrile (B) with the following linear gradient: 0 min 20% B, 25 min 65% B, 27 min 65% B, 28 min 20%B. Injection interval 45 min, flow rate 0.3 ml min^{-1} and column temperature 40 ˚C.

LC-MS. Liquid chromatography combined with mass spectrometry offers unsurpassed selectivity and sensitivity in microcystin and nodularin analyses, although this technique also needs reference materials. An instructive comparison of the appearance of chromatograms obtained with a field sample by HPLC-DAD and LC-MS is presented in Figs. 3.2 and 3.3. The extracted ion chromatograms in LC-MS are much simpler to interpret than the chromatograms obtained with HPLC-DAD. Typical detection limits for microcystins and nodularins in modern LC-MS analyses are in the low picogram range per injection. There are less stringent requirements for HPLC separation when MS detection is used (as compared to DAD). Nevertheless, co-eluting substances as well as mobile phase components can cause ion suppression or enhancement effects that are difficult to control in field samples. Many published papers have adopted a strategy of quantification with HPLC-DAD or ELISA and identification by mass spectrometric techniques.

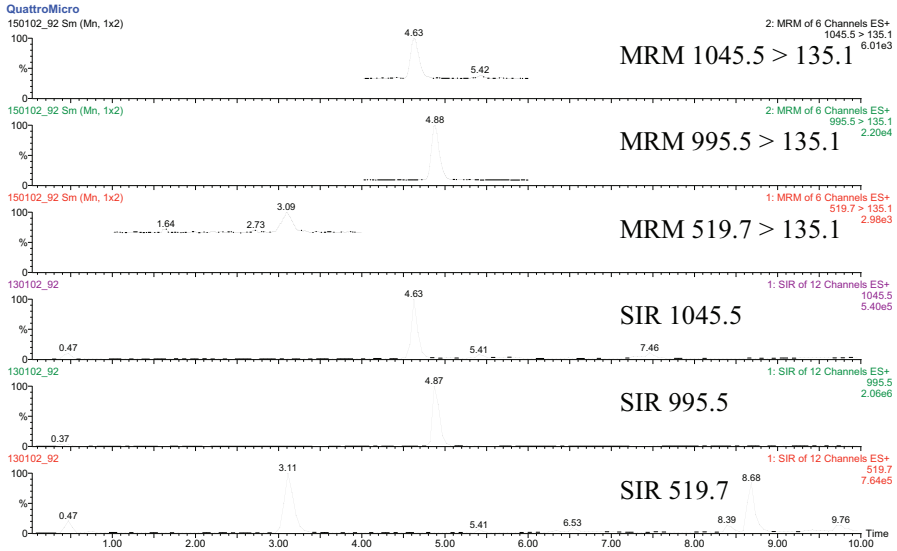

Figure 3.3: Reconstructed ion chromatograms of the same water sample as in Fig. 3.2. MS traces from bottom to top: 1. SIR *m/z* 519.7 (MCYST-RR); 2. SIR *m/z* 995.5 (MCYST-LR); 3. SIR *m/z* 1045.5 (MCYST-YR); 4. MRM *m/z* 519.7 > 135.1 (MCYST-RR); 5. MRM *m/z* 995.5 > 135.1 (MCYST-LR); 6. MRM *m/z* 1045.5 > 135.1 (MCYST-YR). The retention times of the toxins were: microcystin-RR, 3.11 min; microcystin-LR, 4.87 min; microcystin-YR, 4.63 min. Merck Purospher STAR RP-18e column, 30 mm × 4 mm I.D. Mobile phase: 0.5% aqueous formic acid (solvent A) and acetonitrile (solvent B) with the following linear gradient programme: 0 min 25% B, 10 min 70% B, 11 min 70% B, 11.1 min 25%B. Injection interval 17 min, flow rate 0.5 ml min^{-1} and column temperature 40 ˚C. SIR = selected ion recording, MRM = multiple reactant monitoring (see SOP_TOXIC_AAU_10F for a discussion of MS techniques).

3.6.4 Other analytical methods

Thin-layer chromatography (TLC) on silica gel plates has been used as a simple and inexpensive approach for the isolation and detection of microcystins (*52, 120, 146, 147*). Due to the single-use nature of the plates, sample clean-up for TLC does not have to be as rigorous as in many other chromatographic techniques. The relatively high detection limit of the TLC techniques (typically at least several tens of nanograms per spot) requires concentration prior to the analysis. Traditionally the detection of the separated toxins on the TLC plate has been accomplished by UV light (dark spot against the fluorescent background, fluorescence indicator in the silica plate) or by iodine vapour labelling of double bonds. TLC of microcystins and nodularins has been developed further by Ojanperä, Pelander and coworkers. They introduced new solvent systems (*148*), new detection chemistries (*149*) and new instrumental TLC approaches such as overpressured layer chromatography (OPLC) (*150*) for the analysis of microcystins and nodularin.

Capillary electrophoretic (CE) techniques such as capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) are interesting alternatives in the high resolution separation of cyanobacterial peptide toxins (*67, 131, 151-153*). Recently CE has been used for the simultaneous analysis of microcystin-LR, anatoxin-a and cylindrospermopsin by Vasas et al. (*154*). It was concluded that, for complex matrices, a sequential application of both capillary zone electrophoresis and micellar elektrokinetics capillary chromatography is recommended. There are technical obstacles (related to sensitivity and robustness) in the application of the CE techniques to routine analysis, and HPLC can still be regarded as the most powerful analytical tool for cyanobacterial toxins (*153*).

3.6.5 International standardisation of microcystin analyses

ISO, the International Organization for Standardization, has been developing a standard method for the determination of microcystins. The proposed method specifies the extraction of cyanobacterial samples using aqueous methanol, the enrichment of microcystins on reversed-phase SPE cartridges, and the separation of microcystins on reversed-phase HPLC followed by UV detection. For details, the final version of the ISO standard should be consulted. In June 2005, the following information was available on the ISO website, http://www.iso.org : "ISO/FDIS 20179. Water quality -- Determination of microcystins -- Method using solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection. Technical committee / subcommittee: TC 147/SC 2; ISO Standards. ICS: 13.060.50. Status: Under development. Abstract: ISO 20179:2005 specifies a method for the determination and quantification of microcystins in raw water (containing biomass) and treated water, such as tap water. The method described is validated for MCYST-RR, MCYST-YR, and MCYST-LR. It is also applicable for the determination of several structure variants of these microcystins, but an unambiguous identification cannot be made due to the lack of commercially available standards and due to co-elution."

3.7 Method comparison for microcystins

A summary of some general properties of analytical methods for microcystins and nodularins is given in Table 3.4. In critical cases (for example when monitoring drinking water reservoirs) it is beneficial to use complementary methods such as chromatography and ELISA.

Table 3.4: General properties of common methods for the quantitative analysis of microcystins (updated from (*155*))

Method	Low cost	Quick results	Sensitivity	Measures toxicity	Handles simultaneous samples	Also detects novel microcystins	Identifies individual microcystins	No ethical problems
mouse bioassay	+	++		++	+	++		
invertebrate bioassays	++	+		+	++	+		+
PPIA	+	++	++	++	++	++		++
ELISA	+	++	++		++	++		++
HPLC-DAD	+	++	+		++	+	++	++
LC-MS		++	++		++	+	++	++
TLC	++	+	+		++		+	++
MMPB quantitation	+	+	+		+	++		++

++ suitable, property exists; + (minor) problems, has potential; (blank) not suitable, property does not exist

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4 Anatoxin-a

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4.1 Producers, structures and modes of action

Anatoxin-a (Fig. 4.1) is a low molecular weight secondary amine (165 Da). It is a potent neurotoxin and homotropane alkaloid derivative with an enlarged ring (2-acetyl-9-azabicyclo[4,2,1]non-2-ene; *1*). It has been demonstrated to be produced by samples of planktonic freshwater cyanobacteria such as *Anabaena*, *Planktothrix* and *Aphanizomenon*, and by benthic forms (*Oscillatoria*/*Phormidium* sp.), from analysis of laboratory strains and environmental samples (e.g. *2-4*). There have also been reports of the presence of anatoxin-a in brackish waters, such as the Baltic Sea where *Anabaena* can make up part of the bloom of *Nodularia spumigena* (*5*).

Anatoxin-a acts as a post-synaptic cholinergic agonist. Toxicity studies with intraperitoneal injection into mice have determined the LD_{50} to be 200 μg kg^{-1}. Oral toxicity determinations using suspensions of *Anabaena* cells containing anatoxin-a have resulted in LD_{50} values 100-1000 times lower than that obtained with i.p. injection. In addition to anatoxin-a, homoanatoxin-a and 4-hydroxyhomoanatoxin-a have been described (*6, 7*) with modification to the secondary amine and the detection of photodegradation products of anatoxin-a, namely dihydroanatoxin and epoxyanatoxin (*8*). Recognition of the co-occurrence of anatoxin-a with other cyanotoxins has recently been extended. Anatoxin plus microcystins have recently been found in *Arthrospira fusiformis* (*9*) and anatoxin-a with homoanatoxin-a, in *Raphidiopsis mediterranea* (*6*).

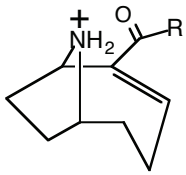

Figure 4.1: Structures of anatoxin-a (R=CH_3) and homoanatoxin-a (R=CH_2CH_3).

4.2 Analytical methods

A number of analytical methods have been used to detect and quantify anatoxin-a and analogues. Due to its high toxicity to mammals via i.p. administration, mouse bioassay via this route has been historically used to assess anatoxin-a. However, with the introduction of more sensitive and specific physicochemical methods, these have been applied to the analysis of anatoxin-a (*10*). As anatoxin-a has an α,β-unsaturated keto group, this allows UV detection of anatoxin-a by HPLC with a lambda max of 227 nm. Using such a method, Edwards et al. (*2*) were able to detect anatoxin-a in stomach contents of dead dogs known to have consumed anatoxin-a-containing *Phormidium* benthic mat material from Loch Insh, Scotland. The use of LC-MS systems for anatoxin-a and homoanatoxin-a has also proved to be successful. Using

an LC-MS system, Viaggiu et al. (*4*) were able to confirm the presence of anatoxin-a in cyanobacterial bloom material (12.1 μg g^{-1}) comprised of *Planktothrix rubescens* in an Italian pond.

In addition to UV absorbance detection, anatoxin-a can be derivatised to a fluorescent product using, for example, pentafluorobenzyl bromide which is substituted onto the secondary nitrogen of anatoxin-a and detected electrochemically after GC, with a limit of detection of 5 ng per ml. Such a method has been applied, for example, to the analysis of anatoxin-a in *Anabaena flos-aquae* cultures and 80 samples from German waterbodies, resulting in the detection of anatoxin-a in 22% of samples (*11*). The ability to make fluorescent derivatives of anatoxin-a has also resulted in the application of solid phase microextraction (SPME) methods for anatoxin-a by fluorescence detection by HPLC. A fluorogenic agent, which reacts with the anatoxin-a was placed on the SPME fibres. The subsequent solutions, when analysed by HPLC resulted in a detection limit of 20 ng ml^{-1} and linearity from 50-1500 ng ml^{-1} (*8*). Furthermore, in addition to SPME methods for anatoxin-a, SPE is also possible for water samples using C_{18} sorbents (*12*).

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5 Cylindrospermopsin

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5.1 Producers, structures and modes of action

Cylindrospermopsin (Fig 5.1) was inferred as being the cause of an outbreak of human hepatic enteritis which occurred on Palm Island in northern Queensland, Australia. Epidemiological analysis indicated that the local water supply was associated with the outbreak. Testing of cyanobacteria from the Palm Island reservoir indicated *Cylindrospermopsis raciborskii* as being toxic by mouse bioassay. Further analysis of cyanobacterial extracts resulted in the elucidation of cylindrospermopsin, a cytotoxic guanidine alkaloid (415 Da). It was initially described as being a hepatotoxin, the liver being the primary organ targeted, although other organs including the kidney, thymus and heart have also been found to be affected by this cyanobacterial toxin. It is now considered that cylindrospermopsin is more of a cytotoxin, with the further ability to cause genetic damage such as DNA strand breakage (*1*).

Of the cyanobacteria tested, *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, *Umezakia natans*, *Raphidiopsis curvata* and *Anabaena bergii* are currently known to include cylindrospermopsin-producers. Similar to anatoxin-a, there are only a limited number of known variants of cylindrospermopsin, namely 7-epicylindrospermopsin (of similar toxicity to cylindrospermopsin) and deoxycylindrospermopsin of lower toxicity. Although first described in Japan in *U. natans* and in Australia in *C. raciborskii*, cylindrospermopsin is now being reported from an increasing number of countries, including Israel, Brazil, Thailand and the USA (see *1*). Although not recognised as a hazard in European water resources until a few years ago, cylindrospermopsin has been reported from two German lakes (*2*) and, in 2004, at one of the EC TOXIC project study sites in Poland. However, cylindrospermopsin production is not always a feature of *C. raciborskii* blooms (*1*). Since examples of cylindrospermopsin-negative blooms from e.g. Portugal, Germany, Hungary and France have been found to be acutely toxic by bioassay (*2-4*) it is clear that blooms of *Cylindrospermopsis* in European waters contain uncharacterised cyanotoxins in addition to potential cylindrospermopsin. Although saxitoxin has been found in Brazilian samples of *C. raciborskii (*e.g. *5*), this neurotoxin does not account for the unknown toxicity in the European *C. raciborskii* bloom samples.

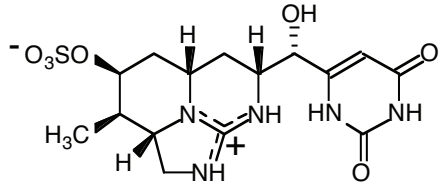

Figure 5.1: Structure of cylindrospermopsin.

5.2 Analytical methods

Concerning the analysis of cylindrospermopsin, intitial studies involved the use of mouse bioassay and indicated the toxicity of cylindrospermopsin to be approximately

200 μg kg^{-1} (e.g. *6*). As with other cyanotoxins, alternatives to the mouse bioassay have been investigated. For cylindrospermopsins, animal invertebrate alternatives such as the Thamnotox kit and the brine shrimp *Artemia salina* provide toxicity assessments with LC_{50} values of 0.7 to 8.1 μg ml^{-1} dependent on the time of exposure to *A. salina* (e.g. *7*). Further toxicity assessment of cylindrospermopsin has found it to be a potent inhibitor of protein synthesis in plants (*8*) and in animals, both *in vitro* (*9*) and *in vivo* (*10*). The ability to inhibit the translation of mRNA into protein by cylindrospermopsin using the rabbit reticulocyte lysate assay system has resulted in the development of sensitive assays for this cyanobacterial toxin with a detection limit of 50 nM cylindrospermopsin (*9*).

In addition to biochemical and biological assessment of cylindrospermopsin, physicochemical methods have also shown promise for the detection and quantification of cylindrospermopsin. One of the most common methods used involves HPLC with UV or diode-array detection. Using diode-array detection cylindrospermopsin can be detected at 262 nm (lambda max) and the characteristic spectrum of this cyanobacterial toxin makes identification relatively easy, as with the microcystins (*11*). The ability to analyse cylindrospermopsin by HPLC has resulted in preliminary exercises concerned with the extraction and analysis of cylindrospermopsin. Of the methods used, all were satisfactory for extraction and analysis (*12*). Other physicochemical methods have employed the use of LC-MS systems and these can be successfully used to monitor the parent and daughter ions of cylindrospermopsin (e.g. *13*).

5.3 Concentration of extracellular cylindrospermopsin

As with other cyanotoxins, methods are required to concentrate cylindrospermopsin from aqueous solution by solid phase extraction. Most cyanotoxins are concentrated by C_{18} sorbents, but these are unsuitable for the concentration of cylindrospermopsin. The most useful solid phase extraction sorbents found to date are graphitised carbon-based and these can successfully concentrate cylindrospermopsin for diode-array detection when present in lake water at a concentration of 1 μg l^{-1} (*14*), a proposed guideline value for drinking water based on the work of Humpage and Falconer (*15*).

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6 Analysis of other cyanobacterial toxins

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6.1 Saxitoxins

Saxitoxins, which comprise a group of at least 21 structurally-related alkaloid neurotoxins are known to be produced by cyanobacteria (strains of *Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Planktothrix*). They are better known as products of certain marine dinoflagellates (*1*) and their main mode of action is via the blockage of mammalian sodium channels resulting in paralysis. Several analytical methods have been applied to their analysis, the most common being the mouse bioassay, as specified in EC shellfish legislation (*2*). As research concerning saxitoxins has progressed, additional analytical methods have been developed. The most commonly employed method is high-performance liquid chromatography (HPLC) with fluorescence detection, using either pre-column (*3*) or post-column oxidation and derivatisation of saxitoxins (*4*). Recent developments have included the application of liquid chromatography-mass spectrometric techniques (LC-MS) including electrospray ionisation-LC-MS (*5*) and hydrophilic interaction LC-MS (*6*). In addition to physicochemical methods, biologically-based methods have also been developed to analyse saxitoxins. These have included the development of antibodies against saxitoxin and neosaxitoxin (*7*), the use of saxiphilin, a saxitoxin-binding protein in an *in vitro* assay (*8*), the use of cell bioassays such as the mouse neuroblastoma cell line (*9*) and more recently, the introduction of a commercial saxitoxin immunoassay, MIST Alert (Jellet Biotek Ltd., Dartmouth, Canada) for the analysis of saxitoxins. A review of saxitoxin research including analytical methods is given by Lehane (*2*).

6.2 Anatoxin-a(S)

Anatoxin-a(S) is a naturally occurring organophosphate molecule, known to be produced by certain *Anabaena* species (*10*). Its mode of action is via the inactivation of acetylcholine esterase, resulting in rapid neurotoxicity and the occurrence of hypersalivation. As with the saxitoxins, assessment of this cyanobacterial toxin has traditionally been by the mouse bioassay, but alternatives have been sought. As there is currently no known chromophore in the anatoxin-a(S) molecule, physicochemical methods including HPLC have rarely been successful for the analysis of this cyanotoxin. Instead, as anatoxin-a(S) is known to inactivate acetylcholine esterase (*11*) this knowledge has been used to develop colorimetric assays which have been successfully applied to the analysis of anatoxin-a(S) (*12*). One drawback of this approach is that all known organosphosphorous pesticides and insecticides will also inhibit this *in vitro* assay. Devic *et al.* (*13*) have taken the approach of engineering mutant acetylcholine esterase enzymes to successfully improve the sensitivity and specificity of the method for anatoxin-a(S) analysis and apply their procedure to the analysis of cyanobacterial bloom samples.

6.3 Lipopolysaccharide (LPS)

Gram negative bacteria including cyanobacteria produce lipopolysaccharide, an outer constituent of the cell wall. Of the 3 main parts of the LPS molecule, the lipid A region is responsible for the production of fever, diarrhoea, vomiting and hypotension

in exposed people. LPS associated with cyanobacterial blooms has been inferred as causing gastrointestinal illness such as in Sewickley, USA that affected 62% of a human population using drinking water from a source containing a cyanobacterial bloom (*14*). For the analysis of cyanobacterial LPS, original methods employed pyrogenic reactions in test animals exposed to bacterial LPS. More recently, the *Limulus* amoebocyte lysate assay (LAL) has been used to detect bacterial (and cyanobacterial) LPS either using a gel clot method or the colorimetric method. Using the latter, Rapala *et al*. (*15*) were able to measure endotoxin (LPS) concentrations in raw waters and examine the effectiveness of drinking water treatment processes to remove these toxins. Cyanotoxin research has also been aided by the use of LAL, such as for determining the most efficient method to extract LPS from cyanobacteria (*16, 17*). Although LPS is considered to be a cyanotoxin, more likely to result in illness rather than death, the increasing use of methods such as LAL will assist in the proper risk assessment of this toxin.

6.4 References

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7 Selection of analytical methodology

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This chapter provides schematic workflows for the analyses of microcystins, anatoxin-a and cylindrospermopsin. High-performance liquid chromatography (HPLC) targets individual analytes and it requires access to authentic toxin standards for analyte identification. Protein phosphatase inhibition assay (PPIA) and enzyme-linked immunosorbent assay (ELISA) are group-specific, highly sensitive screening methods. In addition to microcystins and nodularins, PPIA also detects other protein phosphatase inhibitors. For an introduction to the methods, please refer to Chapters 3-5. The actual SOPs are found in Chapter 9.

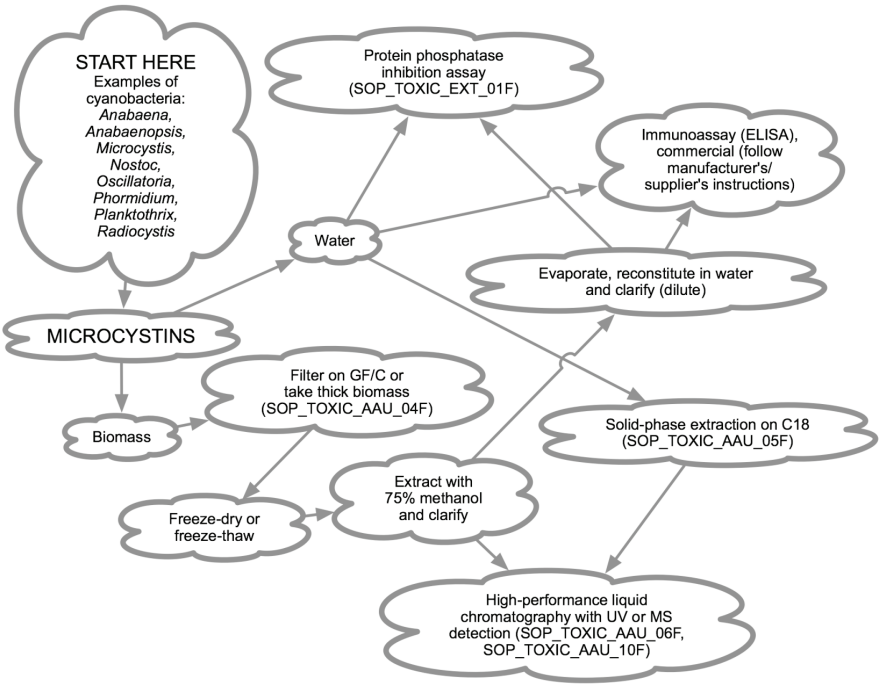

Figure 7.1: Workflow for the analysis of microcystins.

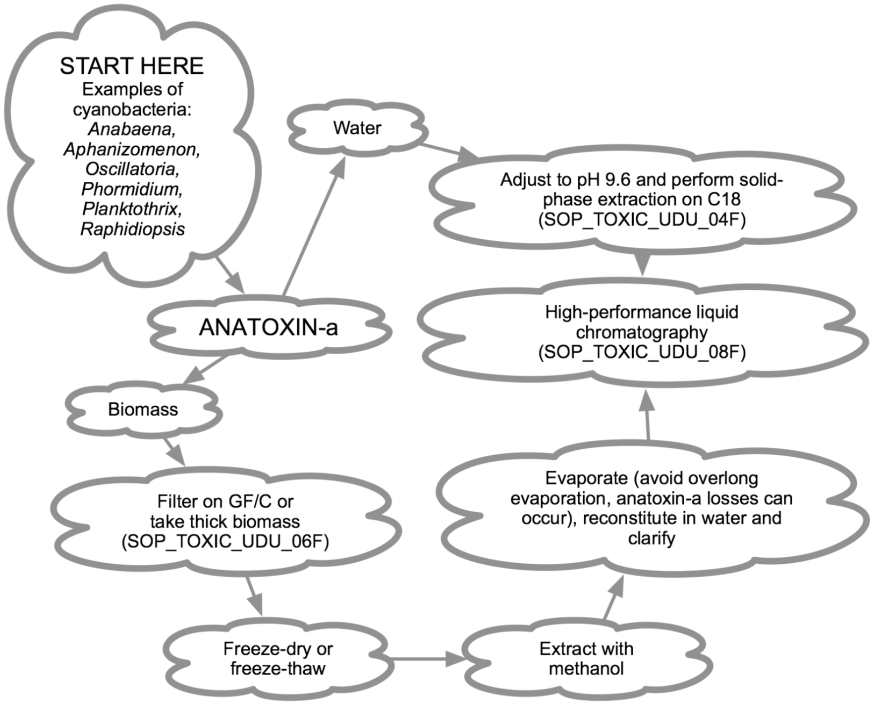

Figure 7.2: Workflow for the analysis of anatoxin-a.

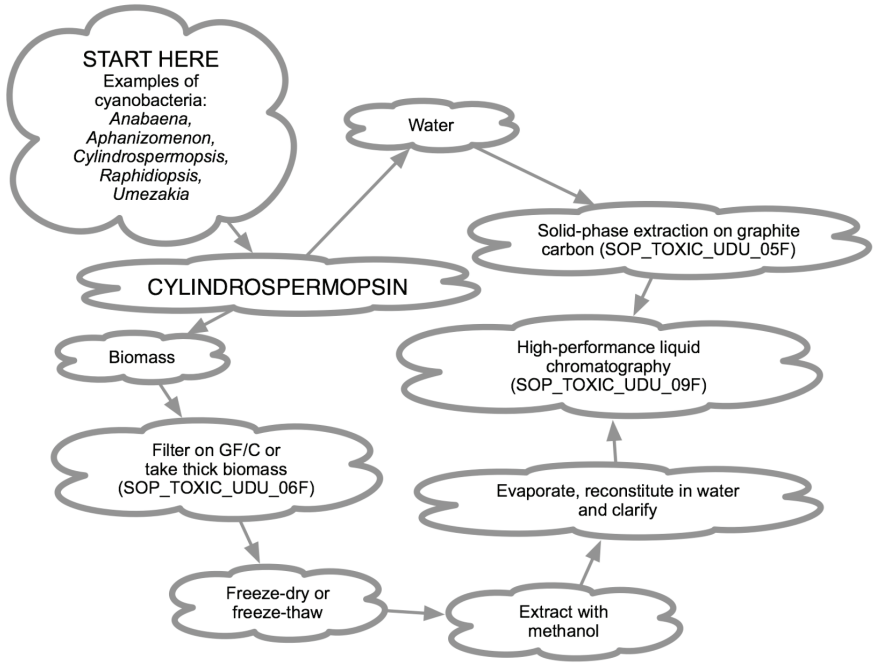

Figure 7.3: Workflow for the analysis of cylindrospermopsin.

8 Applications and significance of results

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8.1 Introduction

The monitoring of waterbodies for cyanobacteria and analysis for cyanotoxins are carried out for several reasons, ranging from basic research, through to quality and safety assessment of raw waters for e.g. recreation, aquaculture and potential potable supply, and finally to that of the quality and safety assurance of treated water for drinking. Monitoring and analysis schemes vary widely in their scope and complexity, from single samples from a waterbody taken e.g. once per year, to intensive programmes. The latter may involve frequent sampling e.g. several times per week, depth profiling and/or multiple sites at an individual waterbody and treatment train. The design of these monitoring and analysis programmes greatly influences the value of the results, both scientifically and in terms for their value for the risk management of raw and treated waters *(1)*. Furthermore, the justifiable interpretation of monitoring and analysis data requires adequate background information on the waterbody, and the details of the monitoring and analytical procedures used.

8.2 Modes of monitoring and analysis

The monitoring of cyanobacterial populations and analysis of cyanotoxins typically requires scientific and technical expertise, transportation, laboratory materials and reagents, and dedicated equipment, from simple to advanced. To justify these capital demands, and help ensure that the results are amenable to interpretation for valid risk management, it is useful to consider the circumstances under which monitoring and analysis are performed (modes) and the reasons why they are carried out. Monitoring can be carried out in a reactive, or proactive mode, although the latter may also be modified in reaction to events, if it is already in place.

Reactive sampling and analysis may be carried out in situations where no routine or proactive system is already in place. This may be in response to:

- the unexpected development of excessive cyanobacterial populations
- health incidents involving humans, animals, birds or fish, which are (suspected to be) associated with cyanobacteria
- changes in performance characteristics during drinking water treatment
- complaints or requests from water-users, including from an increasingly aware public.

Reactive monitoring is more likely than a structured proactive system to be an *ad hoc* procedure. The reactive approach is, by definition post-event, and is more likely to be incomplete due to lack of comprehensive samples and supporting data. Nevertheless, it has been repeatedly shown that reactive sampling and analysis can make valuable contributions to the protection of health and water resources where no proactive system is in place (for examples of reactive investigations to cyanobacteria- and cyanotoxin-associated health incidents, see *2*).

Proactive (structured) monitoring and analysis is a preferable strategy where there has been a history of cyanobacterial mass population development, where associated problems continue, or may be expected in the future. This mode of monitoring is:

- necessary in any system designed to provide warning before a developing cyanobacterial population presents operational problems to water treatment, environmental problems to aquatic biota and to human health via drinking or recreational water.
- required in water management systems based on alert-levels which trigger actions designed to reduce cyanobacterial development, exposure to cyanotoxins, and to protect water supplies and health.
- required in increasing numbers of risk management and health protection policies, from local to national level, which are being defined by guidelines or legislation.

In principle, the validity of the results of cyanotoxin analysis as generated in the laboratory, and increasingly in future at the lakeside, should not be influenced by whether the samples were collected by reactive or proactive sampling: the same analytical reagents, calibrators, methods and equipment may be used. In practice, however, proactive programmes usually provide a more comprehensive situation assessment of past and present conditions and provide greater confidence in the interpretation of results. Examples of the roles of structured cyanobacterial cell and cyanotoxin sampling and analysis in decision-tree frameworks are available (*3,4*). These include alert levels for intervention and action to reduce exposure of water-users and water-consumers to toxin-producing cyanobacteria and cyanotoxins. Databases of the results of proactive cyanobacterial and cyanotoxin analyses are increasing in the environmental agency and water utility sectors and via research programmes, e.g. (*5*).

8.3 Aims of monitoring and analysis and applications of results

Cyanobacterial taxa, their abundance and location have been determined for many decades by classical limnologists. However, the aims of such monitoring with reference to the nuisance and harmful effects of cyanobacterial blooms and cyanotoxins include determination of:

- the taxonomic, temporal and spatial development of the cyanobacterial population. The results provide an indication of the types and concentrations of cyanotoxins which may be potentially produced and of the severity of the risks to health which may occur (Table 8.1).
- the stage of development of the cyanobacterial cells. If the cyanobacteria are at an early stage of vegetative growth and do not show signs of cell breakdown (lysis), then for microcystins and nodularins, it may be assumed that most of the total toxin pool is present in the producer-cells, with relatively little (e.g. <10%) being in extracellular, dissolved form. If, however, microcystin- or nodularin producer-cells are in a state of cell breakdown, whether under natural conditions or due to human intervention (e.g. algicide treatment or mechanical stress) then most or virtually all of the microcystin or nodularin pool can be extracellular. Relations beween cyanobacterial cell structural integrity and the compartmentation of anatoxin-a, anatoxin-a(S) and the saxitoxins are not so clear, although since all of these toxins are small water-soluble products, they are likely to occur naturally in intra- and extracellular

dissolved fractions. Cell integrity cannot be interpreted to indicate whether cylindropsermopsin is likely to be largely intra- or extracellular since a large percentage (e.g. >50%) of the total pool of this toxin is present in extracellular form in apparently intact cultures of producer-cells (*6, 7*). Compartmentation of the cyanotoxins between cells and water is likely to influence the ability to remove or degrade the toxins during water treatment.

- whether warning thresholds or guidelines on maximum acceptable cyanobacterial cell populations are being exceeded, if these are being used in risk management.
- whether responses of water bodies and water supplies to intervention to reduce cyanobacterial populations in raw waters and during water treatment are being effective. The interventions range from catchment or basin-level, to in-lake and water treatment methods (*9, 10*).

Table 8.1: Examples of cyanobacterial genera in fresh- and brackish water as indicators of potential cyanotoxin production

Cyanotoxin	Toxigenic genera[a,b]
Microcystins	*Microcystis, Anabaena, Nostoc, Anabaenopsis, Planktothrix, Oscillatoria, Phormidium, Radiocystis*
Nodularins	*Nodularia*
Cylindrospermopsins	*Cylindrospermopsis, Umezakia, Aphanizomenon, Anabaena, Raphidiopsis*
Anatoxin-a and homoanatoxin-a	*Anabaena, Planktothrix, Oscillatoria, Phormidium, Aphanizomenon, Raphidiopsis*
Anatoxin-a(S)	*Anabaena*
Saxitoxins	*Aphanizomenon, Anabaena, Lyngbya, Cylindrospermopsis, Planktothrix*
LPS	all?

a, See (*2, 8*); b, not all species in a genus include toxigenic members and in some genera only 1 species is so far known to include toxigenic strains.

In locations where resources for cyanotoxin analysis or necessary expertise are not available, or where the number of waterbodies or samples is excessive, or where the waterbodies in question are not priority resources, then cyanotoxin analysis may not be possible or justified. In these cases, the monitoring of cyanobacterial cells assumes an added significance as an indicator of the potential toxicological status of the waterbody and of associated potential health risks. Trigger levels of cyanobacterial cell numbers in action plans (Table 8.3) are presently based upon the potential concentration of microcystins which could be associated with the cyanobacterial cell numbers, and upon a small number of reports of investigations into incidence of human illness (or otherwise) associated exposure to water containing cyanobacteria of known cell number (*11-13*).

The determination of cyanobacterial taxa, cell concentrations, cyanobacterial biovolume and chlorophyll-a concentrations (if cyanobacteria are dominant in the waterbody) can provide early warning of actual cyanobacterial presence and of potential cyanotoxin production. This indicator approach can be complemented by the detection of genes for microcystin and cylindrospermopsin synthesis using the polymerase chain reaction (PCR) with extracted cyanobacterial DNA, and by

fluorescent *in situ* hybridisation (FISH) to detect cyanotoxin genes in cyanobacterial cells by microscopy. However, the use of such surrogates for the actual presence and concentrations of cyanotoxins needs to be treated with caution since: (a) the extent of cyanotoxin production by individual genera and species is not fully understood; (b) knowledge of cyanotoxin concentrations per cyanobacterial cell or cell biovolume is lacking for those toxins other than microcystins; (c) genes for the production of cyanotoxins other than microcystins, nodularins and cylindrospermopsins are not known; and (d) for the latter cyanotoxins, it cannot be assumed that the genes are always expressed. Thus for situation assessment of the actual presence and concentrations of cyanotoxins, cyanotoxin analysis is necessary.

Analytical data on cyanotoxins, if complemented by environmental, water treatment, or health data, can provide information:

- on the presence of individual types, concentrations and compartmentation of cyanotoxins in waterbodies, water treatment plants and drinking water distribution systems.
- on relations between cyanotoxin production and environmental conditions.
- on compartmentation and persistence in raw waters and during water treatment. (In addition to varying distribution between intra- and extracellular compartments, cyanotoxins are susceptible to varying degrees to chemical, bio- and photo-degradation.)
- on relations between cyanotoxins, water quality and health, with reference to exposure levels of humans and animals.
- to contribute to the derivation of cyanotoxin guideline values for drinking water quality and their incorporation into alert level schemes for water monitoring and drinking water abstraction and treatment.
- to determine whether monitoring and decision-making schemes are appropriate or require upgrading.
- to ensure compliance with national or regional guidelines, or cyanotoxin legislation.

8.4 Significance of cyanobacterial cell and cyanotoxin data and health risks

Knowledge of the adverse effects of cyanobacteria and cyanotoxins on human and animal health and on ecosystem "health", including aquatic community biodiversity, includes many gaps. However, the toxicities of examples of all of the cyanotoxin groups listed in Table 8.1 have been investigated to various extents in animal bioassays and to a lesser degree using cell cultures and enzymes. Furthermore, members of all of the listed cyanotoxin groups have been implicated in, or responsible for, deaths or illness in humans and animals via exposure from waterborne exposure (*2, 14, 15*). Detection, therefore, of any of the listed cyanotoxins in analyses of raw or treated water samples requires risk assessment of the significance of the results in terms of the use made of the water, the cyanotoxin concentration and the likelihood and extent of human or animal exposure. Potential exposure media, including water, and exposure routes are summarised in Table 8.2. Guideline levels for cyanobacterial cells and chlorophyll-a concentration in recreational waters are influenced by likely length of exposure and are provisional. They are designed to reduce the risk of potential adverse health outcomes and are summarised in Table 8.3.

Table 8.2: Potential exposure media and exposure routes leading to adverse health effects of cyanotoxins[a]

Exposure medium	Exposure route	At-risk activities/groups
Water	Oral	Drinking raw and treated water, incidental ingestion during water-based recreation
Water	Dermal	Water-based recreation, work practices, showering, bathing
Water	Inhalation	Water-based recreation, work practices, showering
Water	Haemodialysis	Haemodialysis patients
Food	Oral	Consumption of fish, shellfish, waterfowl[b] which may contain waterborne cyanotoxins. Consumption of plant foods previously exposed to cyanotoxins via irrigation
Dietary supplements	Oral	Consumption of cyanobacteria-containing products if containing cyanotoxins

a, Summarised from (*16*); b, (*17*)

Table 8.3: Provisional guideline levels for cyanobacterial cells in bathing waters

Risk	Potential for adverse health outcomes	Guideline level		Potential microcystin concentration
		Cells/ml	µg chl-a / litre	
HIGH	Acute poisoning, long-term illness, short-term or mild illness	Scums, detached mats		>1 mg l^{-1}
MEDIUM	Long-term illness, short-term or mild illness	100000	50	10-20 µg l^{-1} (50 µg l^{-1} possible)
LOW	Short-term or mild illness	20000	10	2-4 µg l^{-1} (10 µg l^{-1} possible)

Condensed from (*15*)

Sufficient oral toxicity data on cyanotoxins in mammals to permit guideline values (GVs) to be derived with some confidence for human drinking water are only available for microcystin-LR (*12*) and more recently, for cylindrospermopsin (*19*). The microcystin-LR GV of 1 µg/litre, derived by working parties of the World Health Organization (*12, 18*) is now widely known. A similar GV for cylindrospermopsin in drinking water may be appropriate (*19*). Since microcystin-LR appears to be one of the most toxic of known cyanotoxins to mammals, and adequate GV for other cyanotoxins are currently lacking, then it seems prudent to apply the microcystin-LR GV to other cyanotoxins until further data are available.

Inevitably, the significance of cyanotoxin data is being interpreted in terms of the microcystin-LR GV. However, the interpretation of such data for risk management is a developing practice. Several countries throughout the world have already adopted or adapted the WHO GV for microcystin-LR into national water legislation. Others prefer to use the GV for guidance only, taking into account that safety factors are built into GV derivation (*12, 15*). It is not appropriate here to favour the regulatory or

guidance approach, but rather to emphasise that the purpose and scope of a GV for drinking water (*18*) needs to be recognised. Thus a cyanotoxin drinking water GV is:

- an estimate of the concentration of cyanotoxin which would not result in a significant risk to a consumer over a lifetime of drinking water consumption.
- advisory.
- derived to accommodate uncertainties and safety factors in its derivation.
- provisional and subject to revision in response to further advances in basic knowledge and practical experience.
- not intended as a recommended concentration to which cyanotoxin-containing water can be allowed to degrade.
- a tool for use in the development and application of cyanotoxin risk management approaches, taking into account practicality, feasibility and the protection of health and water resources.

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9 List of standard operating procedures (SOPs)

SOP: Protein phosphatase 1 inhibition assay, colorimetric

Document identifier: SOP_TOXIC_EXT_01F

Prepared by: Jussi Meriluoto and Lisa Spoof, AAU

Date: 30 June 2005

1 Introduction

Protein phosphorylation-dephosphorylation is a major regulatory mechanism in cell growth, proliferation, differentiation and physiology. Protein phosphatase 1 (PP1) releases phosphate groups from phosphoserine and phosphothreonine residues in proteins. Activity of PP1 is inhibited by microcystins and nodularins. Some recombinant forms of PP1 also have activity towards artificial substrates such as *p*-nitrophenyl phosphate. Additional protein phosphatases, e.g. PP2A, can be used in this form of assay. Cleavage of the phosphate group from *p*-nitrophenyl phosphate results in the yellow product *p*-nitrophenol, and the colour production in this reaction is inversely proportional to the microcystin concentration in the sample.

2 References

Please follow the procedures reported in e.g. the following publications.

An, J. S., Carmichael, W. W.: Use of colorimetric protein phosphatase inhibition assay and enzyme linked immunosorbent assay for the study of microcystins and nodularins. Toxicon **32**, 1494-1507 (1994).

Rapala, J., Erkomaa, K., Kukkonen, J., Sivonen, K., Lahti, K.: Detection of microcystins with protein phosphatase inhibition assay, high-performance liquid chromatography-UV detection and enzyme-linked immunosorbent assay. Comparison of methods. Anal. Chim. Acta **466**, 213-231 (2002).

Ward, C. J., Beattie, K. A., Lee, E. Y. C., Codd, G. A.: Colorimetric protein phosphatase inhibition assay of laboratory strains and natural blooms of cyanobacteria: comparisons with high-performance liquid chromatographic analysis for microcystins. FEMS Microbiol. Lett. **153**, 465-473 (1997).

Wirsing, B., Flury, T., Wiedner, C., Neumann, U., Weckesser, J.: Estimation of the microcystin content in cyanobacterial field samples from German lakes using the colorimetric protein-phosphatase inhibition assay and RP-HPLC. Environ. Toxicol. **14**, 23-29 (1999).

SOP: Control of spectrophotometer absorbance

Document identifier: SOP_TOXIC_EXT_02F

Prepared by: Jussi Meriluoto and Lisa Spoof, AAU

Date: 30 June 2005

1 Introduction

The correct function of the spectrophotometer is essential for quantitative calibration purposes described in the TOXIC SOPs. Spectrophotometry is based on the laws of Lambert and Beer which define the relationship between the radiation intensities incident on and transmitted by a layer of an absorbing medium. The measured absorbance (A) is proportional to the path length (*b*) through which the radiation passes and to the concentration (*c*) of the substance in solution in accordance with the equation:

$A = \varepsilon \times c \times b$

ε = molar absorptivity; if *b* is expressed in centimetres and *c* in moles per litre.

2 Experimental

Calibrate the spectrophotometer function according to official standardised methods. In the TOXIC project the absorbance response of the spectrophotometer was controlled by using the potassium dichromate method described in the European Pharmacopoeia.

3 References

Anon.: Absorption spectrophotometry, ultraviolet and visible. In: European Pharmacopoeia, Fifth ed., Vol. 1, pp. 38-39. Directorate for the Quality of Medicines of the Council of Europe, Strasbourg (2004).

SOP: Preparation of standard solutions of microcystin-LR for HPLC calibration

Document identifier: SOP_TOXIC_AAU_03F

Prepared by: Jussi Meriluoto and Lisa Spoof, AAU

Date: 30 June 2005

1 Introduction

Quantitative calibration of HPLC systems with microcystin-LR is based on the spectrophotometric determination of microcystin-LR concentration. The recorded absorbance is converted into microcystin-LR concentration using the known molar absorptivity.

2 Experimental

2.1 Materials

(a) Microcystin-LR, 2X grade (two times purified on HPLC). N.B. Microcystin-LR, 2X grade can contain some coeluting impurities such as a trace of demethyl-microcystin-LR (usually less than 2% of microcystin-LR concentration). Refer to SOP_TOXIC_AAU_09F for microcystin purification.

(b) Methanol HPLC grade

(c) HPLC grade water purified to 18.2 MΩ cm

(d) Clean borosilicate glass test tubes or vials, 4 ml capacity

(e) Borosilicate glass chromatographic vials: e.g. 1.5 ml clear glass with writing surface. For small sample volumes 0.3 ml polypropylene vials (only for samples in 75% methanol) or borosilicate glass inserts can be used.

2.2 Special equipment

(a) Pipettes capable of accurately dispensing 0.5 ml – 4 ml of 75% methanol.

(b) HPLC instrument, specifications according to the procedure SOP_TOXIC_AAU_06F

2.3 Preparation of standard curve for microcystin-LR

The following standard curve can be applied for samples containing 1-100 ng microcystin-LR / 10 µl injection.

(a) Mix 75 volumes of methanol and 25 volumes of water to make 75% methanol.

(b) Check spectrophotometer absorbance according to the procedure SOP_TOXIC_EXT_02F.

(c) Dissolve ca 30 µg of microcystin-LR, 2X grade, in 3 ml of 75% methanol. Dissolve another sample of 30 µg microcystin-LR, 2X grade, in 4 ml of 75% methanol. Mix well and measure the absorbance of the solutions at 238 nm using 75% methanol as reference. The molar absorptivity of microcystin-LR in 100 % methanol has been reported to be 39800 [Harada et al., 1990], and the molar absorptivity in 75% methanol is practically the same. An absorbance reading of 0.40 equals 10.05 µg microcystin-LR per ml.

(d) Prepare a dilution series of the spectrophotometrically determined solutions of microcystin-LR. Mix thoroughly 500 µl of microcystin-LR solution with 500 µl of 75% methanol. Make further five dilutions, 1 volume + 1 volume, in a similar manner. Your dilutions including the original solution should then cover approximately the range 1-100 ng microcystin-LR / 10 µl injection.

(e) Analyse the samples on the HPLC system in duplicate injections. If you have several columns to calibrate you can divide the microcystin-LR dilutions in aliquots into 0.3 ml polypropylene vials designed for smaller volumes. Store the quantitative calibrants refridgerated and use within one day from preparation.

(f) Calculate the linear regression for your calibration curve using e.g. calculator or Microsoft Excel software.

$y = mx + b$

y = ng microcystin-LR per injection
x = peak area

The slope of the calibration line, m, gives the response factor, which is characteristic for your specific chromatographic conditions. The y-axis intercept, b, should be negligible (typically below 0.3) and the correlation coefficient, R^2, should approach 1.

(g) Monitor column performance regularly by injection of known samples and repeat the calibration as necessary, typically after 1 or 2 months (depending on the amount of samples, solvents used etc).

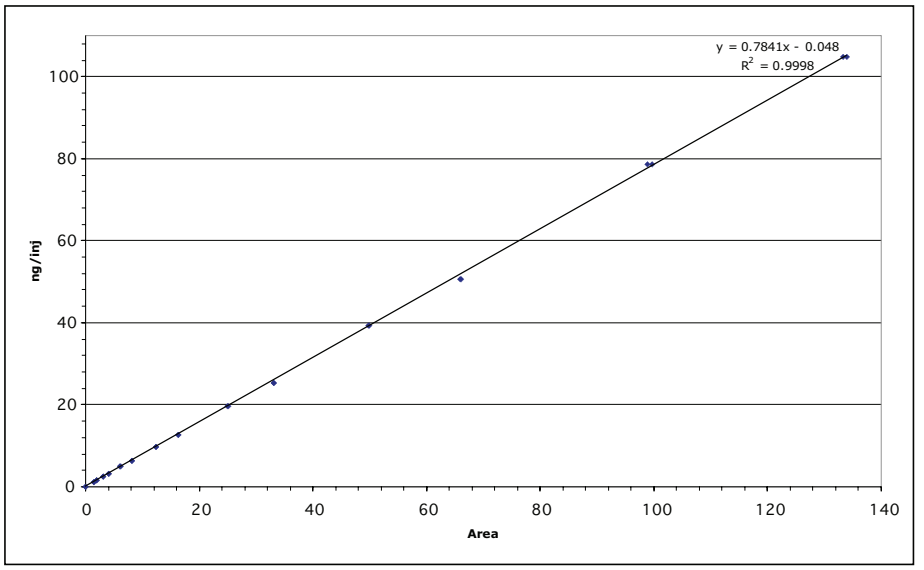

Figure 1: Example of a calibration curve. Column Merck Purospher STAR RP-18e 55 mm x 4 mm I.D. + guard column, run conditions according to TOXIC_SOP_AAU_06F, Agilent 1100 HPLC components and Chemstation software, the diode-array detector equipped with a semi-micro flow cell.

3 References

Harada, K.-i., Matsuura, K., Suzuki, M., Watanabe, M.F., Oishi, S., Dahlem, A.M., Beasley, V.R., Carmichael, W.W.: Isolation and characterization of the minor components associated with microcystins LR and RR in the cyanobacterium (blue-green algae). Toxicon **28**, 55-64 (1990).

SOP: Extraction of microcystins in biomass filtered on glass-fibre filters or in freeze-dried cyanobacterial biomass

Document identifier: SOP_TOXIC_AAU_04F

Prepared by: Jussi Meriluoto and Lisa Spoof, AAU

Date: 30 June 2005

1 Introduction

Water samples containing cyanobacterial cells can be filtered on glass-fibre filters (GF/C, diameter 25-47 mm) for monitoring of intracellular (biomass-bound) toxins. After freeze-thawing or freeze-drying the filters can be extracted by ultrasonication in 75% methanol.

2 Experimental

2.1 Materials

(a) Glass-fibre filters, equivalent to Whatman (Maidstone, UK) GF/C, diameter 25-47 mm

(b) Methanol HPLC grade

(c) HPLC grade water purified to 18.2 MΩ cm

(d) Argon or nitrogen, >99.99%

(e) Borosilicate glass tubes, 12 mm x 75 mm

(f) Parafilm

(g) Microcentrifuge tubes, polyethylene or polypropylene, narrow models preferred

(h) Borosilicate glass chromatographic vials: e.g. 1.5 ml clear glass with writing surface. For small sample volumes 0.3 ml polypropylene vials (only for samples in 75% methanol) or borosilicate glass inserts can be used.

(i) HPLC grade filters showing no adsorption of microcystins from 75% methanol, equivalent to GHP Acrodisc filters, 13 mm in diameter and 0.2 µm pore size, product number 4554, Pall Life Sciences (Ann Arbor, MI, USA), and 2-ml all-plastic (no rubber piston) single use syringes (alternative sample clarification procedure to centrifugation)

2.2 Special equipment

(a) Bath ultrasonicator, comparable to Branson 2510E-MT, Danbury, CT, USA

(b) Probe ultrasonicator, comparable to Sonifier II W-250 from Branson/Emerson Technologies, Dietzenbach, Germany

(c) Freeze-drying equipment (optional)

(d) Microcentrifuge

(e) Heater block with evaporation unit

2.3 Solutions

(a) Mix 75 volumes of methanol and 25 volumes of water to make 75% methanol.

2.4 Extraction procedure

(a) Place the air-dried, frozen filter samples (GF/C, diameter 25mm) in 12 mm x 75 mm borosilicate glass tubes or in 1.5 ml borosilicate glass vials, and freeze-thaw them twice, or, to obtain maximum recovery, freeze-dry the filters. The extraction procedure described below is also suitable for the extraction of 5-8 mg samples of freeze-dried cyanobacteria.

(b) Add 1.2 ml of 75% methanol, mix and extract in the bath ultrasonicator for 15 min. Filters with a diameter of 47 mm are extracted with 2 ml of 75% methanol in the glass tubes. The tubes should be covered with parafilm during the incubation. If vials are used, they should be fitted with caps during the bath ultrasonication.

(c) Ultrasonicate the samples further, one at a time, with the ultrasonic disruptor equipped with a microtip probe, for 1 min. Wash the probe with 75% methanol in between samples. In the case of the Branson Sonifier II W-250, the unit is operated in a pulsed mode with a 30 % duty cycle and with an output control of 2. Avoid extensive fibre loss from the filter. Higher output effects necessitate the use of cooling around the sample with an ice bath.

(d) Centrifuge aliquots of the extracts at 10,000 × *g* for 10 min.

(e) Transfer 500 µl of the supernatants to 1.5 ml borosilicate glass vials or clean 12 mm x 75 mm borosilicate glass tubes and evaporate to dryness in a heating block at 50 ˚C under argon. If desired, take further aliquots.

(f) Reconstitute the dried extracts in 100 µl of 75% methanol, mix well, transfer to 0.3 ml polypropylene vials, centrifuge at 10,000 × *g* for 10 min or filter through the HPLC grade filter to clarify the sample if necessary, and analyse with high-performance liquid chromatography (HPLC) according to the procedure SOP_TOXIC_AAU_06F.

N.B. The efficiency of ultrasonication varies considerably between different ultrasonicator models. Therefore, the above settings and times must be adjusted according to the apparatus in use.

3 References

Lawton, L.A., Edwards, C., Codd, G.A.: Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. Analyst (London) **119**, 1525-1530 (1994).

Fastner, J., Flieger, I., Neumann, U.: Optimized extraction of microcystins from field samples - a comparison of different solvents and procedures. Water Res. **32**, 3177-3181 (1998).

Spoof, L., Vesterkvist, P., Lindholm, T., Meriluoto, J. Screening for cyanobacterial hepatotoxins, microcystins and nodularin, in environmental water samples by reversed-phase liquid chromatography-electrospray ionisation mass spectrometry. J. Chromatogr. A **1020**, 105-119 (2003).

N. B. The reader is also advised to follow up the development of the ISO standard for microcystin analysis.

ISO/FDIS 20179: Water quality - Determination of microcystins - Method using solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection (2005).

SOP: Solid phase extraction of microcystins in water samples

Document identifier: SOP_TOXIC_AAU_05F

Prepared by: Jussi Meriluoto and Lisa Spoof, AAU

Date: 30 June 2005

1 Introduction

Solid phase extraction of microcystins in natural or processed waters is typically performed on C_{18} silica cartridges. Some polymeric materials such as the OASIS HLB can also be useful for the purpose. Chlorinated waters are usually treated with sodium thiosulphate before concentration in order to stop the further degradation of microcystins. Use borosilicate glassware throughout the procedure whenever possible because microcystins in water solutions are easily adsorbed onto plastics commonly used in laboratories. Microcystin-LR in a solution of above 25% methanol was found not to interact with plastics to any great extent [Hyenstrand et al., 2001].

2 Experimental

2.1 Materials

Use analytical reagent grade reagents if not indicated otherwise.

(a) Methanol HPLC grade

(b) HPLC grade water purified to 18.2 MΩ cm

(c) Glass-fibre filters, equivalent to Whatman (Maidstone, UK) GF/C, diameter 25-47 mm

(d) Sodium thiosulphate

(e) NH_4OH

(f) Trifluoroacetic acid (TFA), HPLC or protein sequence analysis grade. TFA should be stored under argon in a desiccator.

(g) Reversed-phase solid phase extraction columns suitable for microcystin work, equivalent to Isolute C18(EC) solid phase extraction columns, size 1 g sorbent in 6 ml reservoir, part number 221-0100-C, from Argonaut Technologies (Mid Glamorgan, UK)

(h) Argon or nitrogen, >99.99%

(i) Borosilicate test tubes or vials, >3 ml capacity

(j) HPLC grade filters showing no adsorption of microcystins from 75% methanol, equivalent to GHP Acrodisc filters, 13 mm in diameter and 0.2 µm pore size, product number 4554, Pall Life Sciences (Ann Arbor, MI, USA), and 2 ml all-plastic (no rubber piston) single use syringes (alternative sample clarification procedure to centrifugation)

(k) Microcentrifuge tubes, polyethylene or polypropylene, narrow models preferred

(l) Borosilicate glass chromatographic vials: e.g. 1.5 ml clear glass with writing surface. For small sample volumes 0.3 ml polypropylene vials (only for samples in 75% methanol) or borosilicate glass inserts can be used.

(m) Acetonitrile gradient HPLC grade

2.2 Special equipment

(a) Vacuum manifold, preferably transparent, equipped with stopcocks, vacuum source and vacuum control

(b) Large volume extraction (LVE) kit for unattended loading of large sample volumes, made of PTFE tubing and adaptors for SPE column connection

(c) pH meter

(d) Filtration unit for 500 ml volume

(e) Heating block, operated at 50 ºC, with evaporation unit

(f) Microcentrifuge (alternative to filtration)

2.3 Solutions

(a) Sodium thiosulphate, 1 g in 100 ml HPLC grade water

(b) TFA, 1% solution in HPLC grade water

(c) NH_4OH, 2 g in 100 ml HPLC grade water

(d) 20% methanol (20 volumes of methanol and 80 volumes of water)

(e) 75% methanol (75 volumes of methanol and 25 volumes of water)

(f) Acetonitrile containing 0.05% TFA (for preparation, see SOP_TOXIC_AAU_06F)

2.4 Procedure

(a) Filter the water sample through the glass-fibre filter if it contains particulates or cyanobacterial cells.

(b) Transfer 500 ml of water sample into a borosilicate glass flask or beaker.

(c) Measure the pH of the water sample, and, if necessary, adjust to pH 5-8 with dilute NH_4OH or TFA.

(d) In case of chlorinated water: add 500 µl of sodium thiosulphate solution (1 g / 100 ml) to 500 ml water. Shake vigorously and allow to stand for 5 minutes.

(e) Add 5 ml of methanol and mix thoroughly.

(f) Condition the solid phase extraction cartridge, C_{18}, 1 g in a 6 ml reservoir, with 10 ml methanol followed by 10 ml of water. Do not let the cartridge dry during conditioning, sample application and wash.

(g) Apply the sample at a flow rate not exceeding 10 ml min^{-1} (visible drops). Regulate the flow with vacuum pressure.

(h) Wash the cartridge with 4 ml of 20% methanol.

(i) Dry the cartridge by drawing air through it for 2 min.

(j) Elute microcystins with 4 ml of acetonitrile containing 0.05% TFA e.g. in 12 mm x 75 mm borosilicate test tube or in borosilicate glass vials. It is advisable that the elution solvent is loaded in two fractions: draw 2 ml solvent into the SPE column, soak the sorbent for 3 min, then load a further 2 ml solvent into the column and collect all the solvent (max. flow rate 4 ml min^{-1}).

(k) Evaporate the acetonitrile eluate at 50 °C using argon or nitrogen.

(l) Resuspend the residue in 500 µl of 75% methanol.

(m) Centrifuge 10 min 10,000 × *g*, or, alternatively, filter, and transfer to HPLC vials.

(n) Analyse supernatant/filtrate on HPLC according to SOP_TOXIC_AAU_06F.

3 References

Hyenstrand, P., Metcalf, J.S., Beattie, K.A., Codd, G.A.: Losses of the cyanobacterial toxin microcystin-LR from aqueous solution by adsorption during laboratory manipulations. Toxicon **39**, 589-594 (2001).

Lawton, L.A., Edwards, C., Codd, G.A.: Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. Analyst (London) **119**, 1525-1530 (1994).

N. B. The reader is also advised to follow up the development of the ISO standard for microcystin analysis.

ISO/FDIS 20179: Water quality - Determination of microcystins - Method using solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection (2005).

SOP: Analysis of microcystins by high-performance liquid chromatography with photodiode-array detection

Document identifier: SOP_TOXIC_AAU_06F

Prepared by: Jussi Meriluoto and Lisa Spoof, AAU

Date: 30 June 2005

1 Introduction

Reversed-phase HPLC on C_{18} phases is a common choice for separating smaller peptides, and the mobile phases for peptides often consist of acetonitrile gradients in the presence of perfluorinated alkyl carboxylic acids, usually trifluoroacetic acid (TFA). Microcystins make no exception, they chromatograph perfectly under these conditions. Neutral ammonium acetate - acetonitrile based eluents are commonly used in preparative toxin separations.

The rapidly growing number of known microcystins, now exceeding 70, has made it impossible to separate and quantitate all microcystins on a single chromatographic run. However, using retention time and spectrum match, the major microcystins, such as microcystin-LR, -RR, -YR with different degrees of demethylation, are fairly easy to identify by HPLC combined with photodiode- array detection. The main chromophores of microcystins, the conjugated diene in the Adda residue and the α,β-unsaturated carbonyl group in *N*-methyldehydroalanine, absorb strongly at 238 nm. UV spectra of microcystins can be divided into three main categories: normal microcystin spectra with a local absorbance maximum at 238-240 nm, the spectra of tyrosine(Y)-containing microcystins with a flatter absorbance maximum at 230-240 nm, and the spectra of tryptophan(W)-containing microcystins which have an absorption maximum at 222-223 nm and a shoulder at 238-240 nm (examples of spectra on page 83, Figs. 6 and 7).

Cyanobacterial blooms can produce very complex microcystin profiles. In order to elucidate the toxin profile and thus indirectly estimate the total toxicity of a complex sample, the chemical methods used for toxin analyses should be able to separate and quantify individual microcystins which have different toxicities. This calls for separation methods of high resolution and good selectivity, i.e. the use of long columns and long run times, or more specific detection methods such as mass spectrometry. On the other hand, many samples in e.g. water treatment trials are rather trivial chromatographically and therefore less resolution may be enough if only a few analytes are to be separated.

2 Experimental

2.1 Materials

(a) Acetonitrile gradient HPLC grade

(b) HPLC grade water purified to 18.2 MΩ cm

(c) Trifluoroacetic acid (TFA), HPLC or protein sequence analysis grade. TFA should be stored under argon in a desiccator.

(d) C_{18} endcapped HPLC column (Purospher STAR RP-18 endcapped, 3 µm particles, LiChroCART 55 x 4 mm I.D., and Purospher STAR RP-18 endcapped, 5 µm particles, LiChroCART 250 x 4 mm I.D., from Merck, Darmstadt, Germany, are mentioned in this SOP as possible alternatives). A compatible guard column is also required.

(e) Borosilicate glass chromatographic vials: e.g. 1.5 ml clear glass with writing surface. For small sample volumes 0.3 ml polypropylene vials (only for samples in 75% methanol) or borosilicate glass inserts can be used.

(f) Microcystin standards

2.2 Special equipment

(a) High-performance liquid chromatograph equipped with high-pressure or low-pressure gradient pump, autosampler, column oven and photodiode-array (PDA) detector

(b) Chromatography analysis software

(c) A positive displacement pipette with a teflon-coated piston working in a glass capillary, capable of accurately dispensing 200 µl of the strong acid TFA

2.3 HPLC mobile phase

(a) HPLC mobile phase component A: HPLC water + 0.05% TFA.

i. Add 800 µl of TFA in 1.6 l of water.

ii. Replace every week.

(b) HPLC mobile phase component B: acetonitrile + 0.05% TFA

i. Take ca 450 µl of TFA in a borosilicate glass vial. Add 400 µl of TFA in 800 ml of ACN. Discard the rest of the TFA.

ii. Replace every week.

Please observe the following:
Work in fume hood with TFA or acetonitrile.
Dispense the TFA under the surface of acetonitrile.
Do not contaminate the original TFA bottle with acetonitrile.
Store undiluted TFA under argon.
Disposal of acetonitrile and TFA should conform to local regulations.

2.4 Chromatography

2.4.1 General procedure

(a) The HPLC system should be set up as described in the manufacturers instructions including degassing, priming and changing columns.

(b) Always use a guard column. Change the guard column if the back-pressure rises or peak forms deteriorate.

(c) Set column oven at 40 ºC.

(d) Change the HPLC gradually up to starting conditions and allow to condition.

(e) Chromatograph the samples and standards as per the recommended HPLC gradients (see below), use 10 µl injections.

(f) Analyse the chromatogram. Compare retention times and spectra to standards.

(g) Calculate the microcystin concentration according to the standard curve procedure described in TOXIC_SOP_AAU_03F.

Procedure for quantitation of microcystins other than microcystin-LR in HPLC-UV work**:** all peaks with microcystin spectra are quantified as microcystin-LR equivalents, i.e. with the same "area to ng/injection" coefficient as for microcystin-LR. If the molecular weight of the other microcystin is known, a correction for molecular weight difference can be applied in critical cases.

2.4.2 HPLC of microcystins on a short column, suitable for samples of medium complexity; column Merck Purospher STAR RP-18 endcapped, 3 µm particles, LiChroCART 55 x 4 mm I.D.

Do not use unnecessarily long HPLC columns with easy samples. The use of a short column saves time and solvents.

Table 1: Suggested gradient programme for Merck Purospher STAR RP-18 endcapped, 3 µm particles, LiChroCART 55 x 4 mm I.D., linear gradient at a flow rate of 1 ml min^{-1}. Injection cycle about 11 minutes.

Time (min)	% A	% B
0.00	75	25
5.00	30	70
6.00	30	70
6.10	75	25
9.00	STOP	

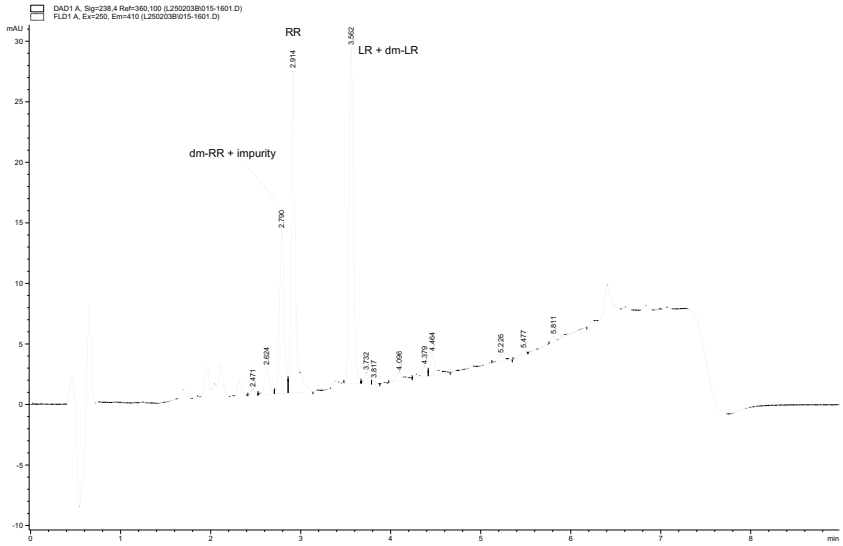

Figure 1: Trace of *Anabaena* extract. Column Merck Purospher STAR RP-18 endcapped, 3 µm particles, LiChroCART 55 x 4 mm I.D., detection at 238 nm. Other parameters as in Table 1. Retention times: microcystin-RR 2.91 min, microcystin-LR 3.56 min.

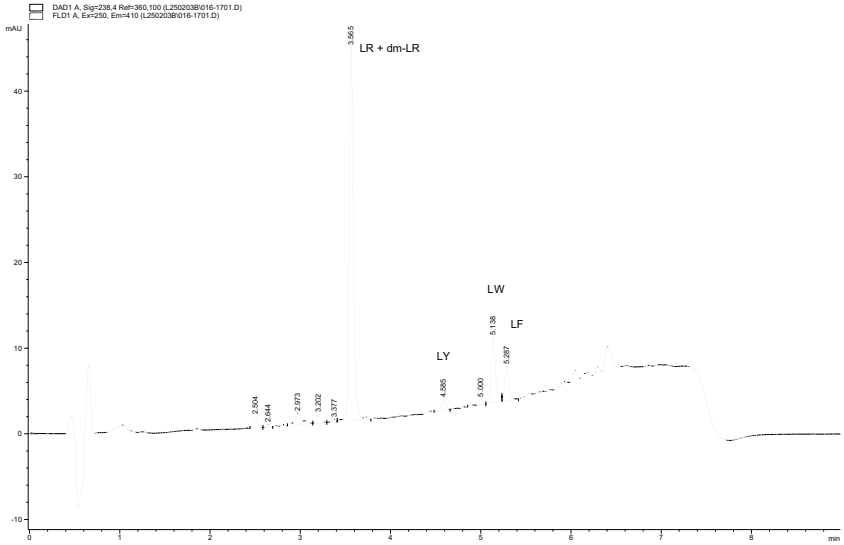

Figure 2: Trace of *Microcystis* extract. Column Merck Purospher STAR RP-18 endcapped, 3 µm particles, LiChroCART 55 x 4 mm I.D., detection at 238 nm. Other parameters as in Table 1. Retention times: microcystin-LR 3.56 min, microcystin-LY 4.58 min, microcystin-LW 5.14 min, microcystin-LF 5.29 min.

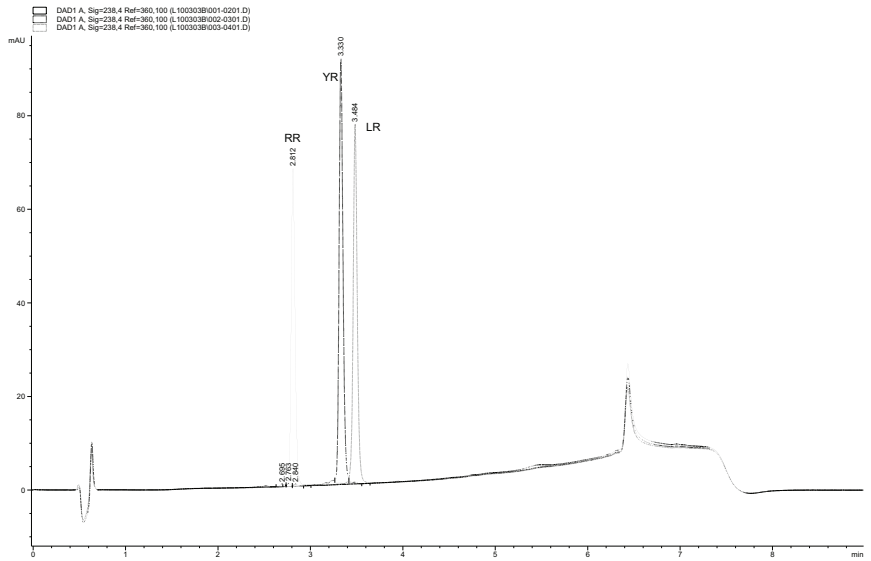

Figure 3: Overlaid traces of commercial microcystin-RR, -YR and -LR samples. Column Merck Purospher STAR RP-18 endcapped, 3 µm particles, LiChroCART 55 x 4 mm I.D., detection at 238 nm. Other parameters as in Table 1. Retention times: microcystin-RR 2.81 min, microcystin-YR 3.33 min, microcystin-LR 3.48 min. This chromatogram was run with an older column and the retention times have been shortened, cf. Figs. 1 and 2. The column should be replaced when the retention times have been shortened by more than 3%. Selectivity changes are possible even before this limit.

2.4.3 HPLC of microcystins on a long column, suitable for complex samples; column Merck Purospher STAR RP-18 endcapped, 5 µm particles, LiChroCART 250 x 4 mm I.D.

Complex field or strain samples may necessitate the use of a long column.

Table 2: One possible gradient programme for Merck Purospher STAR RP-18 endcapped, 5 µm particles, LiChroCART 250 x 4 mm I.D., linear gradient at a flow rate of 0.75 ml min^{-1}. Injection cycle about 62 minutes.

Time (min)	% A	% B
0.00	70	30
10.00	65	35
40.00	30	70
42.00	0	100
44.00	0	100
46.00	70	30
60.00	STOP	

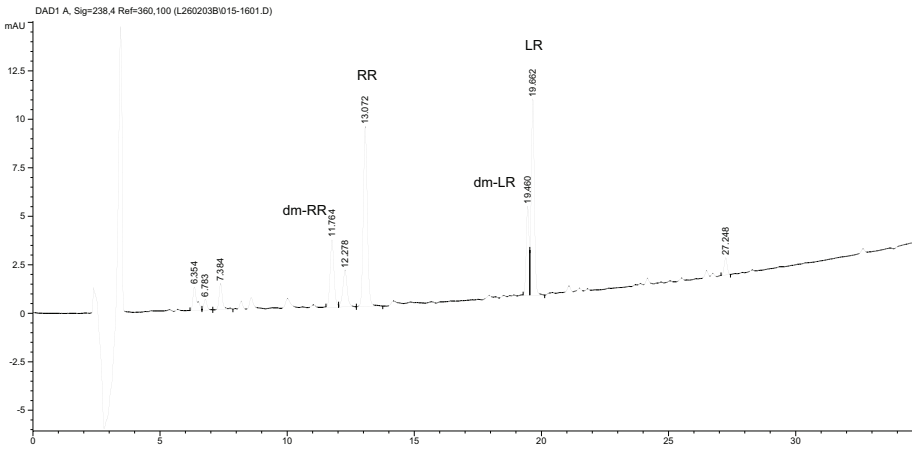

Figure 4: Trace of *Anabaena* extract. Column Merck Purospher STAR RP-18 endcapped, 5 µm particles, LiChroCART 250 x 4 mm I.D., detection at 238 nm. Other parameters as in Table 2. Retention times: microcystin-RR 13.07 min, microcystin-LR 19.66 min.

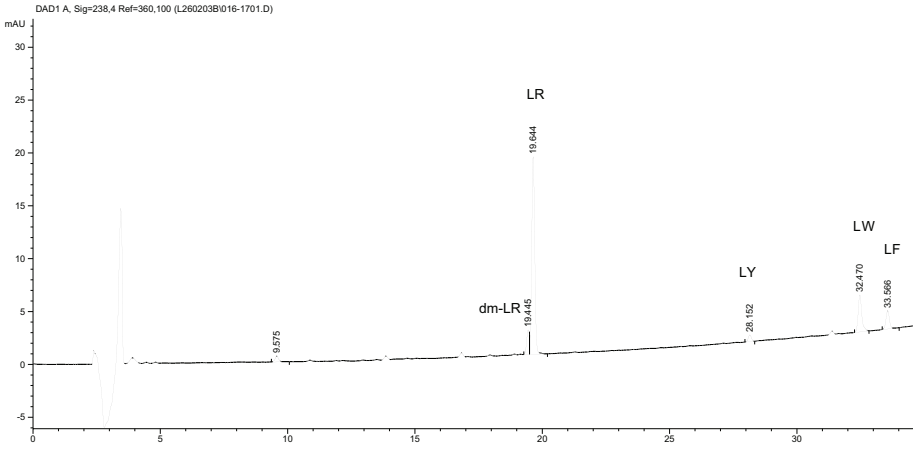

Figure 5: Trace of *Microcystis* extract. Column Merck Purospher STAR RP-18 endcapped, 5 µm particles, LiChroCART 250 x 4 mm I.D., detection at 238 nm. Other parameters as in Table 2. Retention times: microcystin-LR 19.64 min, microcystin-LY 28.15 min, microcystin-LW 32.47 min, microcystin-LF 33.57 min.

2.5 Spectra

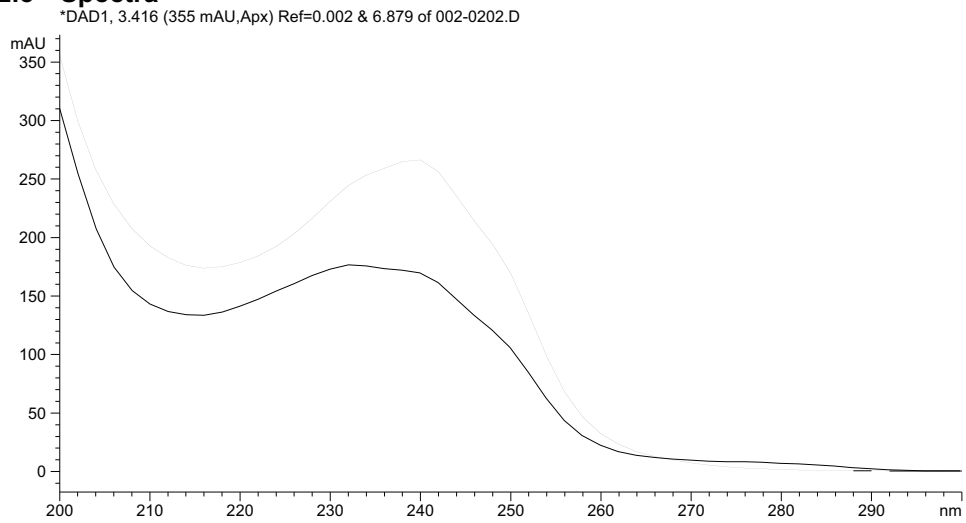

Figure 6: UV spectra of microcystin-LR (upper) and microcystin-YR (lower), chromatographic parameters according to section 2.4.2.

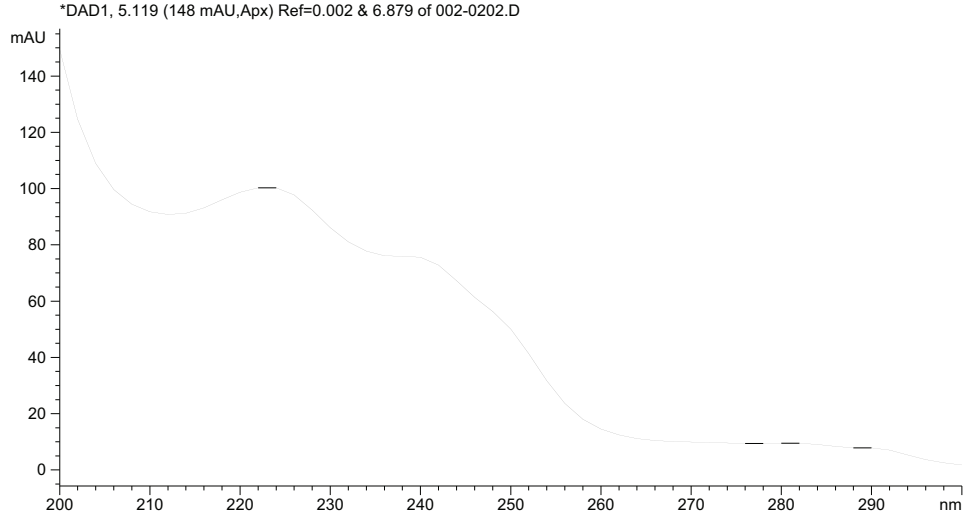

Figure 7: UV spectrum of microcystin-LW, chromatographic parameters according to section 2.4.2.

3 References

Lawton, L.A., Edwards, C., Codd, G.A.: Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. Analyst (London) **119**, 1525-1530 (1994).

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N. B. The reader is also advised to follow up the development of the ISO standard for microcystin analysis.

ISO/FDIS 20179: Water quality - Determination of microcystins - Method using solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection (2005).

Annex to SOP_TOXIC_UDU_08F

Document identifier: SOP_TOXIC_AAU_07F

Prepared by: Peter Backlund and Jussi Meriluoto, AAU

Date: 30 June 2005

Table 1: Modified gradient programme for anatoxin-a runs on Merck Purospher STAR RP-18 endcapped, 3 µm particles, LiChroCART 55 mm x 4 mm I.D., linear gradient at a flow rate of 1 ml min^{-1}. Injection cycle about 14 minutes.

Time (min)	% A (H_2O + 0.05% TFA)	% B (ACN + 0.05% TFA)
0.00	99	1
5.00	93	7
5.10	30	70
7.00	30	70
7.10	99	1
12.00	STOP	

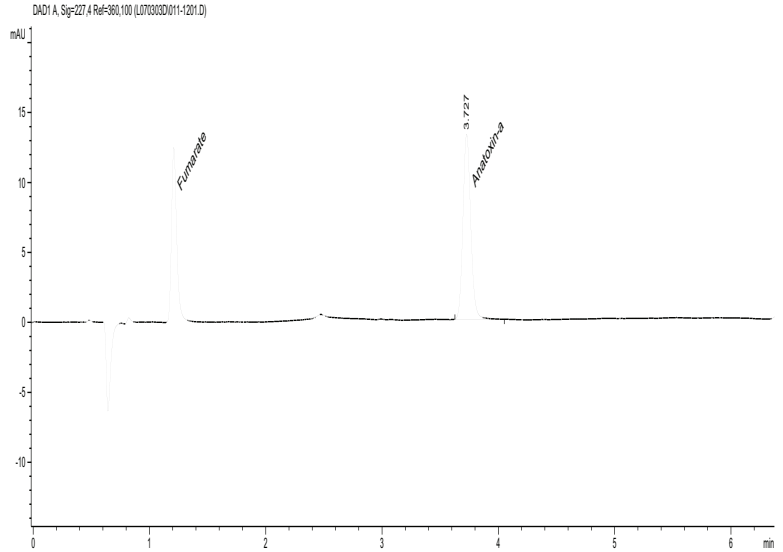

Figure 1: Trace of anatoxin-a fumarate. Column Merck Purospher STAR RP-18 endcapped, 3 µm particles, LiChroCART 55 mm x 4 mm I.D., detection at 227 nm. Other parameters as in Table 1. The retention time of anatoxin-a was 3.73 min.

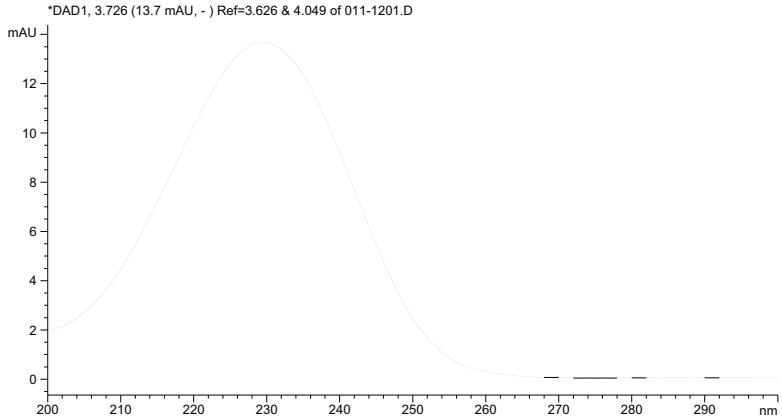

Figure 2: UV spectrum of anatoxin-a, chromatographic parameters according to this annex.

SOP: Cultivation of cyanobacteria in modified Z8 medium

Document identifier: SOP_TOXIC_AAU_08F

Prepared by: Lisa Spoof and Jussi Meriluoto, AAU

Date: 30 June 2005

1 Introduction

The benefits of using cyanobacterial cultures as starting material for toxin purification are apparent. Although it is relatively easy to obtain cyanobacterial material from natural blooms, the coexistence of bacteria and other organisms as well as other extraneous substances can make the purification of toxins from cyanobacterial cells more difficult and also interfere with the purity of the isolated toxins. Furthermore, cyanobacterial cultures constitute a continous and reliable source of cyanobacterial toxins. To ensure success in cultivation, an appropriate medium should be chosen as well as favourable light conditions and temperature, since these affect not only the growth but also the toxin profile.

2 Experimental

2.1 Chemicals

Use analytical reagent grade reagents if not indicated otherwise.

(a) $NaNO_3$

(b) $Ca(NO_3)_2 \times 4\ H_2O$

(c) $MgSO_4 \times 7\ H_2O$

(d) $CaCl_2 \times 2\ H_2O$

(e) $K_2HPO_4 \times 3\ H_2O$

(f) Na_2CO_3

(g) $FeCl_3 \times 6\ H_2O$

(h) HCl

(i) NaOH

(j) EDTA-Na_2

(k) $Na_2WO_4 \times 2\ H_2O$

(l) $(NH_4)_6Mo_7O_{24} \times 2\ H_2O$

(m) KBr

(n) KI

(o) $ZnSO_4 \times 7\ H_2O$

(p) $Cd(NO_3)_2 \times 4\ H_2O$

(q) $Co(NO_3)_2 \times 6\ H_2O$

(r) $CuSO_4 \times 5\ H_2O$

(s) $NiSO_4(NH_4)_2SO_4 \times 6\ H_2O$

(t) $Cr(NO_3)_3 \times 9\ H_2O$

(u) V_2O_5

(v) $Al_2(SO_4)_3K_2SO_4 \times 24\ H_2O$

(w) H_3BO_3

(x) $MnSO_4 \times 4\ H_2O$

2.2 Other materials

(a) Water purified to 18.2 MΩ cm

(b) CO_2 gas, food/cell culture grade

(c) 50 ml polypropylene tubes

(d) GF/A glass-fibre filter material in sheets or ready-cut filter discs

(e) Autoclave bags

(f) Autoclave tape

(g) 50 ml, 250 ml and 5 l borosilicate glass flasks with GL45 mouth

(h) 250 ml and 5 l glass beakers

(i) 1 l measuring cylinder

(j) Aluminium foil

(k) Cotton wool, purified

(l) Teflon and silicon tubing

(m) Filters for sterile air delivery

(n) GL45 screw closures with tubing port/adapter

(o) Sodium hypochlorite, containing 14% active chlorine

N.B. Dedicate the glassware and screw caps for cyanobacterial culture work only. This can be facilitated by marking of the vessels.

2.3 Special equipment

(a) Laminar flow hood

(b) Autoclave

(c) pH meter

(d) Buchner funnel filtration unit for 4000 ml volume

(e) Aquarium pump

(f) Two aquarium lamps, e.g. equivalent to 20 W POWER-GLO (Hagen, Japan)

2.4 Solutions

Store the stock solutions at + 4 ºC. The lifetime of the stock solutions is at least one year.

(a) Macro solution 1A for cultivation of *Microcystis* and *Planktothri*x (non-nitrogen fixing genera)

46.7 g $NaNO_3$, 5.9 g $Ca(NO_3)_2 \times 4\ H_2O$ and 2.5 g $MgSO_4 \times 7\ H_2O$ in 1000 ml ultrapure water

(b) Macro solution 1B for cultivation for *Anabaena* (nitrogen-fixing genus). N.B. Some nitrogen-fixing strains may prefer Macro solution 1A.

3.7 g $CaCl_2 \times 2\ H_2O$ and 2.5 g $MgSO_4 \times 7\ H_2O$ in 1000 ml ultrapure water

(c) Macro solution 2 for cultivation of all strains of *Microcystis*, *Planktothrix* and *Anabaena*

4.07 g $K_2HPO_4 \times 3\ H_2O$ and 2.1 g Na_2CO_3 in 1000 ml ultrapure water

(d) Fe-EDTA solution

i. 0.1 M HCl: Add 1.25 ml 12 M HCl in 100 ml of ultrapure water and make up to 150 ml.
ii. $FeCl_3$ solution: Dissolve 2.80 g $FeCl_3 \times 6\ H_2O$ in 100 ml 0.1 M HCl
iii. 0.1 M NaOH: Dissolve 0.6 g in 100 ml of ultrapure water and make up to 150 ml
iv. EDTA solution: Dissolve 3.9 g EDTA-Na_2 in 100 ml 0.1 M NaOH

Mix 10 ml $FeCl_3$ solution and 9.5 ml EDTA solution and fill up to 1000 ml with ultrapure water

(e) Trace element solution

i. 16.5 mg $Na_2WO_4 \times 2\ H_2O$ in 50 ml ultrapure water
ii. 44.0 mg $(NH_4)_6Mo_7O_{24} \times 2\ H_2O$ in 50 ml ultrapure water
iii. 60.0 mg KBr in 50 ml ultrapure water
iv. 41.5 mg KI in 50 ml ultrapure water
v. 143.5 mg $ZnSO_4 \times 7\ H_2O$ in 50 ml ultrapure water
vi. 77.5 mg $Cd(NO_3)_2 \times 4\ H_2O$ in 50 ml ultrapure water
vii. 73.0 mg $Co(NO_3)_2 \times 6\ H_2O$ in 50 ml ultrapure water
viii. 62.5 mg $CuSO_4 \times 5\ H_2O$ in 50 ml ultrapure water
ix. 99.0 mg $NiSO_4(NH_4)_2SO_4 \times 6\ H_2O$ in 50 ml ultrapure water
x. 20.5 mg $Cr(NO_3)_3 \times 9\ H_2O$ in 50 ml ultrapure water
xi. 4.45 mg V_2O_5 in 50 ml ultrapure water
xii. 237.0 mg $Al_2(SO_4)_3K_2SO_4 \times 24\ H_2O$ in 50 ml ultrapure water
xiii. 155.0 mg H_3BO_3 and 111.5 mg $MnSO_4 \times 4\ H_2O$ in 50 ml ultrapure water

Add 1 ml of stock solutions i.-xii. and 10 ml of stock solution xiii. in 70 ml of ultrapure water, and dilute up to 100 ml with ultrapure water.

2.5 Preparation of culture medium Z8

(a) For *Microcystis* and *Planktothrix*: Add 40 ml of Macro 1A, 40 ml of Macro 2, 40 ml of Fe-EDTA solution and 4 ml of trace element solution to 3000 ml of ultrapure water (in a 5 litre glass beaker),

lower the pH to 6.9 with CO_2 sparging, or alternatively with 0.1 M HCl, and dilute to a final volume of 4000 ml.

(b) For some nitrogen-fixing strains (*Anabaena*): Use 40 ml of Macro 1B solution instead of Macro 1A. Otherwise prepare the culture medium as described in 2.5 (a).

(c) Transfer the medium into a 5 l borosilicate flask equipped with a screw cap. Leave the screw cap on the flask slightly open (half a turn) and cover it with a double layer of aluminium foil and autoclave in a programme for liquids. The temperature of the autoclaving process should be monitored by a probe put in a 4 l reference volume of water.

(d) When the autoclaving process is ready, take the medium flask carefully out of the autoclave. The screw cap should be left half a turn open until the contents have reached room temperature.

(e) Autoclave the dry materials in a separate programme: Autoclave a clean 250 ml borosilicate flask. Put some cotton wool under the cap, leave the screw cap slightly open and cover it with aluminium foil. In addition, autoclave a 250 ml beaker; cover also this with foil or put it in an autoclave bag. Autoclave also the tubing and filter attached to the screw closures via tubing port/adapter, these will be needed to sparge the culture with sterile-filtered air. Put some cotton wool under the screw closure.

N.B. The sterilization of the fresh growth medium and the dry accessories should be performed at least one day before the actual change of the medium as the materials have to reach room temperature before they can be used.

2.6 Transferring cyanobacterial cells to fresh growth medium

The cultures will usually grow to full density in ca 3-4 weeks after which they should be diluted 1:20 in fresh growth medium**.**

(a) Conduct the transfer always in a laminar flow hood cleaned with 70% ethanol.

(b) When you are starting a new culture after receiving a strain from a culture collection, a smaller volume of growth medium is needed. Direct transfer of the received small amount of cell material into culture volumes larger than 50 ml is not recommended. The volume of the medium should be gradually increased with the growth of the culture.

(c) When you maintain a 4 litre culture, use the sterile 250 ml beaker to transfer ca 200 ml of the old culture into the fresh medium. Enough cells should be transferred to make the new medium

slightly green in colour. Use the beaker also to transfer 50-100 ml into a 250 ml sterile flask. The 250 ml flask is for back-up purposes if the transfer does not succeed and cultivation fails. Sterilize the flask mouths by a gas flame before attaching the screw caps. Leave the screw caps slightly loose.

(d) Place the cultures near the aquarium lamps (suggestion: at a 20 cm distance), a dilute culture preferably further away from the lamps. The culture should be illuminated continuously and agitated by sparging sterile-filtered air with the aid of an aquarium pump. The back-up flasks containing dormant cyanobacterial cells do not need air sparging but some low-intensity light.

2.7 Harvesting cyanobacterial cells

(a) Perform the transfer to fresh medium every three to four weeks. If the cultures are kept for toxin production, the cells should be harvested before they start to leak substantial amounts of toxin into the extracellular fraction (check the concentrations of intra- and extracellular toxin, as well as the toxin profile, by HPLC).

(b) Filter the cyanobacterial cells not used as an inoculum on GF/A glass-fibre filters. A suitable filter disc diameter for 4 l cultures is 22.5 cm. Two 22.5 cm filters may be needed per 4 litres of cell suspension. Collect also the filtrate which contains both some toxin and some cells.

(c) Air-dry the GF/A filters and store frozen at –20 ºC.

(d) Waste disposal: Follow the local regulations. If these are not available follow the directions here. Transfer the filtrate to a vessel suitable for autoclaving, e.g. a 10 l polyethylene jerry can. Add 1 part of sodium hypochlorite (containing 14% chlorine) to 19 parts of filtrate. Flush all the glassware etc which has been in contact with cyanobacterial cells with some sodium hypochlorite, pour this to the jerry can containing the filtrate and autoclave in a destruction autoclave.

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Anon.: Instructions for preparation of modified nutrient solution Z8 for algae. Publ. B-11/69, Norwegian Institute for Water Research, Oslo (1972).

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SOP: Purification of microcystins by high-performance liquid chromatography

Document identifier: SOP_TOXIC_AAU_09F

Prepared by: Lisa Spoof and Jussi Meriluoto, AAU

Date: 30 June 2005

1 Introduction

Microcystins are extracted from cyanobacteria preferably with aqueous methanol. After concentration by reversed-phase solid-phase extraction, silica gel chromatography can be used for a preliminary clean-up. Modern purification methods of cyanotoxins are characterised by an intensive use of HPLC. Neutral aqueous ammonium acetate - acetonitrile based eluents on C_{18} have been the first choice in the purification of microcystins by HPLC due to the mild nature of these eluents. Additional purification, when necessary, should be conducted on a chromatographic system with a different selectivity, e.g. using aqueous trifluoroacetic acid - acetonitrile on C_{18}.

The recovery of purified toxins should be monitored with an analytical system after each step of purification to insure that losses of toxins are minimised during the process. A short analytical column allowing fast results is preferred for this purpose. The monitoring of purity, however, calls for separation methods of high resolution and good selectivity, i.e. the use of long columns and long run times, and alternative detection methods such as mass spectrometry.

2 Experimental

2.1 Materials

(a) Acetonitrile gradient HPLC grade

(b) Methanol HPLC grade

(c) HPLC grade water purified to 18.2 MΩ cm

(d) Trifluoroacetic acid (TFA), HPLC or protein sequence analysis grade. TFA should be stored under argon in a desiccator.

(e) Ammonium acetate ($NH_4C_2H_3O_2$), equivalent to product number A7330, Sigma, St. Louis, MO, USA

(f) For evaporation: Nitrogen, preferably >99.999%, or alternatively argon, preferably >99.999%

(g) Reversed-phase solid phase extraction columns suitable for microcystin work, equivalent to Isolute C18(EC) solid phase extraction columns, size 1 g sorbent in 6 ml reservoir, part number 221-0100-C, size 2 g sorbent in 15 ml reservoir, part number 221-0200-D, size 5 g sorbent in 25 ml reservoir, part number 221-0500-E, or corresponding bulk material, from Argonaut Technologies (Mid Glamorgan, UK)

(h) For analytical HPLC: C_{18} endcapped HPLC column (Purospher STAR RP-18 endcapped, 3 µm particles, LiChroCART 55 x 4 mm I.D., and Purospher STAR RP-18 endcapped, 5 µm particles, LiChroCART 250 x 4 mm I.D., from Merck, Darmstadt, Germany, are mentioned as possible alternatives). A compatible guard column is also required.

(i) Silica gel chromatography: column equivalent to Supelclosil PLC-Si, 25 cm x 21.2 mm I.D., 12 µm particles, catalog number 59183 (Supelco, Bellefonte, PA, USA) with a guard column; alternatively a glass column, dimensions approximately 50 cm × 2.5 cm I.D., packed with Si-60 material or equivalent

(j) Preparative HPLC: stationary phase equivalent to Nucleosil C_{18} material, column dimensions at least 150 mm × 21.2 mm I.D., 10 µm particles or smaller, and a C_{18} preparative guard column

(k) Semipreparative HPLC: column equivalent to Discovery C18 250 mm × 10 mm I.D., 5 µm particles. Catalog nr. 569224-U (Supelco, Bellefonte, PA, USA), and a C_{18} semipreparative guard column.

(l) Glass-fibre filters, GF/C, diameter 110 mm

(m) HPLC grade filters showing no adsorption of microcystins at the specified conditions, equivalent to GHP Acrodisc 25 mm syringe filters with 0.2 µm pore size, product number AP-4564, from Pall Life Sciences (Ann Arbor, MI, USA)

(n) 20 ml all-plastic (no rubber piston) single use syringes

(o) Solid phase extraction empty reservoirs, 70 ml

(p) Microcentrifuge tubes, 1.5-2 ml, polypropylene, preferably with screw caps

(q) Centrifuge tubes or bottles with screw caps for large volumes (250-500 ml)

(r) Round bottom flasks 100-1000 ml to be used in rotary evaporation, and flask supports

(s) Borosilicate glass chromatographic vials: e.g. 1.5 ml clear glass with writing surface. For small sample volumes 0.3 ml polypropylene vials (only for samples in 75% methanol) or borosilicate glass inserts can be used.

(t) Borosilicate glass Pasteur pipettes

2.2 Special equipment

(a) Flash chromatography system

(b) For purification: High-performance liquid chromatograph with manual injector (10 ml injector loop) and a UV detector

(c) For analysis: HPLC equipped with high-pressure or low-pressure gradient pump, autosampler, column oven and photodiode-array (PDA) detector

(d) Shaker or a stirring device suitable for large volumes

(e) Vacuum system with vacuum pump, filtering flask (1-2 l) with a hose connection and a filter holder such as a Buchner funnel

(f) Heating block, operated at 50 ºC, with evaporation unit

(g) Preparative centrifuge

(h) Microcentrifuge

(i) Rotary evaporation unit

(j) Fraction collector

(k) Positive displacement pipette with a teflon-coated piston working in a glass capillary, capable of accurately dispensing 200 µl of trifluoroacetic acid (TFA)

(l) 10 ml gastight glass syringe, with a teflon piston

2.3 Solutions

(a) 50% methanol (50 volumes of methanol and 50 volumes of water)

(b) 75% methanol (75 volumes of methanol and 25 volumes of water)

(c) 90% methanol containing 0.1% TFA (90 volumes of methanol, 10 volumes of water and 0.1 volumes of TFA)

(d) Mobile phase for preparative HPLC

i. 1 g of ammonium acetate is dissolved in 1000 ml of HPLC grade water (0.013 M). Filter before proceeding. Suggestion for filtration: Weigh 1 g of ammonium acetate directly in a 20 ml all-plastic (no rubber piston) single use syringe coupled to the 25 mm syringe filter with 0.2 µm pore size. Dissolve the ammonium acetate in the syringe with HPLC grade water and press the solution through the filter into a 1000 ml volumetric flask, repeat this to ensure that all of the salt is dissolved and filtrated into the flask. Dilute the salt in the flask with HPLC grade water up to 1000 ml.
ii. Prepare 1 l of the mobile phase for the hydrophilic microcystins. This mobile phase contains 27% of acetonitrile and 73% of 0.013 M ammonium acetate (270 ml + 730 ml). If required, 1 l of the mobile phase for the more hydrophobic microcystins can be prepared: 31% acetonitrile and 69% 0.013 M ammonium acetate.
iii. Replace every day! Older solutions of the mobile phases cause changes in selectivity.

(e) Mobile phase for semipreparative HPLC

i. Component A: 0.05% TFA in HPLC grade water. Add 800 µl of TFA in 1.6 l of HPLC grade water. Replace daily in the preparative context.
ii. Component B: 100% acetonitrile

(f) Mobile phase for analytical HPLC

The mobile phase is prepared according to SOP_TOXIC_AAU_06F.

Please observe the following:
Work in fume hood with TFA or acetonitrile.
Dispense the TFA under the surface of acetonitrile.
Do not contaminate the original TFA bottle with acetonitrile.
Store undiluted TFA under argon.
Disposal of acetonitrile and TFA should conform to local regulations.

2.4 Procedure of purification

(a) Loosely place 15-20 freeze-thawed glass-fibre filters (diameter 22.5 cm) containing harvested cells from cultured cyanobacteria in a wide container and add 1 liter of 50% aqueous methanol. All filters should be covered with liquid. You can also start with 5-20 g of freeze-dried cyanobacterial cell material and extract this with 250-1000 ml of 50% methanol. The extraction is performed at room temperature under continuous shaking or stirring for 1 hour.

(b) After one hour decant the extract into e.g. a 2 l measuring cylinder and read the volume. Squeeze or press firmly but with great caution the wet filter discs to get as much liquid as possible out of the discs. Take a sample of the extract, centrifuge at 10,000 × *g* for 10 min and dilute (1+10 or 1+100 with 75% methanol) for analysis by analytical HPLC.

(c) Repeat the extraction with smaller volumes of 50% methanol, e.g. 400 ml at a time, until the release of toxins is decreased radically. Usually a total of 3-4 extractions is enough. Remember to monitor the toxin content in the extract by HPLC and also do not forget to measure the volume of the extract at the different steps.

(d) Pool the extracts, measure the total volume and take a sample for HPLC. Centrifuge, dilute and analyse the sample as described earlier.

(e) Transfer ca 500 ml of the extract at a time in a 1000 ml round bottom flask and rotary evaporate this at 50 ºC to 60% of the original volume (e.g. from 500 ml to 300 ml). In the end the total volume of your extract should be ca 60% of the original volume. The methanol concentration in the extract is now under 20%.

(f) Separate the particulate material including filter debris in the extract by preparative centrifugation at 10,000 × *g* or higher for 30 min. Observe the recommendations of the manufacturer in the filling of the centrifuge tubes or bottles.

(g) Decant the supernatant and filter through GF/C filters (110 mm in diameter). You need several filters. Take a sample of the filtrate and analyse it by HPLC as described earlier.

(h) Flash chromatography

Pack a flash cartridge, 10 cm x 2.5 cm I.D., with 10-18 g of C_{18} bulk solid-phase extraction material. Condition the column with 100 ml of methanol followed with 100 ml of HPLC grade water (use air or N_2 with a pressure of about 2.5 bar as a delivery force). After conditioning, apply the extract onto the cartridge with the aid of

pressure (air or N_2, 2.5 bar). Do not let the cartridge dry during conditioning or sample application. Take a sample of the extract after flash chromatography for analytical HPLC to ensure that all toxin is actually retained by the C_{18} material. You do not need to dilute this sample. Dry the cartridge by drawing air or N_2 through it for 2 min. Elute the microcystins with >150 ml of 75% methanol. 75% methanol is preferred over 100% methanol because it elutes microcystin-RR better and there is less chlorophyll-a in the eluate. Take a sample of the eluate and dilute (1+10 or 1+100 with 75% methanol) for analysis by HPLC.

(i) Rotary evaporate the eluate to dryness at 50 ºC .

(j) Preliminary clean-up on silica

By low-pressure chromatography. Redissolve the toxins in 3 ml of methanol (or more if necessary) and apply onto a Si-60 column (50 cm x 2.5 cm I.D.) equilibrated in 100% methanol. Let the sample penetrate the silica gel. Allow the liquid level to approach the upper part of the gel but do not let the gel go dry. Then apply 50 ml of 100% methanol to the column and elute the toxins with 90% aqueous methanol containing 0.1% TFA. Start collection of 4-5 ml fractions before pigments are eluted fom the column and continue until all the toxins are eluted out of column. Monitor the toxin content in the fractions by HPLC as previously described. Wash the column with 75% methanol and store it in 100% methanol.

Alternatively by HPLC. After resuspending the toxins in methanol, divide the extract in 1.2 ml aliquots in screw cap Eppendorf tubes and centrifuge at 10,000 × *g* for 10 min. Pool the supernatants and filter through the GHP 0.2 µm filter with the aid of a glass syringe (10 ml) before injecting in HPLC. Injection volume should be less than 3 ml, use several injections if necessary. The microcystins are eluted with 90% methanol containing 0.1% TFA at a flow-rate of 3 ml min^{-1}. Use of UV-absorbance detector is optional. Note that the UV-absorbance detector can be set at 270 nm in order to attenuate the strong signal at 238 nm. Collect 3 ml fractions with the help of a fraction collector and monitor the microcystin content in the fractions by analytical HPLC.

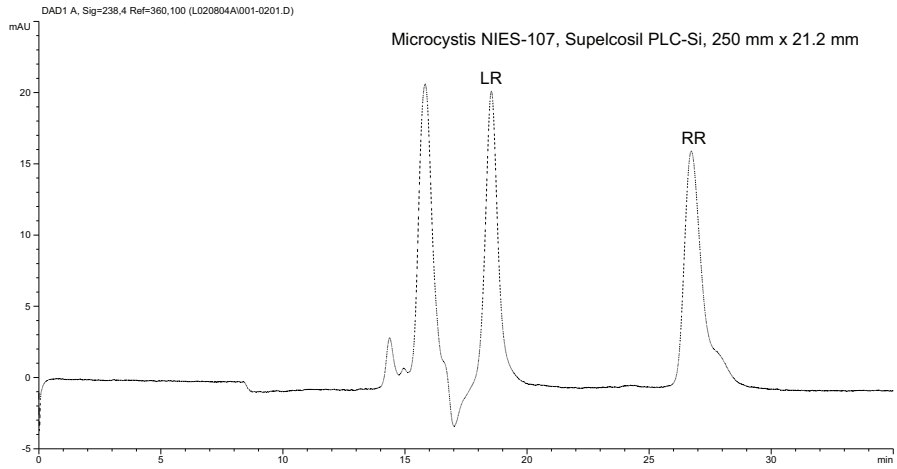

Figure 1: Separation of *Microcystis* NIES-107 extract (75% methanol) on the Supelcosil PLC-Si, 250 mm x 21.2 mm, silica gel column. Isocratic elution with 90% methanol containing 0.1% TFA. Flow rate 3 ml min^{-1}. Detection: absorbance at 238 nm. Ambient temperature (22 ºC). This is a small-scale simulation of the real preparative conditions (1.2 µg microcystin-LR and 2.6 µg microcystin-RR).

(k) The silica gel step usually gives rather low resolution and overlapping fractions. Pool the fractions containing microcystins in 100-250 ml round bottom flasks, and rotary evaporate to dryness at 50 ºC. If there is a good resolution between different microcystins, you could try individual pooling.

(l) Redissolve each pooled and dried fraction in 5-10 ml of 100% methanol by adding 1 ml at a time in the flask and transferring this with a glass Pasteur pipette into a 12 ml glass vial. Take samples for analytical HPLC. Concentrate the methanolic solution to an approximate volume of 1 ml under argon at 50 ºC. Add 6 ml of HPLC grade water to gain a methanol percentage of less than 20% and filter the solution through a GHP 0.2 µm filter with the aid of a 10 ml glass syringe. You may need several filters. Purge the filters with air to minimise the volume retained in the filter. Take again samples for analytical HPLC, dilute them to 1+100 with 75% methanol, and analyse as previously.

(m) Preparative HPLC

The mobile phase consists of 27% acetonitrile - 73% 0.013M ammonium acetate pumped at a flow rate of 4 ml min $^{-1}$. After your preparative HPLC system has been equilibrated you can inject several millilitres of the toxin-containing filtrate in the HPLC.

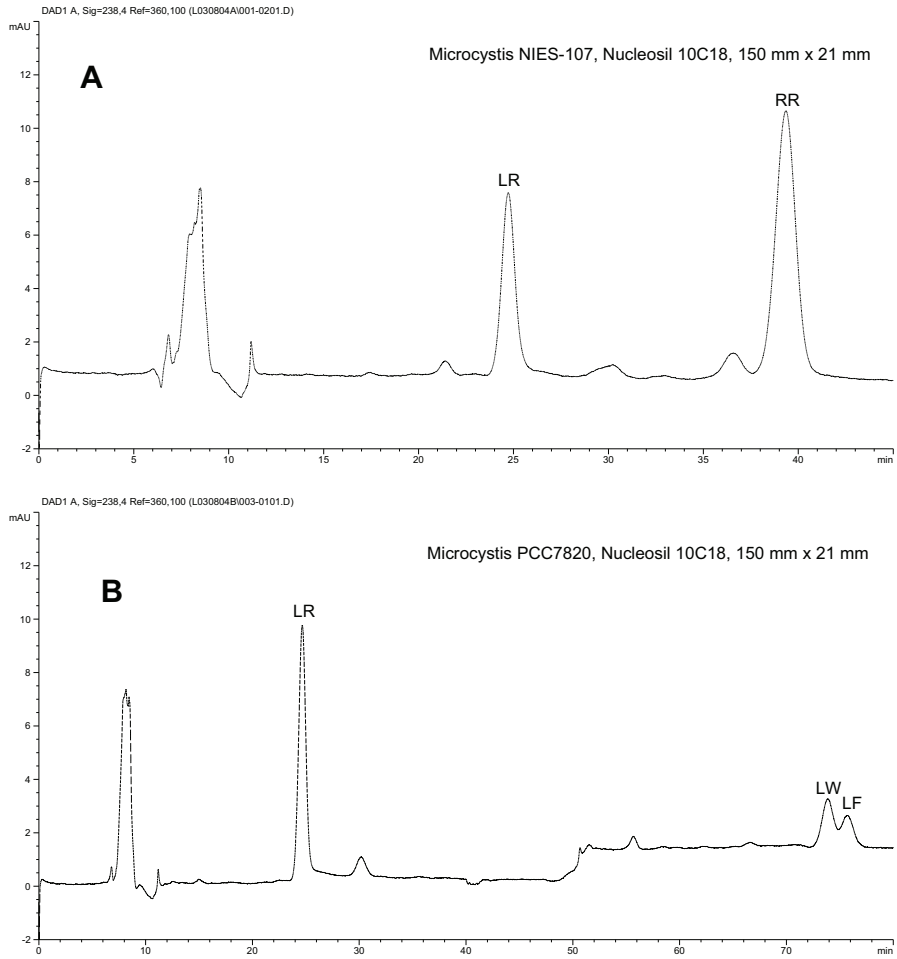

Figure 2: Separation of *Microcystis* NIES-107 extract (Panel A) and *Microcystis* PCC7820 extract (Panel B) on the Nucleosil 10C18 column , 150 mm x 21 mm. Isocratic elution with 27% acetonitrile – 73% 0.013M ammonium acetate (mobile phase A) up to 40 minutes. For PCC7820, the eluent was changed to contain 94% of mobile phase A and 6% of acetonitrile after 40 min. Flow rate 4 ml min^{-1}. Detection: absorbance at 238 nm. Ambient temperature (22 ºC). This is a small-scale simulation of the real preparative conditions (1.2 µg microcystin-LR and 2.6 µg microcystin-RR in NIES-107 and 2.2 µg microcystin-LR in PCC7820).

Note that the UV-absorbance detector should be set at e.g. 270 nm in order to attenuate the strong signal at 238 nm. Collect 4 ml fractions with the help of a fraction collector and monitor the microcystin content in the fractions by analytical HPLC. After about 45 min, when both microcystins-LR and -RR have eluted from the column, change the mobile phase to contain 31% acetonitrile and

69% 0.013 M ammonium acetate. This eluent is suitable for the more hydrophobic microcystins.

(n) Pool fractions containing individual microcystins. Condition several, one for each microcystin, 1 g - 5 g C_{18} SPE cartridges. Use >2 g sorbent per 10 mg toxin (in any case monitor toxin breakthrough). Dilute the microcystin-containing fractions 1+1 with HPLC grade water and apply them with glass Pasteur pipettes onto conditioned (methanol followed by HPLC grade water) SPE cartridges. Do not let the cartridge dry during conditioning, sample application and wash. Apply the sample at a flow rate not exceeding 10 ml min^{-1} (visible drops). Regulate the flow with vacuum pressure. Wash away the ammonium acetate from the cartridge by applying 30 ml of HPLC grade water per g sorbent. Dry the cartridge by drawing air through it for 2 min. Elute the purified microcystins first with 1 ml of 75% methanol per g sorbent and then with 9 ml of 100% methanol per g sorbent. Determine the final microcystin concentration in the eluate, divide in suitable aliquots and evaporate at 50 °C using argon or nitrogen. Store the dry toxin at -20 °C. The resulting purified toxins are marked on vials as microcystin 1X grade.

(o) Second purification for microcystin-LR (microcystin-LR 2X grade)

Resuspend ca 1 mg of the purified microcystin-LR (1X grade) in 100 µl of 75% methanol and add 250 µl of HPLC grade water. Inject the whole volume in a semipreparative HPLC column. Use a mobile phase consisting of A: 0.05% aqueous trifluoroacetic acid (TFA) and B: acetonitrile. Flow-rate 3 ml min^{-1}. Linear gradient programme: 0 min 25% B, 30 min 70% B, 32 min 70% B, 32.1 min 25% B. Total analysis time 60 min at ambient room temperature. Detection: absorbance at 238 nm. Collect 1 ml fractions. Monitor the concentration and the purity of the microcystin in the fractions by analytical HPLC.

Pool the toxin-containing fractions showing no apparent impurities, dilute 1+1 with HPLC grade water and apply with a glass Pasteur pipette onto a conditioned 1 g C_{18} SPE catridge. Flush the cartridge with 30 ml of water, this time to get rid of the TFA, air-dry for 2 min and elute the purified microcystin with 1 ml of 75% methanol followed by 9 ml of 100% methanol. Determine the microcystin concentration in the eluate and divide in suitable aliquots. Evaporate the aliquots as described earlier.

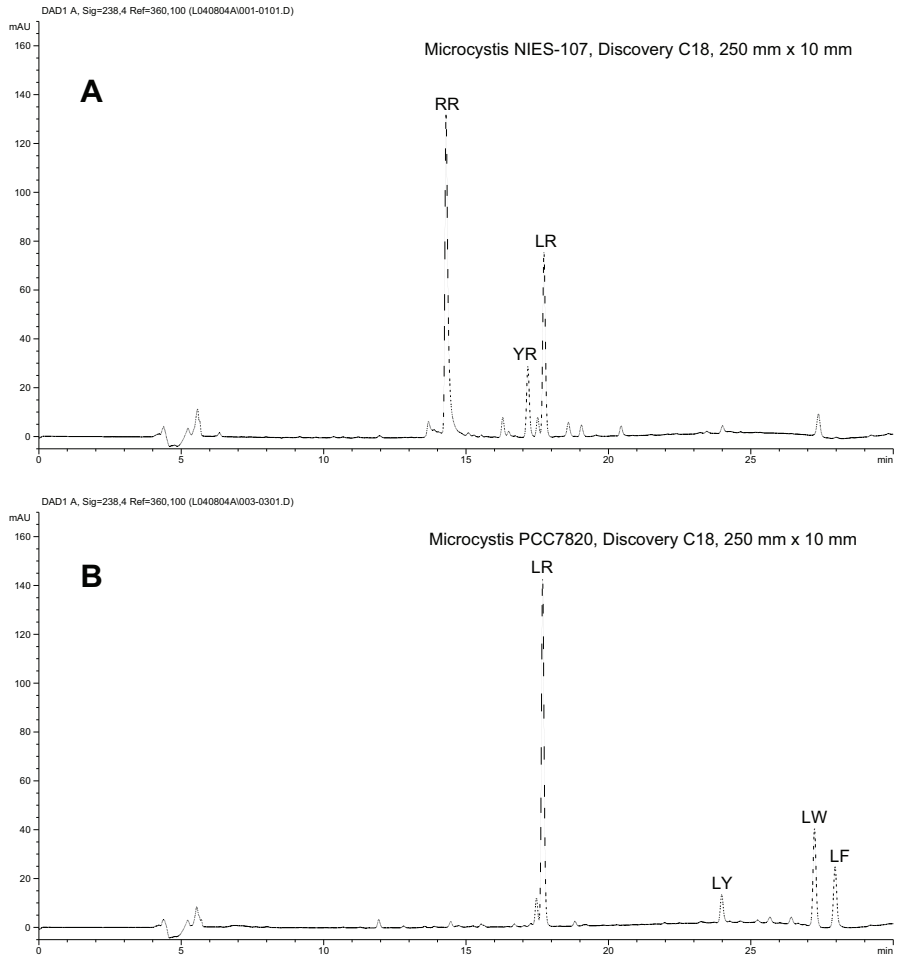

Figure 3: Separation of *Microcystis* NIES-107 (Panel A) and *Microcystis* PCC7820 (Panel B) on Discovery C18, 250 mm x 10 mm I.D., 5 µm particles. Mobile phase TFA-ACN: A: 0.05% aqueous trifluoroacetic acid (TFA), B: acetonitrile. Flow rate 3 ml min^{-1}. Linear gradient programme: 0 min 25% B, 30 min 70% B, 32 min 70% B, 32.1 min 25% B. Total analysis time 60 min. Detection: absorbance at 238 nm. Ambient temperature (22 ºC). This is a small-scale simulation of the real semipreparative conditions (1.2 µg microcystin-LR, 2.6 µg microcystin-RR and 0.5 µg microcystin YR in NIES-107 and 2.2 µg microcystin-LR in PCC7820).

2.5 Purity check procedures, some points to consider

(a) After evaporation of solvent, the toxin is deposited on the vial walls as a thin white film. Inspect the vials for discoloration of toxin. Greenish pigment-derived discoloration is not acceptable.

(b) Use a long column and a slow gradient for purity check by HPLC. Preferably this system should have another selectivity properties compared to the purification system. Use a wider wavelength range in UV-absorbance detection, e.g. 200-300 nm, to reveal peaks not visible at 238 nm. Ideally, there should not be any extra peaks besides the toxin peak and the solvent front at void volume.

(c) For final verification of microcystin properties and structure: perform bioassays and biochemical assays to verify biological activity; check structure by amino acid analysis, MS-MS and NMR.

2.6 General advice

(a) Monitor recovery.

(b) Remember always to vortex thoroughly when making samples for analytical HPLC - vortex before taking a sample and vortex after making a dilution.

(c) Flush all vessels such as round bottom flasks with a small volume of 75% methanol at the end of every step. Often this liquid can be pooled with the sample entering the next step of purification.

2.7 Procedure for microcystin analysis by HPLC

Please see SOP_TOXIC_AAU_06F.

3 References

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SOP: Analysis of microcystins by liquid chromatography - mass spectrometry

Document identifier: SOP_TOXIC_AAU_10F

Prepared by: Olli Sjövall and Jussi Meriluoto, AAU

Date: 14 July 2005

1 Introduction

Modern analyses of environmental and biomedical samples are often performed by liquid chromatography-mass spectrometry (LC-MS). This technique offers unsurpassed sensitivity and selectivity compared to traditional chromatographic techniques. There are several types of mass spectrometer instruments differing in ionisation method and type of mass analyser. Microcystins can be easily detected by e.g. common single-quadrupole, triple-quadrupole (MS-MS) or ion-trap MS instruments. The quadrupole instruments are suitable for quantitative analysis of microcystins. Ion-trap instruments are especially suitable for the qualitative identification and structural analysis of microcystins. With the ion-trap MS instruments multiple steps of fragmentation can be monitored, in practice up to MS^3 or MS^4 depending on the analyte concentration. MS instruments have roughly 100 times higher sensitivity compared to HPLC-UV and the detection limits are commonly in the pg per injection range. LC-MS offers selective detection in complex samples and sometimes possibility to identify toxins or their metabolites without authentic standards.

High-performance liquid chromatography (HPLC) is commonly interfaced with MS instruments via electrospray ionisation (ESI). MS is a universal and sensitive detection technique, which together with a soft ionisation method, such as the ESI operated in the positive ionisation mode, yields molecular ions ($[M+H]^+$ or $[M+2H]^{2+}$) of microcystins. In some cases compounds are detected as adduct ions, e.g. $[M+Na]^+$ ions. LC-ESI-MS has rarely been used in the negative ionisation mode to detect microcystins. The values reported by MS are so called mass-to-charge ratios (*m/z*) of either molecular ions or fragment ions. Fragmentation is useful for a more reliable analyte identification and it can be induced either in the ion source (in-source fragmentation, depending on e.g. cone voltage) or in a collision chamber inside the instrument. The fragmentation, which can be used either for structural investigations or for certain types of quantitative analyses, is accomplished by energy and collision gas addition. Fragmentation analyses are carried out by different MS-MS instruments (tandem mass spectrometry) including the ion-trap instruments.

Identification of microcystins in LC-ESI-MS analyses is based on the retention times in reversed-phase HPLC and the observed *m/z* values (or *m/z* transitions in MS-MS techniques). The fragmentation patterns of microcystins also give valuable information in structural analysis. The most common fragment of microcystins is obtained through the cleavage of the Adda end

group. The resulting ion is detected as *m/z* 135.2 in the positive ion mode (the same ion can be obtained also through in-source fragmentation). Although reversed-phase HPLC efficiently separates microcystins, the high number of known microcystins, ca 80, requires complicated chromatographic steps for complete resolution. In principle, LC-ESI-MS can replace the need for perfect resolution of microcystins, but in practice a good resolution gives invaluable data for the correct identification and quantitation of microcystins from complex matrices.

Mass spectrometric analysis of microcystins can be performed in different ways depending on the purpose. A quick profile of sample can be obtained by *m/z* scanning; a typical scan mass range for microcystins is from 500 to 1200. A sensitive technique for the detection of microcystins is single (selected) ion recording (SIR), which allows monitoring of one or several selected *m/z* values with a high signal-to-noise ratio. The SIR technique can be performed together with the scan technique - this combines the sensitivity of selected ion monitoring with the total profile of ions in a sample. A triple-quadrupole instrument offers a detection technique called multiple reactant monitoring (MRM), in which a pre-chosen (microcystin) ion is accelerated and collided with a collision gas in the second quadrupole, and a cleaved fragment, normally *m/z* 135.2, is monitored. The obtained signal from the chosen transition is lower than the signal of the precursor microcystin in SIR analysis, but the signal-to-noise ratio is high. The MRM technique can be applied to samples with a high noise background or when the correct identification of microcystins is critical.

There are some drawbacks in the quantitation of microcystins by MS instruments compared to UV detection. The response of different microcystins are different and require individual reference compounds for quantitation. The standard curve in LC-MS is not linear and its shape is characteristic for each microcystin. The level of sensitivity may vary greatly from run to run, especially during a long sample sequence. Sensitivity may be affected by e.g. matrix effect and condition of cone. When the analysis of microcystins is started, it usually takes several runs before a stable signal level is reached, usually the sensitivity rises for several runs until it reaches the stable state. During the sequence, the sensitivity may fluctuate between runs and eventually the sample cone (a part of the ion source) becomes so contaminated that signal may drop totally. Quantitation by LC-MS needs continuous monitoring of sensitivity by a set of standards. After cleaning of the ion source componens, new standard curves and stabilisation time are necessary.

Coeluting compounds can cause matrix effects. As a result of ionisation competition between the analytes and matrix components, the matrix can either lower or increase the signal of the analyte. Signal suppression or enhancement in the sample matrix is typically expressed in relation to the signal of the analyte injected in pure solvent. During a run sensitivity may be influenced by the amount of acid present (e.g. formic acid) at the time of elution. Addition of acid (or base) enhances analyte ionisation. Trifluoroacetic acid (TFA) is often added to the mobile phases in HPLC-UV methods in order

to obtain a better separation of microcystins. However, TFA causes ion suppression in LC-MS and it should be replaced by another acid (e.g. formic acid) when acidic mobile phases are used in LC-MS. It is also important to remember that non-volatile buffers such as phosphate cannot be used in LC-ESI-MS.

2 Experimental and practical advice

2.1 Materials

Solvents. Acetonitrile (HPLC gradient grade or better), LC-MS grade water purified to 18.2 MΩ cm and with low organic carbon content, and formic acid (analytical grade). Formic acid is added (0.1-0.5%) to either both mobile phases components or to the aqueous component only. Replace the aqueous component at least every three days to prevent growth of microorganisms.

HPLC column. C_{18} endcapped HPLC column, e.g. 30 mm x 4 mm I.D. 3 µm particles, 55 x 4 mm I.D. 3 µm particles or 250 x 4 mm I.D. 5 µm particles. Some mass spectrometrists prefer narrow-bore columns. Even the shorter columns usually offer adequate separation in LC-MS and the examples in this SOP relate to the use of a 30 mm column. The column should be equipped with a guard column.

Other materials. Borosilicate glass chromatographic vials and screw caps with silicone/PTFE septa. For small sample volumes, 0.3 ml polypropylene vials or glass inserts.

2.2 Special equipment

LC-MS system. High-performance liquid chromatograph equipped with a high-pressure or low-pressure gradient pump system, an autosampler and a column oven. The HPLC is interfaced to the MS with an electrospray ion source. A quadrupole mass analyser can be used for scan and SIR analyses, a triple-quadrupole mass analyser for scan, SIR and MRM analyses, and an ion-trap mass analyser for scan, SIR and MS^n analyses.

2.3 LC-ESI-MS

2.3.1 General procedure

Set up HPLC. The HPLC system should be set up according to good HPLC practice: e.g., unnecessary dead volumes should be avoided and the mobile phases should be degassed. All components and parameters should be monitored and documented. Always use a guard column. Change the guard column if the back-pressure rises or peak forms deteriorate. Set column oven at 40 ºC. Change the HPLC gradually up to starting conditions and allow to condition. The flow is directed to waste while conditioning.

Set up MS. The MS instrument with the electrospray ion source should be set up and tuned as described in the manufacturer instructions. The following

ionisation parameters are given just as an example for one triple-quadupole instrument (Micromass Quattro micro) in the analysis of microcystins. REMEMBER: EVERY INSTRUMENT MODEL MUST BE TUNED INDIVIDUALLY. Examples of ionization parameters: capillary voltage 3.8 kV, cone voltage 80 V (for microcystin-RR and its analogues 40 V if less in-source fragmentation is desired), source temperature 150 ºC, desolvation temperature 300-350 ºC, cone gas flow 50 l h^{-1}, desolvation gas 650 l h^{-1}.

Analytes and standards. Analyse the samples and standards according to the recommended HPLC gradient (Table 2; other column dimensions necessitate adjustment of the gradient and the flow rate). Make 10 µl injections. Higher volumes, e.g. 40-50 µl, may be used when the concentration of microcystins is low and the sample is dissolved in an aqueous solvent. For quantification, the recommended sequence of samples is as follows:

1) 10-20 injections of a microcystin standard (a single microcystin, extract or mixture of microcystins with toxin concentration(s) determined by HPLC-UV, SOP_TOXIC_AAU_06F). The concentration(s) should be close to the concentrations in the actual samples. These initial samples are used to determine when the instrument has received a stable signal.

2) Samples for the standard curve, e.g. a set of 6-10 standards containing different microcystins with concentrations ranging from 0.001 µg ml^{-1} to 20 µg ml^{-1}. The samples with higher microcystin concentrations should be determined by HPLC-UV, SOP_TOXIC_AAU_06F, and the lower standards should be diluted from the higher concentrations.

3) Samples together with standards imbedded in the sample sequence at regular intervals (e.g. a standard of a certain strength after every 3-5 samples, needed for monitoring of signal fluctuation).

4) Again, the samples for standard curve. If the sequence is longer than 24 hours, these samples should be run in the middle of the sequence also.

Matrix effects can be estimated by spiking known amounts of selected microcystins to samples with no microcystins and then evaluating the observed concentrations. Scan, SIR or MRM techniques can be applied depending on the purpose of analyses. Table 1 shows some *m/z* values.

Table 1: Observed *m/z* values of some typical microcystins.

Microcystin	*m/z*	State of molecular ion
Demethyl-MC-RR	512.7 1024.7	$[M+2H]^{2+}$ $[M+H]^{+}$
MC-RR	519.7 1038.5	$[M+2H]^{2+}$ $[M+H]^{+}$
MC-YR	1045.5	$[M+H]^{+}$
Demethyl-MC-LR	981.5	$[M+H]^{+}$
LR	995.5	$[M+H]^{+}$
LY	1002.5 1024.5	$[M+H]^{+}$ $[M+Na]^{+}$
LW	1025.9 1047.5	$[M+H]^{+}$ $[M+Na]^{+}$
LF	986.6 1008.5	$[M+H]^{+}$ $[M+Na]^{+}$

Analysis of data. Identify the microcystins by comparing retention times and obtained *m/z* values to the standards. Calculate the microcystin concentration according to the standard curves. Evaluate possible changes in sensitivity during the sample sequence. Estimate the matrix effect by spiked samples.

2.3.2 LC-ESI-MS sample chromatograms of microcystins

Table 2: Suggested gradient programme for Merck Purospher STAR RP-18 endcapped, 3 µm particles, 30 mm x 4 mm I.D., linear gradient at a flow rate of 0.5 ml min^{-1}. A = 0.5% formic acid in water, B = acetonitrile. For rapid LC-MS of microcystins, see [Meriluoto et al., 2004].

Time (min)	% A	% B
0.00	75	25
10.00	30	70
12.00	30	90
13.00	30	90
13.50	75	25
18.00	STOP	

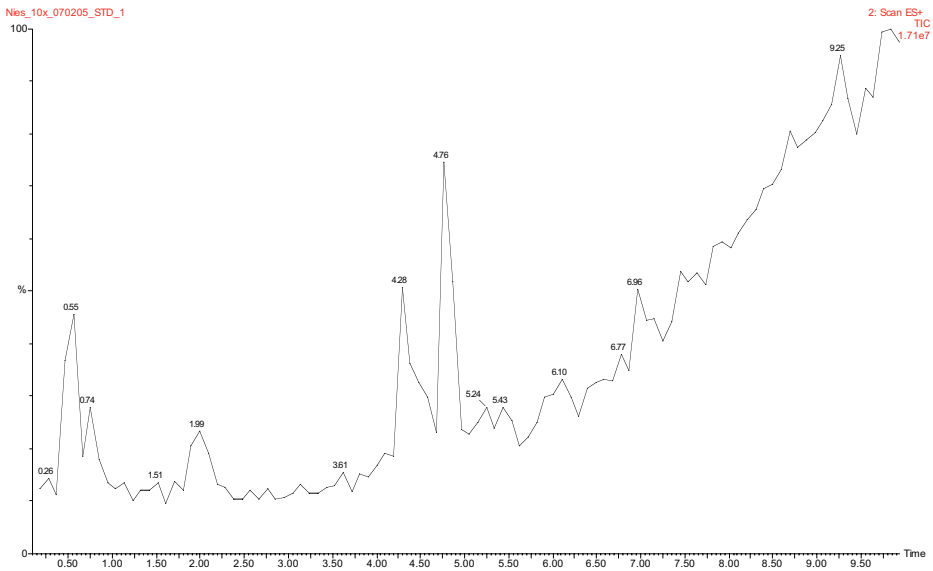

Figure 1: Total ion chromatogram of NIES-107 extract, scan of *m/z* range 500-1200. Retention times: microcystin-RR 1.99 min, microcystin-YR 4.47 min (coelution with an earlier peak), microcystin-LR 4.76 min. Chromatography according to parameters in Table 2.

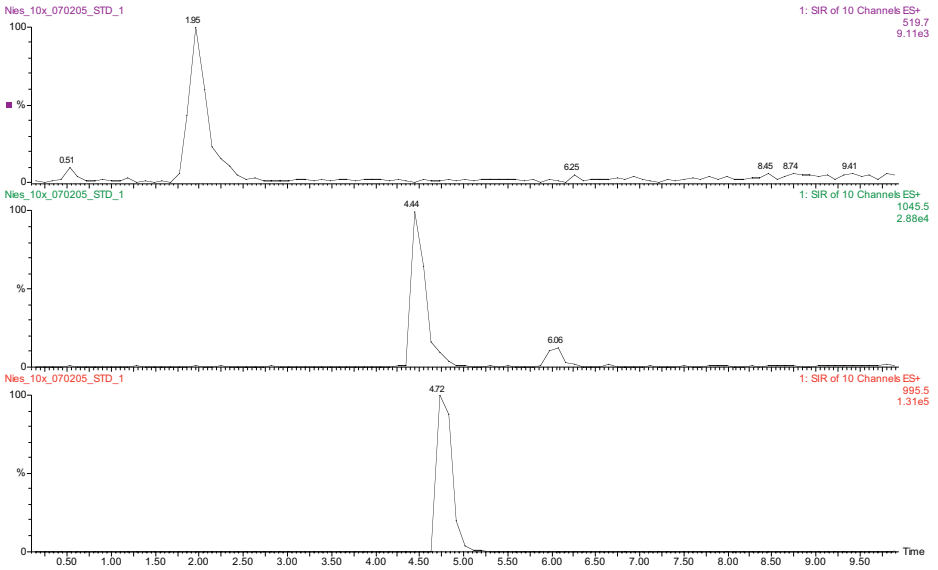

Figure 2: SIR (selected ion recording) chromatograms of microcystin-RR (*m/z* 519.7; 1.95 min), microcystin-YR (*m/z* 1045.5; 4.44 min) and microcystin-LR (*m/z* 995.5; 4.72 min) in NIES-107 extract. Chromatography according to parameters in Table 2.

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(Consult Table 3.3 in Chapter 3, page 27 for more references)

SOP: Field sampling of environmental waters for cyanobacteria and their toxins

Document identifier: SOP_TOXIC_UDU_01F

Prepared by: James S. Metcalf and Geoffrey A. Codd, UDU

Date: 7 July 2005

1 Introduction

In order to obtain reliable, useful information on the production and fates of cyanobacteria and their toxins, environmental monitoring and sampling needs to be performed in the field prior to cyanotoxin analysis. Safety procedures should be carried out, local risk assessments should be performed and national and local regulations should be adhered to.

2 Experimental

2.1 Materials and equipment

(a) Safety equipment (e.g. buoyancy aids, throw ropes, first aid kit, mobile telephone)

(b) Sampling equipment (e.g. dippers)

(c) 2 l sample containers

(d) Thermometer

(e) Dissolved oxygen meter

(f) Conductivity meter

(g) pH meter

(h) Insulated cool-box to hold samples

2.2 General procedure

(a) Always carry out environmental sampling at waterbodies with a minimum of two people and a fully charged, fully credited mobile phone.

(b) At each study site, select the most appropriate place for sampling (e.g. point of water abstraction, highest recreational activity).

(c) Using the sampling equipment, fill the 2 l container with a mixed water sample.

(d) If cyanobacterial scum is present at the site, using the sampling equipment, collect scum material in a separate container.

(e) Measure the temperature, pH, dissolved oxygen and conductivity of the collected water samples at the time of collection if possible.

(f) Store samples in the cool-box and transport the samples back to the laboratory for processing according to SOP_TOXIC_UDU_02F.

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Wetzel, R. G., Likens, G. E.: Limnological Analyses. W. B. Saunders Company, Philadelphia, London, Toronto, 357 pp. (1979).

SOP: Laboratory analysis of samples from environmental waters

Document identifier: SOP_TOXIC_UDU_02F

Prepared by: James S. Metcalf and Geoffrey A. Codd, UDU

Date: 7 July 2005

1 Introduction

After environmental samples have been collected, these require rapid processing to prepare and preserve samples. Samples can be collected for the assessment of pigments and cyanobacterial biovolume. Cyanotoxin analysis can be performed for both the cell-bound and cell-free fractions.

2 Experimental

2.1 Materials

(a) 1 l measuring cylinder

(b) 1 l glass bottles

(c) Filter discs (e.g. Whatman GF/C, 25-70 mm diameter)

(d) Buchner vacuum funnel or similar

(e) Sterile 1.5 ml microcentrifuge tubes

(f) Sterile 1 ml pipette tips

(g) Plastic sterile Petri dishes

(h) Microscope slides and cover slips

(i) Glass Petri dish

2.2 Special equipment

(a) -20 °C freezer

(b) Vacuum filtration apparatus

(c) Oven set at 80 °C

(d) Balance capable of measuring 0.1 mg

(e) Light microscope

2.3 General procedure

(a) In a glass Petri dish, carefully place some GF/C filters in the oven for a minimum of 24 hours.

(b) After the samples have been received in the laboratory a small sub-sample is placed on a microscope slide and cover slip is placed on top to provide an initial indication of the cyanobacterial genera present.

(c) The sample is viewed under the microscope. Cyanotoxins should be analysed according to the genera observed in Table 1. If more than one cyanotoxin needs to be analysed, then multiple filter discs should be prepared.

(d) Determine the weight of a GF/C filter disc to the nearest 0.1 mg.

(e) Measure 1 l of a mixed environmental water sample with the measuring cylinder.

(f) Prepare the vacuum filtration apparatus and filter the 1 l of mixed water sample through the GF/C filter. In the case of high biomass preventing the filtration of 1 l, filter an appropriate reduced volume e.g. 250 ml, 100 ml.

(g) Retain the filtrate in a 1 l glass bottle prior to solid phase extraction (SPE) enrichment for microcystins (SOP_TOXIC_AAU_05F), anatoxin-a (SOP_TOXIC_UDU_04F) or cylindrospermopsin (SOP_TOXIC_UDU_05F).

(h) Place the GF/C filter disc in a labelled plastic Petri dish and store at -20 °C before the extraction of microcystins (SOP_TOXIC_AAU_04F), anatoxin-a (SOP_TOXIC_UDU_06F) or cylindrospermopsin (SOP_TOXIC_UDU_06F).

(i) Prepare a second filter disc in the vacuum apparatus (this does not have to be pre-weighed).

(j) Measure 500 ml of the mixed water sample or an appropriate lesser volume and pour through the vacuum apparatus. The filtrate can be discarded.

(k) Place the filter disc in a labelled plastic Petri dish and store in the freezer for chlorophyll-a analysis (SOP_TOXIC_ULO_01F).

(l) Prepare a Lugol's Iodine sample of each of the mixed water sample according to SOP_TOXIC_UDU_03F.

(m) Into a labelled sterile microcentrifuge tube pipette 1 ml of mixed water sample using sterile pipette tips for microcystin ELISA analysis according to SOP_TOXIC_UDU_10F.

(n) Into a labelled sterile microcentrifuge tube pipette 1 ml of mixed water sample using sterile pipette tips and store in the freezer for PCR analysis.

Table 1: Suggested cyanotoxin analyses according to cyanobacterial genera observed in environmental water samples (TOXIC programme)

Genus	Microcystins	Anatoxin-a	Cylindrospermopsin
Microcystis	+	-[a]	-
Anabaena	+	+	+
Aphanizomenon	-	+	+
Planktothrix	+	+	-
Cylindrospermopsis	-	-	+

a, There is one report of anatoxin-a production by *Microcystis* strains in Japan [Park and Watanabe, 1996]. Analysis of *Microcystis* blooms for anatoxin-a is not routine but may be considered if circumstances merit and resources permit.

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Harada, K. A., Kondo, F., Lawton, L.: Laboratory analysis of cyanotoxins. In *Chorus, I., Bartram, J.* (Eds.), Toxic Cyanobacteria in Water, A Guide to Their Public Health Consequences, Monitoring, and Management, pp. 369-405, E & FN Spon, London (1999).

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SOP: Preservation of cyanobacterial samples in Lugol's Iodine

Document identifier: SOP_TOXIC_UDU_03F

Prepared by: James S. Metcalf, Marianne Reilly and Geoffrey A. Codd, UDU

Date: 7 July 2005

1 Introduction

Although cyanobacteria can easily be identified to the genus level upon microscopic examination, further identification to the species level can be time consuming and differences in the identification of cyanobacterial species are subjective. In order to preserve cyanobacterial samples for future reference and further identification if necessary, Lugol's Iodine solution can be used to preserve cyanobacterial material (although it should be borne in mind that cell shrinkage can occur during storage).

2 Experimental

2.1 Materials

(a) Potassium iodide

(b) Crystalline iodine

(c) Water purified to 18.2 MΩ cm (e.g. Millipore Milli-Q water)

(d) Small glass or plastic containers with caps or lids

2.2 Stock solutions

Store the stock solution at room temperature.

(a) For 1 litre of Lugol's Iodine solution. To 1 l of distilled water, add 100 g potassium iodide and 50 g crystalline iodine and mix together.

2.3 Preparation of cyanobacterial cells in Lugol's Iodine solution

(a) Prepare 50 ml of a water of cyanobacterial sample in a glass or plastic bottle with a sealable lid (e.g. screw cap universal bottle).

(b) To this solution add 3 drops of Lugol's Iodine solution and mix. This should turn the solution to the colour of weak tea.

(c) Store the samples at room temperature, preferably in the dark.

(d) If plastic bottles have been used, the iodine in the Lugol's solution may leach into the plastic. Further additions of Lugol's Iodine or transfer of the samples to new bottles may be necessary.

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SOP: Solid phase extraction of anatoxin-a in filtered water samples

Document identifier: SOP_TOXIC_UDU_04F

Prepared by: James S. Metcalf and Geoffrey A. Codd, UDU

Date: 7 July 2005

1 Introduction

In order to determine the concentration of anatoxin-a in the extracellular fraction of filtered environmental waters, solid phase extraction (SPE) is necessary to concentrate the toxin to concentrations capable of being detectable by HPLC.

2 Experimental

2.1 Materials

Use analytical reagent grade reagents.

(a) C_{18} solid phase extraction cartridges (e.g. Isolute C18(EC) solid phase extraction columns, size 1 g sorbent in 6 ml reservoir, from Argonaut Technologies, Mid Glamorgan, UK)

(b) Glass-fibre filters (e.g, Whatman GF/C), diameter 25-70 mm

(c) Argon or nitrogen, >99.99%

(d) Borosilicate test tubes or vials, >3 ml capacity

(e) Water purified to 18.2MΩ cm (e.g. Millipore Milli-Q water)

(f) Methanol HPLC grade, e.g. HPLC grade methanol, from Rathburn (Walkerburn, Scotland, UK)

(g) Trifluoroacetic acid (TFA), protein sequence analysis grade. TFA should be stored under argon in a desiccator.

(h) Sodium hydroxide

2.2 Special equipment

(a) -20 °C freezer

(b) Vacuum manifold, preferably transparent, equipped with stopcocks, vacuum source and vacuum control

(c) LVE (large volume extraction) kit for unattended loading of large sample volumes, made of PTFE tubing and adapters for column connection

(d) pH meter

(e) Filtration unit (minimum 500 ml)

(f) Rotary evaporator, or hot block and gas apparatus

(g) Microcentrifuge

2.3 Solutions

(a) NaOH, a 1 M solution in Milli-Q water

(b) TFA, 0.1% (v/v) solution in methanol

2.4 Procedure

(a) Filter a minimum of 1 litre of water through a GF/C filter (if not already performed as in SOP_TOXIC_UDU_02F). Volumes may have to be reduced in the case of dense blooms or scum samples.

(b) Measure the pH of the filtered water and change to pH 9.6 by the addition of NaOH.

(c) Condition the C_{18} SPE cartridge with 10 ml methanol containing 0.1% (v/v) TFA followed by 10 ml of water. Apply the sample to the cartridge. Do not let the cartridge dry during conditioning and sample application.

(d) Dry the cartridge by passing air through it.

(e) Elute anatoxin-a with 3 ml 0.1% (v/v) TFA in methanol and collect in a suitable borosilicate container.

(f) Evaporate the methanolic solution at 50 °C under argon or nitrogen.

(g) Resuspend the residue in 500 µl of Milli-Q water.

(h) Centrifuge at full speed (e.g. 10,000 × *g*, 10 mins). Analyse the supernatant for anatoxin-a according to SOP_TOXIC_UDU_08F.

3 References

Rapala, J., Sivonen, K., Luukkainen, R., Niemelä, S. I.: Anatoxin-a concentration in *Anabaena* and *Aphanizomenon* under different environmental conditions and comparison of growth by toxic and non-toxic *Anabaena* strains – A laboratory study. J. Appl. Phycol. **5**, 581-591 (1993).

SOP: Solid phase extraction of cylindrospermopsin in filtered water samples

Document identifier: SOP_TOXIC_UDU_05F

Prepared by: James S. Metcalf and Geoffrey A. Codd, UDU

Date: 7 July 2005

1 Introduction

In order to determine the concentration of cylindrospermopsin in the extracellular fraction of filtered environmental waters, solid phase extraction (SPE) is necessary to concentrate the toxin to concentrations capable of being detected by HPLC.

2 Experimental

2.1 Materials

Use analytical reagent grade reagents.

(a) Polygraphite carbon solid phase extraction cartridges (PGC), e.g. Thermo Hypersil keystone Hypersep Hypercarb 200 mg, 3 ml cartridges, from Thermo Hypersil Keystone, UK

(b) C_{18} solid phase extraction cartridges, e.g. Isolute C18(EC) solid phase extraction columns, size 1 g sorbent in 6 ml reservoir from Argonaut Technologies, Mid Glamorgan, UK

(c) Glass-fibre filters (e.g. Whatman GF/C), diameter 25-70mm

(d) Argon or nitrogen, >99.99%

(e) Borosilicate test tubes or vials, >3 ml capacity

(f) Water purified to 18.2 MΩ cm (e.g. Millipore Milli-Q water)

(g) Methanol HPLC grade, e.g. HPLC grade methanol from Rathburn (Walkerburn, Scotland, UK)

(h) Trifluoroacetic acid (TFA), protein sequence analysis grade. TFA should be stored under argon in a desiccator.

2.2 Special equipment

(a) -20 °C freezer

(b) Vacuum manifold, preferably transparent, equipped with stopcocks, vacuum source and vacuum control

(c) LVE (large volume extraction) kit for unattended loading of large sample volumes, made of PTFE tubing and adapters for column connection.

(d) Filtration unit (minimum 500 ml)

(e) Rotary evaporator or hot block and gas apparatus

(f) Microcentrifuge

2.3 Solutions

(a) TFA, 0.1% (v/v) solution in methanol

2.4 Procedure

(a) Filter a minimum of 1 litre of water through a GF/C filter (if not already performed as in SOP_TOXIC_UDU_02F). Volumes may have to be reduced in the case of dense bloom/scum samples.

(b) Prepare a combined "in series" SPE system with a C_{18} cartridge connected to a PGC cartridge (C_{18} before PGC).

(c) Condition the combined SPE system with 10 ml methanol containing 0.1% (v/v) TFA followed by 10 ml of water. Apply the sample to the cartridges. Do not let the cartridges dry during condition and sample application.

(d) Dry the PGC cartridge by passing air through it.

(e) Elute cylindrospermopsin with 3 ml 0.1% (v/v) TFA in methanol from the PGC cartridge and collect in a suitable borosilicate container.

(f) Evaporate the methanolic solution at 50 °C under argon or nitrogen.

(g) Resuspend the residue in 500 µl of Milli-Q water.

(h) Centrifuge at full speed (e.g. 10,000 × *g*, 10 mins). Analyse the supernatant for cylindrospermopsin according to SOP_TOXIC_UDU_09F.

3 References

Metcalf, J. S., Beattie, K. A., Saker, M. L., Codd, G. A.: Effects of organic solvents on the high performance liquid chromatographic analysis of the cyanobacterial toxin cylindrospermopsin and its recovery from environmental eutrophic waters by solid phase extraction. FEMS Microbiol. Lett. **216**, 159-164 (2002).

Norris, R. L. G., Eaglesham, G. K., Shaw, G. R., Senogles, P., Chiswell, R. K., Smith, M. J., Davis, B. C., Seawright, A. A., Moore, M. R.: Extraction and purification of the zwitterions cylindrospermopsin and deoxycylindrospermopsin from *Cylindrospermopsis raciborskii*. Environ. Toxicol. **16**, 391-396 (2001).

SOP: Extraction of anatoxin-a or cylindrospermopsin from cyanobacterial biomass filtered on glass-fibre filters

Document identifier: SOP_TOXIC_UDU_06F

Prepared by: James S. Metcalf and Geoffrey A. Codd, UDU

Date: 7 July 2005

1 Introduction

For the analysis of anatoxin-a or cylindrospermopsin associated with cyanobacterial biomass, the cells can be recovered on GF/C filters before the extraction of these cyanotoxins.

2 Experimental

2.1 Materials

Use analytical reagent grade reagents.

(a) Glass-fibre filters, e.g. Whatman GF/C, diameter 25-70 mm

(b) Argon or nitrogen, >99.99%

(c) Borosilicate test tubes or vials, >3 ml capacity

(d) Water purified to 18.2MΩ cm (e.g. Millipore Milli-Q water)

(e) Methanol HPLC grade, e.g. HPLC grade methanol, from Rathburn (Walkerburn, Scotland, UK)

(f) Microcentrifuge tubes

(g) Parafilm

2.2 Special equipment

(a) -20 °C freezer

(b) Bath ultrasonicator

(c) Probe ultrasonicator

(d) Freeze-drier unit

(e) Rotary evaporator or hot block and gas apparatus

(f) Microcentrifuge

2.3 Procedure

(a) Remove frozen filter discs in Petri dishes from freezer and place in the freeze-drier unit until they are dry (e.g. after 24-48 hours).

(b) Place the freeze-dried discs in a suitable borosilicate container and add 1.2 ml of 100% methanol, mix and extract in the bath ultrasonicator for 15 minutes. Filters with a diameter greater than 47 mm are extracted with 2 ml methanol. During the incubation, the tubes should be covered with parafilm.

(c) Ultrasonicate each of the samples for a further 1 minute using the probe ultrasonicator, washing the probe with 100% methanol in between samples. When using the probe ultrasonicator, keep the samples on ice so as to prevent excessive heating within the sample.

(d) Centrifuge aliquots of the extracts at a minimum of 10,000 × *g* for 10 minutes.

(e) Transfer 500 µl of the supernatants to 1.5 ml borosilicate glass vials or test tubes and evaporate to dryness using the hot block and gas apparatus at 50 °C.

(f) Reconstitute the aliquots in a total volume of 250 µl of Milli-Q water for each sample and transfer to a microcentrifuge tube.

(g) Centrifuge at a minimum of 10,000 × *g* for 10 minutes.

(h) Analyse the supernatant for anatoxin-a (SOP_TOXIC_UDU_08F) or cylindrospermopsin (SOP_TOXIC_UDU_09F) by HPLC.

3 References

Edwards, C, Beattie, K. A., Scrimgeour, C. M. Codd, G. A.: Identification of anatoxin-a in benthic cyanobacteria (blue-green algae) and in associated dog poisonings at Loch Insh, Scotland. Toxicon **30**,1165-1175 (1992).

Metcalf, J. S., Beattie, K. A., Saker, M. L., Codd, G. A.: Effects of organic solvents on the high performance liquid chromatographic analysis of the cyanobacterial toxin cylindrospermopsin and its recovery from environmental eutrophic waters by solid phase extraction. FEMS Microbiol. Lett. **216**,159-164 (2002).

SOP: Preparation of standard solutions of cylindrospermopsin and anatoxin-a for HPLC calibration

Document identifier: SOP_TOXIC_UDU_07F

Prepared by: James S. Metcalf and Geoffrey A. Codd, UDU

Date: 7 July 2005

1 Introduction

Calibration of HPLC systems with the cyanotoxins anatoxin-a and cylindrospermopsin is based on the spectrophotometric determination of their concentrations. The recorded absorbance is converted into the known concentrations of the cyanotoxins using the known molar absorptivity for each toxin.

2 Experimental

2.1 Materials

Use analytical reagent grade reagents.

(a) Purified cylindrospermopsin (>99% pure by HPLC)

(b) Purified anatoxin-a (>99% pure by HPLC)

(c) Water purified to 18.2 MΩ cm (e.g. Millipore Milli-Q water)

(d) HPLC grade ethanol (e.g. from Rathburn, Walkerburn, Scotland)

(e) Clean borosilicate glass test tubes or vials, 4 ml capacity

(f) Borosilicate glass chromatographic vials

2.2 Special equipment

(a) Pipettes capable of accurately dispensing 0.5 ml – 4 ml of purified water

(b) HPLC instrument, specifications according to the procedure SOP_TOXIC_UDU_08F (anatoxin-a) and SOP_TOXIC_UDU_09F (cylindrospermopsin)

2.3 Procedure

2.3.1 Preparation of standard curves for cylindrospermopsin

(a) Check the spectrophotometer absorbance according to procedure SOP_TOXIC_EXT_02F.

(b) Dissolve ca 30 µg (one vial) of purified cylindrospermopsin in 3 ml purified water. Mix well and measure the absorbance of the solutions at 262 nm using purified water as a reference. The molar absorptivity of cylindrospermopsin has been reported to be 6250 [Banker et al., 2000] and 6100 [Harada et al., 1994]. The mean of these two values is 6175 and an absorbance of 0.4 equals 64.7 µM or 26.8 µg cylindrospermopsin per ml.

(c) Prepare a 10 µg per ml solution of the spectrophotometrically determined cylindrospermopsin using purified water. From this solution, prepare a dilution series of the spectrophotometrically determined solutions of cylindrospermopsin. Mix thoroughly 500 µl of cylindrospermopsin solution with 500 µl purified water. Make five further dilutions, 1 volume + 1 volume, in a similar manner. Your dilutions including the original solution should then cover approximately the range 1-100 ng cylindrospermopsin per 10 µl injection.

(d) Analyse the samples on the HPLC system in duplicate injections. If you have several columns to calibrate you can divide the cylindrospermopsin dilutions in aliquots into 0.3 ml polypropylene vials designed for smaller volumes. Store the calibrants refrigerated (+4 °C) and use within one day from preparation.

(e) Calculate the linear regression for your calibration curve using e.g. a calculator or Microsoft Excel software. An example of a calibration curve for microcystin-LR is shown in SOP_TOXIC_AAU_03F.

$y = mx + b$

y = ng cylindrospermopsin per injection
x = peak area

The slope of the calibration line, m, gives the response factor, which is characteristic for your specific chromatographic conditions. The y-axis intercept, b, should be negligible (typically below 0.3) and the correlation coefficient, R^2, should approach 1.

(f) Monitor column performance regularly by injection of known samples and repeat the calibration as necessary, typically after 1 or 2 months (depending on the amount of samples, solvents used etc).

2.3.2 Preparation of standard curves for anatoxin-a

(a) Check the spectrophotometer absorbance according to the procedure SOP_TOXIC_EXT_02F.

(b) Dissolve ca 30 µg (one vial) of purified anatoxin-a in 3 ml HPLC grade ethanol. Mix well and measure the absorbance of the solutions at 227 nm using ethanol as a reference. The log molar absorptivity of anatoxin-a has been reported to be 4.10 [Harada et al., 1989]. An absorbance of 0.4 equals 31.7 µM or 5.2 µg anatoxin-a per ml.

(c) Prepare a 10 µg per ml solution of the spectrophotometrically determined anatoxin-a using HPLC grade ethanol. Dry a known volume of this solution in the hot block and gas apparatus.

(d) Resuspend the dried film to the same volume with purified water. Prepare a dilution series of the spectrophotometrically determined solutions of anatoxin-a. Mix thoroughly 500 µl of anatoxin-a solution with 500 µl purified water. Make five further dilutions, 1 volume + 1 volume, in a similar manner. Your dilutions including the original solution should then cover approximately the range 1-100 ng anatoxin-a per 10 µl injection.

(e) Analyse the samples on the HPLC system in duplicate injections. If you have several columns to calibrate you can divide the anatoxin-a dilutions in aliquots into 0.3 ml polypropylene vials designed for smaller volumes. Store the calibrants refrigerated (+4 °C) and use within one day from preparation.

(f) Calculate the linear regression for your calibration curve using e.g. a calculator or Microsoft Excel software. An example of a calibration curve for microcystin-LR is shown in SOP_TOXIC_AAU_03F.

$y = mx + b$

y = ng anatoxin-a per injection
x = peak area

The slope of the calibration line, m, gives the response factor, which is characteristic for your specific chromatographic conditions. The y-axis intercept, b, should be negligible (typically below 0.3) and the correlation coefficient, R^2, should approach 1.

(g) Monitor column performance regularly by injection of known samples and repeat the calibration as necessary, typically after 1 or 2 months (depending on the amount of samples, solvents used etc).

3 References

Harada, K.-I., Kimura, Y., Ogawa, K., Suzuki, M., Dahlem, A. M., Beasley, V. R., Carmichael, W. W.: A new procedure for the analysis and purification of naturally occurring anatoxin-a from the blue-green alga *Anabaena flos-a*quae. Toxicon **27**, 1289-1296 (1989).

Harada, K.-I., Ohtani, I., Iwamoto, K., Suzuki, M., Watanabe, M. F., Watanabe, M., Terao, K.: Isolation of cylindrospermopsin from a cyanobacterium *Umezakia natans* and its screening method. Toxicon **32**, 73-84 (1994).

Banker, R., Teltsch, B., Sukenik, A., Carmeli, S.: 7-epicylindrospermopsin, a toxic minor metabolite of the cyanobacterium *Aphanizomenon ovalisporum* from Lake Kinneret, Israel. J. Nat. Prod. **63**, 387-389 (2000).

SOP: Analysis of anatoxin-a by high-performance liquid chromatography with photodiode-array detection

Document identifier: SOP_TOXIC_UDU_08F

Prepared by: James S. Metcalf and Geoffrey A. Codd, UDU

Date: 7 July 2005

1 Introduction

Anatoxin-a and related toxins are nicotinic agonists produced by some strains of cyanobacteria. A number of methods have been used for the determination of these toxins and high-performance liquid chromatography (HPLC) is commonly used. Although HPLC has been used for the detection of anatoxin-a, UV and PDA detection, MS detection and fluorescence detection have also been successful. Although HPLC-PDA detection is not as sensitive as fluorescence or MS detection, this HPLC method can be successfully used to determine these alkaloid neurotoxins.

2 Experimental

2.1 Materials

Use analytical reagent grade reagents.

(a) Anatoxin-a, Antx-a

(b) Water purified to 18.2 MΩ cm (e.g. Millipore Milli-Q water)

(c) Acetonitrile, ACN

(d) Trifluoroacetic acid, TFA

(e) C_{18} Reversed phase HPLC column (e.g. Phenomenex Luna 5 µm, 150 mm × 4.6 mm I.D.)

2.2 Special equipment

(a) Freezer, -20 ˚C

(b) Dual pump high-performance liquid chromatography equipment including either a UV or photodiode-array (PDA) detector

(c) Chromatography analysis software (e.g. Waters Millennium)

2.3 Stock solutions

Store the stock solutions at -20 ˚C.

(a) Anatoxin-a in water (concentrated stock, e.g. 1 mg ml^{-1}) or stored dry at -20 ˚C unless previously used

2.4 Working solutions and HPLC mobile phase components

(a) Anatoxin-a for analysis. Diluted from stock to make e.g. 100 µl of a 1 µg ml^{-1} solution with Milli-Q water

(b) Samples should be prepared in water and clarified for HPLC analysis (e.g. centrifugation)

(c) HPLC mobile phase component A: Milli-Q water + 0.1% (v/v) TFA

(d) HPLC mobile phase component B: ACN + 0.1% (v/v) TFA

Please observe the following:

Work in fume hood with TFA or acetonitrile.
Dispense the TFA under the surface of acetonitrile.
Do not contaminate the original TFA bottle with acetonitrile.

2.5 Chromatography

2.5.1 General procedure

(a) The HPLC system should be set up as described in the manufacturer's instructions including degassing, priming and changing columns.

(b) Always use a guard column. Change the guard column if the back-pressure rises or peak forms deteriorate.

(c) If you have a column oven on the HPLC, set at 40 °C.

(d) Change the HPLC gradually up to starting conditions and allow to condition.

(e) Chromatograph the samples and standards as per the HPLC gradient (see below), use 10 to 25 µl injections.

(f) Analysis of the chromatogram. Compare retention times and spectra to standards.

(g) Compare the chromatograms of samples to the standard using the spectra and peak area.

(h) Peaks that have the same spectra as the standard can be calculated as anatoxin-a equivalents using the equation

$$\text{Antx-a } (\mu g\ ml^{-1}) = \frac{\text{Sample area}}{\text{Standard area}} \times \text{concentration of standard } (\mu g\ ml^{-1})$$

2.5.2 HPLC of anatoxin-a on a medium column, suitable for small to medium complexity

Table 1: Suggested gradient for Phenomenex Luna column (Section 2.1), linear gradient at a flow rate of 1 ml min^{-1}. Injection cycle takes about 27 minutes.

Time (mins)	% A (H_2O + 0.1% TFA)	% B (ACN + 0.1% TFA)
0	100	0
15.0	85	15
15.5	0	100
16.5	0	100
18.0	100	0
25.0	100	0

N.B. Due to differences in HPLC systems, this gradient is given as an example and may require modification

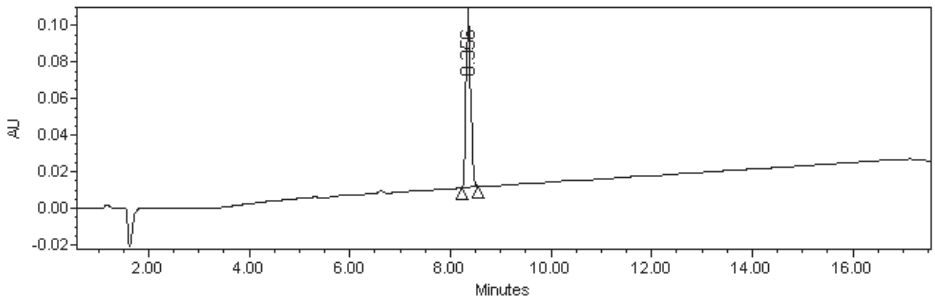

Figure 1: Trace of anatoxin-a. Column, Phenomenex Luna C_{18}, 5 µm particles, 150 × 4.6 mm I.D., detection at 227 nm. Other parameters as in Table 1.

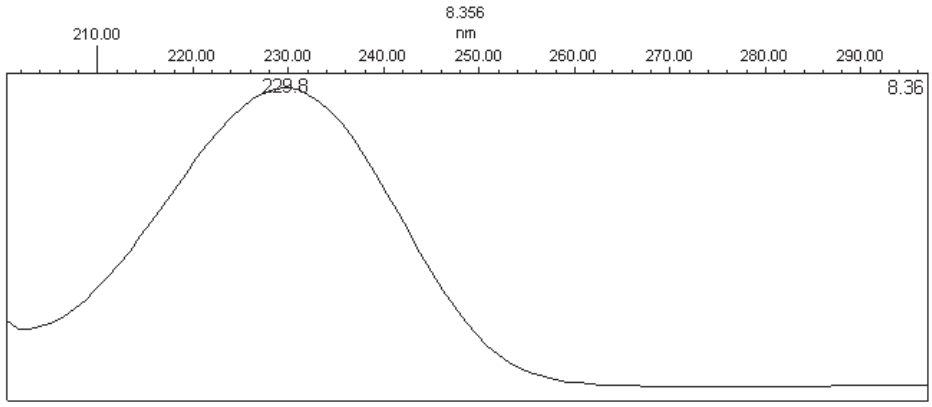

Figure 2: UV spectrum of anatoxin-a, chromatographic parameters as in section 2.5.2.

3 References

Edwards, C, Beattie, K. A., Scrimgeour, C. M., Codd, G. A.: Identification of anatoxin-a in benthic cyanobacteria (blue-green algae) and in associated dog poisonings at Loch Insh, Scotland. Toxicon **30**, 1165-1175 (1992).

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James, K. J., Sherlock, I. R., Stack, M. A.: Anatoxin-a in Irish freshwater and cyanobacteria, determined using a new fluorimetric liquid chromatographic method. Toxicon **35**, 963-971 (1997).

James, K. J., Furey, A., Sherlock, I. R., Stack, M. A., Twohig, M., Caudwell, F. B., Skullberg, O. M. Sensitive determination of anatoxin-a, homoanatoxin-a and their degradation products by liquid chromatography with fluorimetric detection. J. Chromatogr. A **798**, 147-157 (1998).

SOP: Analysis of cylindrospermopsin by high-performance liquid chromatography with photodiode-array detection

Document identifier: SOP_TOXIC_UDU_09F

Prepared by: James S. Metcalf and Geoffrey A. Codd, UDU

Date: 7 July 2005

1 Introduction

Cylindrospermopsins are guanidine alkaloid hepatotoxins produced by some strains of cyanobacteria. A number of methods have been used for the determination of these toxins and high-performance liquid chromatography (HPLC) is commonly used. Although HPLC has been used for the detection of cylindrospermopsins, UV, photodiode-array (PDA) detection and MS detection have all been successful. Although HPLC-PDA detection is not as sensitive as MS detection of cylindrospermopsin, this HPLC method can be successfully used to determine this alkaloid hepatotoxin.

2 Experimental

2.1 Materials

Use analytical reagent grade reagents.

(a) Cylindrospermopsin, Cyn

(b) Water purified to 18.2 MΩ cm (e.g. Millipore Milli-Q water)

(c) Methanol (MeOH)

(d) C_{18} Reversed phase HPLC column (e.g. Naclai Tesque Cosmosil 5 µm, 150 mm × 4.6 mm I.D.)

2.2 Special equipment

(a) Freezer, -20 ˚C

(b) Dual pump high-performance liquid chromatography equipment including either a UV or photodiode-array (PDA) detector

(c) Chromatography analysis software (e.g. Waters Millennium)

2.3 Stock solutions

Store the stock solutions at -20 ˚C.

(a) Cylindrospermopsin in water (concentrated stock, e.g. 1 mg ml^{-1}) or stored dry at -20 ˚C unless previously used

2.4 Working solutions and HPLC mobile phase components

(a) Cylindrospermopsin for analysis. Diluted from stock to make e.g. 100 µl of a 1 µg ml^{-1} solution with Milli-Q water.

(b) Samples should be prepared in water and clarified for HPLC analysis (e.g. by centrifugation).

(c) HPLC mobile phase A: Milli-Q water

(d) HPLC mobile phase B: Methanol

If acetonitrile or TFA is to be used:

-work in fume hood.
-dispense the TFA under the surface of acetonitrile.
-do not contaminate the original TFA bottle with acetonitrile.

2.5 Chromatography

2.5.1 General procedure

(a) The HPLC system should be set up as described in the manufacturers instructions including degassing, priming and changing columns.

(b) If you have a column oven on the HPLC, set at 40 °C.

(c) Change the HPLC gradually up to starting conditions and allow to condition.

(d) Run samples and standards as per the HPLC gradient with 100 µl of standard or sample per vial and a 25 µl injection to the HPLC.

(e) Analysis of the chromatogram

(f) Compare the chromatograms of samples to the standard using the spectra and peak area.

(g) Peaks that have the same spectra as the standard can be calculated as cylindrospermopsin equivalents using the equation

$$\text{Cyn } (\mu g\ ml^{-1}) = \frac{\text{Sample area}}{\text{Standard area}} \times \text{concentration of standard } (\mu g\ ml^{-1})$$

2.5.2 HPLC of cylindrospermopsin on a medium column, suitable for small to medium complexity

Table 1: Suggested gradient for a Naclai Tesque Cosmosil column (Section 2.1), linear gradient at a flow rate of 1 ml min^{-1}. Injection cycle takes about 35 minutes.

Time (mins)	% A (H_2O)	% B (MeOH)
0	99	1
24	88	12
25	88	12
26	0	100
28	0	100
29	99	1
35	99	1

N.B. Due to differences in HPLC systems, this gradient is given as an example and may require modification.

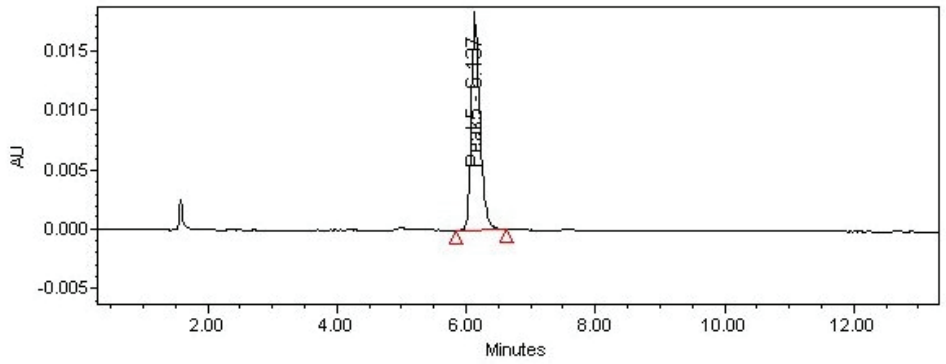

Figure 1: Trace of cylindrospermopsin. Column, Naclai Tesque Cosmosil C_{18}, 5 µm particles, 150 × 4.6 mm I.D., detection at 262 nm. Other parameters as in Table 1.

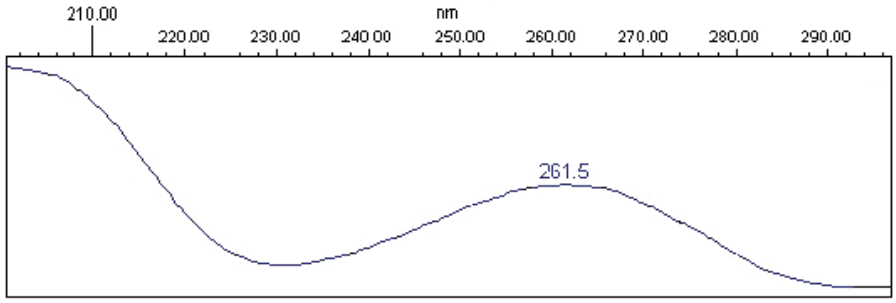

Figure 2: UV spectrum of cylindrospermopsin, chromatographic parameters as in section 2.5.2.

3 References

Eaglesham, G. K., Norris, R. L., Shaw, G. R., Smith, M. J., Chiswell, R. K., Davis, B. C., Neville, G. R., Seawright, A. A., Moore, R. E.: Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue-green algal toxin, for public health purposes. Environ. Toxicol. **14**, 151-154 (1999).

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Metcalf, J. S., Beattie, K. A., Saker, M. L., Codd, G. A.: Effects of organic solvents on the high performance liquid chromatographic analysis of the cyanobacterial toxin cylindrospermopsin and its recovery from environmental eutrophic waters by solid phase extraction. FEMS Microbiol. Lett. **216**, 159-164 (2002).

SOP: Analysis of environmental cyanobacterial samples by ELISA for microcystins

Document identifier: SOP_TOXIC_UDU_10F

Prepared by: James S. Metcalf and Geoffrey A. Codd, UDU

Date: 7 July 2005

1 Introduction

Immunoassays including ELISAs are increasingly being used for the detection and quantification of microcystins in environmental samples and clinical material. Sample preparation is important to obtain meaningful quantitative data using these sensitive, specific tests.

2 Experimental

2.1 Materials

Use analytical reagent grade reagents.

(a) Water purified to 18.2 MΩ cm (e.g. Millipore Milli-Q water)

(b) Microcentrifuge tubes

(c) Sterile microcentrifuge tubes

(d) Sterile pipette tips (1000 µl)

(e) Beakers (200-500 ml)

(f) Ice

2.2 Special equipment

(a) -20 °C freezer

(b) Microcentrifuge

(c) Bunsen burner

(d) Tripod and gauze

(e) Autoclave

(f) Plate reader

(g) Sterile plastic disposable syringes (1-5 ml)

(h) Microcystin ELISA kit

2.3 Procedure

2.3.1 Preparation of the samples

(a) Take the microcystin ELISA samples (SOP_TOXIC_UDU_02F) and collapse the gas vacuoles in the cyanobacteria, either by banging the filled container on the bench or by taking the sample up in a sterile plastic syringe, sealing the end, pulling back the syringe plunger and releasing two or three times.

(b) Centrifuge at 10,000 × *g* for 5 to 10 minutes.

(c) Using sterile pipette tips, remove the supernatant and place in a fresh microcentrifuge tube.

(d) The pellets and the supernatant can be stored at -20 °C until ready for analysis.

2.3.2 Extraction of microcystins from pelleted cyanobacterial material

(a) If frozen, allow the samples to thaw and warm to room temperature.

(b) Boil 100 to 300 ml of Milli-Q water with the Bunsen burner, tripod and gauze.

(c) Resuspend the pellet to a volume of 200 µl.

(d) Close the microcentrifuge tube lid and place in a rack in the boiling water bath for 60 seconds.

(e) Cool on ice and centrifuge at 10,000 × *g* for 5 to 10 minutes.

2.3.3 Microcystin ELISA analysis

(a) Analyse the supernatant from sections 2.3.1(c) and 2.3.2(e) according to the manufacturer's or producer's instructions.

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SOP: Determination of biomass using chlorophyll-a analysis

Document identifier: SOP_TOXIC_ULO_01F

Prepared by: Malgorzata Tarczynska, ULO

Date: 31 March, 2003

1 Introduction

Chlorophyll-a is a widely used and accepted indicator of cyanobacterial biomass. It is considerably less time-consuming than microscopic biomass determination but also less specific and less precise. However, it is a useful measure during cyanobacterial blooms, when the phytoplankton chiefly consists of cyanobacteria.

2 Experimental

2.1 Materials

(a) Aqueous acetone solution

(b) Saturated magnesium carbonate solution

(c) Hydrochloric acid

(d) Distilled water

(e) Glass fibre filters (e.g. Whatman GF/C), 25 to 70 mm diameter

(f) Centrifuge test tubes with screw caps, capacity for at least 8 ml

2.2 Special equipment

(a) Spectrophotometer

(b) Glass cuvettes, typically of 1 cm path length

(c) Centrifuge

(d) Pipettes

(e) Filtration apparatus

(f) Vacuum pump

(g) Freezer, -20 °C

2.3 Stock solutions

(a) Saturated magnesium carbonate solution, add 1.0 g finely powdered $MgCO_3$ to 100 ml distilled water

(b) Aqueous acetone solution, 90 volumes of acetone and 10 volumes of saturated magnesium carbonate solution

(c) Hydrochloric acid, add 83 ml HCl to 917 ml distilled water.

2.4 Procedure

(a) Filter the water sample through a GF/C glass-fibre filter (SOP_TOXIC_UDU_02F).

(b) If extraction cannot be performed immediately, filters should be placed in individual, labelled centrifuge test tubes and stored at -20 °C in the dark.

(c) Add 6 ml 90% aqueous acetone solution. Place the cap on the tube and store in the dark at +4 °C for 24-48 hours.

(d) Centrifuge for 15 minutes at 3,000-5,000 × *g* to clarify samples.

(e) Blank spectrophotometer with 90% aqueous acetone solution at each wavelength.

(f) Transfer 3 ml clarified extract to a 1-cm cuvette and read the optical density at 750 nm and at 664 nm.

(g) Acidify the extract in the cuvette with 0.1 ml 1 M HCl. Adjust the volume added according to the volume used, calculating approximately 0.003 ml of 1 M HCl per ml of acetone solution.

(h) Gently agitate the acidified extract for 90 seconds.

(i) Read the optical density at 750 nm and at 665 nm.

2.5 Calculation

(a) Correct for turbidity by subtracting absorbance:
664_b-750_b = corrected 664_b absorbance
665_a-750_a = corrected 665_a absorbance

(b) Using the corrected values calculate chlorophyll-a and pheophytin-a per cubic meter as follows:

$$\text{Chlorophyll-a, mg m}^{-3} = \frac{26.7\ (664_b - 665_a) \times V_1}{V_2 \times L}$$

$$\text{Pheophytin-a, mg m}^{-3} = \frac{26.7\ [1.7(665_a) - 664_b] \times V_1}{V_2 \times L}$$

where:
V_1 = volume of extract, ml
V_2 = volume of sample, l
L = light path length or width of cuvette, cm
664_b, 665_a = absorbances of 90% acetone extract before and after acidification, respectively

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Lawton, L., Marsalek, B., Padisak, J., Chorus, I.: Determination of cyanobacteria in the laboratory. In *Chorus, I., Bartram, J.* (Eds.), Toxic Cyanobacteria in Water, A Guide to Their Public Health Consequences, Monitoring, and Management, pp. 347-367, E & FN Spon, London (1999).

SOP: Determination of cyanobacterial biomass using *in vivo* phycocyanin fluorescence

Document identifier: SOP_TOXIC_ULO_02F

Prepared by: Katarzyna Izydorczyk, ULO and Wido Schmidt, TZW

Date: 7 July 2005

1 Introduction

The pigmentation of cyanobacteria differs from that of eukaryotic algae. Cyanobacteria contain phycocyanin, and phycocyanin content in phytoplankton can provide a useful index for the presence and biomass of cyanobacteria in water samples. *In vivo* phycocyanin analysis is the detection of phycocyanin fluorescence in living cyanobacterial cells present in water. A flow-through fluorimeter can be used for the *on-line* monitoring of cyanobacteria. *In vivo* fluorescence measurements may usefully contribute to the early warning of drinking and recreational waters.

2 Experimental

2.1 Materials

(a) Water purified to 18.2 MΩ cm (e.g. Millipore Milli-Q water)

(b) C-Phycocyanin standard (extracted from *Spirulina platensis*, Sigma Chemicals, product number P6161, 1 mg)

2.2 Special equipment

(a) Filter fluorometer with adequate optical filters, e.g. a 10-AU Field Fluorometer, Turner Designs with The Phycocyanin Optical Kit (P/N: 10-305) including a Cool White Mercury Vapor Lamp, a 630 nm excitation filter, and a 660 nm emission filter

(b) Luminescence spectrometer, e.g. PerkinElmer model LS50. The light source is a xenon discharge lamp. Wavelength range of use: excitation 620 nm and emission 645 nm for phycocyanin. Spectral bandpass of the monochromator determined by excitation slit 10 nm to emission slit 10 nm, optimised for best analytical results.

(c) Glass cuvettes or flow cell

(d) Pump when a flow cell is used. It is recommended that the flow rate does not exceed 600 millilitres per minute. The minimum flow of 50 millilitres per minute is safe. Keep in mind that the presence of air bubbles will affect measurement.

2.3 Solutions

Store the stock solutions at +4 °C in the dark.

(a) 1 mg/l phycocyanin in water (concentrated stock): dissolve 1 mg of phycocyanin standard in 1000 ml distilled water.

(b) 100 μg/l phycocyanin in water (solution A.): 10 ml of concentrated stock in 100 ml distilled water

(c) 10 μg/l phycocyanin in water (solution B.): 10 ml of solution (A) in 100 ml distilled water

(d) 1 μg/l phycocyanin in water (solution C.): 10 ml of solution (B) in 100 ml distilled water

2.4 Procedure

(a) The fluorimeter should be set up as described in the manufacturers instructions.

(b) Place the cyanobacterial sample in the cuvette or start sample water flowing through the fluorimeter when flow cell is used.

(c) Read the current fluorescence intensity [RAW - raw fluorescence signal].

2.5 Calibration

Filter fluorimeters can be calibrated by a number of different techniques. The most common calibration of a filter fluorimeter consists of compensating with blanks (solution containing zero concentration of the substance to be read) and adjusting the instrument to reflect a known concentration of sample (the standard).

(a) Place solutions A., B. and C. in the cuvette and measure the fluorescence intensity of each solution.

(b) Plot the fluorescence against the phycocyanin concentration to create a calibration curve.

(c) Measure environmental water samples and determine the concentration of phycocyanin from the calibration curve.

Further calibration by correlation between the raw fluorescence signal and the cell number or biomass of cyanobacteria is recommended.

Generally, a linear relationship between fluorescence intensity and pigment concentration is found. However, when the concentration of cyanobacteria is too high, light cannot pass through the sample to cause excitation; thus very high cyanobacterial concentrations can result in a very low fluorescence readout. To test for linearity, simply take a reading for a high concentration of the sample; dilute by a factor (1:1, 1:10, etc.); and measure the dilute samples.

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Annex 1. Validation of microcystin-related SOPs through intercalibration exercises

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The purpose of intercalibrations is to quarantee coherent analytical information between laboratories. Two microcystin intercalibration exercises (MC-IC1 and MC-IC2) have been organized within TOXIC by Åbo Akademi University. The MC-IC1 was carried out in 2003-2004 and consisted of two phases. The first phase of the MC-IC1 was focussed on the quantitative analysis of microcystins by high-performance liquid chromatography (HPLC) coupled with UV-detection and the recovery of microcystins in water samples by solid-phase extraction (SPE). The second phase enabled implementation of SOP adjustments and other corrective measures to be made to obtain high and reproducible analyte recoveries. The results of the MC-IC1 exercise have been published in internal reports.

The MC-IC2 organised in 2005 was planned to evaluate extraction procedures of microcystins. The exercise tested analyte identification as well as accuracy and precision of quantitative determination of microcystins, and the limit-of-quantitation. The MC-IC2 also assessed the effect of glass-fibre filters on the recovery, the effect of evaporation and reconstitution, and the solid-phase extraction recovery of microcystins spiked in commercial bottled drinking water and in local tap water. The MC-IC2 exercise contained five different targets intended to evaluate the above mentioned objectives. Test materials contained freeze-dried cyanobacterial material, cyanobacterial extracts which were dissolved in water at two different concentration levels, and purified microcystin-LR.

Eight laboratories from seven countries participated in the MC-IC2. The limit-of-quantitation was calculated for microcystin-LR during the calibration of the HPLC instruments. The limit-of-quantitation was typically 0.5-1 ng/10 µl injection with some variation depending on the type of column and HPLC instrumentation used.

One of the tasks was to determinate concentrations of microcystin-RR, -YR and -LR in freeze-dried cyanobacterial material in the absence and presence of a glass-fibre filter (SOP_TOXIC_AAU_05F, SOP_TOXIC_AAU_06F). Table 1 shows obtained reproducible values from the organising laboratory as an example.

Table 1: Concentrations of microcystin-RR, -YR and -LR in freeze-dried cyanobacterial material (*Microcystis*), an example from the MC-IC2 exercise

Microcystin	Presence of glass-fibre filter	Microcystin concentration µg g^{-1}	Standard deviation (n=5) µg g^{-1}
RR	No	321	15
	Yes	327	9
YR	No	171	10
	Yes	171	5
LR	No	292	12
	Yes	296	8

The recoveries of microcystin-RR, -YR and -LR after solid-phase extraction of water samples and subsequent HPLC determination are shown in Table 2 (values from the organising laboratory). This intercalibration task was intended to test the performance of SOP_TOXIC_AAU_05F and SOP_TOXIC_AAU_06F at naturally found toxin concentrations.

Table 2: Recovery of microcystins from spiked commercial bottled drinking water (500 ml) and spiked local tap water (500 ml) after solid-phase extraction and HPLC analysis. The amounts of spiked microcystins in water: microcystin-RR 6.0 µg l^{-1}, -YR 2.8 µg l^{-1} and -LR 5.8 µg l^{-1}. The toxins were spiked as an extract of the cyanobacterium *Microcystis*.

Type of water	Microcystin-RR		Microcystin-YR		Microcystin-LR	
	Rec. (%)	s.d. (n=3)	Rec.%	s.d. (n=3)	Rec.%	s.d. (n=3)
Bottled	77.5	6.1	86.6	6.7	82.1	6.8
Tap	84.7	6.9	92.7	4.5	89.1	5.9

Full details of the MC-IC2 intercalibration exercise will be published in a scientific journal. The authors can be contacted for more information.

Annex 2. Best practice guidance for management of cyanotoxins in water supplies

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One of the main outputs from the TOXIC project is a manual to provide operational guidance for management of cyanotoxin risk to water supplies. The objectives of this separately available document are:

- to review the techniques and technologies for management of risk from cyanotoxins in water supplies, in particular the capabilities of water treatment processes to remove or destroy toxins,
- to provide best-practice guidance for cyanotoxin risk management for water supplies.

The manual is aimed primarily at scientists and engineers responsible for operational management of water supply systems, or for selection of technology for reservoir management and water treatment. The contents of the manual are reviewed below.

The **INTRODUCTION** provides an overview of the issues related to cyanotoxins in water supplies. This includes information on the nature and properties of the toxins, and their implications for treatment selection and operation.

Section 2, **RAW WATER MANAGEMENT AND MONITORING,** reviews techniques that can be used for monitoring and managing raw water risk. This includes elements of catchment management in relation to nutrient input control, but greater emphasis is given to reservoir management, particularly for preventing algal blooms.

Section 3, **WATER TREATMENT PROCESS DESIGN AND OPERATION FOR CYANOTOXIN REMOVAL**, reviews the performance of a wide range of water treatment processes for removal or destruction of cyanotoxins, and provides specific guidance on design and operation. This assumes prior knowledge of water treatment, but generic information on each of the processes is provided in appendices for those unfamiliar with the technologies.

Section 4, **RISK MANAGEMENT AND MINIMISATION**, provides strategies and procedures for assessing and mitigating risk, including formal risk assessment techniques such as Hazard Analysis and Critical Control Point (HACCP), and Failure Modes and Effect Analysis (FMEA). Particular emphasis is given to implementing the best practice guidance with a Water Safety Plan framework.

Section 5, **PROCESS SELECTION**, provides information to help select the most suitable processes or combinations of processes for specific applications.

APPENDICES provide:

- generic information on appropriate water treatment technologies, including a glossary of water treatment terminology, and a review of performance for cyanotoxin removal,
- test procedures to assist in process selection, design and operation.

The Best Practice Guidance Manual is distributed by WRc plc.

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