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METHODS FOR CYANOTOXINS DETECTION

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Abstract

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Global occurrence and concern about microcystin contamination, the potential consequences of exposure to cyanobacterial toxins in recreational and drinking waters promoted the development of numerous methods to detect the toxin and their producers as well as identification and quantification of toxins. In current study we overview numerous methods that have been developed for the cyanotoxin analysis. We discuss advantages and shortages of their applications to solve different questions.

Keywords: Cyanobacteria, harmful algal blooms, human health risk

INTRODUCTION

The ongoing anthropogenic eutrophication and global climate warming rapidly change environment and promote intense development of potentially toxic cyanobacteria in inland waters that are important as a source of drinking water and of human recreation. Global occurrence of toxic cyanobacteria together with concern about contamination and potential consequences of exposure to cyanobacterial toxins in recreational and drinking waters promoted the development of numerous methods to detect, identify and quantify the toxins and their producers (Kurmayer & Christiansen, 2009; Lawton et al., 2010). It is impossible to distinguish toxic cyanobacteria strains from non-toxic under a light microscope (Ouellette & Wilhelm, 2003; Joung et al., 2011). Thus, several efficient, sensitive chemical analytical methods and bioassays have been developed to determine the concentrations of cyanotoxins in environmental samples and in cultures (Oehrle et al., 2010). Some of the methods are currently applied for the routine monitoring; the others are mainly used as scientific tool for the harmful algae investigations. The aim of this study was to overview the various assessment methods developed for cyanotoxin analysis and to discuss the advances and shortage of their applications.

METHOD REVIEW

Analytical methods

One of the most sensitive methods for detection of cyanotoxins is **high performance liquid chromatography** (HPLC) that allows distinguishing between toxins types and variants (Poon et al., 2001; Spoof et al., 2001). The method is widespread as quantitative and qualitative tool, however, it is expensive, time consuming, require sample concentration and purification (Tsutsumi et al., 2000; Nicholson & Burch, 2001). There are few available certified standards for cyanotoxins variants also. To determine cyanobacterial toxins in animal tissues the reversed phase high performance liquid chromatographic method (HPLC, LC) coupled with UV, photodiode array (PDA) and/or mass spectrometer (MS)

detectors was applied (Triantis et al., 2010; Spoof et al. 2010). The method allows more precise identification based on retention time and mass-to-charge. However, the method requires expensive equipment, highly qualified staff. Other shortcomings are relatively long analysis time due to the need of samples concentration or clean and a relatively small number of available commercially cyanotoxins standards (Triantis et al., 2010). Therefore, the method is not optimal for rapid detection of low toxin concentrations and routine sample analysis. HPLC-MS/MS analysis with direct aqueous injection without sample clean up can be useful for rapid detection of different cyanobacterial toxins and may be applied for monitoring of cyanobacterial toxins (Hedman et al., 2008). Recently, a solid-phase extraction (SPE) – liquid chromatography (LC) – mass spectrometry (MS) method was developed to concentrate and detect nine cyanotoxins simultaneously, including six microcystins congeners, and was found to be appropriate for cyanotoxins monitoring (Yen et al., 2011). The matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) method allows the identification of known microcystin variants. The method is less time-consuming due to exclusion of extraction or purification processes and requires lower quantities (microgram vs. milligram) of the sample to compare with HPLC or bioassays (Fastner et al., 2001; Welker et al., 2002).

Gas chromatographic (GC) method is based on oxidation of microcystins, which splits the Adda ((2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-pheny1deca-4, 6-dienoic acid) side chain to produce 3-methoxy-2-methyl-4-phenylbutyric acid (MMPB), which is then determined, either by GC, GC/MS or by HPLC/fluorescence detection (Sano et al., 1992; Kaya & Sano, 1998). However, individual toxins are not determined. This method cannot be used to monitor water samples according to proposed guidelines, because it is not possible to get microcystin-LR toxicity equivalents as a result. On the other hand, Kaya & Sano (1998) found detection limit of this approach to be about 0.4 ng of total microcystin concentration expressed in microcystin-LR, but detection limit depends on the water concentration.

Capillary electrophoresis (CE) is used for the separation and quantification of the biological mixtures including cyanotoxins, however, it has lower sensitivity compared with HPLC (Nicholson & Burch, 2001; Vasas et al., 2004). CE separates positively/negatively charged and neutral components differently from HPLC and GC separating components based on polarity or size. CE is not sufficiently robust for use in a routine analytical laboratory or water bodies monitoring. Method sensitivity has been increased during CE separation of microcystins derivatized with a highly fluorescent dienophile and detection using a laser-induced fluorescent detector. Quantitative results of toxin are possible to obtain, but the method should be considered as a screening procedure until more development is carried out. Vasas et al. (2004) indicated that CE application for complex matrices analyses should be combined with other analytical methods like micellar electrokinetic chromatography on necessity.

Using **thin layer chromatography** (TLC), microcystins can be identified in a manner analogous to PDA detection in HPLC based on their characteristic of UV spectra (Nicholson & Burch, 2001). With appropriate detection systems, UV spectra of the separated components can be recorded. Different TLC procedures for the separation of microcystins have been reported by Poon et al. (1987), AlLayl et al. (1988), Ojanpera et al. (1995) and Pelander et al. (1996, 1998). TLC can give quantitative results of toxin, but this approach should be considered as a screening procedure first until more development is carried out.

Bio-analytical methods

Enzyme-linked immunosorbent (ELISA) and protein phosphatase inhibition assays (PPIA) are widely used for screening and toxicity evaluation in many laboratories (Dörr et al., 2010). ELISA is based on monoclonal or polyclonal antibody actions against cyanotoxin structure (Metcalf et al., 2000; Zeck et al., 2001; Yu et al., 2002; Figueiredo et al., 2004). The method is rapid, relatively cheap, requires minor equipment and personal training. ELISA allows within minutes to few hours effectively detect very low concentrations (ng/L) of cyanotoxins in water samples, organisms and tissues (Lawton & Edwards, 2008; Sivonen, 2008). However, the method evaluates only total value of cyanotoxin in a sample, but does not identify individual isoforms as well as does not assess the toxicity (Lawton et al., 2010). An

& Carmichael (1994) found that some toxic microcystins were poorly or not detected. The evaluation of cross-reactivity of a number of microcystins using ELISA showed a poor correlation between reactivity and acute toxicity that indicate possible underestimation of some microcystins variants concentration (Triantis et al., 2010). Because of high cross reactivity leading to false positives, ELISA can be used only as a semi-quantitative screening tool (Nicholson & Burch, 2001). In addition, ELISA test kits manufactured by different companies can give different results (Metcalf et al., 2002). Recently, the production of robust and sensitive recombinant antibodies has facilitated the development of a lateral flow immunoassay (ImmunoStrip), which can rapidly detect microcystins and nodularins from water and cells in the field with minimal equipment or processing (Lawton et al., 2010).

Toxic effect evaluation of microcystins and nodularin using **PPIA** assay is based on specific inhibition of the serine and threonine phosphatase enzymes (type 1 (PP-1) and 2A (PP-2A) protein phosphatases) responsible for the dephosphorylation of intracellular phosphoproteins (Figueiredo et al., 2004; Msagati et al., 2006). Two variations of PPIA – colorimetric and fluorescent assays – have been developed. Radiolabelled PPIA has been applied in water monitoring with a detection limit less than 0.1 μg/L (Lambert et al., 1994; Xu et al., 2000). This sensitive method suffers from reasonably complicated preparation of labelled proteins due to short half-life of ^{32}P isotope (Nicholson & Burch, 2001). The drawbacks of the method are related with costliness of radioactive ATP and enzymes used, commercial unavailability of ^{32}P isotope and strict regulations for the laboratories working with radioactive substances. The colorimetric PPIA assay is based only on inhibition of PP1. This screening method is rapid, easy and sensitive requiring less equipment and cheaper compared to ELISA or radiolabelled PPIA assays (Nicholson & Burch, 2001). The assay correlates positively with HPLC and detects micocystins LR with limits below the World Health Organization guideline. PPIA assay can be applied as a screening method, although it is not yet available as a kit and needs a solution preparation (Triantis et al., 2010). Also, the method can't provide enough accurate quantitative results, because some microcystins variants do not react with protein phosphatase enzymes to a similar extent (An & Carmichael, 1994; Heresztyn & Nicholson, 2001). It was also shown that PPIA overestimate the toxin concentration in a sample (Wirsing et al., 1999; Metcalf et al., 2001; Bouaícha et al., 2002).

Molecular methods

Cyanotoxins can be monitored indirectly determining the toxin producers in the water body (toxic genotypes of cyanobacteria). A recent approach for the detection of microorganisms in natural environments based on molecular methods was proposed for rapid determination whether a cyanobacterial bloom or a determined species are potentially toxic as well as to quantify toxic cyanobacteria by designing primers based on *mcy* genes (Rudi et al., 1998; Tillett et al., 2001; Pan et al., 2002). Due to the high sensitivity of PCR-based methods, toxic genotypes in water may be detected for the long time before the occurrence of a cyanobacterial bloom with detectable toxin concentrations. Thus, the risk of toxic bloom formation could be identified early in the growth season and could be adopted in parallel with routine microscopic inspection of phytoplankton. Genetic methods are able to indicate the potential of toxin synthesis. This early warning could result in more efficient surveillance with monitoring effort being focused on those water bodies that have been found to have both toxin-producing genotypes and a high risk of cyanobacterial bloom formation.

Quantitative real-time PCR assays were developed in order to directly quantify the toxin genes in a given volume of water (Kurmayer & Kutzenberger, 2003; Vaitomaa et al., 2003; Rinta-Kanto et al., 2005; Koskenniemi et al., 2007). The real-time PCR technique is the only quantitative technique available. The Taq nuclease assay (TNA) has been introduced to control for uncertainties in quantifying toxic genotypes. Estimation of genotype proportions is based on the semi-logarithmic calibration curves, so there are some limitations for the accuracy of this technique. Scorzetti and co-authors (2009) developed a rapid accurate and simultaneous qualitative molecular technique, which can provide detection of multiple species within the harmful algae bloom community. It was designed for 14 species-specific probes and 4 sets of specific primers. Multiple-simultaneous detection was achieved with a bead ar-

ray method using a flow cytometer and color-coded microspheres, which are conjugated to the developed probes. Following a parallel double PCR amplification, which employed universal primers in a singleplex reaction and a set of species-specific primers in multiplex detection, was performed in a cost-effective and highly specific analysis. This multi-format required less than 4 h to complete sample collection and up to 100 different species can be identified simultaneously in a single sample.

Biological assays

Bioassays based on bacteria (*Aeromonas hydrophila*, *Bacillus cereus*, *B. subtilis*), plants (*Spirodela oligorrhiza, Solanum tuberosum, Sinapis alba, Lemna minor*), crustaceans (*Daphnia* spp., *Artemia salina*, larvae of *Thamnocephalus platyurus*), insects (*Locusta migratoria*), fish embryo tests (*Danio rerio*) have been shown to be sensitive to microcystins and may be used to assess toxicity of cyanobacteria-produced toxins (Nicholson & Burch, 2001; Figueiredo et al., 2004; Kováts et al., 2011). According to Nicholson & Burch (2001), mammalian assays like mouse (sometimes rat or swine) bioassays are usually used to distinguish between different types of hepatotoxins/neurotoxins and for determination of LD_{50} values. Bioassays are an important tool for assessing the toxicity level of the unknown cyanotoxins. By now these bioassays do not give the quantitative results, can't distinguish between microcystin variants and have low sensitivity, thus it is problematic to apply them for routine monitoring of water samples. The adopted *in vitro* studies for toxicity assessment using rat hepatocyte bioassay are more ethical to compare with *in vivo* mammal bioassays and show a strong correlation with the HPLC analysis (Heinze et al., 2001; Zegura et al., 2003). Hisem et al. (2011) noted that *in vitro* mammal cells are better than *Artemia salina* toxicity test to assess health risk for humans, because cyanobacterial metabolites targets various basal metabolic pathways in the eukaryotic cell rather than being a specific mechanism against a complex multi-cellular organism.

So, for the determination of cyanotoxins in water, a variety of methods have been developed in the last decade, including enzyme-linked immunosorbent assays, high performance liquid chromatography (HPLC) with an ultraviolet detector (UV), mass spectrometry (MS) or molecular methods (Yen et al., 2011). However, the majority of protocols for cyanotoxin detection are relatively time consuming, expensive and require high laboratory expertise (Lawton et al., 2010). Thus, it is of primary importance to choose the reliable screening method for cyanotoxins that may differ from country to country because of the financial strengths and the magnitude of concern on environmental problems.

As an example could be a system of analytical processes (ELISA, PPIA, HPLC/PDA, LC/MS) developed by Triantis and co-authors (2010) in order to serve as a cost-effective scheme for the monitoring of cyanobacterial toxins (microcystin-LR, -RR, -YR, -LA and nodularins) on a quantitative basis in surface and drinking waters. Being sufficiently specific and sensitive, enzyme-linked immunosorbent assay was validated as primary quantitative screening tools with detection limits of 0.1 mg/L. To serve as a quantitative screening method for the detection of the toxic activity associated with hepatotoxins, the PPIA method was applied in microplate format with detection limit of >0.2 mg/L of MC-LR equivalents. Fast HPLC/PDA method has been developed for the determination of microcystins by using a short C18 column (50 mm) with 1.8 mm particle size. This method allows achieving 10-fold reduction of sample run time (less than 3 min) and sufficient separation of microcystins. Finally, the analytical system includes an LC/MS/MS method developed for five target compounds determination, which allows to evaluate extremely low limits of detection (<0.02 mg/L).

CONCLUSIONS

Most analytical methods are time consuming, expensive, require high quality laboratory expertise and are mainly used to solve scientific problems dealing with quantitative and/or qualitative results. In our opinion, ELISA, ImmunoStrip, colorimetric PPIA assay or PCR-based methods are applicable to routine monitoring. ELISA and colorimetric PPIA assay has low equipment requirements and allow rapid, easy, effective and sensitive detection of total microcystin amount, but individual microcystins are not identified and toxicity is not assessed. ImmunoStrip assay rapidly detects microcystins and nodularins from wa-

ter and cells with minimum equipment or processing. Molecular methods have a potential to detect toxic genotypes in the water long time before the occurrence of a cyanobacterial bloom and detectable toxin concentrations. Quantitative real-time PCR assays directly quantify the toxin genes in a given volume of the water and are also helpful for the bloom toxicity determination. Such an approach could be adopted in parallel with routine microscopic inspection of phytoplankton. Bioassays are still an important tool for assessing the toxicity level of the known cyanotoxins or the presence of additional unknown toxic substances.

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CIANOTOKSINŲ NUSTATYMO METODAI

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Santrauka

Vandens telkinių, naudojamų geriamo vandens tiekimui ar rekreacijai, užteršimas melsvabakterių sintetinamais toksinais ir cianotoksinų kaupimasis gyvuose organizmuose šiuo metu yra ypač aktuali problema visame pasaulyje. Kylantis didelis susirūpinimas paskatino toksinių melsvabakterių nustatymo ir jų sintetinamų cianotoksinų kokybės, kiekio ir toksiškumo įvertinimo metodų kūrimą. Šioje apžvalgoje aptariami sukurti cianotoksinų analizės metodai, atskleidžiami šių metodų privalumai ir trūkumai, jų taikymas sprendžiant skirtingus probleminius klausimus, susijusius su toksinių žydėjimų įvertinimu.