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Laboratory analysis of cyanobacterial toxins and bioassays.

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INTRODUCTION AND GENERAL CONSIDERATIONS

Cyanobacterial toxins or cyanotoxins are a diverse group of compounds with differing chemistries; hence, a single analytical method can rarely be used to evaluate all potential compounds. The general steps required to detect, identify, quantify and monitor the different classes of toxins do follow a common approach (Figure 14.1).

Before committing to a major cyanotoxin sampling campaign, it is important to evaluate the information and level of detail required to make appropriate management decisions (see also Chapters 11 and 12). Planning the work therefore is best done by a group including the different aspects involved, for example, field samplers, laboratory support, analysts, as well as

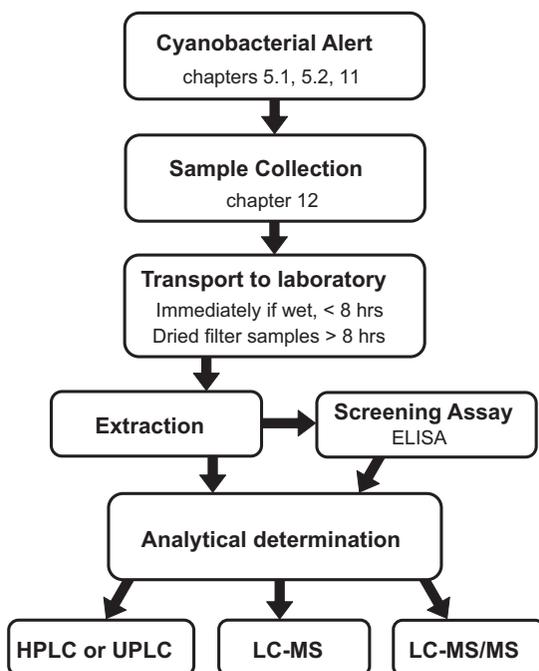


Figure 14.1 General scheme from cyanobacteria alert (see Chapter 5) to analytical determination of cyanotoxin concentrations.

managers leading the work, possibly in contact with the authority responsible for public health. This way a range of perspectives come together to design the most suitable and effective approach. An audit by others with expertise in the field (in-house or external) serves to ensure that all important areas have been considered and nothing essential is overlooked. Some of the key questions and topics which should be covered in planning and auditing are included in Checklist 14.1, and it is important to take time to make sure that all important requirements can be fulfilled.

The available methods for analysing cyanobacterial toxins are very diverse. The criteria for selecting the appropriate analytical method for a given monitoring programme include the consideration of reproducibility, sensitivity and selectivity (see Box 14.1) as well as factors such as the time to result, required training, capital investment and laboratory conditions needed, and consumable running costs per sample.

BOX 14.1: PARAMETERS OF ANALYTICAL PERFORMANCE

Before any analysis is done, it is important to understand the nature of the problem to be addressed and the type of data required. A number of terms are often used to describe the performance of an analytical method. Of these terms, the most important are accuracy, precision, sensitivity, repeatability and reproducibility. For any analytical method that is set up to quantify cyanotoxins, these parameters should be determined and well documented to support the validity of the resulting data.

Accuracy defines the closeness of measured amount of a compound in a sample to the true amount in the sample. The actual or true value has to be determined by a validated method using reference material.

Precision defines the closeness of repeated measurements of a single sample to each other. To achieve high accuracy of a method, it needs to be highly precise but high precision does not guarantee high accuracy because measurements could be biased by a systematic error.

Sensitivity defines the capability of a method to avoid false-negative results, that is, to detect a compound in a sample when it is present but to not give a result when the compound is absent. In practice, the higher the sensitivity, the lower the limit of detection.

Repeatability characterises the consistency of measured values obtained from a single sample by one person applying one method on one analytical system. To test repeatability generally includes all sample preparation steps, and usually a predetermined limit of variability is set in which the measured values should lie for the method to be accepted.

Reproducibility characterises the consistency of measured values obtained from a sample by different persons on different analytical systems. Reproducibility of a method that is applied in different laboratories is preferably validated with interlaboratory comparison tests, where a single sample of known toxin content is split and analysed by different laboratories.

Exact definitions of these and further terms related to analytical chemistry can be found at <http://goldbook.iupac.org/index.html>

Trained staff are needed, especially to operate complex analytical systems, primarily for the establishment of methods for analytes not yet analysed in the particular laboratory and their initial validation phase. For routine analyses, modern analytical systems generally offer some degree of automation that can be made use of once a method has been established.

As a rule of thumb, the most sensitive and specific methods tend to be the most demanding ones in terms of investment (capital, method optimisation and validation), required personnel training and running costs – but often with a substantial delay of result delivery of hours to days. On the other hand, methods that are less sensitive and selective may deliver results very fast. This can be essential in situations where an analytical result is needed to trigger immediate management actions to mitigate risks, for example, in Alert Level Frameworks (see Chapters 5.1 and 5.2).

CHECKLIST 14.1 CYANOTOXIN DETECTION

- Which toxin classes are to be analysed?
- From what type of samples (e.g., water, cells, tissue, sediments)?
- Does the detection have to be quantitative? If so, what detection limit is required?
- Do drinking-water samples need immediate quenching (e.g., sodium thiosulphate or ascorbic acid) to eliminate the continued action of oxidising agents like chlorine?
- What instrumentation and expertise is available (i) in-house or (ii) external?
- What capacity is available, that is, which number of samples can currently be analysed (including laboratory space, appropriate sample storage)?
- What training is needed – for sampling, for sample processing, for analysis and for data interpretation? How are the data going to be used and reported?

14.1 SAMPLE HANDLING, STORAGE AND SHIPPING

Following a sampling trip, the samples arriving in the laboratory need to be processed further for analysis or storage. Three aspects are important for sample handling and storage: safety, sample processing to ensure stability and traceability.

14.1.1 Safety

Laboratory staff handling samples potentially containing toxic cyanobacteria and cyanobacterial toxins are potentially exposed to health hazards (see also section 5.2), and appropriate protective measures need to be implemented. These measures will be based on two aspects: implementing general safety measures for hazardous material defined in national and international occupational health and safety guidelines, and an assessment of the risk of exposure to toxic cyanobacterial material potentially given in the specific procedures to be carried out. Any staff member handling potentially toxic cyanobacterial samples has to be accordingly trained and equipped with adequate protective equipment. Depending on the work to be done, this protective equipment will range from standard laboratory coats, gloves and safety glasses to – where there is a risk of inhalation exposure – breathing masks (Stewart et al., 2009). For water samples taken in monitoring programmes, the quantities of toxins are generally low, that is, in the low microgram per litre range, likely posing no risk of intoxication. Nonetheless, skin and eye contact, inhalation and ingestion have to be avoided by wearing an appropriate safety wear. Risks of exposure tend to be higher if larger quantities of bloom material are handled, for example, for toxin purification, with toxin quantities potentially in the low milligram range. The highest risk of exposure to toxic material is likely through the handling of dried bloom material, that is, exposure to dust, requiring the wearing of a breathing mask and/or the handling of sample material in an exhaust hood. Powdery freeze-dried bloom material, often statically charged, easily escapes containment, and this may cause a risk of exposure not only during laboratory work but also for cleaning staff.

14.1.2 Sample processing for storage

Samples that are not analysed immediately upon arrival at the laboratory need to be stored properly to avoid degradation of the cyanotoxins to be quantified (see Figure 12.3). Generally, cyanobacterial toxins are rather stable compounds, and storage at -20°C largely prevents degradation. As guidance, the stability – or breakdown – as described in Chapter 2 may serve as a first orientation for conducting stability testing under the actual conditions, including all steps in transportation, storage and shipping. Since

degradation mostly occurs through microbial activity, lower temperatures generally increase stability.

However, simply freezing entire samples may not be appropriate for practical and analytical reasons. For example, when extracellular and intracellular toxins are to be analysed separately, this requires prior separation of both fractions, which can no longer be done once samples have been frozen. Therefore, sample processing steps may be required that allow a reliable and efficient preservation of samples or portions of samples for later analyses.

If the toxin content in the particulate fraction is to be analysed, cyanobacterial cells and other particulate material (“seston”) are best collected on a filter, thus concentrating the sample for space-efficient storage. One criterion for the choice of the type of filter (glass fibre, membranes of different types, cellulose) is the appropriate pore size to retain cyanobacteria. As most toxigenic taxa occur in colonies or filaments, pore sizes < 2 µm are generally acceptable. Smaller pore sizes may slightly increase retention efficiency but at the cost of more rapid clogging of the filters – which is a factor that determines to a large extent the time needed to process samples. Further, it is important that the filter material be compatible with the downstream processing, in particular with the extraction procedure to avoid the release of any compounds that interfere with the downstream analysis during the extraction step in organic solvents; also, it must not be dissolved by these solvents. A protocol for the handling of such samples needs to be validated and tested with negative controls, that is, filters through which pure water has been passed.

For the filtration step, the water sample is well mixed immediately before measuring a volume to be filtered, for example, with a calibrated cylinder, because even within a very short time (i.e., minutes), buoyant cyanobacteria can float to the top (and other phytoplankton can settle), potentially leading to biased analytical results. Preferably, the total volume to be filtered is portioned into several smaller volumes to avoid increasingly longer filtration times on a gradually clogged filter. The volume to be filtered for an individual sample depends on the density of cells – that can hence be highly variable – and on the detection limit of the downstream analytical procedure. For the latter, the extraction and concentration steps need to be considered.

The filter should hold only residual moisture before it is frozen for storage or processed further for analysis, respectively, as discussed in the following section.

Filtrates are used for the analysis of dissolved (i.e., extracellular) cyanotoxins. To produce larger volumes of particle-free samples, it may be helpful to use a combination of two filters, for example, a glass fibre filter to retain larger particles and a second filter with smaller pores to remove small particles.

14.1.3 Sample storage and shipment

If filter samples are to be stored, they are folded loaded-face on loaded-face and placed in an appropriate container, for example, chemically inert reaction tubes or enveloped in aluminium foil and labelled correctly. For their long-term storage or shipment, it is advantageous to dry the loaded filters; otherwise, shipment on dry ice is recommended. Drying can be achieved by freeze drying, in a vacuum centrifuge or at moderate temperatures (<80°C for microcystins and cylindrospermopsins) in a drying oven when lyophilisation is not available (Welker et al., 2005). For the latter, stability tests are recommended to check whether the drying process (that may last for several hours) causes any degradation. It is generally good practice to test the stability of the samples under the chosen storage conditions with a series of identical samples that are analysed at the different processing steps and after varying storage times. Performing the extraction procedure in the storage tube can allow safe handling, reduce processing time and minimise the risk of sample confusion.

For the storage of particle-free filtrate or extract, toxin adsorption to labware may be relevant. A few studies on this issue show that microcystin congeners differ in their tendency to adsorb to materials (Hyenstrand et al., 2001; Kamp et al., 2016; Altaner et al., 2017). These studies cover only some of the possible combinations of materials, solvents and toxins, and it is recommended to test the material used in an individual laboratory under the actual sample processing procedures to assess suitability. Once labware of a particular material and manufacturer has been found appropriate, it should not be changed without corresponding verification.

Shipping of samples requires consideration not only of stability but also of compliance to legal aspects, in particular declaration rules for transborder shipments (see Metcalf et al. (2006) for an overview).

14.1.4 Traceability

It is critically important to label all field samples arriving in the laboratory in a way that allows the results to be tracked back to the sampling site and sampling date at a later point in time. Similar considerations also apply for individual steps in sample processing, dilution of standards and quality control (QC) samples. For certified laboratories, the traceability of samples and materials is essential and generally follows guidelines such as ISO 9001 (ISO, 2015). Although a thorough quality management system according to ISO 9001 may not be necessary for non-certified laboratories, some principles can be implemented to ensure proper sample management.

As outlined in Chapter 11, a first important point is the labelling of individual samples. Sample names need to be unique, not only for a recent set of samples but also with respect to all samples that are expected in the future in a given laboratory and in cooperating laboratories. A naming system is therefore best defined prior to sampling campaigns and followed by

the entire staff. Whichever system of sample naming is agreed upon, it is important to register all samples received in a laboratory in a central repository that is in turn backed up regularly. As barcode labelling and reading is becoming affordable, the introduction of these respective systems is now an option. Furthermore, labels need to be stable, that is, lastingly attached to sample containers under the actual storage conditions. Especially when samples are stored in the freezer, adhesive labels or markings need to be tested for resilience to repeated freeze–thaw cycles.

14.2 GENERIC METHODOLOGIES USED IN CYANOTOXIN ANALYSIS

14.2.1 Sample extraction for analysis

Most cyanotoxins are retained in the cell-bound fraction of samples taken from waterbodies (Chapter 2). Only when cyanobacterial populations experience cell lysis, for example, in surface scums exposed to high temperatures and light intensities, substantial amounts of toxins are released to the surrounding water. An exception is cylindrospermopsin that can be released from viable cell and found in large proportions in the cell-free fraction (section 2.2 Box 5.1 and Chapter 10). Further, extraction and sample clean-up is of critical importance for the analysis of cyanotoxins in foods to avoid both under- and overestimating concentrations (see section 5.3.4).

To make cell-bound cyanotoxins accessible to chemical analysis, they need to be extracted from the cells with an appropriate solvent. The solvent needs to be selected to efficiently extract toxins from cells in a few (maximum of three) extraction cycles and needs to be compatible with downstream analytical methods. Extraction procedures for individual classes of toxins will be discussed in the respective sections.

There is no single method available that is suitable for extracting all classes of cyanotoxins; hence, it may be necessary to collect several samples or subdivide samples prior to processing (see Chapter 12).

14.2.1.1 Solid-phase extraction (SPE)

While some immunoassay (ELISA) methods and LC-MS/MS (see below) may be sufficiently sensitive for monitoring in compliance with guideline values, trace analysis of cyanotoxins in water (i.e., concentrations below 1 µg/L) typically requires sample concentration using solid-phase extraction (SPE). This processing involves passing a known volume (typically 100–500 mL) of a (particle-free) water sample through a solid-phase cartridge to concentrate dissolved cyanotoxins. SPE requires a vacuum manifold system, PTFE (polytetrafluorethylen) connectors and tubing (for a minimum adsorption of analytes) and a vacuum pump. The equipment for SPE can be

used for different classes of cyanotoxin; however, specific cartridges need to be used which retain the cyanotoxin of interest. A large selection of ready-to-use SPE cartridges is available with different sorbent materials and varying sorbent volumes. Sorbent materials primarily differ in the degree of hydrophobicity and have to be selected in correspondence with the cyanotoxin of interest, for example, C18 for microcystins or graphitised carbon for highly hydrophilic cylindrospermopsins.

Filtering water prior to SPE is essential to minimise clogging of the cartridges. Nonetheless, during times of blooms, the higher load of very small particles may significantly increase loading times. In this case, it is advised to note the volume of sample that has already passed and discontinue the sample loading. Once the water sample has passed through the cartridge, a washing step follows before the toxin adsorbed on the cartridge is eluted in a small volume (typically 3–5 times the sorbent bed volume) of solvent into a collection tube (Figure 14.2). This can be analysed directly or further concentrated by drying to enhance the detectability. It is tempting to load as large a volume of water sample as possible to allow the detection of low levels of cyanotoxin; however, this is not always appropriate. Larger volumes can take many hours to load onto the cartridge and matrix contaminants are also being concentrated; hence, larger sample volumes increase matrix interference. Loading samples onto SPE cartridges can be time-consuming so it is wise to determine the most appropriate volume of water. This can be done by processing some test samples of spiking water (preferably similar to the samples) and determining how long the process takes. Typically, a volume is chosen that takes no more than 3 h to pass through the SPE cartridge, allowing time for sample preparation, elution and processing before analysis.

It is useful to validate methods by spiking known amounts of the cyanotoxin of interest into water samples. Spiking should not be performed with high-purity water (e.g., Milli-Q) as this may lead to poor recoveries, and as this is in no way representative of the samples being processed. Using the typical raw or tap water to be tested is best (if tap water is chlorinated, using a chlorine-quenching agent, i.e., sodium thiosulphate or ascorbic acid) as this will provide a good indication of expected performance.

14.2.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Immunoassays are based on the binding interaction between a highly specific antibody and the analytes of interest. The most common of these assays is the ELISA kit using antibodies raised to specific cyanotoxins. The toxins are detected by the modification in the colour reaction with the intensity of the colour being inversely related to the amount of toxin.

ELISAs can offer rapid results with a relatively low investment in capital equipment. As these assays do not identify specific cyanotoxin variants of a toxin class and give an indication of total toxin concentration – total

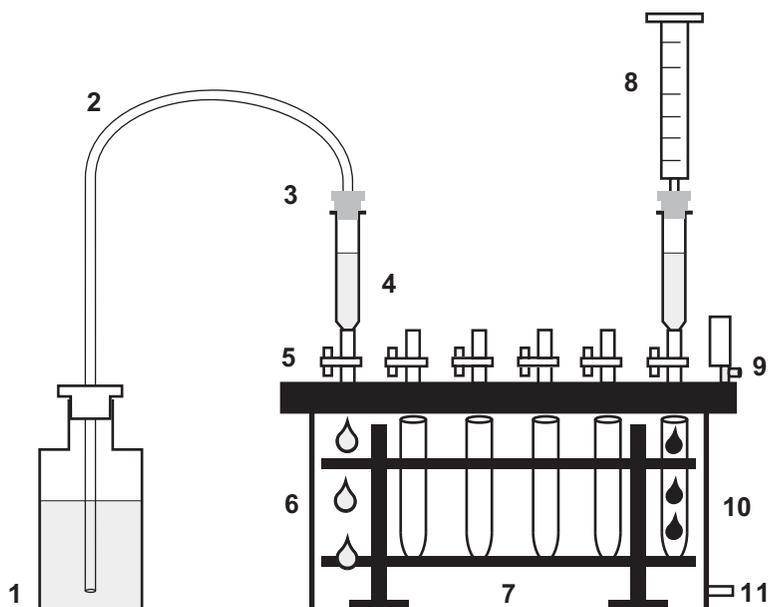


Figure 14.2 Setup for solid-phase extraction (SPE) for concentrating trace amounts of cyanotoxins dissolved in water. 1. Filtered water sample in stoppered glass bottle; 2. PTFE tubing carrying water under vacuum; 3. PTFE tubing; 4. SPE cartridge with sorbent; 5. PTFE stopcocks used to stop and start flow; 6. water flowing to waste while cyanotoxins are adsorbed on the cartridge; 7. vacuum manifold system with removable rack; 8: reservoir used to introduce solvents for conditioning and eluting the cartridge (syringe); 9. pressure gauge with needle valve; 10. concentrated sample eluted into a sample collection tube or vial; 11. vacuum line connection.

microcystins, for example – they are often used as a screening method. It is recommended to confirm toxin content and to routinely check for false negatives using instrumental methods (HPLC, LC/MS; Gaget et al., 2017). Where such methods are not available, periodic shipping of a few selected samples to a support laboratory elsewhere may be an option.

ELISA kits are very popular for a rapid, straightforward detection of most classes of cyanotoxins, although an individual kit is required for each class of cyanotoxin and even different kits may be necessary to cover the variants within one class. The kit-based formats provide a straightforward guidance on how to perform, calibrate and interpret the results. Multiple samples can be evaluated at one time, and results can typically be reported in less than a day. As with all biochemical test kits, care has to be taken with storage, since ambient or elevated temperature during prolonged transportation can reduce their reliability. The 96-well plate format allows samples to be read and quantified in a plate reader, facilitating the analysis of many samples and the calibration in a short space of time.

Kits often come with a removable strip format so that not all wells need to be used at the time of analysis thus increasing the cost-effectiveness of the assay. If only a few samples are to be assayed, it is advisable to confirm the format before making a purchase.

ELISA kits with sensitivity in the range of the WHO lifetime cyanotoxin guideline values are commercially available for almost all classes of cyanotoxins (see below). However, it is important to remember that cell-bound toxins need to be extracted prior to performing the ELISA. Also, care has to be taken to quench oxidants used in water treatment (chlorine or chlorine dioxide; see above) and to ensure the pH of the sample is appropriate for the specifications of the assay. Filtration or centrifugation may be required to remove particulates, and dilution may prove necessary to ensure that the quantification is in the approved range given in the instructions. Where the cost of these kits is a problem and access to producing antibodies is available, an option may be to produce antibodies in-house or to have this provided through co-operation with an external institution (university, company, etc.), as demonstrated in the case study described in Box 15.1 in Chapter 15.

14.2.3 High-performance liquid chromatography (HPLC)

HPLC has become well established for the routine analysis of environmental pollutants. These systems consist of a solid-phase chromatography column through which analytes dissolved in liquid solvents are pumped and separated due to differences in the interaction of individual analytes with the solid phase. The flow then passes through a detector, for example, UV absorbance or fluorescence detectors, with the absorption proportional to the amount of analyte, with data collected on a computer. Most systems now include an autosampler to allow a set of samples to be loaded and automatically analysed. The number of samples that can be analysed in a given space of time depends primarily on the duration of a single sample run. For example, the run time for microcystins with a conventional HPLC is around 1 h per sample. Analyses with fewer target compounds, that is, less structural variants such as cylindrospermopsin or anatoxin-a, generally require shorter run times and hence allow a higher sample throughput. The separation of the analytes can be achieved by isocratic elution; this is when the solvent composition remains the same throughout the analysis. Isocratic elution is suitable for analyses that target only a few analytes and with a limited matrix interference, that is, with relatively low amounts of other, nontarget compounds. To allow for better separation of target analytes, gradient elution is commonly applied, where the proportions of the solvents change over the run time. This allows a wide range of analytes to be separated, such as multiple variants of microcystins. To ensure that analytes and contaminants are not carried over to the next sample, a washing step with 100% solvent is often included in the analytical run.

The most common detector used on HPLC systems is the photodiode array (PDA), which will provide an adsorption spectrum (200–600 nm) for the compounds being analysed. This is useful for the analysis of cyanotoxins as many of them have characteristic UV absorption spectra (Figure 14.4 and 14.5), thus providing an indication even of cyanotoxins in the sample for which no standard reference material is available (see Box 14.2).

BOX 14.2: REFERENCE MATERIAL FOR CALIBRATION

According to IUPAC, reference material is defined as “a substance or mixture of substances, the composition of which is known within specified limits [...] to be used for the calibration of an apparatus”. For cyanobacterial toxins to be used as reference material for establishing a calibration curve for the quantification of these cyanotoxins, two criteria need to be fulfilled:

1. Purity defines the share of an individual compound of the total material. Purity is generally expressed in gravimetric percent that should be at least 95% in reference material.
2. Amount is generally defined in gravimetric units, and ideally with the specified limits, that is, a range of amount that should be as narrow as possible.

In this sense, not all cyanotoxins that are commercially available are reference materials. Hence, these compounds cannot be used directly to establish calibration curves. In particular, the nominal amount in a vial may deviate considerably from the true amount. In consequence, this means that a calibration curve established with such a “standard” would introduce a systematic error to all subsequent analytical quantifications.

The true amount hence needs to be determined. This can be done either by weighing with a sufficiently precise and sensitive balance or by spectrophotometric analysis (ISO, 2005). For the latter, wavelength-specific extinction coefficients need to be available, which is the case for some but not all cyanotoxin variants. Extinction coefficients are specific for solvents and temperature; that is, a compound dissolved in water cannot be quantified by using an extinction coefficient established for the compound dissolved in methanol.

For cyanobacterial toxins sold as certified reference material, the purity as well as the amount is well defined, and its can be used directly as standard for calibration.

The typical capital investment required for an HPLC is around \$30,000 USD with relatively modest costs for maintenance, including replacement UV lamps and columns.

Training of staff and adoption of a standard protocol is easily achievable, while interpretation of samples and cyanotoxin identification (especially unknowns from their spectra alone) requires more time to develop confidence. This applies equally to UPLC (ultra-performance liquid chromatography) discussed below.

14.2.3.1 Ultra-performance liquid chromatography (UPLC)

UPLC offers a considerable advantage over conventional HPLC as it allows very rapid separation of analytes (run times of around 10 min) and a greatly reduced solvent usage, typically 0.3 mL/min compared to 1 mL/min for conventional HPLC systems. For example, these systems can achieve the separation of multiple microcystin variants in run times little over 10 min, thus providing high throughput of samples, substantial saving and results on the same day for samples with short extraction times (e.g., bloom material and filter discs with cells). For samples requiring longer extraction (SPE of water samples or tissue samples), it can yield results within 24 h.

UPLC systems are highly reliable with the response factor for microcystins in the UV detector changing little over time. The capital investment should typically be around \$50 000 USD for the complete system at relatively low levels of maintenance, with the main component that needs replacement being the UV lamp (it is useful to have a spare in stock).

14.2.4 Liquid chromatography with mass spectrometry (LC-MS)

The addition of a mass detector to chromatography systems makes a very powerful tool for the analysis of cyanotoxins. Mass spectra can provide an indication of the elemental composition and structure of an analyte along with determining the quantity of analytes for which reference materials are available with high sensitivity (see Box 14.2). A range of differing systems is available, and very careful consideration is required to determine which fulfils analytical requirements and is within the budget available. Different ion sources are available with positive electrospray ionisation (ESI) most commonly used in the analysis of cyanotoxins. The type of mass analyser (Caixach et al., 2017) also varies and can have a significant impact on cost and the data obtained; hence, it is essential that background evaluation is carried out to ensure the system suits the needs defined during planning (see section 14.1.1). In general, LC-MS will provide data relating to chromatographic retention times, the parent ion masses and fragmentation patterns

for each compound as they are eluted. More complex LC-MS/MS systems combine a series of more than one mass detector (e.g., a triple quadrupole mass detector). As analyte ions pass through mass analysers, the former allow the selection of an analyte based on parent ion mass, while the latter allow the selective detection of fragment ions. This makes LC-MS/MS a highly specific analytical technique.

Robust protocols are required for LC-MS/MS as the signal from the MS can be either enhanced or suppressed by matrix interference (salts, organics, etc.). Furthermore, the response (i.e., signal strength relative to analyte amount) can drift over a relatively short time, necessitating a regular and frequent calibration. A routine maintenance protocol is advisable with the interval for cone cleaning determined for different sample matrixes; for example, in studies for the analysis of mussel tissue, cone cleaning was required after 40 samples (Waack, 2017). This interval was determined by spiking an extracted sample and then carrying out repeated, identical sample injections and determining after how many samples the reliability of the detection and quantification diminished.

While LC-MS provides very powerful sensitivity and detection capabilities, the more advanced systems (LC-MS/MS) require a capital investment of around \$500 000 USD and an annual running cost of \$20–40 000. Furthermore, a high level of staff training is required to use, interpret and maintain these systems, but once established, they provide unrivalled analytical capabilities. It is advisable, where possible, to see one or several systems in operation and have an opportunity to analyse specific samples from the area to be monitored prior to committing to this significant capital investment.

14.2.5 Selecting an analytical system

The lack of suitable analytical equipment is typically a barrier to monitoring cyanotoxins, and a strong case is often made for capital investment. The influence of current scientific publications frequently draws attention to the significant capabilities of very advanced instrumentation. However, while these systems provide impressive capability, a robust evaluation of the analytical requirement, running costs and infrastructure should be made to inform purchasing decisions. Checklist 14.2 provides some key points to discuss both in-house and with those who have recently invested in cyanotoxin analysis before making decisions. In particular, it is advantageous to develop a regional network, sharing expertise and resources, for example, through a regional centre of competency. This may lead to a decision to use simpler techniques such as ELISA while validating the results periodically by having a small set of samples analysed with advanced techniques elsewhere. Where training is required, it is often more efficient to invite an expert to provide an in-house workshop as this ensures analyses

are operational and staff develops confidence using the in-house system. (Note: small grants are often available for this, e.g., through international exchanges and workshop funding.) A further benefit of this approach is continued support from experienced international collaborators ensuring ongoing development of monitoring programmes. Support provided by the system's vendor generally is charged for. This should be considered in the budget for investment and running costs.

CHECKLIST 14.2 EVALUATING INSTRUMENTAL ANALYTICAL REQUIREMENTS AND SUITABILITY

- What information is needed? Which class of toxins will be the main focus? Is the main target monitoring compliance of cyanotoxin guideline levels?
- Check the cost of consumables, for example, vials, columns, SPE cartridges and solvents in relation to the number of samples expected over time. In many countries, solvents and even high-purity water can be prohibitively expensive (the benefits of ultra-performance liquid chromatography (UPLC) are low flow rates and short runtimes requiring little solvent).
- What are the costs of waste solvent disposal required by environmental legislation?
- Is the infrastructure appropriate? This includes a stable power supply as fluctuating or intermittent power can rapidly destroy equipment. If not, what are the costs of installing effective power surge protection such as uninterruptible power supply (UPS) systems?
- Can room temperature be kept within the range needed by the instruments and analyses (results can be affected by high or fluctuating temperature, so air conditioning is often required)?
- Do all purchases include installation and initial training, ensuring that there are available engineers in the area?
- What is the cost of a service contract, and is it essential?
- For planning to purchase a LC/MS (it requires a nitrogen generator and cannot run efficiently on regular laboratory gas cylinders), ensure that the contract includes either annual service – that is, the cost of service engineers visits – or, if you have technical expertise to carry out the service, the purchase of a service kit.
- Talk to users of different instrument manufacturers regarding their experience of service and support especially in your location.
- Consider the benefits of partnering with others rather than buying own equipment.

14.3 QUANTIFICATION OF MICROCYSTINS AND NODULARINS

Of all the cyanotoxins, most experience exists with the methodology for the extraction and detection of microcystins. Furthermore, due to its chemical similarity, many of the methods for microcystins will also readily detect nodularin (Lawton et al., 1994b); hence, it will be included in this section. In general, the term “microcystin” will be taken to refer to both these related classes of toxins unless the differentiation is required.

Methods range in complexity and sophistication, spanning the well-established “tried and tested” approaches through to preliminary research findings on novel detection strategies. While many of these novel methods offer exciting opportunities for the future, this chapter focuses on a few of the most relevant approaches for establishing routine methods suitable for the more widely available resources and common requirements.

14.3.1 Extraction methods for microcystins and nodularins

14.3.1.1 *Cyanobacterial cells*

All cell/bloom samples will require extraction as these toxins tend to be retained inside healthy cells. Many extraction protocols for microcystins have been described (e.g., various solvent combinations, cycles of freeze/thawing, sonication, freeze drying, including combined methods). Among these solvent combinations, aqueous methanol (typically 50–80%; (Barco et al., 2005)) has proven to be very effective for extracting microcystins in face of their wide range of polarities. This solvent can be used for extracting cell pellets once a sample has been centrifuged (and the supernatant discarded or assayed for extracellular microcystin) as well as for extracting cells concentrated on filters. Depending on the volume of cells, around 90% recovery of microcystins (Barco et al., 2005) can be achieved with the first extraction. Often this is sufficient, as this has to be balanced against the further time required for processing a second extraction, as this will typically yield less than 10% of the total microcystin; also if the two extractions are combined, this reduces the detection limit due to the additional volume of solvent used in the second extraction. Extraction time of around 1 h is sufficient for good recovery. With the increased availability of dispersive extractor systems (automated vortexers that shake samples vigorously at defined speeds and timed duration), however, extraction can be achieved in just a few minutes and with high reproducibility. Where samples are extracted in centrifuge tubes (typically 1.5-mL microfuge), these can be spun and the supernatant then directly analysed using instrumental methods.

When designing an extraction protocol, it is good to keep it as simple as possible as this will limit error and also potential workplace exposure to microcystins: for example, freeze drying is sometimes reported as a step during sample preparation if a specific dry weight of cells is to be determined, but this can produce powders that are difficult to contain and prone to static charge. Other methods also reported the use of a sonicator probe which may cause cross-contamination, but also produce aerosols.

The use of organic solvents (e.g., methanol) is not compatible with biochemical assays such as ELISA and enzyme inhibition tests. Some ELISA kit manufacturers provide a cell lysis kit, while other analysts have advocated aqueous extraction or dilution to limit the concentration of solvent: for example, a 1 in 10 dilution of a 50% aqueous methanol extract may be tolerated but should be checked with controls for the specific kit used. Since microcystins demonstrate high temperature stability, a brief exposure (5 min) of a small sample (e.g., 1 mL) to about 80°C in a water bath followed by centrifugation (13 000×g; microfuge) can result in simple solvent-free extraction (Metcalf & Codd, 2000). Extracts can then be diluted in water or buffer as required.

Similarly, high organic solvent content in extracts to be analysed by chromatographic systems needs to be tested for compatibility, in particular when gradient elution is applied that generally starts with hydrophilic conditions.

14.3.1.2 Water samples

Some very sensitive methods (e.g., LC-MS/MS) may be able to detect microcystins at environmental concentrations. However, even then it may be desirable to carry out solid phase extraction (SPE) to limit matrix effects.

The most commonly used SPE material is end-capped C18 cartridges, which have demonstrated high recovery and reliability. Some users prefer newer resins (e.g., polymeric phases), which are good where MS is the detector of choice; however, the high recovery of polar compounds by these cartridges can interfere with the more polar microcystins (e.g., microcystin-RR) if detection is with photodiode array (PDA). Several published methods provide a good detail on establishing SPE extraction of microcystins (Lawton et al., 1994b; Triantis et al., 2017c).

Some researchers have developed online sample concentration for fully automated extraction and analysis of microcystins. This is typically an advanced option including LC/MS(MS) and a quite specialised approach; however, it may be desirable particularly for laboratories that need a high throughput, such as those of public authorities monitoring compliance to regulations or of drinking-water suppliers.

Recoveries are best if sample handling is limited, processing time is kept to a minimum and samples are analysed immediately or stored at -20°C when this is not possible. There is some evidence that samples may change

when stored for longer periods of time even at -20°C , but further studies are required to clarify the extent of this problem. If samples are stored, they should be vortexed if a subsample is to be removed after storage.

14.3.1.3 Tissue samples

It is becoming increasingly important to evaluate microcystins in more complex matrixes such as animals that have become intoxicated, fish and aquaculture products that may be contaminated or even plant materials. Much work is still required to fully understand the efficiency of different extraction and toxin recovery protocols. This is particularly challenging for microcystins and nodularins as they are known to bind to proteins; furthermore, microcystins, in particular, can bind covalently to certain protein phosphatases in living cells. Further, the recovery of standards spiked to the material to be tested will only represent unbound toxin recovery efficiency.

While a range of processing strategies with varying degrees of complexity have been used, all of these strategies need to be tested and tailored to the specific requirements of the material to be studied. Simple blending of fresh tissue (mussels) followed by a single aqueous methanolic (80% methanol) extraction was found to give good recoveries in the range of 61–97% for 11 microcystins and nodularin (Turner et al., 2018). Very poor recovery was observed for hydrophobic microcystins when either the samples were acidified or water alone was used. The solvent extracts can be directly analysed by instrumental systems. In contrast, for biochemical tests (ELISA or protein phosphatase inhibition), samples will need to be dried to remove the solvent or sufficiently diluted with water or buffer.

Due to the difficulties in detecting bound microcystin, a method was developed which is designed to cleave part of the microcystin at the first double bond of the ADDA moiety liberating 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB; see Figure 14.3). The assumption behind this approach is that one molecule of MMPB is liberated for each molecule of microcystin, hence predicting the total microcystin content. An oxidation step is used to liberate the MMPB fragment from the parent microcystin, which is assumed to be simpler than digesting the microcystin bound to protein. While this

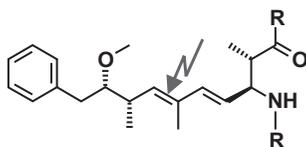


Figure 14.3 ADDA moiety of microcystins and nodularins with an indication of the site of MMPB cleavage. For the full structure, see section 2.1.

method has been used in a range of studies, it is very difficult to determine the degree of sample recovery as spiking will only represent free toxin. Most reported studies currently use MS detection of MMPB (m/z 208); however, this mass is not unique to this oxidation product (ChemSpider shows >6300 compound with this or very similar mass). Others have augmented the method to search for a product ion at 131, which again may not provide confident detection. However, Foss and Aubel (2015) have successfully used the MMPB method in comparison with the ADDA-ELISA, indicating good agreement.

In summary, the detection of microcystins and nodularins in tissue is important for assessing their possible role in animal poisoning or occurrence in food (fish, shellfish, vegetables, etc.), and while no current method will recover the total amount of microcystins, aqueous methanol extraction will give a good indication of whether microcystin is present.

14.3.2 Quantification of microcystins and nodularins by biochemical methods

14.3.2.1 Quantification by protein phosphatase inhibition assay

Microcystins and nodularins are known to be potent inhibitors of protein phosphatases PP1 and PP2A. This activity is central to their toxicity, and hence, detection of inhibition also indicates the potential biological activity of a sample. The assay can be performed relatively easily where the facilities are available for biochemical work. All the reagents and enzymes can be purchased for the colorimetric assay, which detects the enzymatic hydrolysis of the substrate (*p*-nitrophenyl phosphate) that liberates the coloured product *p*-nitrophenol (detected at 405 nm). The assay can be performed in a microtitre plate with the temperature, mixing and timing controlled by the plate reader and relatively straightforward protocol, typically resulting in good reproducibility.

Some challenges can arise if the sample inhibits the enzyme (depending on pH, solvents or other contaminants) or if it contains background colour; however, this rarely occurs as the enzyme assay is highly sensitive, allowing a significant dilution of, for example, a cell extract. Detection limits as low as 0.0039 µg/L have been reported, which is well below the WHO guideline value (Sassolas et al., 2011). This assay is also available as a commercial kit, which has a quantification range between 0.25 and 2.5 µg/L and an analysis time of only 30 min. The manufacturers also provide a tube format that could be used in the field and eliminate the need for investment in a plate reader, although users repeatedly analysing multiple samples will benefit from the multiwell plate format as this can be automatically analysed and is more practical for multiple samples and calibration points.

14.3.2.2 Immunoassays for microcystin and nodularin detection

The ADDA-based ELISA is particularly popular as it has been designed to detect the ADDA moiety, which is both very specific to these toxins and present in all variants, regardless of other chemical diversity. Sensitivity is reported as around 0.1 µg/L with the assay time of 2.5 h. There are a range of ELISA kits (usually with antibodies raised to microcystin-LR), and it is worthwhile checking which would be most appropriate for use in a specific situation. Considerations may be cost, format, location of suppliers and experience of other users in the vicinity. ELISA has been used for a range of samples demonstrating a good cross-reactivity for a number of variants and different sample matrices (Heussner et al., 2014); however, for tissue samples, sample processing needs a careful consideration as some studies have reported false positives and concentrations which are greatly in excess of the levels detected by LC-MS/MS (Brown et al., 2018).

14.3.3 Instrumental analytical methods for microcystins and nodularins

While a range of analytical approaches has been explored over the past 30 years, the central method of choice revolves around liquid chromatography (LC). Microcystins and nodularins can be separated very readily on C18 reverse-phase columns, although some closely eluting variants (e.g., microcystin-LR and desmethyl-microcystin-LR) require a careful column selection. Different methods of detection have also been evaluated, with a general consensus on UV detection and/or mass spectrometry, including MS/MS.

14.3.3.1 Analysis of microcystins and nodularins by HPLC-PDA

High-performance liquid chromatography with photodiode array (HPLC-PDA) provides an accessible, robust method for the detection and quantification of all microcystins by virtue of their distinct UV absorption spectra (Lawton et al., 1994b). Even in the absence of standards for every microcystin, confident quantification of total microcystins can be achieved. Most microcystins have very similar absorption spectra (although the overall characteristics between 200 and 300 nm can vary with concentration) with a maximum at 238 nm from the conjugated double bond in the ADDA moiety (Figure 14.4). The exception to this are microcystins that contain the variable amino acid tryptophan (e.g., microcystin-LW), which have an absorption maximum at 223 nm, and those that contain the variable amino acid tyrosine (e.g., microcystin-YR), which have an absorption maximum at 231 nm (Figure 14.4). A simple approach is to use microcystin-LR calibration for total microcystin quantification, assuming a similar molar

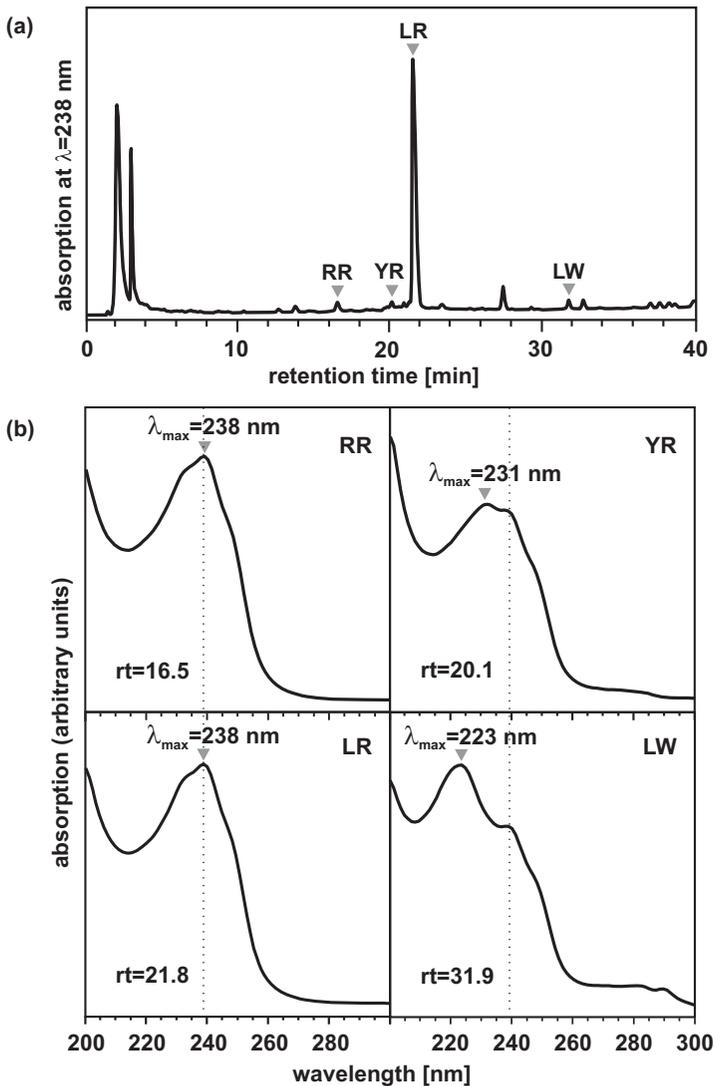


Figure 14.4 HPLC-PDA chromatogram of a bloom sample dominated by *Microcystis* sp. (a) and UV-absorption spectra of selected microcystin peaks (b). The individual variants are indicated by the standard two-letter code. rt: chromatographic retention time. The dotted line in the spectral plots indicates the wavelength $\lambda = 238$ nm; all microcystins show an absorption band at this wavelength that results in a shoulder in the spectra of variants containing tyrosine (Y) and tryptophan (W). A second absorption band at $\lambda = 246$ nm can be seen as shoulder in spectra of all microcystins. Both absorption bands are related to the conjugated double bond of the conserved ADDA moiety. For analytical details, see Welker et al. (2003).

absorption coefficient at 238 nm, that is, a similar response in PDA detection. An advantage of HPLC-PDA analysis is the fact that microcystin variants for which no reference material is available can be recognised based on a peak's absorption spectrum. Verification can be achieved by collecting the eluting peak and performing an offline analysis, for example, by MALDI-TOF MS (Welker et al., 2002b). Beyond this, it is preferable to set up the method with a range of microcystin variants of differing polarities, spanning from early to late retention, to provide confidence that a wide range of microcystins can be detected should they occur in samples.

The typical analytical set-up will be a gradient separation using high-purity water plus trifluoroacetic acid (TFA; 0.05%) and acetonitrile with TFA (0.05%). A gradient from 30% to 70% acetonitrile is usually required to separate all microcystins, and a rapid wash to 100% will eliminate carryover between runs.

14.3.3.2 Analysis of microcystins and nodularins by LC-MS(MS)

Both LC-MS and LC-MS/MS are powerful instruments for the analysis of microcystins. For developing a new method for microcystin analysis by LC-MS/MS, *the Handbook of Cyanobacterial Monitoring and Cyanotoxin Analysis* (Meriluoto et al., 2017) provides good guidance and a number of standard operating procedures (SOPs). Different approaches are possible, including the detection only of microcystins for which standards are available, using selected reaction monitoring (SRM), which is very sensitive and accurate (Turner et al. 2018). The drawback of this approach, however, is that a significant proportion of the microcystins in a sample could go unreported – those variants for which no calibration has been established based on standards. For example, USEPA Method 544 (Shoemaker et al., 2015) is limited to six microcystins, while other published methods have extended this to over 10. Birbeck et al. (2019) reported 40% of samples had more than 20% of their total microcystin variants not detected by the USEPA Method 544 and a number of these variants were the dominant microcystins. It is therefore advised to consider the taxonomic composition of the sample that allows a tentative prediction of the structural variants to be present. A way forward for routine monitoring is a detailed initial analysis to identify the spectrum of microcystins, and as long as bloom composition stays stable, such SRM can be a robust and sensitive approach, provided periodic checks are carried out to confirm that the overall microcystin profile is still covered by the standards used.

If microcystin variants are to be quantified for which no purified quantitative standards are available, estimates on the basis of their retention characteristics, mass and fragmentation pattern can serve for an initial assessment. However, as detector response intensities vary substantially between individual variants, a quantitation can be at best tentative; for

example, MC-RR has a several-fold higher response than MC-LR, probably due to a higher ionisation efficiency (Krause et al., 1999). A valuable approach is to add a PDA detector to the system along with the MS detection: this will greatly enhance confident detection and in particular quantification of microcystins for which no specific calibration could be established. Since the PDA is robust and quantification varies little over time, it provides an excellent confirmation and quantification.

14.4 QUANTIFICATION OF CYLINDROSPERMOPSINS

Cylindrospermopsin (CYN), including its small number of variants, is highly polar due to its zwitterionic nature, and hence, is readily soluble in water. Unlike most of the other cyanotoxins, it is often found in significant concentrations outside the cell as well as within the cell (section 2.2). It appears to be relatively stable and to some extent resistant to a rapid biodegradation. Detection appears to be limited to either ELISA or chromatography (with photodiode array (PDA) and/or MS).

14.4.1 Extraction of cylindrospermopsins

14.4.1.1 *Cyanobacterial cells*

Extraction of cylindrospermopsins from dried cells can be simply achieved in water (Welker et al., 2002a). A known amount of freeze-dried cells can be weighted into a microcentrifuge tube and extracted with 1 mL of water added by vortexing intermittently for 1 h or placing in a dispersive extractor for 2 min at a full speed. The sample should then be centrifuged ($13\,000\times g$), and the supernatant can be directly analysed by either ELISA or chromatography. With fresh cells, a similar protocol can be followed by first centrifuging the fresh sample and retaining the supernatant for analysis. The cell pellet can then be extracted in 50% aqueous methanol, although care has to be taken either to remove the methanol by drying the sample or to dilute it, since the methanol will interfere with both ELISA and peak shape in chromatography (Metcalf et al., 2002a).

14.4.1.2 *Water samples*

Low concentrations of cylindrospermopsins in water will require sample concentration by solid phase extraction (SPE; Triantis et al., 2017a). Due to the polarity of the molecule, it is poorly retained by C18 and other solid media typically used for water analysis. Good recoveries can be achieved by the specialised cartridges such as graphitised carbon or polymeric resins. The typical protocol requires the filtration of a water sample (the filter should be extracted for cell-bound cylindrospermopsins) and then passing

the sample through the preconditioned cartridge. Recovery can be significantly affected by the loading speed so this should be carefully controlled and optimised for the protocol being used. Cylindrospermopsins are eluted from the cartridge with methanol. It is useful to spike some samples of the water to be analysed as well as tap water (quenching any oxidant such as chlorine) to both become familiarised with the process and to define a standard operating procedure (SOP) within the laboratory.

14.4.1.3 Tissue and urine samples

Most accounts of studies investigating the localisation of cylindrospermopsins in experimental animals have indicated that unaltered cylindrospermopsins can be excreted in the urine (Norris et al., 2001); hence, methods to detect it in this matrix can be useful where human exposure is suspected. This has been successfully achieved through salt removal and SPE (carbo-graph or other hydrophobic analyte recovery solid-phase) clean-up and concentration (Foss & Aubel, 2013). Similarly, cylindrospermopsin has been recovered from serum samples although in these samples the focus is on protein precipitation with solvent (methanol) prior to SPE.

Good recovery of cylindrospermopsin from tissue (e.g., fish, mussels and vegetables) has been shown in a limited number of studies (Prieto et al., 2018) using aqueous solvents (typically methanol, aqueous methanol or acetic acid), although these methods may require further testing to determine the optimum protocol. Depending on the matrix and concentrations, direct analysis without SPE may give satisfactory results.

14.4.2 Quantification of cylindrospermopsins by ELISA

Cylindrospermopsin is a protein synthesis inhibitor, and as such, biological assays can be relatively slow and nonspecific. Therefore, the favoured assay is the cylindrospermopsin-specific ELISA kit. Several ELISA kits are commercially available for cylindrospermopsin with detection limits well below 1 µg/L, and these kits can be used directly on water samples. As the proportion of extracellular to cell-bound cylindrospermopsin can vary significantly, it is important to test for both the cell bound and free toxin. Use of kits will require relatively modest investment of a plate reader and the expense of the purchase of the kits. Full instructions for performing the assay, calibration and validation are provided with each kit. When establishing the use of ELISA, matching results with HPLC for selected samples and matrices will be valuable for determining the level of confidence as false positives have been shown with low-positive concentrations (Metcalf et al., 2017).

14.4.3 Instrumental analytical methods for cylindrospermopsins

As cylindrospermopsin is a very polar analyte, it is poorly retained on many C18 columns. Some columns have become available, which are better suited to the retention and separation of cylindrospermopsin and its variants (e.g., polar retention C18, graphitised carbon); therefore, it is advisable to select a column that is specifically designed for highly polar compounds (de la Cruz et al., 2013).

14.4.3.1 Analysis of cylindrospermopsins by HPLC-PDA

Cylindrospermopsins have a characteristic UV spectrum with an absorption maximum at 262 nm (Figure 14.5). This spectrum can be used to distinguish cylindrospermopsin from other peaks on the chromatogram in a way similar to that for microcystins. Chromatography is either carried out isocratically

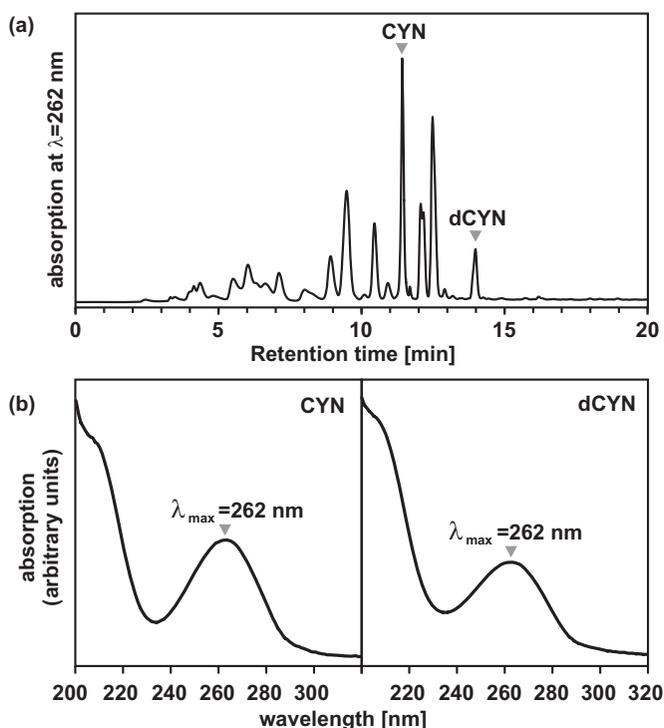


Figure 14.5 HPLC-PDA chromatogram of a bloom sample dominated by *Cylindrospermopsis* sp. (a) and absorption spectra of the two cylindrospermopsin variants (b), cylindrospermopsin (CYN) and 7-deoxy-cylindrospermopsin (dCYN), showing an absorption maximum at $\lambda = 262$ nm. For analytical details, see Welker et al. (2002a).

(5% organic solvent, methanol or acetonitrile with 95% water) or using a slow gradient (e.g., from 0% to 10% methanol) with 100% solvent wash, which limits carryover of other contaminant peaks especially from crude extracts (e.g., cells, tissue). Extracts can be analysed directly, while water samples will require SPE-concentration. Where samples are in a high proportion of methanol, this can affect chromatography. It is therefore advisable to either dry and resuspend the extract in water or dilute in water (e.g., 1 in 10, if concentrations in relation to the detection limit are sufficiently high). Using a guard column can eliminate the negative impact of methanol on the chromatography.

Hydrophilic interaction liquid chromatography (HILIC) columns have also been evaluated for the analysis of cylindrospermopsin separation due to their suitability for highly polar compounds. These columns tend to be less robust and may not separate cylindrospermopsin as well as the high-polarity C18 phases; however, they are continually improving and increasingly becoming the column of choice for the polar cyanotoxins (cylindrospermopsin, anatoxin-a and saxitoxin), allowing the analysis of these cyanotoxins together in one run (Haddad et al., 2019).

14.4.3.2 Analysis of cylindrospermopsins by LC-MS(MS)

Cylindrospermopsin is readily detected by mass spectrometry using chromatography conditions similar to those for HPLC-PDA. Electrospray in positive ionisation mode yields the parent ion with m/z 416 and product ions with m/z 194, 176, 336 and 274. Selected reaction monitoring (SRM) can provide highly specific detection (Triantis et al., 2017a). Detection of cylindrospermopsin in drinking-water by this method with prior SPE concentration gave good recoveries at 0.01 µg/L (67%) and 0.1 µg/L (85%). Since there are only few other cylindrospermopsin variants, it is much less likely that MS detection will miss structural variants as in the case of microcystins. US EPA Method 545 based on LC-MS/MS has a minimum reporting level of 0.06 µg/L for finished drinking-water (US EPA, 2015); for ambient freshwaters, Shoemaker and Dietrich (2017) give a minimum reporting level of 0.23 µg/L. The analysis of cylindrospermopsin along with other more polar cyanotoxins (deoxycylindrospermopsin, anatoxin-a and saxitoxin) has been successfully achieved using HILIC-MS, demonstrating a robust detection of these toxins in cultured samples and bloom extracts (Dell'Aversano et al., 2004).

14.5 QUANTIFICATION OF ANATOXINS

Anatoxin-a and its analogues, homoanatoxin-a and dihydro-anatoxin-a, can readily be detected by both HPLC and LC-MS/MS, and while GC/MS can also be used, this is not commonly done. As with many other cyanotoxins, ELISA kits are available for rapid detection (less than 2 h).

14.5.1 Extraction of anatoxins

Anatoxin-a can often be found in benthic cyanobacteria growing on surfaces of, for example rocks, riverbeds and submerged macrophytes. Anatoxins produced by these cyanobacteria has been implicated in animal fatalities. Addressing the occurrence of benthic cyanobacteria requires different sampling strategies than for pelagic cyanobacteria (Chapter 12).

14.5.1.1 Cyanobacterial cells

Anatoxin-a has been successfully extracted efficiently from cyanobacterial cells using acidified solvents, either just water or methanol or a mixture of both (e.g., 50% acidified aqueous methanol). This provides good recoveries and a relatively clean sample, although if microcystins are also going to be analysed from the same extract, it is advisable to omit the acid as it will adversely affect the recovery of more hydrophobic microcystins. Where samples are used for biological tests that would be sensitive to acid and/or organic solvents, extraction by multiple freeze/thawing cycles in water is preferable and has been successfully used.

14.5.1.2 Water samples

While anatoxins are largely cell-bound, it has also been observed to occur dissolved in water (Wood et al., 2018). Anatoxins are increasingly included in drinking-water monitoring during bloom episodes. ELISA kits can be used to determine toxin concentration without further extraction, merely after filtering the sample for cell removal. For instrumental analytical methods, particularly HPLC, concentration by SPE is required, and both C18 and graphitised carbon have been successfully used (Triantis et al., 2017b). Where mass spectrometry is going to be used for detection, a stable isotope-labelled phenylalanine-*d*5 can be used as an internal standard to determine the recovery efficiency.

14.5.1.3 Tissue samples

Very few studies have determined the recovery of anatoxins from tissue samples, generally applying methods similar to those applied for analysis of anatoxins in cyanobacterial cells. Using acidified aqueous methanol can help provide a cleaner extract since both the solvent and acid will precipitate proteins. Further sample clean-up and concentration can be achieved by an additional SPE step.

14.5.2 Quantification of anatoxins by ELISA

ELISA kits are commercially available for the detection and quantification of anatoxin-a and homoanatoxin-a with quantification reported in the range

of 0.15–5.0 µg/L. Typically, the assay takes around 2 h and is suitable for extracts of cells and toxins dissolved in water. These kits have been used to determine anatoxin-a concentrations in field samples (John et al., 2019) and throughout the water treatment train (Almuhtaram et al., 2018). As always, when applying such kits to finished drinking-water, care should be taken to quench oxidants when sampling.

14.5.3 Instrumental analytical methods for anatoxins

The most commonly used analytical system for the detection, identification and quantification of anatoxin-a is HPLC-PDA or LC/MS(MS). The advantage of ultra-performance liquid chromatography (UPLC) columns and systems is that they allow short retention times, providing a rapid analysis. One of the main challenges for the analysis of anatoxin-a is the ubiquitous co-occurrence of phenylalanine, which has a very similar retention time and mass. It is important to ensure that the selected chromatography column and elution profile can separate the two compounds (see Box 5.3 for an example of this misinterpretation of analytical results).

14.5.3.1 Analysis of anatoxins by HPLC-PDA

Chromatography for anatoxin analysis is the same for HPLC-PDA and the LC-MS(MS), for example, a gradient mobile phase consisting of water/ acetonitrile (both acidified with 0.1% formic acid) where the organic phase is increased from a low proportion of organic solvent (e.g., 2–5% to around 35% over 5 min (for UPLC)) at a flow rate between 0.3 and 0.4 mL/min. Samples can be separated on a suitable UPLC C18 column typically maintained at 40 °C (Colas et al., 2020). For photodiode array (PDA) detection, scanning between 200 and 300 nm can be sufficient, with anatoxin-a showing a distinct absorption maximum at 227 nm. Spiking of separate samples with both anatoxin-a and phenylalanine helps to ensure that both compounds are well separated and to establish a specific retention. A “similar retention time” is not sufficient to assign a peak to anatoxin-a as described in Box 5.3. Notably, dihydroanatoxin-a and dihydrohomoanatoxin-a do not show a distinct UV absorption spectrum due to the lack of the double-bond in the molecule, which makes it difficult to distinguish these variants from the background matrix by photometric detection without fluorescence derivatisation (James et al., 1998).

14.5.3.2 Analysis of anatoxins by LC-MS(MS)

The same chromatographic conditions are also appropriate for chromatographic separation prior to mass spectral detection via positive ESI. The parent ion $[M+H]^+$ 166.1 is identical for anatoxin-a and phenylalanine, although,

as described above, with the suitable column the two compounds can be distinctly separated. This has been achieved on both C18 and hydrophilic interaction liquid chromatography (HILIC) columns, however, not necessarily on all brands. For anatoxin-a dissolved in water, spiking with stable isotope-labelled phenylalanine-*d5* allows estimates on recovery during SPE. The labelled phenylalanine elutes at the same retention time as phenylalanine occurring in the sample, but can be accurately quantified due to its altered mass (m/z 171). US EPA drinking and ambient water ATX method based on LC-MS/MS has minimum reporting levels below 0.1 µg/L (Shoemaker & Dietrich, 2017). Parent ions of homoanatoxin-a and dihydroanatoxin-a are $[M+H]^+$ 180.1 and $[M+H]^+$ 168.1, respectively (see section 2.3).

14.6 QUANTIFICATION OF SAXITOXINS

The identification and quantification of saxitoxins is challenging although there is a lot to be learned from the analysis of this toxin class in marine harmful algal blooms (HABs). In shellfish, monitoring with a mouse bioassay developed in the 1930s was still the benchmark until the validation of an analytical method in 2005 (Box 14.4). At least 57 saxitoxin variants have been reported (Wiese et al., 2010), but not all of these variants have been found in cyanobacteria (section 2.4). Furthermore, accurate information on the prevalence of different variants has been hampered by the complexity of analysis. It is known that saxitoxins can transform into different analogues (Wiese et al., 2010); hence, care has to be taken to ensure the stability of samples and standard solutions. In general, acidic solutions (e.g., HCl) are considered suitable (Alfonso et al., 1994).

14.6.1 Extraction of saxitoxins

Saxitoxins are highly polar, and extraction protocols tend to use acidic conditions. The extraction methods for saxitoxins extensively studied for marine shellfish are also suitable for freshwater saxitoxins. A protocol to extract STXs from a range of matrices is available from AOAC (AOAC, 2005b). Solid phase extraction (SPE) using graphitised carbon or HILIC resins, for example, can be used to concentrate STXs for achieving lower detection limits or for sample clean-up (Humpage et al., 2010; Testai et al., 2016).

14.6.1.1 Cyanobacterial cells

Saxitoxins typically occur cell bound, but up to 40% of the total amount has also been found extracellularly. As for cylindrospermopsin, it is therefore important to include both fractions – toxin dissolved in water and cell-bound toxin – in the determination of total toxin. Separation of fractions

can be done either by filtration during sampling or soon afterwards in the laboratory by centrifugation or filtration. The cells preferably are extracted in acidified solvent, and the supernatant can be directly used for analysis.

14.6.1.2 Water samples

Saxitoxins in water need to be concentrated, which can be achieved by SPE (Imhof & Schmidt, 2017) with cartridges suitable for very polar compounds (e.g., porous graphitised carbon). It is recommended to evaluate the suitability of the selected cartridges and protocol by determining recovery using spiked (dechlorinated) tap water or filtrated lake water.

14.6.1.3 Tissue samples

Extraction of saxitoxins has been well described for marine shellfish samples. It involves homogenising tissue in a blender with the addition of acidified solvent, typically 1% acetic acid solution (AOAC, 2005b; 2011; Van De Riet et al., 2011). Prior to testing in a biological system, for example, ELISA, the pH will need to be adjusted to around 7.

14.6.2 Quantification of saxitoxins by biochemical methods

Several biochemical assays have been developed in the past but with the exception of ELISA, most have not been widely adopted, mainly because of their complexity and specialist expertise required to perform them.

14.6.2.1 Quantification of saxitoxins by ELISA

There are a number of (in 2019 at least six) manufacturers of different ELISA kits for saxitoxins. Most have been configured to detect saxitoxin, achieving good correlation with analytical results; however, there are challenges with cross-reactivity with other saxitoxin variants. This is of particular concern for neo-saxitoxin and gonyautoxin (GTX) variants since these variants represent as high a risk to health as saxitoxin (Papageorgiou et al., 2005). Some innovative methods are available which add an additional sample preparation step (e.g., incubation in the presence of *L*-cysteine) to transform most of the GTX variants to detectable saxitoxin or neo-saxitoxin (McCall et al., 2019). Some manufacturers produce an ELISA for saxitoxin and another kit for neo-saxitoxin. Recently, a multiplex ELISA has been demonstrated which detected nine saxitoxins in human plasma (Eangoor et al., 2019).

Careful selection of the most appropriate kit for screening purposes is important and should consider regional availability, cross-reactivity and the saxitoxin variants prevalent in the area of sampling (Harrison et al., 2016). As with all ELISA screening methods for cyanotoxins, it is wise to periodically

confirm findings using an established instrumental analytical method. This may be achieved through collaboration with a centre of excellence rather than own investment in equipment for instrumental analytical methods and the corresponding expertise, as this can be considerable (see below).

14.6.3 Instrumental analytical methods for saxitoxins

The analysis of saxitoxins has been fraught with many difficulties as saxitoxins do not contain a chromophore (do not adsorb light) nor natural fluorescence; hence, typical HPLC detectors cannot be used to identify or quantify them. Further, they are very polar molecules that are not easily retained by reverse-phase chromatography (e.g., using C18 columns). Derivatisation to form a fluorescent analyte has proven valuable, with both precolumn and postcolumn (i.e., the saxitoxins are first chromatographically separated and then mixed with the derivatisation reagents before detection) methods developed and the precolumn method becoming an AOAC Official Method (AOAC, 2005b; 2011). While this method is validated for the analysis of paralytic shellfish poisoning in shellfish, it is also suitable for the analysis of saxitoxins from cyanobacteria in cells, water and tissues.

14.6.3.1 Prechromatographic oxidation and liquid chromatography with fluorescence detection

To overcome the difficulties of detecting saxitoxins, a preanalysis oxidation method was developed by Lawrence et al. (1995) allowing the saxitoxins to be analysed using a fluorescence detector (fluorescence excitation 340 nm and emission 395 nm). Around the same time, Oshima (1995) proposed a postcolumn derivatisation method. The so-called *Lawrence method* with precolumn sample oxidation has now been adopted for regulatory monitoring purposes and is being used in many laboratories as part of routine monitoring programmes for saxitoxins in shellfish. The attention this method has received has ensured significant performance testing, including inter-laboratory studies (Turner et al., 2019). This analysis requires a significant commitment to setting up and maintaining the method, including purchasing a wide range of standards. Where intermittent confirmatory analysis of ELISA results is required, it is beneficial to approach a laboratory which is already well established in this field rather than committing to the onerous task of developing this method in-house.

14.6.3.2 Analysis of saxitoxins by LC-MS/MS

Ultra-performance liquid chromatography (UPLC) with MS/MS provides a very powerful analytical tool for the detection, identification and quantification of saxitoxins at very low limits of quantification in the sub-ng/mL

range. Several chromatographic approaches can be used, one being the more traditional C18 columns with a high proportion of water in the gradient elution. Alternatively, by using hydrophilic interaction liquid chromatography (HILIC), which is well suited to highly polar analytes, a rapid analysis has become possible with fast UPLC columns (e.g., 11 min compared to a previous 40 min). Furthermore, the solvent-rich mobile phase used in HILIC can provide a significant advantage. One recent study demonstrated the separation, identification and quantification of 14 saxitoxins in less than 10 min (Turner et al., 2015).

Another promising approach to the detection of saxitoxins is the use of inline SPE coupled with C18 UPLC-MS/MS. This approach effectively combines sample extraction and concentration from a water sample which is then directly injected onto the analytical system. The full automation has many advantages in reducing staff time, handling errors and sample loss as well as giving a low limit of quantification (Imhof & Schmidt, 2017).

14.7 DETECTION AND QUANTIFICATION OF ANATOXIN-A(S)

Work on anatoxin-a(S) has been hampered by the limited availability of cultures which produce this cyanotoxin, which subsequently limits the availability of the purified toxin. This is further restricted by the difficulty in detecting anatoxin-a(S) in natural samples. As an organophosphate inhibitor, anatoxin-a(S) can potentially be detected with a biochemical screening assay using an acetylcholine esterase inhibition assay. However, since organophosphate pesticides may be present in environmental samples, confirmation is required. Furthermore, in the absence of any authentic purified anatoxin-a(S) and confirmatory analytical methods, few reports of anatoxin-a(S) have been confirmed.

With greater availability and use of HILIC columns along with MS/MS (Dörr et al., 2010), it may be expected that gradually anatoxin-a(S) will become more widely detected, isolated and investigated. When planning sampling campaigns, it may be useful to give consideration to the possible presence of anatoxin-a(S), preferably in collaboration with expert groups able to screen samples with advanced multitoxin methods.

14.8 METHODS FOR SYNCHRONOUS DETECTION OF MULTIPLE TYPES OF CYANOTOXIN

14.8.1 Multiplex antibody systems

Methods are likely to become available which allow the detection and quantification of multiple cyanotoxins in a single system (Eangoor et al., 2019). These systems are referred to as bioarray, microarray or multiplex systems.

One such system using fluorescence detection of antibody binding signals has been found to be very sensitive, allowing the detection of microcystins, saxitoxins and cylindrospermopsin along with two of the marine shellfish toxins, okadaic acid (OA) and domoic acid (McNamee et al., 2014). Assay time was around 15 min per sample, providing results for all five biotoxins at once and in the sub- $\mu\text{g/L}$ range. While these systems are yet to become widely available, this type of screening is likely to become increasingly adopted in the near future with applications in screening of drinking-water quality and recreational waterbodies.

14.8.2 Multi-cyanotoxin analytical methods

An increasing number of instrumental methods which can analyse cyanotoxins in a single analytical chromatography run have been published (Dell'Aversano et al., 2004; Greer et al., 2016; Zervou et al., 2017). With the use of the optimal UPLC column and MS/MS detection, multitoxin methods are convenient where advanced analytical systems are routinely employed. Pekar et al. (2016) demonstrated the separation of anatoxin-a, cylindrospermopsin and microcystin variants, achieving the analysis of 22 cyanotoxins in both raw water and drinking-water Haddad et al. (2019) have added saxitoxin to the analysis, allowing the separation of four classes of cyanotoxin in a single analytical method.

14.9 FUTURE DEVELOPMENTS

With increasing legislation and pressure on water resources causing more demand to test for cyanobacterial toxins, rapid simple screening tests are increasingly likely to be required. These advances will most likely employ immunological, biosensors and related technologies to permit a rapid simple assessment of cyanobacterial and water samples after extracting cell-bound toxins. Immunological strip detection systems have been commercially developed (e.g., Kim et al., 2013; Weller, 2013), and such technologies are being adapted to furthering our understanding of cyanobacteria and their toxins in the environment.

Increasingly, cyanotoxin analytical methods will be required to analyse a wider complexity of novel materials that may require method development (e.g., in food; see section 5.3). In the case of cyanotoxins that do not covalently bind to proteins, simple extraction and clean-up methods with SPE should permit accurate analysis, although verification will still be required. Combined single-step SPE methods for sample preparation of, for example, cylindrospermopsins, anatoxin-a and saxitoxins should be developed to extract such cyanotoxins in an effort to reduce sample preparation for their subsequent measurement with, for example, mass spectrometry (Fayad et al., 2015).

Although more specialised analytical methods such as mass spectrometry will continue to be required to verify cyanotoxins, future needs will be to develop methods for not yet identified bioactive compounds produced by cyanobacteria and to develop robust multitoxin analytical methods.

Further advances may come with the use of biosensors, such as with recombinant PP1 α showing an increased sensitivity for microcystin-LR (Catanante et al., 2015), 3D-graphene-based biosensors (Zhang et al., 2017) for the detection of microcystin-LR or DNA-based aptamer systems, either for the detection of *Microcystis* (Tong et al., 2015) or for the detection of microcystin (Li et al., 2016). Assay systems such as this show good promise and may be useful in future to provide quantitative and toxicological assessment of cyanobacterial toxins.

14.10 BIOASSAYS AND THEIR USE IN THE SURVEY OF TOXIC CYANOBACTERIA

Assessments of potentially toxic environmental samples, including blooms of cyanobacteria, most commonly rely on chemical monitoring of individual chemicals, that is, the targeted analytical or bioanalytical identification and quantification of known toxins as outlined above. With respect to quality assurance/quality control (QA/QC) criteria and straightforward interpretation of the results, chemical monitoring is an approach generally applied in all regulatory settings, including water quality and safety. The obvious limitation of chemical monitoring is the fact that many analytical methods detect only those toxins that they target, which could often be only a single or a few structural variants, while others remain undetected, thus potentially underestimating the sample's toxicity.

Several studies showed that field cyanobacterial samples may cause stronger toxicity in comparison with the effects of pure toxins when tested at equivalent concentrations, indicating the presence of other toxic components (see section 2.10). These may, in addition to diverse cyanobacterial metabolites, also be toxic metals or compounds of anthropogenic origin such as pesticides, polycyclic aromatic hydrocarbons and other emerging contaminants present in complex environmental samples. Based on chemical analysis alone, it is not possible to evaluate the overall toxicity of complex mixtures. To cope with these limitations, some environmental monitoring programmes have implemented toxicity testing with bioassay(s), for example, whole effluent toxicity testing in USA and Germany (Escher & Leusch, 2011) or the EU-supported effect-based monitoring programmes (Tousova et al., 2017).

This section firstly introduces some toxicology principles with respect to the interpretation of bioassay results; then summarises the existing experience; and critically discusses current state, limitations and recommendations

on the applicability of bioassay for the monitoring of toxic cyanobacterial blooms with respect to possible impacts on human health. Other aspects such as testing for ecotoxicity with invertebrates or other aquatic biota are addressed only briefly because of their limitations for assessing human health end-points. This chapter does not cover other applications of bioassays such as the toxicological characterisation of individual toxins. The focus of this chapter is on bioassays employing cells, tissues or whole animals; other subcellular bioanalytical tools (ELISA or enzyme inhibitory assays such as the protein phosphatase assay) do not fit the “bioassay” criteria and are discussed in section 14.2.

14.10.1 Insights into interpretation of toxicity results

Before discussing examples and the practical applications of bioassays, those who plan to implement them in monitoring programmes need a good and common understanding of the terminology and how individual terms are used. Firstly, there is a central paradigm of toxicology, that is, “All things are poison and nothing is without poison. Solely, the dose determines that a thing is not a poison” (Paracelsus, 1493–1541). Whenever “toxicity” or “effect” is considered (e.g., animal death due to anatoxin-a neurotoxicity, microcystin-induced liver injury or decreased cell viability *in vitro* through cytotoxicity of cylindrospermopsin), observed effects are related to the defined test conditions. Most importantly, whether the effect manifests always depends on the dose, duration of exposure and biological system (organism). In this sense, toxicity of a compound is widely understood as causing adverse effects upon exposure as expected under normal conditions. For example, compounds like vitamins can cause adverse health effects when applied in high doses (hypervitaminoses) but vitamins are generally not considered a toxin, because, under normal conditions, an exposure leading to adverse effects is improbable (Hathcock et al., 1990; Vieth, 2007).

Current toxicology aims to establish links between the adverse health outcome (i.e., *in vivo* manifestation of the toxic effect) with the exposure to a toxicant through a chain of causal events formalised as an “adverse outcome pathway” (AOP; Patlewicz et al., 2015). Examples of relevant adverse health outcomes may be, for example, the death of an animal caused by a high dose of anatoxin-a, disruption of neurobehavioral abilities after chronic exposures to lower doses of anatoxin-a or eye irritation and skin rash after direct acute exposure to high doses of cyanobacterial biomass. “Toxicity” always starts at the molecular level; propagates through cells, tissues and organs; and eventually becomes apparent *in vivo* as systemic toxicity. The ultimate adverse outcome manifests only under specific preconditions (the toxin can reach the target, it is present in sufficiently high doses, etc.) and when the chain of events is not repaired by

detoxification mechanisms (ADME – absorption, distribution, metabolism and excretion). An AOP relevant for cyanobacteria could be, for example, “inhibition of protein phosphatases (PPase) leading to hepatic hypertrophy and tumour promotion activity”.

The interaction between the toxic chemical and its biological counterparts (molecular initiating event, MIE) can be either specific or nonspecific. Examples of specific interactions (key–lock principle) include binding of MC-LR to PPase, preferentially in liver cells, cylindrospermopsin interferences with the machinery of protein synthesis with no apparent preference to the cell type or binding of anatoxin-a to the nicotinic acetylcholine receptor on neuronal cells. Nonspecific interactions, when a chemical does not have a “specific target”, are common and include, for example, the disruption of cell membrane function after the accumulation of chemicals also known as narcotic or basal toxicity, damage to proteins, membrane phospholipids or nucleic acids by reactive oxygen species or denaturation of proteins by acidic chemicals. One chemical may act through several modes of action that may lead to a single or multiple different adverse outcomes. For example, cylindrospermopsin may inhibit protein synthesis, react with DNA or induce oxidative stress leading to death or various chronic effects depending on the concentration, exposure duration, life stage, age or sex of the organism (Pichardo et al., 2017).

Toxicity of natural samples of cyanobacteria may thus be a complex response to – for example – unfavourable pH, presence of ions and metals, saccharides, peptides (including toxins, amino acids, nucleotides, phospholipids), components of other plankton organisms (Palíková et al., 2007a; Palíková et al., 2007b) or compounds of anthropogenic origin.

14.10.2 Bioassays in the assessment of toxic cyanobacteria

Bioassays have primarily been developed for the testing of chemical substances based on different regulatory frameworks. Most of these tests went through a validation process with standardisation by ISO or OECD assuring good characterisation of the studied chemical and testing conditions. Bioassays are mainly based on animal testing but there is an increasing demand to reduce animal experiments and use alternative methodologies such as *in silico* and *in vitro* methods often combined into so-called *integrated testing strategies (ITS)* or *integrated approaches to testing and assessment (IATA)*. Toxicity bioassays have been adapted to assess the toxicity of cyanobacterial samples. However, when testing complex samples like cyanobacterial crude extracts, the causative agent(s) inducing the toxicity cannot be easily identified.

Nevertheless, many studies explored the use of bioassays in toxicity screenings of natural cyanobacterial samples or explored their potential to

serve as early warning tools. Positive bioassay responses could then trigger chemical analysis of cyanotoxins for more precise characterisation of the hazard. Testing with bioassays is expected to show whether the sample contains toxic substances and how toxic these substances may potentially be. Researchers can combine multiple bioassays to cover various end-points ranging from acute cytotoxicity and mortality to complex organ or systemic effects such as reproduction toxicity. Specific *in vitro* assays have been used to assess mechanisms of action, potential genotoxicity or endocrine disruptive effects. Unfortunately, complex research approaches can hardly be implemented for routine monitoring or screenings of potential health hazards. However, at least four cases can be listed in which toxicity testing remains relevant:

1. if illness of animals or humans is suspected to have been caused by cyanobacteria but symptoms cannot be attributed to known cyanotoxins found by chemical analyses;
2. for testing whether specific cyanobacterial strains show toxicity not attributable to known cyanotoxins;
3. to characterise the toxicity and/or mechanism of action of newly identified toxins or congeners of previously known cyanotoxins (Fischer et al., 2010);
4. to establish the data needed to derive guideline values for the concentrations of substances to which humans may be exposed, for example, cyanotoxins in drinking-water or in waterbodies used for recreation.

14.10.2.1 Nonmammalian bioassays

Ecotoxicity assays using bacteria, protozoans, invertebrates, plants or aquatic vertebrates such as fish or amphibians have been used in many studies for detecting cyanotoxins.

Bacterial bioassays have been used to screen complex cyanobacterial samples such as the Microtox bioluminescence assay using *Aliivibrio* (formerly *Vibrio*) *fischeri* or *Photobacterium* (*Vibrio*) *phosphoreum* (Lawton et al., 1994a; Vezie et al., 1996) but with poor correlations between the reduction of the measured end-point (emitted light) and the sample's content of cyanotoxins. Poor correlations were also revealed in the bioassay with *Serratia marcescens* despite promising original studies with pure saxitoxins and microcystins (Lawton et al., 1994a).

Cyanobacterial samples were also tested with protozoan assays such as *Tetrahymena thermophila* (commercially available as Protoxkit-F; Protoxkit-F, 1998), *T. pyriformis* and *T. thermophila* (Maršálek & Bláha, 2004) or *Spirostrum ambiguum* (Tarczyńska et al., 2001). Further, bioassays with aquatic or terrestrial plants were explored (Kós et al., 1995; Pflugmacher et al., 2001; Vasas et al., 2002).

Among the bioassays with aquatic invertebrate animals, cladocerans have been widely used due to their easy maintenance, small size, wide distribution and rapid growth rates. These bioassays include standardised 24- and 48-h immobilisation assays with *Daphnia magna* (OECD, 2004; 2012) or commercially available test kits (Daphtoxkit-F, 1995; Ceriodaphtoxkit-F, 1995). Complex cyanobacterial samples have been tested with species of *Daphnia* (DeMott et al., 1991; Okumura et al., 2007; Ferrão-Filho et al., 2009), *Ceriodaphnia* (Maršálek & Bláha, 2004; Okumura et al., 2007) or *Moina* (Ferrão-Filho et al., 2009). Further model organisms include *Artemia salina* (Kiviranta et al., 1991; Metcalf et al., 2002b; Beattie et al., 2003; Lindsay et al., 2006) or mosquito adults and larvae (Kiviranta et al., 1993). Also extensively used was the bioassay with larvae of fairy shrimp *Thamnocephalus platyurus* commercially available as Thamnotoxkit-F (MicroBioTests Inc., Mariakerke, Belgium). Box 14.3 provides more information and illustrates the difficulties and limitations in the interpretation of results for the *Thamnocephalus* bioassay and, correspondingly, for all invertebrate bioassays.

BOX 14.3: THE THAMNOCEPHALUS PLATYURUS BIOASSAY

The bioassay with larvae of fairy shrimp *Thamnocephalus platyurus* has been discussed in the past as a potential tool for routine screening of bloom toxicity. It has a number of advantages such as allowing for simple and practical use even in a format of a commercially available kit called *Thamnotoxkit*. It provides fast 24-h response with a possible reduction of exposure to 1 h (Törökné et al., 2007). The assay has been standardised (ISO, 2011), and it showed good performance in an interlaboratory test with cyanobacterial samples (Törökné et al., 2000a).

With respect to individual cyanobacterial metabolites, *T. platyurus* was generally reported to be highly sensitive. However, the reported IC₅₀ values were surprisingly within a narrow – low micromolar – range for all the studied and structurally diverse cyanobacterial metabolites and toxins (including microcystins, cylindrospermopsin, microginin, aeruginosins, spumigins, cyanopeptolin, eucyclamides, oscillapeptin J) as well as fluoro-conjugated MCs or odour compounds such as sesquiterpenes (Blom et al., 2001; Blom & Jüttner, 2005; Portmann et al., 2008; Höckelmann et al., 2009; Gademann et al., 2010; Kohler et al., 2014; Grundler et al., 2015; Mazur-Marzec et al., 2015; Scherer et al., 2016; Bober & Bialczyk, 2017), while the assay was reported to be less sensitive to the neurotoxin anatoxin-a (Sieroslawska, 2013). With regard to screening of complex bloom samples, the literature provides a conflicting picture. One study (Tarczyńska et al., 2000) compared seven extracts and found

a statistically significant relationship between the observed toxicity and the microcystin-LR content. On the other hand, several other studies showed high toxicity in *T. platyurus* irrespective of the content of major cyanotoxins (Maršálek et al., 2000; Törökné et al., 2000a; Törökné et al., 2000b; Keil et al., 2002; Nałęcz-Jawecki et al., 2002; Maršálek & Bláha, 2004; Törökné et al., 2007; Ács et al., 2013; Sieroslawska, 2013).

Despite apparently high sensitivity of the *T. platyurus* assay, its implementation into routine monitoring of bloom toxicity would not provide a major added value to toxin analyses with chemical or biochemical methods because it is generally not able to discriminate between toxic (in the sense of “containing cyanotoxins”) and nontoxic cyanobacterial samples. Further, its responses were not correlated with toxicity observed with other organisms, including mouse *in vivo* assay (Tarczyńska et al., 2000; Tarczyńska et al., 2001).

In addition to invertebrates, fish and frog bioassays have also been explored. With respect to ethical concerns associated with the use of adult fish (namely, zebrafish *Danio rerio*, Japanese medaka *Oryzias latipes* or fat-head minnow *Pimephales promelas*), assays with fish embryos have become popular (Berry et al., 2009). The zebrafish *Danio rerio* fish embryo toxicity (FET) assay has been standardised (OECD, 2013). The embryo fish tests were used in many studies of cyanobacteria (Oberemm et al., 1999; Wang et al., 2005; Lecoz et al., 2008), but some concerns related to the uptake of toxins through the chorion barrier or limited toxicokinetics in developing embryos have been raised. With respect to amphibians, frog embryo teratogenesis assay *Xenopus* (FETAX) (ASTM, 2017) using *Xenopus laevis* (African clawed frog) has also been explored but showed rather low correlation with the content of known cyanotoxins (Oberemm et al., 1999; Fischer & Dietrich, 2000; Burýšková et al., 2006).

14.10.2.2 Mouse bioassay

For many years, the mouse *in vivo* bioassay was used to determine toxicity of cyanobacterial blooms (Carmichael, 1992; Fastner et al., 2003; Sotero-Santos et al., 2006) or to detect phycotoxins in shellfish (Box 14.4). In testing of toxic cyanobacteria, male Swiss Albino mice were the most commonly used animals. Effects are assessed after intraperitoneal injection (i.p.) of 0.1–1.0 mL of a lysate of the cyanobacterial sample. Mice are observed for 24 h, sacrificed by an approved method and submitted to postmortem examination of tissue injury (Falconer, 1993). The observation period could be extended when the possibility of protracted symptom manifestation is expected, as it may be the case with cylindrospermopsin (see Chapter 2.2).

When more than one type of cyanotoxin is present, the more rapid-acting toxin may mask the symptoms of the others. Acute toxicity is expressed as the dose at which one half of the treated animals has died within the determined time period, that is, usually 24 h (LD_{50} in mg extract dry weight/kg mouse body weight). According to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UNECE, 2017), five acute toxicity categories are recognised based on oral LD_{50} (mg/kg b). The most toxic is Category 1 with oral $LD_{50} < 5$ mg/kg bw, while for Category 5 acute oral LD_{50} ranges between 2000 and 5000 mg/kg. LD_{50} higher than 5000 mg/kg b is considered as absence of acute toxicity.

BOX 14.4: MOUSE BIOASSAY IN TOXICITY TESTING OF MARINE BIVALVES

Biotoxins produced by marine (phyto)plankton which may accumulate in seafood remain a major public health issue in some parts of the world. Some regulatory approaches also refer to the use of mouse bioassays, but the bioassay is no longer used very often with regard to recent technology developments of chemical-specific analytical methods as well as ethical concerns.

For example, okadaic acid (so-called *OA toxins*, that is, OA and its analogues), the dinophysins toxins (DTX1, DTX2 and DTX3) can be found in tissues of molluscs such as oysters, mussels, scallops and clams, and cause diarrhetic shellfish poisoning (DSP). The inhibition of serine/threonine phosphoprotein phosphatases is their main mode of action, similar to, for example, cyanobacterial microcystins. To control for DSP, the mouse and the rat *in vivo* bioassays have been official reference methods in the EU (Commission Regulation (EC) No. 2074/20054) using the intraperitoneal (i.p.) injection of mussel tissue extract followed by 24-h monitoring of test animals. Despite the advantages of the bioassay (whole-organism toxicity response, no need for complex analytical equipment), an official opinion of the European Food Safety Authority (EFSA, 2009) highlighted several disadvantages, that is, a high variability and labour demand, needs of specialised animal facilities, false-positive results due to interferences with, for example, free fatty acids, not selective for solely the OA-group toxins, not quantitative, inappropriate i.p. administration route and ethical reasons. The EFSA therefore concluded that they are inappropriate for assessing compliance to the regulatory limit set for seafood by the EU. The same report also concluded that the phosphoprotein phosphatase assays and LC-MS/(MS)-based methods have the greatest potential to replace the mammalian assays, due to sufficient sensitivity and satisfactory validation performance.

A recommendation to replace the mouse bioassay for the assessment of broader groups of marine toxins (AZA, BTX, DA, OA, PTX, SXT, YTY) by alternative chemico-physical methods such as LC/MS has been prepared by German Federal Institute for Risk Assessment (BfR, 2005). The mouse bioassay is only envisaged as an additional analytical step when a positive result has been obtained and further clarification is needed in the interests of consumer protection (suitability of the test results for use in court, etc.). The LC/MS method has, for example, been recognised by the New Zealand Food Safety Authority (FSA) as an official method and successfully tested in an interlaboratory trial.

Nevertheless, for control of marine paralytic shellfish poison (PSP) biotoxins, the mouse bioassay remains a standardised method of the European Union Reference Laboratory for Marine Biotoxins (EURLMB) at Agencia Española de Consumo, Seguridad Alimentaria y Nutrición (AECOSAN, 2014), which is in line with the Association of Official Analytical Chemists Official Method (AOAC, 2005a). The mouse bioassay is mentioned in the context of PSP biotoxins in the food of animal origin within the frame of the European Regulation (EC) N° 853/2004, and related methodological regulations (Commission Regulation (EC) N° 2074/2005 amended by EC N° 1664/2006, EC N° 1244/2007 and EU N° 15/2011).

Among the other standardised methods for marine biotoxin detection, HPLC method – so-called *Lawrence method* (Lawrence et al., 2005) – is mentioned in the EU regulation and immunochemistry approaches remain to be discussed as an alternative for the future, after undergoing validation through interlaboratory exercises (Burrell et al., 2016; Dorantes-Aranda et al., 2018; Turner et al., 2019).

Using this mammalian model, observations of the target organs can help extrapolating to the effects in humans. However, the mouse bioassay is generally done through intraperitoneal injection, which may not be the most relevant route of exposure for such extrapolation to humans. Also, other aspects such as low sensitivity and selectivity, high rates of false positives, variability and ethical concerns created a demand for alternative tests. Nevertheless, in some countries, the mouse bioassays can still provide some guidance for managers, for example, to determine toxicity of marine bivalves considered for human consumption or when a bloom occurs in the raw water but chemical analyses do not reveal any known cyanotoxins.

For deriving WHO guideline values or regulatory standards issued by public authorities worldwide, if data on human populations are inadequate or insufficient, a preferred basis is chronic exposure studies with rodents,

with toxin applied orally, that is, via food, drinking-water or gavage, and animal health observed during extended periods of time, particularly those based on standardised guidelines such as OECD Test No. 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents), Test No. 407 (Repeated Dose 28-day Oral Toxicity Study in Rodents). One-generation reproduction studies (OECD Tests No. 415 or No. 443) are particularly comprehensive and thus valuable, but rare because of high costs and demands. In practice, toxicological data from such chronic whole-animal studies may not be available, and risk assessors need to include other toxicological data, including those from acute oral exposure tests (e.g., OECD Tests No. 420, 423 or 425). These and further guidelines are freely available under <https://www.oecd-ilibrary.org/environment> (Book Series).

14.10.3 *In vitro* assays for determining toxicity and genotoxicity

In vitro bioassays using cell cultures have received wide attention for replacing animal tests. However, a single *in vitro* bioassay alone cannot cover all of the biological targets or processes found within an organism. Therefore, a hierarchic *in vitro* test strategy is necessary for characterising the type of toxicity induced by the unknown toxicants as proposed in different strategic documents and recommendations for water quality assessment (enHealth, 2012; Grummt et al., 2013). The following paragraphs provide examples as well as a summation of the advantages and disadvantages of *in vitro* bioassays, which could form part of such a hierarchic *in vitro* test strategy specifically for cyanotoxins.

The hepatotoxicity of microcystins triggered the use of hepatocytes (liver cells) from different fish or mammalian species (Aune & Berg, 1986). Freshly isolated hepatocytes may – for a certain period – retain necessary liver characteristics like active bile acid transport or phase I and II metabolising enzymes, and many studies showed high sensitivity to cyanotoxins in rat or mouse hepatocytes (Fladmark et al., 1998; Li et al., 2001; Boaru et al., 2006). A need for fresh isolation can be overcome by using cryopreserved hepatocytes, preferably of human origin or a specific cell line such as HeparG (Bazin et al., 2010), which maintains most of the *in vivo* features. A basic prerequisite for microcystin uptake into the cells seems to be the presence of certain organic anion-transporting polypeptides (OATP) within the cell membrane, as a study with genetically modified OATP-competent HEK293 cells has shown (Fischer et al., 2010). Many other cell lines, such as HepG2, CaCo2, and V79, have been used to study cyanobacterial samples (Lawton et al., 1994a; Fastner et al., 2003; Žegura et al., 2003; Lankoff et al., 2006; Žegura et al., 2008; Fischer et al., 2010). Besides having a tumoral origin in most cases, these cell lines may lack certain metabolic enzymes important for activation and in particular for

detoxification; therefore, they are prone to giving misleading results, thus limiting their informative value.

For assessing neurotoxicity, the Neuro-2A neuroblastoma cell test has been developed and used for monitoring of saxitoxins in freshwater cyanobacteria (Gallacher & Birkbeck, 1992; Humpage et al., 2007). Endocrine activity can be examined by oestrogen or androgen receptor-specific reporter gene assays (OECD, 2016a; b) and steroidogenesis assay in H295R cells (OECD, 2011). Within the OECD framework, these *in vitro* tests are part of the first tier and are considered as a screening tool, which is not sufficient to categorise a substance as an endocrine disruptor.

Genotoxicity and mutagenicity are important end-points for human and environmental hazard evaluation, and a number of assays, often adopted as ISO or OECD guidelines, are used in the assessment of toxic cyanobacteria. Among the bacterial assays, the Ames assay (OECD, 1997) showed the mutagenicity of various cyanobacterial extracts (Huang et al., 2007), while pure cyanotoxins were mostly negative in the assay (Žegura, 2016). Palus et al. (2007) showed genotoxicity of various extracts or cyanobacterial toxins in SOS chromo test with *Escherichia coli* PQ37 but negative results were reported with the SOS/umu-test (ISO, 2000) with *Salmonella* Typhimurium TA1535/pSK1002. However, the caveat of many publications is that cytotoxicity (resulting in DNA fragmentation) is not accounted for, which may lead to false positives or overestimation of the relevance of genotoxicity.

With regard to eukaryotic cell models, the mammalian cell gene mutation assay (OECD, 2016c) demonstrated that MC-LR preferentially induces clastogenic effects on DNA rather than point mutations (Zhan et al., 2004). Various cyanobacterial extracts induced micronuclei in the *in vitro* cytokinesis-block micronucleus assay (micronucleus test) (OECD, 2016d) in human lymphocytes (Palus et al., 2007). Cylindrospermopsin was shown to have clastogenic and aneugenic activities in human WIL2-NS lymphoblastoids (Humpage et al., 2000) and hepatic cells (Bazin et al., 2010; Štraser et al., 2011). The comet assay, also known as the single-cell gel electrophoresis (SCGE) assay – which detects DNA damage (in the form of strand breaks, or other lesions that are converted into strand breaks under alkaline conditions) and DNA repair activity, and gives an indication of the genotoxic insult – has gained broad attention in genetic toxicology of toxic cyanobacteria (Ding et al., 1999; Žegura et al., 2003; Humpage et al., 2005; Palus et al., 2007). Cyanobacterial extracts (Palus et al., 2007) and several pure cyanotoxins, including MC-LR, cylindrospermopsin and nodularin, have been shown to induce DNA strand breaks (see Žegura et al., 2011 for a review). MC-LR induces transiently present DNA strand breaks that can be repaired and most probably occur indirectly due to oxidative stress (Žegura, 2016), while CYN induces DNA strand breaks in metabolically active cells (Humpage et al., 2005; Hercog et al., 2017).

Since the 2010s, hazard identification shifted towards mechanistic assessment that enables predictions of adverse outcome pathways (AOPs) (Ankley et al., 2010; Schroeder et al., 2016). The “omic” biomarker approaches (Li et al., 2017) using high-throughput molecular biology and mass spectrometry tools allow us to identify biological targets and pathways affected by the toxic compounds, including cyanotoxins (Štraser et al., 2013; Hercog et al., 2017). The “omic” biomarkers complement the standard toxicity and genotoxicity assays but how to use these complex data in the risk assessment procedure remains to be clarified.

14.10.4 Summary

In summary, toxicity testing of complex samples such as cyanobacterial blooms, raw or tap water provides rather minor additional value to current risk assessment of cyanobacterial toxicity, particularly where known cyanotoxins are present and sensitive instrumental methods for the detection of multiple toxins, as discussed above, are available (Meriluoto et al., 2017; Zervou et al., 2017). Alternatively, immunoassays such as ELISA or enzyme-inhibitory assays may serve for semiquantitative and sufficiently selective screenings. Toxicity testing has its place in bloom situations in which targeted analyses do not reveal any known cyanotoxins and uncertainty about the safety of the water remains, as, for example, in the case of a South Australian water supply with an unidentified toxin from *Phormidium* (Baker et al., 2001). In such a situation, the results of bioassays with mammalian cells *in vitro* or animals *in vivo* (mouse test) are most likely to provide some immediate guidance for managers regarding the acute toxicity of water.

Bioassays are important for further exploring the effects of yet unknown or not sufficiently characterised substances produced by cyanobacteria. A wide range of bioassays is available at many different levels from molecular to cell cultures or whole organisms. However, one single test will rarely be sufficient to fully characterise the toxicity of a cyanobacterial bloom; this usually requires a set of assays. Bioassays can give rapid responses, but a thorough validation process is needed for testing their performances, particularly if they are to be applied in the investigation of complex samples such as blooms, raw or treated drinking-water. *In vitro* bioassays are useful for developing an understanding of the biochemical processes underlying toxicity, whereas *in vivo* studies, despite technical and ethical concerns, continue to have a key role in supporting risk assessment, including in guideline value derivation. For the identification of unknown toxic agents from cyanobacterial blooms, effect-based monitoring or effect-directed analyses (EDA) (Escher & Leusch, 2011; Tousova et al., 2017) efficiently combine both bioassays and chemical analyses.

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