Methods for detection and quantification of cyanobacterial toxins – a review

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Abstract

Cyanobacteria (blue-green algae) forming massive water blooms and surface scums in eutrophic waters produce wide range of toxins (the cyanotoxins), among them hepatotoxic and tumor promoting microcystins and neurotoxic alkaloids. This article reviews both physico-chemical and biological methods for detection of the cyanotoxins. Short overview of the most often reported modifications of HPLC and GC methods for the cyanobacterial toxins is provided. Since chemical analyses do not describe the real toxicity of the sample, biological methods are used as an addition. Various types of biotests for assessment of cyanobacterial toxicity, as well as enzyme inhibition tests and ELISAs methods allowing quantification of the cyanotoxins are also reviewed.

Introduction

In the middle of twentieth century, eutrophication was recognised as a pollution problem in many western European and North American countries (ROHDE, 1969). Eutrophication is the process of biological production enhancement, caused by increasing levels of nutrients (especially nitrogen and phosphorus) loaded into the environment. In eutrophic waters, fast growth of primary producers can develop into floating biomass called water blooms, or can concentrate in the surface level and form scums. Cyanobacteria (cyanophyceae, blue-green algae) often form or dominate water blooms and scums worldwide. Several adverse effects linked with occurrence of cyanobacterial water blooms have been reported. Among them deaths of fish due to depletion of dissolved oxygen (JACOBSEN, 1994), impairment of recreational use of water bodies due to production of scums and unpleasant odour substances (PILOTTO et al., 1997), and also production of toxic compounds. Cyanobacterial toxins (the cyanotoxins) have become important for human and
animal health (FALCONER, 1998). Up to now many metabolites with various biological activities have been identified and isolated from cyanobacteria (CARMICHAEL, 1997). Neurotoxic alkaloids (anatoxin-a, anatoxin-a(S), saxitoxins; CODD & POON, 1988) and cyclic hepatotoxic peptides (microcystins and nodularins; DAWSON, 1998) are the most often reported cyanotoxins. The health significance of the toxic cyanobacteria results from many reports on injuries and deaths of animals all over the world (LAWTON & CODD, 1991; CODD et al., 1992; PREISING et al., 1996; THOMAS et al., 1998). Moreover, reports on toxicity and tumor promotion in experimental animals (JACKSON et al., 1984; FALCONER, 1991; CARBIS et al., 1994; FALCONER et al., 1994), as well as fatal accidents of human deaths (JOCHIMSEN et al., 1998; POURIA et al., 1998) increased the need for monitoring and rapid identification of toxic cyanobacteria in water blooms. This contribution reviews the methods for detection and quantification of cyanobacterial toxins. Both physico-chemical instrumental methods and biological methods are reviewed. Advantages and possible disadvantages of the methods are discussed.

**Physico-chemical methods for detection of the cyanotoxins**

Selection of chemical method for detection of the cyanotoxins depends on chemical structure of respective compound. Several different methodological approaches have been developed for chemical detection and quantification of cyanotoxins. High performance liquid chromatography (HPLC) and gas chromatography (GC) are now the most commonly used techniques. These methods coupled with mass spectrometry (MS) or its modification (fast atom bombardment, FAB), and/or nuclear magnetic resonance (NMR) can serve as a tool for identification and structure determination of new cyanotoxins. This section provides an overview of the physico-chemical methods used for detection of the cyanotoxins. The greatest interest is focused on the methods for detection of known cyanobacterial hepatotoxins (microcystins) and neurotoxins (anatoxin-a).
Methods for detection of microcystins

Several analytical methods were developed for detection of peptide microcystins. However, HPLC is now used most frequently. Principally, three different types of chromatography arrangements are now used for detection with HPLC:

1. Reversed phase HPLC (RP-HPLC) is the most often used method for microcystin analyses. UV detector or UV photodiode array (PDA) detector are usually used, due to typical absorption spectra of microcystins. Several modifications of RP-HPLC conditions were published (Harada et al., 1988; 1991; Namikoshi et al., 1992). Mobile phases composed from various combinations of acetonitrile with other solvents are the most widely used (Meriluoto & Eriksson, 1988; Sivonen et al., 1990; Vasconcelos et al., 1993; Luukainen et al., 1993; 1994; Lawton et al., 1994b). Beside UV-detection, other detection systems for linked with RP-HPLC have been successfully used. Poon et al. (1993) first reported use of electrospray ionization mass spectrometry for microcystin identification. Employment of electrochemical detector following HPLC was published recently by Meriluoto et al. (1998). Other improved detection techniques by derivatization with the fluorogenic (Shimizu et al., 1995) or chemiluminescent reagents (Murata et al., 1995) were also reported.

2. Internal surface reversed-phase HPLC (ISRP-HPLC) allow injections of samples containing proteins directly to chromatographic column without previous step of protein removal as usual in RP-HPLC. ISRP-HPLC method for microcystin detection in cyanobacterial biomass was reported by Meriluoto & Eriksson (1988).

3. Ion-exchange chromatography was occasionally used for microcystin detection (Gathercole & Thiel, 1987). Since the selectivity of ion-exchange HPLC is different from RP-HPLC, link of both methods can be suitable for preparative isolation or purification of microcystins.

Among other analytical methods used for detection of microcystins thin layer chromatography (TLC) or high performance thin layer chromatography (HPTLC) should be noticed (Harada et al., 1988; Lanaras & Cook, 1994). However, TLC methods are usually limited for semipreparative purposes or check of the sample purity. Capillary zone electrophoresis (CZE) was also reported as the useful tool for detection of cyanobacterial toxins. Improved detection method based on laser
fluorescence linked with CZE was successfully used for detection of microcystins and cyanobacterial neurotoxin saxitoxin (WRIGHT et al., 1989).

Methods for detection of neurotoxins

Several instrumental methods for detection of neurotoxins in cyanobacterial biomass or produced into the raw waters have been proposed. Although several types of structurally different cyanobacterial neurotoxins are known, most of the work have been done with anatoxin-a, the oldest known cyanobacterial neurotoxin.

Gas chromatography (GC) coupled with various detection systems is used for detection of anatoxin-a. The most often reported is electrochemical detection (GC-ECD; SMITH & LEWIS, 1987; STEVENS & KRIEGER, 1988; HAUGEN, 1994), or link GC with mass spectrometry (GC-MS; HIMBERG, 1989; BRUNO et al., 1994). Another modification employing electrospay ionization and MS (ESI/MS) for anatoxin-a and saxitoxin determinations was also reported (POON et al., 1993).

Although GC is the most widely used chromatographic method for cyanobacterial neurotoxins, several modifications of HPLC have also been proposed (ASYTACHAN & ARCHER, 1981; WONG & HINDIN, 1982). Recent modifications of HPLC methods for anatoxin-a use both simple UV (POWEll, 1997) or fluorescence detection after pre-column derivatization (JAMES et al., 1997; 1998). Thin layer chromatography (TLC) was also proposed for rapid semiquantitative analysis of cyanobacterial neurotoxins (OJANPERA et al., 1991).

Methods for determination of new cyanobacterial metabolites

Reports on new bioactive metabolites produced by cyanobacteria are published every year (e.g. MARQUEZ et al., 1998; NAMIKOSHI et al., 1998; PRINSEP et al., 1998). Following preparative isolations of new cyanotoxins, molecular weight and structures are determined. To assign molecular weight, fast atom bombardment MS (FAB-MS) is usually used in the first step. Subsequently, high resolution FAB-MS has been successfully used for obtaining possible formulas. In the third step, nuclear magnetic resonance (NMR) is used for the structure determination. Various modifications based on $^{13}$C or $^1$H 2D-NMR were used for the structure determination of new cyanotoxins. Final step in determination of the structure of peptide products is the aminoacid stereochemistry.
Only limited number of examples and recent references can be mentioned here. SHIN et al. (1997a; 1997b; 1997c) used modified sequence of above described methods for detection of structures of novel enzyme inhibitors from *Anabaena circinalis* and *Oscillatoria agardhii*. Other reports on determination of the structure of new bioactive compounds from the strains of genera *Microcystis* or *Nostoc* have been published by MATSUDA et al. (1996), MURAKAMI et al. (1997a; 1997b), OKINO et al. (1993; 1997), and ISHIDA et al. (1997; 1998). The most recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) seem to be promising tool for rapid identification of the cyanotoxin structures. “Finger print” spectra obtained from MALDI-TOF MS are toxin specific and they can successfully be used for rapid assessment and structural analyses of environmental mixtures of cyanotoxins (ERHARD et al., 1997).

**Biological methods for detection of the cyanotoxins**

Beside chemical analyses of cyanobacterial toxins, wide range of biological laboratory methods has been developed for detection and identification of the cyanotoxins in cyanobacterial biomass and raw waters. Since analytical analysis cannot describe the "real toxic effect" of complex mixture of chemicals, relatively simple and low cost methods provide necessary additional information on overall toxicity, and allow rapid evaluation of the potential hazard for humans, animals or ecosystems.

Specific biological screening method can be selected, depending on the the type of information required. Additionally, facilities and expertise availability are another criteria for selection of appropriate biotest. However, it is important to notice that there cannot be one single method providing adequate information on toxicity of all types of cyanotoxins: rodent bioassay can serve as a method for risk assessment of cyanotoxins for animals and man; microbial or crustacean assays can be adopted in ecosystem health policy; tests for specific toxicity (e.g. enzyme inhibitions) can serve as an alternative for analytical methods, etc.

Current knowledge on biological methods for detection of cyanobacterial toxins is reviewed in this section. Advantages, disadvantages and recommendations for use in various fields of risk assessment are discussed.
Methods for toxicity assessment

Many biological methods have been developed for testing toxicity of cyanobacteria. A lot of them search for specific bioactivity of known toxins - hepatotoxicity, neurotoxicity, cytotoxicity or enzyme inhibiting activity. On the other hand, for many years the mouse bioassay alone has been used to determine overall toxicity of cyanobacterial water blooms. Considerable research efforts have been made to find suitable alternative methods for routine monitoring assay of cyanotoxins and many novel and also sensitive biological methods have become available in recent years.

Bacterial bioassays

Several reports on use of bacteria as a model for cyanobacterial toxicity testing have been reported. Microtox® bioluminescence assay is one of the most widely studied bacterial toxicity tests. Method is based on measuring of the light intensity emitted by the test bacteria (*Photobacterium phosphoreum*). First reports suggested that Microtox should be used as a sensitive test for detection of microcystins in cyanobacterial samples (LAWTON et al., 1990). However, recent works showed, that the response of *Photobacterium* to cyanobacterial samples is non-specific and does not correlate with the content of known cyanotoxins (CAMPBELL et al., 1994; LAWTON et al., 1994a; VEZIE et al., 1996). Similar results were obtained with genetically engineered luminescent *Pseudomonas putida* (LAHTI et al., 1995). Although toxic effects were observed, no correlation with the content of hepatotoxins or neurotoxins was determined.

Another bacterial bioassay, based on changes in pigment formation of *Serratia marcescens* was discussed for cyanobacterial toxicity assessment (DIERSTEIN et al., 1989). This bioassay was proposed to be useful for analysis of hepatotoxins (nodularins and microcystins). However, detailed comparisons showed only poor correlation between content of the cyanotoxins and toxic response (LAWTON et al., 1994a).

Bacterial toxicity kits based on measurement of de novo biosynthesis of β-galactosidase in *Escherichia coli* mutants (Toxi-Chromotest™, Toxi-Chromopad™) were used for screening cyanobacterial toxicity in our laboratory (MARŠÁLEK &
Detailed study of different fractions of cyanobacterial biomass showed high molecular fraction, containing proteins and pigments being the most toxic. Our results again indicate only poor correlation between the response and the content of known cyanotoxins.

Although bacterial bioassays were shown to be not sensitive to known hepatotoxins, they should be recommended for the screenings of ecotoxicological risks of other cyanobacterial toxins. Moreover, cost-effectiveness and easy laboratory performance must be pointed out among advantages of bacterial bioassays.

**Invertebrate bioassays**

Number of invertebrate bioassays have been studied in recent years for assessment of cyanobacterial toxicity.

Brine shrimp (*Artemia salina*) bioassay is the most often reported crustacean test. Several studies showed good correlations between crustacean lethality and the content of microcystins (Campbell *et al*., 1994; Lahti *et al*., 1995; Kiviranta and Abdel-Hameed, 1994; Maršálek *et al*., 1995). The shrimp bioassay became very popular, since no culture maintenance or special equipment is required and the test is commercially available as standardized test kit. On the other hand, ecological limitation of marine crustacean *Artemia salina* for testing of freshwater samples must be noted. Moreover, no evaluation of the test for screening of neurotoxins and other cyanotoxins was reported.

Another crustacean bioassay using *Daphnia pulex* was shown to detect successfully microcystins in cyanobacterial samples (Arnold, 1978; De Mott *et al*., 1991). However, maintenance of *Daphnia* cultures is labour intensive and problems with interlaboratory standardization of the bioassay are well known.

Thamnotox-kit A, commercially available toxicity test kit employing freshwater crustacean *Thamnocephalus platyurus* showed good sensitivity to a number of cyanotoxins (Kozma, 1997). Moreover, much better correlation with the content of microcystin-LR and sensitivity was observed when compared to *Artemia salina* bioassay (Maršálek *et al*., 1995).

Smaller and less developed species of zooplankton seem to be less sensitive to cyanotoxins than larger invertebrates. Copepod *Bosmina longirostris* toxicity test
showed resistance to the toxic cyanobacterial biomass (Fulton, 1988). Acute response of rotifer *Brachionus calyciflorus* to microcystin-LR was very low (Maršálek & Bláha, 1998). However, prolonged exposure of rotifers to toxic cyanobacteria was shown to cause significant changes in the reproduction success (Snell, 1980).

On the other hand, bioassays employing protozoans were shown to be sensitive to cyanobacterial toxins. Good response of *Paramecium caudatum* (Ransom et al., 1978), *Tetrahymena pyriformis* (Slabert & Morgan, 1981) and *Spirostomum ambiguum* (Maršálek et al., 1995) have been reported.

Mosquito (*Aedes aegypti*) adults and larvae have been studied as a model for testing effects of cyanobacterial samples to insects (Turell & Middlebrook, 1988; Kiviranta, 1992; 1993). Good sensitivity for hepatotoxic cyanotoxins was found. However, the test is not used very often due to difficulties in handling the test organism. Successful use of *Drosophila melanogaster* for detection of cyanobacterial neurotoxins has been reported by Swoboda et al. (1994). Although *Drosophila* flies are easy to maintain in laboratory, they are not widely used for toxicity testing of cyanobacterial samples.

Another two insect bioassays were recommended for detection of cyanobacterial neurotoxins. Adult house flies (*Musca domestica*) microinjected with the toxic samples showed good correlation with mouse bioassay (Ross et al., 1985). However, handling house flies and performance of the test is relatively difficult.

Most recently, newly developed test with the desert locust (*Schistocerca gregaria*) was proposed as a useful model for detection of saxitoxins in cyanobacterial samples (Hiripi et al., 1998; McElhiney et al., 1998).

**Vertebrate bioassays**

Results of the toxicity tests with vertebrate laboratory animals provide the most complex toxicological information. However, only limited information on the mode of toxic action is usually available. Several experimental models have been exploited for testing cyanobacterial toxicity. Mouse bioassay is the most often reported test allowing extrapolation of the toxicity results to domestic animals and man. Additionally, fish bioassays are of importance for ecological risk assessment of cyanobacterial water blooms.
Mouse bioassay

Rodent acute toxicity test is commonly used in many countries for testing of cyanobacterial toxicity. Albino mice are commonly used for routine testing of cyanotoxins. Toxicity of intraperitoneal (i.p.) injected crude cyanobacterial extract is assessed. Treated animals should be observed for 5, 10, 30 minutes and 2 hours. If characteristic symptoms appear within this period, acute intoxication is proved. Death in several first minutes after i.p. injection indicates presence of neurotoxic metabolites. Vertigo, muscle cramps and death by suffocation are the most common neurotoxic symptoms. No apparent damage of organs is observed. On the other hand, first symptoms of acute hepatotoxicity are represented by strained, quick and confused movement of animals. Bristling hair, fast and short breathing characterize the second period of hepatotoxic poisoning. Finally, letargy, bulging eyes and breath slowing down are usually followed by death within an hours. Post-mortem dissection shows swollen liver with accumulated blood enlarging the liver volume by 60-70%, other organs are apparently pale (FALCONER et al., 1981; JACKSON et al., 1984).

Toxicity of cyanobacterial biomass to mouse is expressed as 50% lethal dose (LD50) in mg of dry biomass per kg of body weight. LD50 values can range from >1000 (non-toxic); 500-1000 (low toxicity); 100-500 (medium toxicity); LD50 less than 100 mg/kg indicates high toxicity (LAWTON et al., 1994a). Testing of purified toxins carried out simultaneously with cyanobacterial samples, allows description of the toxicity in “toxic equivalents”. LD50 of pure microcystin-LR in the mouse bioassay ranges from 50 to 100 µg/kg body weight.

Mouse i.p. acute toxicity bioassay is fast method for screening overall toxicity of cyanobacterial biomass (results are obtained in minutes or hours). Moreover, possible human injuries can be successfully predicted from rodent model. On the other hand, limitation of ethic claims from using laboratory animals, and relatively high costs should not be overlooked. Additionally, in the samples with more than just one type of cyanotoxins (e.g. neurotoxins together with hepatotoxins), the more rapid acting toxin may mask effects of other compounds.
Fish bioassays

Although fish as an important component of water ecosystem should be a good representant for assessment of ecological risk of toxic cyanobacteria, their use in toxicity testing have an important limitation. Model species usually used in routine toxicity testing (e.g. rainbow trout) belong among predator fish, which do not graze on phytoplankton. However, since other routes of intoxications cannot be neglected, several methodological approaches have been reported for purposes of testing toxicity of cyanobacteria.

Several assays are based on lethality observation, or on measurement of physiological changes after injection of freezdried biomass or pure toxins. LD50 of microcystin-LR to rainbow trout following i.p. injection was 550 µg/kg body weight (TENCALLA et al., 1994). Selective damage of liver and changes in biochemical parameters were observed after exposure via dorsal aorta (BURY et al., 1997).

Another experimental design use exposures of fish to lysed cyanobacterial cells. Exposure of Eastern rainbowfish (Melanotaenia duboulayi) to lysed cyanobacteria caused significant changes in respiratory system (JOHNSTON et al., 1994). Similarly, BURY et al. (1996) reported changes in cortisol, glucose and ions plasma levels at brown trout (Salmo trutta). Field observations with common carp (Cyprinus carpio; Carbis et al., 1997) or brown trout (Salmo trutta, RODGER et al., 1994) exposed to toxic water blooms, showed histopathological changes and affected several biochemical parameters.

Experimental arrangements allowing testing of cyanobacterial toxicity after oral exposure or exposure via gills were also developed. TENCALLA et al. (1994) compared different uptake routes for hepatotoxic cyanotoxins. Gastrointestinal tract was found to be the main route for microcystin. Significant liver damage was observed. Additionally, activities of fish liver protein phosphatases were studied by SAHIN et al. (1995). Oral exposure of rainbow trout to microcystins, caused strong enzyme inhibitions, although no apparent histopathological changes were observed.

Fish behavior and activity observations were also used as the sensitive endpoint for discrimination between toxic and non-toxic water blooms. Both reported phytoplanktivorous fish species - Hypophthalmichthys molitrix (BEVERIDGE et al., 1993) and Oreochromis niloticus (KESHAVANATH et al., 1994) were sensitive to low
concentrations of microcystins in the cyanobacterial biomass. Similarly, behaviour of zebrafish (*Danio rerio*) was recorded and used for assessment of long term sublethal effects of the cyanotoxins (BAGANZ et al., 1998). Significant dose relations were observed in spawning activity and success, locomotor activity and daytime motility after exposure to pure microcystin-LR.

The effects of cyanobacterial hepatotoxins and neurotoxins (both purified and in crude cyanobacterial extracts) on the fish embryos (hatching, mortality, malformalities and morphological effects) showed, that these effects cannot be attributed to cyanotoxins content alone (OBEREMM et al., 1999).

*Bioassays using cell cultures in vitro*

Methods for assessment of cyanobacterial toxicity with several types of cell cultures have been evaluated. Since microcystins and anatoxins are the most widely studied cyanotoxins, the most of the work was focused on development of methods for detection of hepatotoxicity and neurotoxicity. However, methods allowing detection of other biological activities of cyanobacterial metabolites are also discussed in this section.

Principally, two different approaches are exploited in *in vitro* toxicity testing:

1. Use of the cells which are freshly isolated from target tissue. Biological properties and regulatory pathways of the cells are only slightly changed compared to activities in intact organism. On the other hand, maintenance of the cells is limited for only few days, and the isolation of the primary cells is relatively difficult.

2. Use of permanent cell lines. The maintenance of the cell lines (usually isolated from tumor tissues) is relatively easy. However, some biological properties of the tumor cells can be significantly changed when compared to *in vivo* system.

*In vitro hepatotoxicity*

Previous studies have revealed, that active uptake of microcystins to the cells is the first limiting step of their toxicity (ERIKSSON et al., 1990b). It was shown, that the uptake of microcystins to cells is realized via multispecific transport system for bile acids (RUNNEGAR et al., 1981; ERIKSSON et al., 1990a). Thus, freshly isolated hepatocytes carrying such transport system seem to be appropriate tool for *in vitro*
hepatotoxicity assessment of cyanobacteria. On the other hand, several cell lines derived from liver carcinoma (e.g. hepatocarcinoma cell line Hep G2) were shown to lack the bile acid transport system (ERIKSSON et al., 1990a). Therefore, their use for toxicity testing of microcystins is limited.

Toxicity testing of cyanobacterial samples with freshly isolated hepatocytes has been widely investigated. First reports showed good correlation between in vitro toxicity and the mouse bioassay results (AUNE & BERG, 1986). Measurement of lactate dehydrogenase (LDH) leakage from the cells is conventionally used as the endpoint.

Many other studies on effects of microcystin in vitro used various physiological endpoints, as well as microscopical investigation of the treated cells (RUNNEGAR et al., 1981; RUNNEGAR & FALCONER, 1986; ERIKSSON et al., 1988). Flow cytometric analyses of the cell proliferation, DNA synthesis and DNA content were recently reported to be useful tool for assessment of microcystin tumor promoting activities (HUMPAGE & FALCONER, 1999). Although the flow cytometry analyses are very sensitive for detection of in vitro cytotoxicity (BLÁHA & MARŠÁLEK, 1999), expensive laboratory equipment (flow cytometer) is required.

Time and cost effective method for testing cell viability: MTT test (MOSMANN, 1983) was modified for assessment of cyanobacterial in vitro hepatotoxicity. The colorimetric method is based on measurement of the cell dehydrogenase activities; yellow tetrazolium salt (MTT) is reduced to blue formazan. The colour change is quantified with microplate spectrophotometer. Use of the MTT test for detection of microcystin effects in hepatocytes showed good correlation with the results of mouse bioassay (HEINZE, 1996). Due to low costs, MTT test seems to be promising tool for rapid assessment of in vitro toxicity of cyanobacteria.

In vitro neurotoxicity

Several cell culture methods were developed for neurotoxicity testing of cyanobacteria, depending on current knowledge of mode of neurotoxic action. Since anatoxin-a acts as a mimic of neurotransmitter actetylcholin in extracellular matrix (CARMICHAEL et al., 1979; SPIVAK et al., 1980), no sensitive in vitro assay was developed for this toxin.
Other cyanobacterial toxins acting as sodium channel blockers (so called paralytic shellfish poisons, PSPs; Ikawa et al., 1982; MAHMOOD & CARMICHAEL, 1986) can successfully be detected with \textit{in vitro} cell assay. Three different approaches have been evaluated.

Firstly, a neuroreceptor binding assay employing radio-labelled saxitoxin has been evaluated. The method is based on competitive displacement and measurement of radioactivity (DAVIO & FONTELO, 1984; DOUCETTE et al., 1994). The results showed good correlation with the mouse bioassay (CEMBELLA et al., 1995).

Secondly, a sensitive method based on measurements of single sodium channel ion fluxes was proposed for cyanobacterial neurotoxicity assessment (POMATI et al., 1998). However, the efflux of isolated channels in only single cell is measured in this patch clamp technique. Therefore the use and reproducibility of the method for \textit{in vitro} neurotoxicity testing is problematic.

Thirdly, a technique based on indirect detection of sodium channel blockers was developed (GALLACHER & BIRKBECK, 1992; JELLET et al., 1992). The method employes competition between blocking activity of toxins and model ion eflux enhancers. Neuroblastoma cells treated with model enhancer loose their viability. However, addition of the sample containing sodium channel blocker (e.g. cyanobacterial saxitoxin) stops the ion eflux, and the cell viability stays unchanged. On the basis of viability measurement (e.g. with MTT test) neurotoxic potential of the cyanobacterial sample is evaluated. This method seems to be promising for rapid \textit{in vitro} neurotoxicity screening and is now available as a commercial test kit.

\textit{Methods for screening of various bioactive compounds}

Several reports revealed, that many biological effects of cyanobacteria do not correlate with the concentrations of known cyanotoxins (BLÁHA & MARŠÁLEK, 1999). Therefore, various \textit{in vitro} cellular methods can be proposed for screening of other not known cyanobacterial toxins.

Use of fibroblast cell line as an alternative tool for screening of microcystin toxicity has been investigated by CODD et al. (1989). However, other report employing V79 hamster fibroblast cell line showed only poor correlation with mouse bioassay (LAWTON et al., 1994a).
Another assay based on agglutination of blood cells has been reported by CARMICHAEL & BENT (1981). Although this bioassay seemed to be a fast method with promising results, it was found to be a poor indicator of microcystin toxicity. However, use of such method can provide additional data on biological activities of other various cyanobacterial products.

The model for mutagenicity testing of cyanobacterial metabolites with the huma RSa cells has recently been reported (SUZUKI et al., 1998).

Another interesting biological activity of cyanobacterial metabolite has been reported recently (MORLIERE et al., 1998). Tolyporphin (TP), a porphyrin extracted from cyanobacteria, was found to be a very potent photosensitizer in both in vitro tests with EMT-6 tumor cells, and in vivo experiments with immunodeficient mice. The photokilling effectiveness of TP against the tumor cells was 5000 times higher than the activity of Photophrin II, the only photosensitizer approved for clinical trials.

The immunosuppressive effects of cyanobacterial extracts were also studied with the extracts of four cyanobacterial strains (Nodularia spumigena, Synechocystis aquatilis, Oscillatoria redekei and Microcystis aeruginosa; MUNDT et al., 1991). Antimicrobial and antiviral activities of cyanobacterial extracts have recently been reported. Antiviral activity of an aqueous extract of Microcystis aeruginosa towards the replication of influenza A virus in MDCK cells was reported by NOWOTNY et al. (1997). Similarly, antibacterial activities of methanol and aqueous extracts of various cyanobacteria were also examined by OSTENSVIK et al. (1998). The results of the agar diffusion test showed no correlation with the content of known cyanotoxins.

Biological and biochemical methods for quantification of the cyanotoxins

Previously discussed biological methods allow detection of various types of cyanobacterial toxicity. Although the response of some of the bioassays discussed above can be calibrated for purified toxins, and the toxicity of the samples could be explained as "toxic equivalents", several other sensitive biological and biochemical methods have been developed for quantification of the cyanotoxins.
Two groups of the methods will be discussed in this section:
1. Biochemical methods - methods based on knowledge of toxin action at molecular level. Methods measure enzyme inhibitions or specific biochemical changes at cellular and subcellular level.
2. Immunoassays - methods based on molecular recognition. Antibodies prepared selectively for various toxins are used in immunospecific assays.

**Biochemical assays**

Enzyme inhibitory assays were developed for microcystins and neurotoxin anatoxin-a(S).

The biochemical assay of microcystins use their inhibitory effects toward protein phosphatases. Several methodological modifications of the protein phosphatase inhibition assay have been proposed.

The most widely used modification is based on the quantitation of the radioactivity of $[^{32}\text{P}]$-phosphate released from a radiolabelled protein substrate by the activity of the protein phosphatases PP1 and PP2A (Holmes, 1991). The method was shown to be sensitive to low microcystin levels (ng). Several applications of the method for detection of microcystins in marine, freshwater and raw drinking waters have been reported (Andersen et al., 1993; Craig et al., 1993; Lambert et al., 1994). Although the method has successfully been used for microcystin toxicity screening, the adoption for routine monitoring is limited due to need of special laboratory equipment and regulations linked with the use of radioactivity.

Non-radioactive modifications of protein phosphatase inhibition assay were reported more recently. The colorimetric substrates avoiding the complications of laboratory work with radioactive materials were proposed (An & Carmichael, 1994; Ward et al., 1997). Additionally, the luminescence endpoint was also successfully used for measurement of protein phosphatase inhibition (Isobe et al., 1995).

The results of the protein phosphatase inhibition assay help to confirm the real microcystin toxicity linked with the cyanobacterial water blooms. Therefore, the non-radioactive modifications of the method should be more routinely used for screening of microcystin toxicity as an addition to HPLC analyses.
The activity of cyanobacterial neurotoxins can also be analyzed with the enzyme inhibition method. Anatoxin-a(S) was shown to be a potent inhibitor of acetylcholinesterase. Measurement of acetylcholinesterase inhibition by cyanobacterial samples has been reported by MAHMOOD & CARMICHAEL (1987) and COOK et al. (1989). The method is very sensitive, and is the only currently available alternative to the mouse bioassay. However, the presence of other acetylcholinesterase inhibitors, e.g. organophosphorous pesticides, can alter the results of the bioassay.

**Immunoassays**

Use of specific antibodies for detection of microcystins have been reported. Both radioimmunoassays (RIA) and enzyme linked immuno sorbent assays (ELISA) were successfully used. However, the routine use of RIA methods due to work with radioactivity is limited. Non-radioactive ELISA methods usually based on colorimetric measurement seem to be the most promising alternative to chemical analyses of microcystins.

First monoclonal antibodies against microcystin (microcystin-LA) were developed by KFIR et al. (1986). Sensitive sandwich ELISA based on polyclonal antibodies raised against microcystin-LR was developed later by CHU et al. (1989). The method was successfully used for detection of microcystins in environmental samples (CHU et al., 1990). Sensitivity of the method with the detection limit 1 ng/ml was sufficient for screening of microcystins in both cyanobacterial biomass and raw waters.

More recently, several other monoclonal antibodies showing better cross-reactivity with different forms of microcystins were developed (NAGATA et al., 1995; 1997). Based on these antibodies, sensitive competitive ELISA with 50 pg/ml detection limit has been developed by UENO et al. (1996) and is now commercially available.

Another approach in development of ELISA techniques for screening of microcystins was proposed by TSUTSUMI et al. (1998). They developed an ELISA technique based on anti-idiotype antibodies raised up against the anti-microcystin antibodies. The detection limit for microcystin-LR was 100 pg/ml.

Development of immunoassays for cyanobacterial neurotoxins (saxitoxins) have also been reported (CEMBELLA et al., 1995). Since the mouse assay is the only
widely used bioassay for screening of saxitoxins, development of alternative method for routine monitoring is in active progress. Although both polyclonal and monoclonal antibodies have been raised against saxitoxin, no good cross-reactivity with wide spectrum of the known saxitoxin variants was observed. In spite of the fact, the immunoassays are still being developed (KRALOVEC et al., 1996) and they should serve as a suitable routine monitoring system for saxitoxins in the future.

Conclusions

Based on the review of detection methods for cyanobacterial toxins following conclusions and recommendations are proposed:

1. Chromatographic methods (HPLC, GC) are currently the most widely used for detection of known cyanotoxins. However, interlaboratory round tests should be realized and the methods should be more standardized.

2. Although instrumental analytical methods allow sensitive detection of known cyanotoxins, bioassays should be used as a preliminary/additional step (screening or the verification of biological activity). The results of both chemical and biological tests provide complex information on real cyanobacterial toxicity potential.

3. ELISA seem to be promising and relatively inexpensive alternative to HPLC method of known cyanotoxins. ELISA for microcystins could be recommended for monitoring of drinking waters.

4. Since several reports indicate that many still not recognized toxins and bioactive compounds are produced by cyanobacteria, in vitro methods for screening of such activities should be developed and widely used.

References


