Chapter 23: Conventional laboratory methods for cyanotoxins

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Introduction

Over recent years it has become apparent that toxic cyanobacterial blooms are on the increase, presenting a hazard to animal and human health (Appendix A, Table A.1). The importance of algal toxins is reflected in their inclusion of EPA recognised contaminants in water (Richardson and Ternes 2005). Microcystins have been extensively studied and reported over recent years. Despite the number of microcystin variants and lack of standards, a large number of biological and chemical methods have been optimised for a variety of matrices, usually cells, water and tissue. Data on chronic and acute toxicity have led to the WHO to set a guideline maximum of 1 μg per litre in drinking water. Methods developed for microcystins are suitable for the pentapeptide nodularins, although these cyanotoxins usually occur in brackish water.

In contrast, relatively little work has been done on methods detection of other known toxins, anatoxins, cylindrospermopsins, BMAA and aplysiatoxins. Saxitoxins being the exception, as they occur widely in the marine environment and many methods have been developed for their detection in shellfish. However, there has been only limited application of these methods to freshwater samples. There are many challenges in assessing and selecting suitable methods since blooms can not only be composed of co–occurring species but it is also known that some species produce multiple classes of toxins.

This paper reviews methods presented in the literature, many of which are currently used for routine monitoring and in research. We discuss the application, validation, cost and practicability of a range of techniques. Priorities, future needs and challenges are addressed.

Analysis of microcystins

Microcystins are the most commonly reported cyanobacterial toxins and this is reflected by the large number of methods for their detection and analysis summarised in Table A.2. Although nodularins are less of a problem in freshwater, most methods developed for microcystins are suitable for nodularins. By far the greatest challenge in analysing microcystins is the fact that there are in excess of 65 variants characterised to date and most likely others yet to be identified. It is essential that any method used has the ability to detect all variants, regardless of availability of standards. Equally important, extraction and separation procedures must be suitable for the chemical range of variants in order to obtain accurate qualitative and quantitative data.

HPLC methods

There are many liquid chromatography based methods in the literature, utilising a range of stationary phases, mobile phases and detectors for both isocratic and gradient separations (Meriluoto 1997). However, reversed–phase chromatography with diode array detection (HPLC–PDA) has been the most widely used approach over the last two decades, as it enables detection of all microcystins based on their characteristic UV spectra (Lawton et al. 1994). Use of a gradient helps to ensures microcystins variants will be separated and despite lack of standards or certified reference materials, quantification of approximate total microcystin content is possible based on purified MC–LR to give MC–LR equivalence. Inter–laboratory validation data supports this approach combined with concentration and clean–up on SPE (Isolute C18) for the monitoring of intra and extra cellular microcystins in water samples as recommended in a "Blue Book" publication in the UK (Environment Agency 1998). Limits of quantification reported are 1–10 ng on column (achieving sub–µg per litre). A recent inter–laboratory trial highlighted the need for certified reference materials as commercial material that is currently available is essentially a laboratory reagent not a standard. When this material is used as a standard it results in varying responses for the same samples in different laborato-

ries (Fastner et al. 2002).This study also highlighted that despite variation in material which is used as standards, a variety of analytical systems and methods yielded similar responses, extraction procedures used for real samples was more problematic, emphasising the need for complete method optimisation. Detection limits have been improved by the use of immunoaffinity SPE for concentration, however, there are still limitations on binding capacity and the volumes loaded which must be overcome if this is to be a practical solution (Lawrence and Menard 2001, Aranda–Rodrigues et al. 2003). Recent advances, using recombinant antibody fragments, have demonstrated potential for the development of cost effective, robust and reproducible immunoaffinity cartridges (McElhiney et al. 2002).

As technology has evolved, LC–ESI–MS or LC–ESI–MS/MS is becoming the preferred technique as it offers greater selectivity and sensitivity than diode array detection. Good sensitivity was achieved using a single quadrupole (LC–ESI–MS), LOD of 11, 72, 21 and 6 pg for MC–LR, MC–RR, MC–YR and nodularin respectively on column (1 mm I.D.) using selected ion monitoring (SIM (Barco et al. 2002)). However, most methods published in the literature use tandem MS, which enables noise reduction and thus greater sensitivity, multiple reaction monitoring, and the removal of the need for complete separation of analytes. This approach enabled the development of a high through–put method which analysed ten microcystins in 2.8 minutes, without the need for complete resolution (Meriluoto et al. 2004). However, although the potential of LC–MS/MS is unequivocal, much work is still needed since most methods have been developed with a limited number of microcystins and there is no way to guarantee detection of unknown microcystins as fragmentation patterns vary considerably with conditions and microcystin chemistry itself. Fig. 1 illustrates the diversity of ionisation under typical reversed phase conditions. Microcystins containing no arginine are more susceptible to the formation of sodium and potassium adducts which is far from ideal in a quantitative application. Therefore, for a robust LC–MS/MS method, there is a requirement for ionisation optimisation and a thorough study on the effects of a wider variety of sample matrices, their effects and overcoming/understanding them. For suppression of sodium and potassium adducts, Yuan et al. demonstrated that the addition of oxalic acid biased the formation of the molecular ion thus increasing the sensitivity although this is seldom used and adduct ions are regularly monitored (Yuan et al. 1999). This work also showed that storage led to increases in adducts.

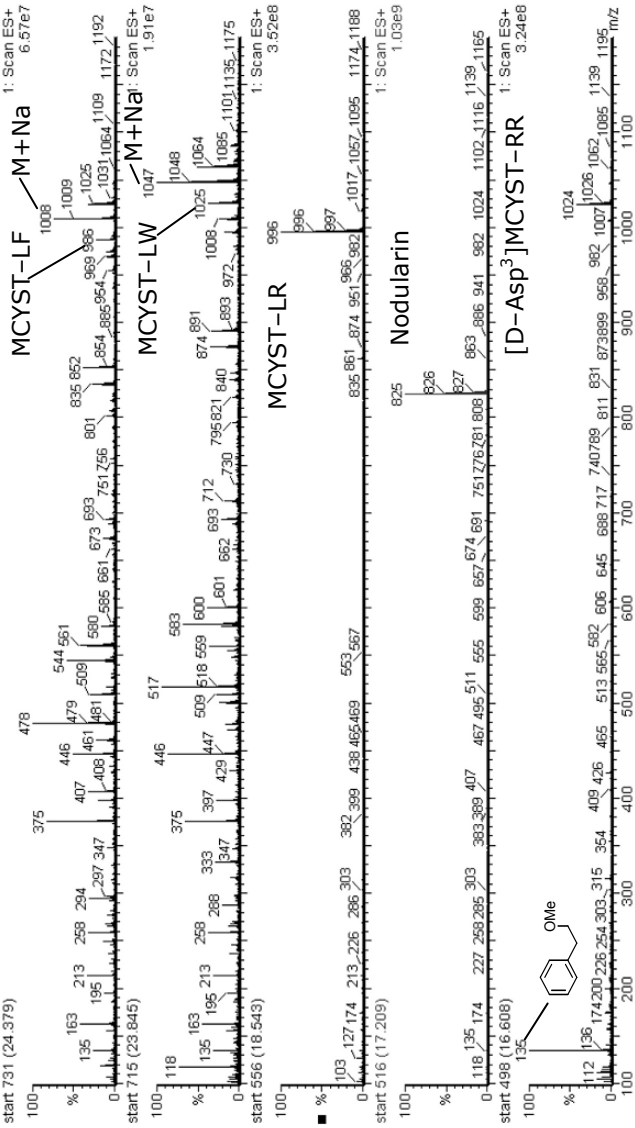

Fig. 1. Mass spectral of microcystin–LF, –LW, –LR, –D–Asp3 RR and nodularin performed using a Waters Micromass LC–ESI–MS. Separation was performed on a Symmetry C18 column using a water/acetonitrile plus 0.05%TFA gradient. Eluent was monitored by diode array (200–400 nm) and positive ion electrospray (100–1200 amu; cone voltage of 80 ev).

Maximizing individual microcystin sensitivity can be achieved by complex methods utilising time scheduled selected reaction monitoring conditions as demonstrated by Bogialli et al (2005). Several reports have examined matrix effects on analysis of tissue samples, illustrating the importance of the inclusion of this work for any method under development and in subsequent validation. Ruiz et al. demonstrated a 15% over estimation of MC–RR in extract from kidney compared to a 37% decrease in detection in liver (Ruiz et al. 2005). From these findings they recommended the use of matrix matched standards for use when quantifying unknown samples.

Matrix–assisted laser desorption (MALDI) has been used in conjunction with TOF analysers for the detection of microcystins and unknown variants in small samples (Welker et al. 2002). Characteristic fragmentation was achieved by post–source decay, which results in destruction of the peptide bonds. Whilst rapid, this offline technique requires some extraction to eliminate matrix/sample interferences, but, as improved matrices are developed, there is future potential for an approach eliminating time consuming sample preparation and chromatography. This is illustrated in a recent publication, describing the use of MALDI linked to a triple quadruple for the qualitative and quantitative determination of spirolide toxins (Sleno and Volmer 2005). The combination of this ionisation technique with sensitive multiple reaction monitoring, proved to be precise and accurate without the need for extensive sample preparation.

Another exciting approach which is rapid and eliminates time consuming SPE, where the microcystins were captured on a hydrophobic chip and subsequently ionised by surface–enhanced laser desorption ionisation–time–of flight MS (SELDI–TOF–MS) enabled determination of 2.5 pg MC–LR in 2 µl (1.2 µg L^{-1}) water (Yuan and Charmichael 2004). However, severe matrix effects were experienced when more complex samples were analysed, and it was not possible to monitor the characteristic m/z 135 due to background interference. Future chip developments could present the way forward although may prove costly.

In–vitro bioassays

To compliment the large number of physico–chemical methods there exists a significant number of bioassays for detection of microcystins. Microcystins and nodularins are strong inhibitors of protein phosphatases, PP–1, PP–2A and PP–3, PP–2A being the most sensitive. This functionality has been exploited to develop assays which provide a direct measure of toxicity. A range of substrates have been used but the most commonly used are

p–nitrophenol phosphate (*P*–NPP), 4–methylumbelliferyl phosphate (MUP) and 6,8–difluoro–4–methylumbelliferyl phosphate (DiFMUP). The latter has been successfully validated against HPLC and mouse bioassay for the detection of okadaic acid in shellfish (González et al. 2002). This approach has been adapted to a rapid microplate assay for screening microcystins in drinking water without the need for pre–concentration, achieving a detection limit of 0.1 μg L^{-1}, which is well below the provisional guideline value (Bouaïcha et al. 2002). This assay provides a useful pre– or post analytical screen for bioactivity although false positives may be obtained from other phosphatase inhibitors, which may occur in environmental samples. Many researchers have reported good correlation of data obtained by protein phosphatase inhibition assay and HPLC–PDA (Ward et al. 1997, Wirsing et al. 1999). The necessary components are available commercially although there is batch variation in enzyme activity.

Immunoassays

Immunoassays, exploiting polyclonal, monoclonal antibodies and recombinant antibody fragments, are widely used as screening tools for microcystins and nodularins and are well reviewed elsewhere (McElhiney and Lawton 2005, Metcalf and Codd 2003). Several kits are commercially available, in microtitre plate or tube format. Many of the assays/kits use antibodies raised against MC–LR and subsequently may have limited cross reactivity (EnviroLogix Inc, Portland, ME, USA), whereas kits using antibodies raised against ADDA provide improved sensitivity and excellent cross–reactivity (Abraxis LLC, PA, USA: Biosense Laboratories AS, Bergen, Norway). However, the behaviour of non–toxic degradation products including free ADDA is as yet unknown These ELISA kits are supplied in a 96–well microplate format with ready to use reagents enabling screening of up to 96 samples in 2.5 hours with a consumable cost of $400.00. All of these commercial kits are simple to use, rapid and economical for screening. As with phosphatase inhibition assays, immunoassays can be used for detection of microcystins below the WHO guideline without the need for sample pre–concentration.

Other useful methods

A cost effective, rapid, thin layer chromatography (TLC) method has also been developed which enables detection of microcystins to meet the WHO 1 μg L^{-1} guideline. This method relied on visualisation of the microcystins

on the developed TLC plate using N,N–dimethyl—1,4–phenylendia–monium dichloride (N,N,–DPDD) and good correlation was achieved compared to protein phosphatase and ELISA assay (Pelander et al. 2000). However, without sophisticated spotting and scanning devices, this is not quantitative, but would serve as a useful screen for known microcystins, although it does require improvements in sample concentration to remove interfering contaminants.

A method for determination of total microcystins relies on oxidation to produce 2–methyl–3–methoxy–4–phenyl–butyric acid (MMPB) from ADDA, which is detected by GC–MS (Kayo and Sano 1999), HPLC–Fl (Sano et al. 1992) or HPLC–TSP. Whilst this method has been demonstrated to be useful for complex samples such as sediments, the need for oxidation, and the fact that only total microcystin is determined, make it a complex, time consuming and expensive screen. Despite these disadvantages, this could be a useful confirmatory method and can be used with a wide range of instrumentation without the need for microcystin standards. Most methods described determine free microcystins, this method will also detect bound microcystin, thus providing a complete picture in metabolism studies.

Capillary electrophoresis based methods exploit high efficiency columns to separate variants often problematic in LC separations such as MC–LR and [D–Asp (Lawton et al. 1994)] MC–LR providing a useful complimentary technique (Bateman et al. 1995). Issues such as sensitivity and interfering compounds have been overcome by improved online and offline sample clean–up.

Combined methods

HPLC–UV/PDA has been shown to be a powerful tool in combination with protein phosphatase inhibition or ELISA assay. HPLC–PP2A was first reported in 1991 as a highly sensitive bioscreen for okadaic acid along with related polyether toxins (Holmes 1991) and later applied for the detection of microcystins in freshwater environments (Boland et al. 1993). These approaches are still used, often along side mass spectrometry to determine complete structure/activity profiles of unknown samples (Ortea et al. 2004).

Fractionation into 96 well plates was used to increase automation and extending the assay to include an immunoassay providing LC–UV/ELISA /PP2A data, achieving detection limits 1000 x more sensitive than UV (Zeck et al. 2001). This paper also compared the response of the same sample to PP2A, and ELISA, using three commercially available antibod-

ies, highlighting huge variation in cross reactivity. Several groups have reported the use of ELISA alongside PP2A inhibition, providing a measure of total microcystins and toxicity, however, the most elegant use of these techniques is the immunophosphatase assay.

Analysis of saxitoxins

Saxitoxins (also known as paralytic shellfish poisons, PSPs) are another complex group of compounds which have presented a challenge over the last two decades. Until June 2005, the only validated method available was the mouse bioassay, routinely used for screening shellfish and phytoplankton. However, there has been much progress in development of methods as summarised in Table A.3 of Appendix A, reflecting their importance in the shellfish industry and the fact that many countries have rigorous guidelines and monitoring requirements.

HPLC analysis

In June 2005 an HPLC method relying on fluorescence detection of the oxidised saxitoxins was approved by AOAC after inter–laboratory validation (Lawrence et al. 2004). Whilst this method is robust, the sample processing is complex and two pre–column oxidation reactions/separations may be needed for quantification of the complete range of saxitoxins. A further problem is that oxidation of some GTXs, dcGTXs, dcSTX and dcNEO results in the production of two fluorescent compounds, thus requiring a broad range of standards. Despite the reported robustness, this is a time consuming and therefore expensive method. Automation of the derivatization procedure would reduce manual processing, however it must be noted the fluorescent products are not stable after a few hours.

An alternative approach using post–column derivatization has been preferred in many labs as it benefits from simple automation. However, three, more recently two, separations are needed to accurately quantify all toxins. This method is sensitive to changes in flow rate, reagent age and temperature. With both pre– and post column derivatization methods, it is ideal to run a sample without oxidisation to confirm peaks are not interfering contaminants.

Several methods using capillary electrophoresis have been reported although, they are not widely used and suffer from low sensitivity due to the low volume injected and the requirement for a very clean sample in order

to obtain reproducible chromatography. It is reported that LOD is an order of magnitude greater than HPLC–FL/MS.

A recent publication described a single gradient separation for all saxitoxins with MS/MS detection for qualitative analysis and future optimisation of quantitation provides a promising alternative analytical method (Dell'Aversano et al. 2005). This will provide a simpler, although more expensive method, without the need for oxidation.

Many assays have been described which exploit the functionality of the saxitoxins, i.e. sodium channel blocking activity. Most of these rely on the use of cultured cell lines and specialist techniques/facilities, thus not practical for routine monitoring purposes and out with the scope of this review.

Immunoassays

An immunoassay kit, RIDASCREEN®, is available from R–Biopharm AG (Darmstadt, Germany), which is used widely by commercial organisations for screening shellfish. This is a sensitive (LOD of 50 ppb), quantitative, plate based kit, which requires a microtitre plate reader (450 nm). Each 48 plate allows analysis of up to 42 samples providing results after a one–hour incubation. This is generally used as a rapid screen, providing a yes/no response, providing good correlation with the mouse bioassay for the detection of saxitoxins in shellfish (Inami et al. 2004). This kit has a lot of potential for screening saxitoxins in water, cells and tissues, but the only published report was analysis of crude cyanobacterial cell extracts (Teneva et al. 2005). It must be remembered that there is poor cross reactivity with related compounds, e.g. 12% with neosaxitoxin which is often a major component produced by cyanobacteria.

One of the most promising, commercially available screens, is the Jellet Rapid Test (JRPT: formerly MIST Alert) which is a lateral flow immuno–chromatographic test approach based on antibodies raised to multiple, structurally diverse saxitoxins, providing good cross reactivity and therefore accuracy (Jellet et al. 2002). The JRPT functions in a manner similar to a pregnancy testing kit, providing a yes/no answer within twenty minutes. This has been widely tested across the world in parallel with the mouse bioassay and HPLC, and in many areas now serves as the primary screening tool. Potential use of this system for monitoring saxitoxins in freshwater has yet to be investigated, although, it must be remembered that the level of detection is aimed at the shellfish and some modification for freshwater application would be necessary or a sample concentration step added.

Analysis of cylindrospermopsins

Compared to microcystins and saxitoxins, relatively few methods have been developed for detection of cylindrospermopsins (Table A.4 in Appendix A). This may be due to the fact this is a more recently discovered toxin which was easily detected by HPLC–PDA/MS and/or that events have been limited. HPLC–PDA is good for detection of cylindrospermopsins and its analogues as they have characteristic UV spectra (λ max at 262 nm) however, sample cleanup is necessary to remove co–eluting contaminants (Welker et al. 2002) . HPLC–PDA was used by five out of six laboratories during a recent inter–laboratory comparison of cylindrospermopsin analysis (Törökné et al. 2004). Cylindrospermopsin was extracted from freeze–dried cells by a variety of procedures followed by HPLC analysis to determine method suitability. Whilst all methods were successful for crude extraction/analysis of cylindrospermopsin, further refinements would be necessary if any of these was to be used for monitoring purposes. LC–MS/MS is currently the most favoured method of analysis, providing structural confirmation and sensitive quantification by monitoring the transition from M+H ion (m/z of 416) to the major fragment m/z of 194, achieving a range of 1–600 μg L^{-1} without sample concentration (Eaglesham et al. 1999). Although cell and invertebrate assays have been used to detect cylindrospermopsin, these are non–specific and insensitive. The development of a sensitive, selective rapid screen for monitoring is essential. However, it is important to remember with cylindrospermopsins in water samples, that these compounds are excreted from the cyanobacterial cell during growth, thus necessitating robust sampling protocols and analysis of extra– and intracellular toxin.

Analysis of anatoxin–a

Apart from the mouse bioassay, all reported methods of detection of anatoxin–a are based on chromatography, with or without derivatization as summarised in Appendix D. LC–UV has been widely used but suffers from limitations such as sensitivity and interferences in complex sample matrices. In recent years sensitive, qualitative and quantitative methods which rely on some form of derivatization procedure included GC–MS, GC–ECD and HPLC with fluorescence detection have been the preferred methods (Himberg 1989, Stevens and Krieger 1988, James et al. 1998). As with most applications, improvements in LC–MS and LC–MS/MS technology have led to increasing use for detection of anatoxin–a and its ana-

logues, eliminating the need for derivatization (Furey et al. 2005, James et al. 2005). However, LC–MS was the sole method used to confirm the presence of anatoxin–a as the most likely cause of a young man's death in 2002, but as it transpired the compound was in fact phenylalanine, but due to the fact that the two compounds are isobaric and have similar retention characteristics, LC–MS alone was insufficient to distinguish between them (Furey et al. 2005). This case, illustrates the need for multiple, robust and complimentary methods and /or detectors. A diode array detector in series would have shown the difference in UV spectra of anatoxin–a and phenylalanine, having maximum absorption at 227 nm and 257 nm respectively. A similar approach, using LC–PDA and LC–MS was recently used to unequivocally identify anatoxin–a associated with a dog poisoning in France (Gugger et al. 2005).

In the short term, the only option for a low cost, rapid screen, could be the TLC method where the anatoxin is reacted with the diazonium reagent, Fast Black K salt, to form an orange–red product (Ojanperä et al. 1991). Although this method is sufficiently sensitive for determination of anatoxin in algal cells (10 $\mu g\ g^{-1}$), pre–concentration of water samples would be necessary. This method should also be suitable for detection of anatoxin analogues.

Analysis of anatoxin–a(s)

The occurrence of this alkaloid cholinesterase inhibitor is rare, as is reflected by the number of methods published. Despite its rarity, anatoxin–a(s) is highly toxic (LD_{50} in mice is 50 $\mu g\ kg^{-1}$ body weight) and has been responsible for several livestock and bird poisonings thus necessitating reliable methods of detection. Lack of a chromophore, limits the use of conventional HPLC methods although mass spectrometry would be an ideal means of detection. Colorimetric bioassays based on acetylcholinesterase inhibition have been the most reliable methods to date, although false positives can be obtained from organo–phosphorus insecticides (Ellman et al. 1961). This assay is a rapid and sensitive laboratory screen, with all necessary enzymes and reagents available from general laboratory suppliers. Biosensors, incorporating enzymes of different sensitivities, have been developed which facilitate specific detection of anatoxin–a(s) below $\mu\ L^{-1}$ level (Devic et al. 2002). A similar biosensor used oxime reactivation of the enzyme to differentiate between anatoxin–a(s) and insecticide inhibition (Villatte et al. 2002). Refinement and commercialisation of these bio-

sensors would be an ideal screen for anatoxin–a(s), being rapid, inexpensive and simple.

Analysis of ß–N–methylamino–L–alanine (BMAA)

A recent publication indicated that this neurotoxic amino acid is produced by a diverse range of cyanobacteria (Cox et al. 2005), a potential hazard, obviating the need for further investigation. Several HPLC methods have been reported including derivatization with 6–aminoquinolyl–*N*–hydroxylsuccinimidyl carbamate followed by RP–HPLC with fluorescence detection with a limit of quantitation reported as 1.2 μg L^{-1}. MS detection of this derivative was also used for additional confirmation. GC–MS has also been used to detect BMAA in cycad seeds as an N–ethoxy carbonyl ethyl ester derivative (Pan et al. 1997). Although these methods have been used to detect BMAA in cycads, flying foxes and brain tissue, further work is needed to provide robust methods, encompassing extraction, concentration/clean–up and quantitative/qualitative analysis to support necessary research and monitoring programs.

Conclusions and Summary

It is clear from the literature that numerous methods are available for most cyanotoxins, although many publications on monitoring data indicate that the favored approach is the use of proven, robust methods for individual toxins. The most effective approach is the utilization of a robust rapid screen, where positive samples are followed up by qualitative and quantitative analysis to provide the essential decision making data needed for successful management strategies (Fig. 2). Currently, rapid screens are available for microcystins, saxitoxins and anatoxin–a(s), whilst optimisation and validation is needed, many publications report good correlation with the mouse bioassay and HPLC.

There is an urgent need for rapid, simple, and inexpensive assays for cylindrospermopsins, anatoxin–a and BMAA. Although methods exist for analysis of BMAA, the fact that a recent study showed 95% of cyanobacteria producing this, some at levels >6,000 μg g^{-1} dry wt, is of concern and rapid screening followed by robust analysis is needed.

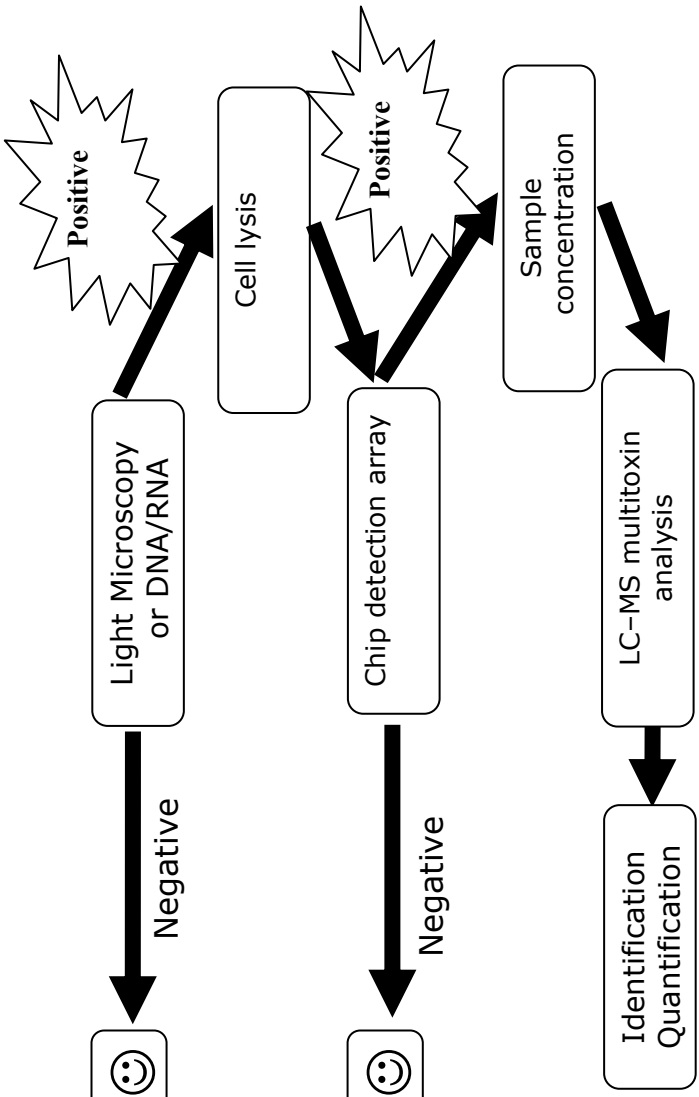

Fig. 2. Future strategy for cyanotoxin monitoring including initial screen to identify the presence of harmful cyanobacteria either by established light microscopy or novel molecular techniques, followed by rapid multitoxin array with the ability to highlight the presence of any of the known classes of cyanotoxins above a pre–determined threshold and where necessary analytical confirmation by LC–MS.

An ideal approach would be a single method capable of extracting and detecting all cyanotoxins. Several publications describe such approaches using LC–MS, but as expected from a group of compounds with diverse chemistry, there are obvious limitations in recoveries during sample processing, chromatographic performance and sensitivity (Dahlmann et al. 2003, Dell'Aversano et al. 2004, Pietsch et al. 2001).

Selection of methods must be based on the application requirements, equipment available and cost. For many organisations it may be more cost effective to out–source the occasional analysis. However, as the incidence of blooms appears to be increasing, the need for more rigorous monitoring is needed, sensible investment is needed to meet recommended guidelines. Most of the methods discussed in this paper are suitable for achieving this goal, although clean–up and concentration is usually necessary for physicochemical methods.

References

Aranda–Rodriguez R, Kubwabo C, Benoit FM (2003) Toxicon 42 587–599

Barco M, Rivera J, Caixach J (2002) J Chromatogr A 959 103 –111

Bateman KP, Thibault P, Douglas DJ, White RL (1995) J Chromatogr A 712 253-268

Bogialli S, Bruno M, Curini R, Di Corcia A, Lagana A, Mari B (2005) J Agric Food Chem 53 6586–6592

Boland MP, Smillie MA, Chen DZX, Holmes CFB (1993) Toxicon 31 1393–1405

Bouaïcha N, Maatouk I, Vincent G, Levi Y (2002) Food Chem Toxicol 40 1677–1683

Chianella I, Lotierzo M, Piletsky SA, Tothill IE, Chen B, Karim K, Turner APF (2002) Anal Chem 74 1288–1293

Cox PA, Banack SA, Murch SJ, Rasmussen U, Tien G, Bidigare RR, Metcalf JS, Morrison LF, Codd GA, Bergman B (2005) PNAS. 102 No 14 5074–5078

Dahlmann J, Budakowski WR, Luckas B (2003) J Chromatogr A 994 45 –57

Dell'Aversano C, Eaglesham GK, Quilliam MA (2004) J Chromatogr A 1028 155–164

Dell'Aversano C, Hess P, Quilliam MA (2005) J Chromatogr A 1081 190 –201

Devic E, Li D, Dauta A, Henriksen P, Codd GA, Marty JL, Fournier D (2002) Appl Environ Microbiol 68 No 8 4102–4106

Eaglesham GK, Norris RL, Shaw GR, Smith MJ, Chiswell RK, Davis BC, Neville GR, Seawright AA, Moore MR (1999) Environ Toxicol 14 151–154

Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) Biochem Pharmacol 7 88–95

Environment Agency (1998) Methods for the examination of Waters and Associated Materials. The Environment Agency, Bristol UK

Fang X, Fan X, Tang Y, Chen J, Lu J (2004) J Chromatogr 1036 233–237

Fastner J, Codd GA, Metcalf JS, Woitke P, Wiedner C, Utkilen H (2002) Anal Bioanal Chem 374 437–444
Fastner J, Heinze R, Humpage AR, Mischke U, Eaglesham GK, Chorus I (2003) Toxicon 42 313–321
Furey A, Crowley J, Hamilton B, Lehane M, James KJ (2005) J Chromatogr A 1082 91– 97
González JC, Leira F, Fontal OI, Vieytes MR, Arévalo FF, Vieites JM, Bermúdez–Puente M, Muniz S, Salgado C, Yasumoto T, Botana LM (2002) Anal Chim Acta 466 233–246
Gugger M, Lenoir S, Berger C, Ledreux A, Druart JC, Humbert JF, Guette C, Bernard C (2005) Toxicon 45 919–928
Harada KI, Nagai H, Kimura Y, Suzuki M, Park HD, Watanabe MF, Luuklkainen R, Sivonen K, Carmichael WW (1993) Tetrahedron 49 9251–9260
Himberg K (1989) J Chromatogr A 481 358–362
Hoeger SJ, Shaw G, Hitzfeld BC, Dietrich DR (2004) Toxicon 43 639–649
Holmes CFB (1991) Toxicon 29 469–477
Inami GB, Crandall C, Csuti D, Oshiro M, Brendan RA (2004) J AOAC Int 87 1133–1142
James HA, James CP (1991) Report No Ro224. Foundation for Water Research, Allen House, The Listons, Liston Road, Marlow UK
James KJ, Furey A, Sherlock IR, Stack MA, Twohig M, Caudwell FB, Skulberg OM (1998) J Chromatogr A 798 147–157
James KJ, Crowley J, Hamilton B, Lehane M, Skulberg OM, Furey A (2005) Rapid Commun Mass Spectrom 19 1167–1175
Jellet JF, Roberts RL, Laycock MV, Quilliam MA, Barrett RE (2002) Toxicon 40 1407–1425
Kayo K, Sano T (1999) Anal Chim Acta 386 107–112
Kim YM, Oh SW, Jeong SY, Pyo DJ, Choi EY (2003) Environ Sci Technol 37, 1899–1904
Kondo F, Ikai Y, Oka H, Matsumoto H, Yamada S, Ishikawa N, Tsuji K, Harada KI, Shimada T, Oshikata M, Suzuki M (1995) Nat Toxins 3 41–49
Lawrence JF, Menard C (2001) J Chromatogr A 922 111–117
Lawrence JF, Niedzwiadek B, Menard C (2004) J AOAC Int 87 No 1 83–100
Lawton LA, Edwards C, Codd GA (1994) Analyst 119 1525–1530
Locke SJ, Thibault P (1994) Anal Chem 66 3436–3446
Manger RL, Leja LS, Lee SY, Hungerford JM, Kirkpatrick MA, Yasumoto T, Wekell MM (2003) J AOAC Int 86 540–543
McElhiney J, Lawton LA (2005) Toxicol Appl Pharmacol 203 219–230
McElhiney J, Lawton LA, Edwards C, Gallacher S (1998) Toxicon 36 417–420
McElhiney J, Drever M, Lawton LA, Porter AJ (2002) Appl Environ Microbiol 68 5288–5295
Meriluoto J (1997) Anal Chim Acta 352 277–298
Meriluoto J, Karlsson K, Spoof L (2004) Chromatographia 59 291–298
Metcalf JS, Codd GA (2003) Chem Res Toxicol 16 103–112
Metcalf JS, Bell SG, Codd GA (2001) Appl Environ Microbiol 67 904–909

Metcalf JS, Lindsay J, Beattie KA, Birmingham S, Saker ML, Törökné AK, Codd GA (2002) Toxicon 40 1115–1120
Mirocha CJ, Cheong W, Mirza U, Kim YB (1992) Rapid Commun Mass Spectrom 6 128–134
Ojanperä I, Vuori E, Himberg K, Waris M, Niinivaara K (1991) Analyst 116 265–267
Ortea PM, Allis O, Healy BM, Lehane M, Shuilleabháin AN, Furey A, James KJ (2004) Chemosphere 55 1395 – 1402
Oshima Y (1995) In: .M Hallegraeff, DM Anderson and AD Cambella [eds] Manual on Harmful marin Algae, IOC Manuals and Guides, 33 81–94
Pan M, Mabry TJ, Cao P, Moini M (1997) J Chromatogr A 787 288–294
Pelander A, Ojanperä I, Lahti K, Niinivaara K, Vuori E (2000) Water Res 34 2643–2652
Pietsch J, Fichtner S, Imhof L, Schmidt W, Brauch HJ (2001) Chromatographia 54 339–344
Richardson SD, Ternes TA (2005) Anal Chem 77 3807–3838
Ruiz MJ, Cameán AM, Moreno IM, Picó Y (2005) J Chromatogr A 1073 257–262
Sano T, Nohara K, Shiraishi F, Kaya K (1992) Int J Environ Anal Chem 49 163–170
Seawright AA, Nolan CC, Shaw GR, Chiswell RK, Norris RL, Moore MR, Smith MJ (1999) Environ Toxicol 14 135–142
Sleno, L, Volmer DA (2005) Anal Chem 77 1509–1517
Stevens DK, Krieger RI (1988) J Anal Toxicol 12 126–131
Takino M, Daishima S, Yamaguchi K (1999) J Chromatogr 862 191–197
Teneva I, Dzhambazov B, Koleva L, Mladenov R, Schirmer K (2005) Toxicon 45 711–725
Törökné A, Asztalos M, Bánkiné M, Bickel H, Borbély G, Carmeli S, Codd GA, Fastner J, Huang Q, Humpage A, Metcalf JS, Rábai E, Sukenik A, Surányi G, Vasas G, Weiszfeiler V (2004) Anal Biochem 332 280–284
Villatte F, Schulze H, Schmid RD, Bachmann TT (2002) Anal Bioanal Chem 372 322–326
Ward CJ, Beattie KA, Lee EYC, Codd GA (1997) FEMS Microbiol Lett 153 465—473
Welker M, Bickel H, Fastner J (2002) Water Res 36 4659–4663
Wirsing B, Flury T, Wiedner C, Neumann U, Weckesser J Environ Toxicol 14 23–29
Yuan M, Carmichael WW (2004) Toxicon 44 561–570
Yuan M, Namikoshi M, Otsuki A, Watanabe MF, Rinehart KL (1999) J Am Mass Spectrom 10 1138–1151
Zeck A, Weller MG, Niessner R (2001) Anal Chem 73 5509–5517
Zhang L, Ping X, Yang Z (2004) Talanta 62 193–200
Zotou A, Jeffries TM, Brough PA, Gallagher T (1993) Analyst 118 753–758

Appendix A

Table A.1 Toxin producing cyanobacteria and their relative human–health risk.

Cyanotoxin	Cyanobacteria	Health Risk	Occurrence
Microcystins	*Microcystis, Anabaena, Planktothrix, Nostoc, Anabaenopsis, Snowella, Woronichinia*	Acute: Liver toxin Chronic: Tumour promoter	Reported globally Very common in Freshwater blooms
Nodularins	*Nodularia* Cyanobacterial symbiont of marine sponge (?)	Acute: Liver toxin Chronic: Tumour promoter and possible carcinogen	Brackish waters Freshwater thermal spring
Saxitoxins	*Anabaena, Planktothrix, Aphanizomenon, Lyngbya, Cylindrospermopsis*	Acute: Neurotoxin	Freshwater reported in North and South America, Australia and Europe
Anatoxin–a	*Anabaena, Planktothrix, Aphanizomenon, Cylindrospermum, Raphidiopsis*	Acute: Neurotoxin	Freshwater, low incidence of reports
Anatoxin–a(s)	*Anabaena*	Acute: Neurotoxin	Freshwater, very low incidence of reports
Lipopolysaccharide	All cyanobacteria	Acute: Inflammation May reduce the bodies detoxification mechanisms	Global and present in all cyanobacterial blooms
Cylindrospermopsin	*Cylindrospermopsis, Anabaena, Umezakia, Aphanizomenon, Raphidiopsis*	Acute: Organ and tissue damage particularly liver Chronic: Possible carcinogen	Increasingly reported especially in warmer climates

Cyanotoxin	Cyanobacteria	Health Risk	Occurrence
Aplysiatoxins	*Lyngbya, Schizothrix, Planktothrix*	Acute: Inflammation affecting skin and GI tract Chronic: tumour promoter	Benthic marine cyanobacteria
Lyngbyatoxin–a	*Lyngbya*	Acute: Inflammation affects skin and GI tract	Benthic marine cyanobacteria
B–methylamino–L–alanine (BMAA)	Present in c. 95% of cyanobacteria tested although the amount of BMAA varies by several orders of magnitude.	Chronic: Neurotoxin (Parkinson–like and dementia)	Global

Table A.2. Methods for the detection of microcystins and nodularins. LoQ – limit of quantitation given as amount on the system and/or concentration; Cost/sample–quoted contract as opposed to "in–house"; Time/sample – run time excluding sample concentration/preparation.

Method	LoQ	Cost/ Sample	Time/ sample	Multi–variant	Comments
HPLC–UV (Meriluoto 1997)	1–10 ng	n/a	30 min	Limited	Very limited identification, dependent on retention time of known standards.
HPLC–PDA (Lawton et al. 1994)	1–10 ng 250 g/l	$300	30–60 min	Yes	Utilizes characteristic spectra to identify microcystins and nodularins. Validated and widely used.
LC–MS (Barco et al. 2002)	50 pg	$400	30 min	Yes	SIM allows low level detection. TIC required for the detection and identification of multiple variants.
LC–MS/MS (Zhang et al. 2004)	2.6 ng/l	$400	30 min	Yes	Fragment analysis provides 'finger print' for individual variants including those with identical masses. MRM enhanced selectivity and sensitivity.
MALDI–TOF (Welker et al. 2002)	1–100 µg/l	n/a	< 1 min	Yes	Identification and structural elucidation. Problems with matrix interference.
SELDI–TOF (Yuan and Carmichael 2004)	2.5 pg/2 µl water	n/a	300 /day	Yes	New technology with a lot of potential
Frit–FAB (Kondo et al. 1995)	20–50 ng	n/a		Yes	Low sensitivity now superseded
MMPB with LC–MS or GC–MS or LC–Fl (Kayo and Sano 1999, Sano et al. 1992)	0.02 µg/l	n/a		Yes	Detects oxidation product of microcystin, no identification of individual variants. Good for complex matrices and bound toxin.

Method	LoQ	Cost/ Sample	Time/ sample	Multi–variant	Comments
TLC (Pelander et al. 2000)	10 ng	n/a	30 min	Yes	Requires automation for reliable quantification. Limited by number of standards available.
CE–UV (Bateman et al. 1995)	3 µg m/l	n/a	12 min	Yes	Limited sample loading volume results may poor LoQ. This can be improved with inline concentration
CE–MS	4 pg 0.2 µg m/l	n/a	12 min	Yes	High speed separation with identification from mass data
Mouse	50–500 µg/kg	$500	24 hr	Yes	Non–specific, specialized facilities and license required
ELISA–adda (McElhiney and Lawton 2005)	20 pg/ml	$250	2.5 hr per plate	Yes	No pre–concentration required. Good sensitivity and cross reactivity. Commercially available.
ELISA (McElhiney and Lawton 2005)	20–3000 pg/ml	$200	3 hr per plate	Yes	Several kits commercially available. No pre–concentration required.
Dipstick (Kim et al. 2003)	95 pg/ml	n/a	12 min	some	Not tested with non–arginine containing microcystins.
Inhibitor immunoassay (Metcalf et al. 2001)	IC_{50} 45 ng	n/a	3 hr	Yes	Good combination of structural detection and bioactivity

Method	LoQ	Cost/ Sample	Time/ sample	Multi–variant	Comments
Protein phosphatase (Ward et al. 1997, Wirsing et al. 1999)	0.1–0.25 µg/L	n/a	1 hr per plate	yes	Provides total toxicity. Components commercially available. No sample concentration needed.
Molecular Imprinted polymers (Chianella et al. 2002)	0.2 µg/L	n/a	25 min	No	Different polymers need for each variant. Very stable with great potential for biosensor.

Table A.3 Methods for the detection of saxitoxins. LoQ – limit of quantitation given as amount on the system and/or concentration; Cost/sample – quoted contract as opposed to "in–house"; Time/sample – run time excluding sample concentration/preparation.

Method	LoQ	Cost/ sample	Time/ sample	Multi–variant	Comments
HPLC–Fluorescence, pre–column derivatization (Lawrence et al. 2004)	80 µg/kg	$400	40 min	Yes	Relatively reliable but two oxidation/separations needed. Complex sample processing would benefit from automation. Validated.
FAB–MS (Mirocha et al. 1992)	200 pg		10 min	Not shown yet	Potential interference issues
LC–QTOF–MS (Fang et al. 2004)	0.1 µg/g	$400	25 min	DcSTX and STX only	Confirmation and quantitation Simple sample treatment and good recoveries.
HILIC–MS/MS (Dell'Aversano et al. 2005)	50–1000 nM (single quad)	$400	30 min	Yes	Confirmation and quantitation Improved sensitivity using SIM (5–30 nM)
HPLC– Fluorescence, pre–column derivatization (Oshima 1995)	65 µg/100 g	$400	1.5 hr	Yes	Three separation methods needed. Some users focus on species dominant group of compounds.
CE–UV/MS (Lock and Thibault 1994)	0.2–1 µg/ml	$400	40 min	Yes	Good sample clean–up essential as it is sensitive to contaminants.
Mouse	0.2 µg/ml	$500	0.5 –2 hr	Yes	Specialized facilities and license required.
Sodium channel receptor (Manger et al. 2003)	2 µg/100 g	$500	6 hr	Yes	Cells commercially available. Requires cell culture facilities

Method	LoQ	Cost/ sample	Time/ sample	Multi–variant	Comments
MIST ALERT/ JRPT (Jellet et al. 2002)	40 µg/100g	$150	20 min	Yes	Rapid and multi–variant Optimised for field use and rapid response for shellfish. LoQ based on regulatory guidelines for shellfish.
ELISA	50 ppb		42 samples/1.5 hr	Yes	Limited cross reactivity. Commercially available.
Locust (McElhiney et al. 1998)	2.5 µg/ml	$30	90 min	Yes	Robust and simple. Locust is a controlled species in many countries, bush cricket is suitable alternative.

Table A.4 Methods for the detection of cylindrospermopsins. LoQ – limit of quantitation given as amount on the system and/or concentration; Cost/sample – quoted contract as opposed to "in–house"; Time/sample – run time excluding sample concentration/preparation.

Method	LoQ	Cost/ sample	Time/ sample	Multi–variant	Comments
HPLC–UV (Törökné et al. 2004)			10–65 min	Not discussed	Limited use
HPLC–PDA (Welker et al. 2002)	1–300 ng	$400	20 min	Yes	Potential matrix effects from extraction procedure
LC–MS/MS (Eaglesham et al. 1999)	1–600 µg/L	$400	10 min	Yes	Minimal processing. Characterization and quantitation obtained. Used for crawfish samples.
Mouse (Seawright et al. 1999, Fastner et al. 2003)	0.2 mg/kg (IP LD_{50})	$500	24 hr	Yes	Specialized facilities and license required. Lengthy assay time.
Brine shrimp (Metcalf et al. 2002)	8.1 µg /ml	$50	24 hr	Not reported	Insensitive and lengthy assay time. Sensitivity increases with incubation time.

Table A.5 Methods for the detection of anatoxin–a. LoQ – limit of quantitation given as amount on the system and/or concentration; Cost/sample – quoted contract as opposed to "in–house"; Time/sample – run time excluding sample concentration/preparation.

Method	LoQ	Cost/ sample	Time/ sample	Multi–variant	Comments
HPLC–UV (Zotou et al. 1993)	1 µg/ l	$300	20	Not discussed	Limited data – isocratic analysis
GC–MS (Himberg 1989)	0.1 ng	$400	10 min	Yes	Derivatization to N–acetyl anatoxin
HPLC–Fluorescence (James et al. 1998)	10 ng/l	$300	35	Yes	Derivatization necessary. Good complementary confirmation technique
LC–MS (Takino et al. 1999)	15 ng/l	$400	25 min	Yes	No derivatization needed. Qualitative and quantitative
LC–QIT–MS (James et al. 2005)	25–1000 µg/l	$400	20 min	Yes	Characterisation and quantification without loss of sensitivity.
GC–ECD (Stevens and Krieger 1988)	2 pg 3 ng/ l	$300	10 – 30 min	Yes	Derivatized with trichloroacetic anhydride. Sensitive but no structural confirmation
LC–TSP–MS (Harada et al. 1993)	1–40 ng	$400	10 min	Yes	Isocratic – not ideal for complex samples without sample specific clean–up. SIM needed. Superseded by ESI and APCI
TLC (Ojanperä et al. 1991)	10 µg/g cells	$20	1 hr multiple samples	Yes	Useful as a rapid screen. Sample concentration needed for water samples.