Chapter 24: Emerging high throughput analyses of cyanobacterial toxins and toxic cyanobacteria

Kaarina Sivonen

Department of Applied Chemistry and Microbiology, P.O.Box 56, Viikki Biocenter, FI–00014 Helsinki University, Finland, e–mail: kaarina.sivonen@helsinki.fi

Introduction

Toxic cyanobacterial mass occurrences (blooms) are commonly found in fresh, brackish, and marine waters (Sivonen and Jones 1999). Cyanobacteria growing in benthic environments have also been shown to contain toxins (Sivonen and Jones 1999; Edwards et al. 1992; Mez et al. 1997; Surakka et al. 2005). The most common cyanobacterial toxins (cyanotoxins) are hepatotoxins (microcystins and nodularins), neurotoxins [anatoxin–a, anatoxin–a(S) and saxitoxins], cytotoxins (cylindrospermopsins), and dermatotoxins (aplysiatoxin and debromoaplysiatoxins) (Sivonen and Jones 1999). Microcystins in freshwaters are most frequently produced by *Microcystis*, *Planktothrix* (formerly *Oscillatoria*) and *Anabaena* (Sivonen and Jones 1999). Microcystin production has been also proven for *Nostoc* (isolates from water and terrestrial environment; Sivonen et al. 1992; Oksanen et al. 2004), terrestrial isolates of *Hapalosiphon* (Prinsep et al. 1992) and *Phormidium* (Izaguirre and Neilan 2004). In brackish waters such as the Baltic Sea or saline lakes and estuaries in Australia and New Zealand, nodularin producing cyanobacterium, *Nodularia spumigena,* frequently occur (Rinehart et al. 1988; Sivonen et al. 1989; Heresztyn and Nicholson 1997). Neurotoxins are commonly produced by *Anabaena* and less frequently by *Aphanizomenon, Lyngbya and Oscillatoria* (Sivonen and Jones 1999). *Cylindrospermum raciborskii*, *Aphanizomenon, Umezakia, Anabaena* and *Radhidiopsis* produce cylindrospermopsins (Fergusson and Saint 2003), whereas *Lyngbya, Schizothrix* and *Oscillatoria* are primarily implicated as main producers of dermatotoxins (Sivonen and Jones 1999;

see also current listings of toxins and toxin producers in this volume; e.g., Humpage). Cyanobacterial toxins have caused hundreds of animal poisoning cases worldwide (Ressom et al. 1994) and are threat to human health (Kuiper–Goodman et al. 1999). It has become evident that efficient methods to detect cyanobacterial toxins as well as toxic cyanobacteria are needed. This paper will evaluate the potential of the current and emerging high throughput methods for analysis of cyanobacterial toxins and toxin producing organisms.

Detection of toxins

To protect water users from poisoning and exposure to the toxins, it is important to know the identity and quantity of the toxins. Such cases include drinking water, dietary supplements, important areas for recreation, animal–poisoning cases, etc. Several papers in the current issue deal with toxin analyses in detail (Meriluoto and Spoof 2006; Lawton 2006) thus, only the potential for developing high throughput methods are considered here.

The structures of the most wide spread cyanotoxins are known and that has made possible the development of high throughput analysis methods such as enzyme–linked immunosorbent assay (ELISA), protein phosphatase inhibition assay (PPIA), high–performance liquid chromatography (HPLC) and liquid chromatography – mass spectrometry (LC/MS). ELISA is available for cyanobacterial hepatotoxins and saxitoxins whereas, PPIA is used to detect microcystins and nodularins. Both methods have been evaluated to be sensitive and fast screening methods (Harada et al. 1999; Rapala et al. 2002; Hilborn et al. 2005). Both of these analyses can be carried out in multi–well plate formats. The ELISA analyses can be fully automated by using new robotic technologies. The PPIA analysis involves the use of enzyme which readily inactivates without precautions. Both of these methods give total toxin concentrations but do not differentiate the compounds. Matrix–assisted laser desorption/ionisation time–of–flight mass spectrometry (MALDI–TOF) has been proven as a fast screening method for the detection of cyanobacterial peptide toxins and bioactive compounds (Erhard et al. 1997; Fastner et al. 2001; Welker et al. 2004).

To identify individual toxins, separation is needed and identification methods such as HPLC combined with UV, FT or mass detection are required. There are good applications for many toxins and they can be accurately determined (Harada et al. 1999; Sivonen 2000; Meriluoto et al. 2004; Meriluoto and Codd 2005). LC/MS is an excellent method to identify the individual toxins. Improvement of the instruments has also made

the method very sensitive. All chromatographic methods are currently rather fast. The analysis times are in minutes and the equipment with auto samplers can be run continuously on nights and weekends. Thus, the limiting step still is sampling and sample preparation/extraction. The availability of standards of certain toxins is still a problem. New developments in this field leading to on–line measurements and fast and easy to use kit detection systems are likely to improve the rapid detection of cyanotoxins in the future (in this monograph, see Lawton 2006, Wilhelm 2006).

Detection of cyanobacteria and genes involved in toxin production

In order to implement any rational mitigation scheme, further research is needed on the biology, ecology and proliferation of cyanobacteria producing toxins. The major toxin producers have been identified by isolating the organisms and showing their toxin production capability (Sivonen and Jones 1999 and the cases thereafter, in this monograph). This has created invaluable culture collections, which contain toxic and non–toxic planktonic cyanobacteria as well as benthic strains. Such culture collections have been valuable sources for physiological studies on toxin production, taxonomic/phylogenetic analyses of toxin producers as well as studies on the biosynthesis of toxins. We cannot identify toxin producers by microscopy since toxic and non–toxic strains of the same species are known to occur (Ohtake et al. 1989; Vezie et al. 1998). The gene clusters involved in microcystin and nodularin production are known. Microcystins (nodularins) are produced non–ribosomally by the multi–enzyme complex consisting of polyketide synthases and peptide synthetases, which was verified by Dittmann et al. (1997) by gene knockout experiments. These biosynthetic gene clusters have been fully sequenced from *Microcystis* (Nishizawa et al. 1999 and 2000; Tillet et al. 2000), *Planktothrix* (Christiansen et al. 2003), Anabaena (Rouhiainen et al. 2004) and *Nodularia* (Moffitt and Neilan 2004). This has made possible to develop molecular methods to identify these toxin producers in samples. However, the biosyntheses of neurotoxins and other cyanobacterial toxins remains to be elucidated and verified by gene knockout experiments before molecular analysis can be developed with high certainty. Toxin analyses will not reveal the producers in most cases. The only exception is nodularin, which is produced thus far, only by Nodularia spumigena. Detection of toxic cyanobacteria by conventional or real–time PCR can serve as an early warning method since PCR detects the presence of toxin producers in very low concentrations.

These and microarray (DNA chip) technologies are the only methods currently available to study toxin producing cyanobacteria in situ.

Conventional PCR

Conventional PCR is a fast and cheap method to detect potentially toxin–producing strains in samples. This method requires only PCR and gel documentation facilities, which are common nowadays in laboratories. Like the methods for toxin analysis, the most time consuming task using molecular methods is the environmental sample collection, DNA isolation and purification. Conventional PCR can be used to detect microcystin/nodularin producers since the gene clusters are now known and several primers are thus, available (Table 1). These gene clusters are large (55 kb for microcystins and 47 kb for nodularin) and offer a wealth of information and possibilities for probe and primer design. In the recent years, it has become evident that cyanobacteria often produce plenty of non–ribosomal cyclic or linear peptides other than microcystins (Rouhiainen et al. 2000; Fastner et al. 2001; Welker et al. 2004) and nodularins (Fujii et al. 1997) and this should be taken into consideration in primer design.

Microcystis aeruginosa is found worldwide, the most frequently occurring microcystin–producing cyanobacterial species and its biosynthetic genes have been known for the longest time. For this reason, most studies have focused on the detection of toxic *Microcystis* strains (Table 1) but more recently extended to include other producers such as *Planktothrix* and *Anabaena*. Simultaneous occurrence of several potential microcystin producers in a lake is not uncommon (Vaitomaa et al. 2003). There are few general primers designed to detect several different producers of microcystins (Hisbergues et al. 2003; Rantala et al. 2004) and to produce sequence information to design genus specific primers (Vaitomaa et al. 2003). In the development of genus–specific primers the testing with all possible microcystin–producing cyanobacteria is required in addition to *in silico* analyses. The other option, to detect specific microcystin producers, was presented by Hisbergues et al. (2003). PCR products generated by general primers were digested with restriction enzymes and producer genera were identified based on the differences in resulting bands. The gene region chosen in that study was *mcy*A, a region missing from the *Nodularia,* thus those primers were not suitable for detection of nodularin producers. Vaitomaa et al. (2003) and Rantala et al. (2004) successfully used *mcy*E primers to get PCR product from all known microcystin and nodularin producers. This gene region is involved in the construction of Adda and activation and condensation of glutamate. Adda and glutamic acid are present in both microcystins and nodularins and are shown to be the most

important determinants (excluding the cyclic nature of the compounds) for toxicity of these compounds (Harada et al. 1990; Rinehart et al. 1994; Goldberg et al. 1995). Structural variations of Adda and glutamic acid in microcystins and nodularins are less frequent than other parts of these molecules (L– amino acids in microcystins see Sivonen and Jones 1999), which makes this gene region attractive for primer and probe design. The microcystin and nodularin synthetase sequence information has also been used to study the evolution of these genes (Rantala et al. 2004). The microcystin synthetase genes were ancient and the non–toxic strains have lost these genes during evolution. This also implies that individual strains of cyanobacteria may have retained these genes and new toxin producers among the cyanobacteria currently regarded as non–producing species may still be found.

Conventional PCR is prone to typical PCR based method problems (Wintzingerode et al. 1997) and requires some understanding of basic molecular biology. The primers are always designed and tested only with a subset of strains and thus, may not be readily applicable to new strains/species without further analysis. Environmental samples may also contain PCR inhibitors and this should be controlled especially in the case of negative results. Multiplex–PCR when developed may further improve this method and make it faster.

DNA–based detection methods are only able to identify potential toxin producers. The analysis of high number of *Microcystis* strains and also environmental samples have found only few cases where microcystin synthetase genes were detected but the organism was unable to produce microcystins possibly due to mutations in the large gene cluster (Kaebernick et al. 2001; Tillett et al. 2001; Mikalsen et al. 2003). The study of Kurmayer et al. (2004) showed that a rather high frequency of *Planktothrix* strains/filaments contained the genes but were not producing microcystins in Alpine lakes of Austria (see Table 1).

Molecular methods to detect producers of cylindrospermopsins have been developed (Schembri et al. 2001; Fergusson and Saint 2003). These detection methods are based on polyketide synthase (PKS) and peptide synthetase (PS) gene clusters present only in cylindrospermopsin producing strains, but verification of the involvement of these genes in cylindrospermopsin biosynthesis by knockout is yet to be done. Ferqusson and Saint (2003) developed multiplex PCR assay for *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum* and *Anabaena bergii* based on the PKS and PS genes which correlated well with the actual production of cylindrospermopsin.

Lack of information on the biosynthesis and biosynthetic genes of other toxins such as the cyanobacterial neurotoxins limits the use of molecular methods to detect the producers of these toxins.

Table 1. Conventional PCR analyses of microcystin and nodularin producing cyanobacteria.

Target organisms	Gene	Application and results	Reference
Several cyanobacteria common in cyanobacterial blooms	General primers for peptide synthetases and microcystin synthetase	Several strain of Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Microcystis, Nodularia, Nostoc, Planktothrix (Oscillatoria), Plektonema, Pseudanabaena and Synechococcus were tested (isolates from Australia, Europe and Japan) – generally strains producing microcystins gave positive results with microcystin synthetase primers	Neilan et al. 1999
Microcystis	NMT region of gene mcyA	Tested with 38 microcystin producing and non–producing strains of Microcystis mostly from Australia and North America	Tillett et al. 2001
Microcystis	mcyB three primer pairs	Tested with whole cells of 30 strains of Microcystis, 4 strains of Planktothrix/(Oscillatoria) 2 Anabaena and 1 Aphanizomenon as well as 200 samples from Chinese lakes. One of the primers pairs (TOX2P/TOX2M) gave consistent results with the toxins analyses.	Pan et al. 2002
Microcystis	mcyA	Some bloom samples were mcyA positive	Baker et al. 2002
Microcystis	mcyB	Applied to water samples collected from 476 channel catfish production ponds in USA, 31% gave positive results	Nonneman & Zimba 2002
Microcystis	mcyB – A1 domain	Single colonies from L. Wannsee (Berlin Germany), 75% of M. aeruginosa, 16% of M. ichtyoblabe and 0% of M. wesenbergii gave positive results. Restriction analysis of PCR products gave 7 restriction types, which differed also by nucleotide sequence. The largest colonies contained highest proportion of the microcystin producing genotypes	Kurmayer et al. 2002, 2003

Target organisms	Gene	Application and results	Reference
Anabaena, Microcystis	*mcyE*	Tested with 13 strains of *Microcystis*, 14 strains of *Anabaena*, 8 strains of *Planktothrix* and a strains of *Nostoc* (isolates mostly from Finland). Primers were genus–specific.	Vaitomaa et al. 2003
Anabaena, Microcystis, Planktothrix (Nostoc)	*mcyA* + RLFP analysis of PCR products	Primers tested with 24 strains of *Microcystis*, 8 *Anabaena*, 11 *Planktothrix*, 2 *Nostoc* and 7 *Nodularia* strains and a lake sample. Consistent results with the microcystin analyses – no product with *Nodularia*. Identification of producer by restriction profile.	Hisbergues et al. 2003
Microcystis	*mcyA* (condensation domain), *mcyB* A1 domain	A total of 244 *Microcystis* colonies from 9 different European lakes were analyzed: 75% of *M. aeruginosa* and *M. botrys*, less than 20% of *M. ichtyoblabe* and *M. viridis* contained mcy genes. *M. wesenbergii* did not contain mcy genes. The maximum proportion of mcy–PCR positive colonies was found among the largest colony group.	Via–Ordorika et al. 2004
Anabaena, Microcystis, Planktothrix, Nostoc, Nodularia	General primers *mcyE, mcyD*	Observed amplification of all tested producers of microcystins and nodularin. Microcystin synthetase genes are ancient. Nodularin biosynthetic genes evolved from microcystin synthetase genes.	Rantala et al. 2004
Planktothrix	*mcyA*	All 49 strains of red–pigmented *P. rubescens* contained mcyA and 23 strains of green–pigmented *P. agardhii* were either with or without mcyA. One strain of *P. agardhii* and 8 strains of *P. rubescens* had *mcyA* genes but were unable to produce microcystins. The population of inactive microcystin genotypes was 5% in Irrsee and 21 % in Mondsee.	Kurmayer et al. 2004

Target organisms	Gene	Application and results	Reference
Planktothrix	*mcyA, B* and *C*	Adenylation domains of 21 strains of *P. agardhii* and *P. rubescens*, were compared to produced microcystin variants. *McyAAd1* with NMT had N–methyl–dehydroalanine and strains without NMT had dehydrobutyrine as amino acid no 7. *McyBAd1* genotype had homotyrosine and other genotype arginine in position 4.	Kurmayer et al. 2005
Microcystis	*mcyA, B, C, D, E, G*	Nine *Microcystis* strains (Spain, USA, Australia, Canada, South–Africa, Morocco) and 8 field colonies (Spain) were tested. Simultaneous detection of several genes using whole cells gave expected results	Ouahid et al. 2005
Planktothrix	*mcyT, TD, A, EG, B, E, CJ, HA*	Primers were tested with 47 non–toxic and toxic *Planktothrix* strains (European isolates), primers targeted to *mcyE* gave the most reliable results	Mbedi et al. 2005
Lake samples – tested with primers designed for *Microcystis*	*mcyB* and *mcyD*	Lake Oneida, NY, USA. Mcy genotypes were present in the water from mid–June to October. 88% of samples were positive for *mcyB* and 79% for *mcyD*	Hotto et al. 2005

Quantitative real–time PCR

The real–time PCR yields quantitative information and answers the question regarding which organism is a major producer of the toxins in a sample. The real–time PCR needs specific instrument that are more expensive than the standard PCR machines and have higher running costs. However, the benefits of this technology being quantitative are obvious compared with the conventional PCR. The current real–time PCR machines are designed for high throughput analyses since they use multi–well plates or carry cells with multiple capillaries. Currently, only few applications of this method for detection of cyanotoxin producers are found in literature (Table 2). Two major methodologies, SyberGreen and TaqMan methods are available; the latter is regarded to be more specific. The optimization of real–time PCR is often difficult and time consuming. The primers should preferably target amplification of short sequences (100 –200 bp) to be efficient. The prerequisite for this method is that the biosynthetic genes are known which restricts the current use of this method to microcystin and nodularin producers.

The quantitative real time PCR method is likely to produce valuable information on the toxin producers. Since it is quantitative, it can be associated with other environmental parameters possibly giving new insights to what factors promote the selection of toxic cyanobacteria and why especially toxic blooms occur. In case where several producers are found in environmental samples, it will reveal which organism is the major producer. It may have great importance when mitigation schemes are designed – *Microcystis* and nitrogen fixing cyanobacteria such as *Anabaena* or low light adapted *Planktothrix* or *Cylindrospermopsis* require different approaches.

Table 2. Quantitative real –time PCR analyses of toxic cyanobacteria

Target/Gene	Method	Major findings	Reference
Microcystis mcyA (122pb) f (mcyB (850pb))/	TaqMan	Primers were specific to *Microcystis* and three copies of the target genes per sample was detected within 2 hours	Foulds et al. 2002
Microcystis/mcyB Intergenic spacer region of phycocyanin (PC)	TaqMan	The proportion of mcy genotypes ranged from 1 to 38% of the all *Microcystis* genotypes (determined by PC genotypes) in Lake Wannsee (Germany).	Kurmayer and Kutzenberger 2003
Anabaena and *Microcystis/mcyE* genus specific primers	SyberGreen	Both potentially toxic *Anabaena* and *Microcystis* co–occurred in both lakes studied (Finland). *Microcystis* was dominant toxin producer in L. Tuusulanjärvi in summer 1999	Vaitomaa et al. 2003
Microcystis/mcyD	TaqMan	Lake Erie: the results indicated presence of other toxin producers in the lake in addition to *Microcystis*	Rinta–Kanto et al. 2005

DNA chips

DNA chips (microarrays) offer new insights into cyanobacterial populations in natural environmental settings. This methodology is very new and has not been widely used in environmental analysis, yet. It is an attractive method for monitoring since large amount of data can be created fast and the method can be automated (the data consist of hybridization results which are analyzed by computer). Current monitoring of cyanobacteria in lakes is based on microscopic identification and cell/colony/filament counting. This microscopic method is prone to pitfalls: the identification of organism is subjective, it requires extensive training, and in addition, microscopic counting is very time consuming and tedious. DNA chip technology can identify all cyanobacteria that are present in a sample accurately and identify the toxin producers depending on the designed probes. It should be emphasized that toxic cyanobacteria cannot be identified by microscopy, thus this new technology is superior when it comes to the identification of potentially toxic cyanobacteria in samples. At present, applications of DNA chip technology for the detection of cyanobacteria are scarce. Rudi et al. (2000) developed a microarray based on 16S rRNA genes for a few groups of cyanobacteria (*Phormidium, Microcystis*, *Planktothrix*, *Anabaena, Aphanizomenon, Nostoc +Anab., Aph.),* Eubacteria (+ chloroplasts). The array was based on membrane bound probes. The environmental DNA was isolated, amplified by PCR, labeled and hybridized with the complementary probes on the membrane. The method was used to detect cyanobacteria in eight Norwegian lakes.

Another recent example of development of microarray to detect cyanobacteria comes from the European Union project MIDI–CHIP (http://www.cip.ugl.ac.be/midichip/). The technology used in the MIDI–CHIP project was so called the universal microarray method (Gerry et al. 1999). This method combines hybridization with ligation detection reaction (LDR), which improves identification. In this method, two probes, a discrimination and a common probe, are needed. The universal array method avoids the limitation of hybridization. It is very difficult to establish standard conditions for different polymorphic DNA targets to be analyzed at the same time. The method consists of oligos (ZipCodes), which have similar thermodynamic behavior and are unrelated to probes. This makes array very flexible and new probes easy to add. The prototype microarray was based on 16S rRNA genes (Castiglioni et al. 2004). Sequences from data bases (281) and strains isolated in MIDI–CHIP project were aligned and phylogenetic groupings were based on Neighbour joining three made in ARB–program (Ludwig et al. 2004). The arrays detected 19

major cyanobacteria groups and were tested with strains and environmental samples and found to be specific. These arrays have been developed further to include a few more important phylogenetic groups as well as the detection of microcystin/nodularin producing cyanobacteria (MIDI–CHIP project, unpublished results).

Genome projects

In recent years, the whole genome sequencing projects have also been extended to cyanobacteria. The first cyanobacterial genome sequenced was *Synechocystis* PCC 6803, which is a model organism to study photosynthesis, as well as stress or high light acclimation processes (Kaneko and Tabata 1997; Marin et al. 2003; Hihara et al. 2001). This strain does not produce any of the known cyanobacterial toxins. Since then, tens of cyanobacterial genomes have been fully or partly sequenced (e.g. http://www.kazusa.or.jp/cyano/; http://genome.jgi–psf.org/; http://www.moore.org/microgenome/). Most recently, toxin producing cyanobacteria genome sequencing projects have been started: two microcystin producing cyanobacteria *Microcystis aeruginosa* (Pasteur Institute) and *Anabaena* strain 90 genome (at the authors' laboratory in collaboration with Beijing Genomics Institute, China) as well as nodularin producing *Nodularia spumigena* from the Baltic Sea (Moore Foundation). Detailed annotation of these genomes is likely to reveal genes associated with toxin production and regulation. Comparison of these genomes will yield information on the metabolic versatility of these organisms and the differences between the various producers of toxins. Knowledge of genome sequences makes it possible to design expression arrays to study the gene expression of these organisms in various conditions as was carried out with *Synechocystis* PCC 6803 or *Anabaena* PCC 7120 strains (Marin et al. 2003, Hihara et al. 2001; Katoch et al. 2004). Gene knockout experiments with planktonic cyanobacteria which will give important information about the function of the yet uncharacterized genes, have been difficult to accomplish. The whole genome sequence of an organism provides wealth of information, which can also be utilized in proteome research. The whole set of proteins of an organism can be separated by 2D gel electrophoresis and identified by mass spectrometry (Simon et al. 2002). Genome sequencing, as well as gene expression and proteome analyses are all high throughput methods. Combination of all these methodologies in case of toxin producing, bloom–forming cyanobacteria is likely to reveal key aspects of the biology of these important organisms. However, several ecologically relevant, mass–occurrence forming and toxin producing cyanobacteria such as

Planktothrix, *Cylindrospermopsis* and all of the neurotoxin producers still wait for ushering into the genome sequencing programs.

Conclusion and Summary

The common occurrence of toxic cyanobacteria causes problems for health of animals and human beings. More research and good monitoring systems are needed to protect water users. It is important to have rapid, reliable and accurate analysis i.e. high throughput methods to identify the toxins as well as toxin producers in the environment. Excellent methods, such as ELISA already exist to analyse cyanobacterial hepatotoxins and saxitoxins, and PPIA for microcystins and nodularins. The LC/MS method can be fast in identifying the toxicants in the samples. Further development of this area should resolve the problems with sampling and sample preparation, which still are the bottlenecks of rapid analyses. In addition, the availability of reliable reference materials and standards should be resolved.

Molecular detection methods are now routine in clinical and criminal laboratories and may also become important in environmental diagnostics. One prerequisite for the development of molecular analysis is that pure cultures of the producer organisms are available for identification of the biosynthetic genes responsible for toxin production and for proper testing of the diagnostic methods. Good methods are already available for the microcystin and nodularin–producing cyanobacteria such as conventional PCR, quantitative real–time PCR and microarrays/DNA chips. The DNA–chip technology offers an attractive monitoring system for toxic and non–toxic cyanobacteria. Only with these new technologies (PCR + DNA–chips) will we be able to study toxic cyanobacteria populations *in situ* and the effects of environmental factors on the occurrence and proliferation of especially toxic cyanobacteria. This is likely to yield important information for mitigation purposes. Further development of these methods should include all cyanobacterial biodiversity, including all toxin producers and primers/probes to detect producers of neurotoxins, cylindrospermopsins etc. (genes are unknown). The on–going genome projects concerning toxin producing cyanobacteria combined with future gene expression and gene knockout experiments as well as proteome research will yield a wealth of information on the biology and metabolic regulation of these organisms in near future.

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References

Baker JA, Entsch B, Neilan BA, McKay DB (2002) Monitoring changing toxigenicity of a cyanobacterial bloom by molecular methods. Appl Environ Microbiol 68:6070–6076

Castiglioni B, Rizzi E, Frosini A, Sivonen K, Rajaniemi P, Rantala A, Mugnai MA, Ventura S, Wilmotte A, Boutte C, Grubisic S, Balthasart P, Consolandi C, Bordoni R, Mezzelani A, Battaglia C, De Bellis G (2004) Development of a universal microarray based on the ligation detection reaction and 16S rRNA gene polymorphism to target diversity of cyanobacteria. Appl Environ Microbiol 70(12):7161–7172

Christiansen G, Fastner J, Erhard M, Börner T, Dittmann E (2003) Microcystin biosynthesis in *Planktothrix*: genes, evolution, and manipulation. J Bacteriol 185:564–572

Dittmann E, Neilan B, Erhard M, Von Döhren H, Börner T (1997) Insertional mutagenesis of peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. Mol Microbiol 26:779–787

Edwards C, Beattie KA, Scrimgeour CM, Codd GA (1992) Identification of anatoxin–a in benthic cyanobacteria (blue–green algae) and in associated dog poisonings at Loch Insh, Scotland Toxicon 30:1165–1175

Erhard M, von Döhren H, Jungblut P (1997) Rapid typing and elucidation of new secondary metabolites of intact cyanobacteria using MALDI–TOF mass spectrometry. Nature Biotechnol 15:906–909

Fastner J, Erhard M, von Döhren H (2001) Determination of oligopeptide diversity within a natural population of *Microcystis spp*. (cyanobacteria) by typing single colonies by matrix–assisted laser desorption ionization–time of flight mass spectrometry. Appl Environ Microbiol 67:5069–5076

Fergusson KM, Saint CP (2003) Multiplex PCR assay for *Cylindrospermopsis raciborskii* and cylindrospermopsin–producing cyanobacteria. Environmental Toxicology 18:120–125

Foulds IV, Granacki A, Xiao C, Krull UJ, Castle A, Horgen PA (2002) Quantitation of microcystin–producing cyanobacteria and E. coli in water by 5'–nuclease PCR. J Appl Microbiol 93:825–834

Fujii K, Sivonen K, Adachi K, Noguchi K, Sano H, Hirayama K, Suzuki M, Harada KI (1997) Comparative study of toxic and non–toxic cyanobacterial

products: novel peptides from toxic *Nodularia spumigena* AV1. Tetrahedron Letters 38:5525–5528

Gerry NP, Witowski NE, Day J, Hammer RP, Barany G, Barany F (1999) Universal DNA microarray method for multiplex detection of low abundance point mutations. J Mol Biol 292: 251–262

Goldberg J, Huang HB, Kwon YG, Greengard P, Nairn AC, Kuriyan J (1995) Three–dimensional structure of the catalytic subunit of protein serine/threonine phosphatase–1. Nature 376:745–753

Harada KI, Matsuura K, Suzuki M, Watanabe MF, Oishi S, Dahlem AM, Beasley VR, Carmichael WW (1990) Isolation and characterization of the minor components associated with microcystins–LR and –RR in the cyanobacterium (blue–green algae). Toxicon 28:55–64

Harada KI, Kondo F, Lawton F, Lawton LA (1999) Laboratory analysis of cyanotoxins. In: Toxic Cyanobacteria in Water: a Guide to Public Health Significance, Monitoring and Management. I. Chorus and J. Bertram (Edn). The World Health Organization. ISBN 0–419–23930–8. E and FN Spon, London, UK, pp 369–405

Heresztyn T, Nicholson BC (1997) Nodularin concentrations in Lakes Alexandrina and Albert, South Australia, during bloom of the cyanobacterium (blue–green alga) *Nodularia spumigena* and degradation of the toxin. Environ Toxicol Water Qual 12:273–281

Hihara Y, Kamei A, Kanehisa M, Kaplan A, Ikeuchiv M (2001) DNA microarray analysis of cyanobacterial gene expression during acclimation to high light. The Plant Cell 13:793–806

Hilborn DE, Carmichael WW, Yuan M, Azevedo SMFO (2005) A simple colorimertic method to detect biological evidence of human exposure to microcystins. Toxicon 46:218–221

Hisbergues M, Christiansen G, Rouhiainen L, Sivonen K, Börner T (2003) PCR–based identification of microcystin–producing genotypes of different cyanobacterial genera. Arch Microbiol 180:402–410

Hotto A, Satchwell M, Boyer G (2005) Seasonal production and molecular characterization of microcystins in Oneida Lake, New York, USA. Environ Toxicol 20:243–248

Izaguirre G, Neilan BA (2004) Benthic *Phormidium* species that produces microcystin–LR, isolated from three reservoirs in Southern California. Sixth International Conference on Toxic Cyanobacteria, Abstract book pp 50

Kaebernick M, Rohrlack T, Christofferssen K, Neilan BA (2001) A spontaneous mutant of microcystin biosynthesis: genetic characterization and effect on Daphnia. Environ Microbiol 3:669–679

Kaneko T, Tabata S (1997) Complete genome structure of the unicellular cyanobacterium *Synechocystis sp* PCC6803. Plant and Cell Physiology 38:1171–1176

Katoh H, Asthana RK, Ohmori M (2004) Gene expression in the cyanobacterium *Anabaena sp* PCC7120 under desiccation. Microbial Ecol 47:164–174

Kuiper–Goodman T, Falconer I, Fitzgerald J (1999) Human health aspects. In: Toxic cyanobacteria in water. A guide to their Public Health Consequences,

Monitoring and Management. I. Chorus and J. Bartram (edn) The World Health Organization. ISBN 0–419–23930–8 E and FN Spoon, London, pp 113–153

Kurmayer R, Kutzenberger T (2003) Application of real–time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium Microcystis sp Appl Environ Microbiol 69:6723–6730

Kurmayer R, Dittmann E, Fastner J, Chorus I (2002) Diversity of microcystin genes within a population of the toxic cyanobacterium *Microcystis spp* In Lake Wannsee (Berlin, Germany). Microb Ecol 43:107–118

Kurmayer R, Christiansen G, Chorus I (2003) The abundance of microcystin–producing genotypes correlates positively with colony size in *Microcystis sp* and determines its microcystin net production in Lake Wannsee. Appl Environ Microbiol 69:787–795

Kurmayer R, Christiansen G, Fastner J, Börner T (2004) Abundance of active and inactive microcystin genotypes in populations of the toxic cyanobacterium *Planktothrix spp* Environ Microbiol 6:831–841

Kurmayer R, Christiansen G, Gumpenberger M, Fastner J (2005) Genetic identification of microcystin ecotypes in toxic cyanobacteria of the genus *Planktothrix*. Microbiology 151:1525–1533

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32:1363–1371

Marin K, Suzuki I, Yamaguchi K, Ribbeck K, Yamamoto H, Kanesaki Y, Hagemann M, Murata N (2003) Identification of histidine kinases that act as sensors in the perception of salt stress in *Synechocystis sp* PCC 6803. Academy of Sciences of the United States of America 100:9061–9066

Mbedi S, Welker M, Fastner J, Wiedner C (2005) Variability of the microcystin synthetase gene cluster in the genus *Planktothrix* (Oscillatoriales, Cyanobacteria). FEMS Microbiol Lett 245:299–306

Meriluoto J, Codd GA (edn) (2005) TOXIC: Cyanobacterial Monitoring and Cyanotoxin Analysis. Åbo Akademi University Press (Turku), ISBN 951–765–259–3, pp 149

Meriluoto J, Karlsson K, Spoof L (2004) High–throughput screening of ten microcystins and nodularins, cyanobacterial peptide hepatotoxins, by reversed–phase liquid chromatography–electrospray ionisation mass spectrometry. Chromatographia 59:291–298

Mez K, Beattie KA, Codd GA, Hanselmann K, Hauser B, Naegeli H, Preisig HR (1997) Identification of a microcystin in benthic cyanobacteria linked to cattle deaths on alpine pastures in Switzerland. Eur J Phycol 32:111–117

Mikalsen B, Boison G, Skulberg OM, Fastner J, Davies W, Gabrielsen TM, Rudi K, Jakobsen KS (2003) Natural variation in the microcystin synthetase operon *mcyABC* and impact on microcystin production in *Microcystis* strains. J Bact 185:2774–2785

Moffitt MC, Neilan BA (2004) Characterization of the nodularin synthetase gene cluster and proposed theory of the evolution of cyanobacterial hepatotoxins. Appl Environ Microbiol 70:6353–6362

Neilan BA, Dittmann E, Rouhiainen L, Bass RA, Schaub V, Sivonen K, Börner T (1999) Nonribosomal peptide synthesis and toxigenicity of cyanobacteria. J Bact 181:4089–4097

Nishizawa T, Asayama M, Fujii K, Harada KI, Shirai M (1999) Genetic analysis of the peptide synthetase genes for a cyclic heptapeptide microcystin in *Microcystis spp* J Biochem 126:520–529

Nishizawa T, Ueda A, Asayama M, Fujii K, Harada KI, Ochi K, Shirai M (2000) Polyketide synthase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. J Biochem 127:779–789

Nonneman D, Zimba PV (2002) A PCR–based test to assess the potential for microcystin occurrence in channel catfish production ponds. J Phycol 38:230–233

Ohtake A, Shirai M, Aida T, Mori N, Harada KI, Matsuura K, Suzuki M, Nakano M (1989) Toxicity of *Microcystis* species isolated from natural blooms and purification of the toxin. Appl Environ Microbiol 55:3202–3207

Oksanen I, Jokela J, Fewer D, Wahlsten M, Rikkinen J, Sivonen K (2004) Discovery of rare and highly toxic microcystins from lichen associated cyanobacterium *Nostoc sp* strain IO–102–I. Appl Environ Microbiol 70:5756–5763

Ouahid Y, Pérez–Silva G, del Campo FF (2005) Identification of potentially toxic environmental *Microcystis* by individual and multiple PCR amplifications of specific microcystin synthetase gene regions. Environ Toxicol 20:235–242

Pan H, Song L, Liu Y, Börner T (2002) Detection of hepatotoxic *Microcystis* strain by PCR with intact cells from both culture and environmental samples. Arch Microbiol 178:421–427

Prinsep MR, Caplan FR, Moore RE, Patterson GML, Honkanen RE, Boynton AL (1992) Microcystin–LA from a blue–green alga belonging to the Stignonematales. Phytochemistry 31:1247–1248

Rantala A, Fewer DP, Hisbergues M, Rouhiainen L, Vaitomaa J, Börner T, Sivonen K (2004) Phylogenetic evidence for the early evolution of microcystin synthesis. Proceedings of the National Academy of Sciences of the United States of America 101(2):568–573

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

Ressom R, Soong FS, Fitzgerald J, Turczynowicz L, El Saadi O, Roder D, Maynard T, Falconer I (1994) Health effects of toxic cyanobacteria (blue–green algae). National Health and Medical Research Council, Australian Government Publishing Service, Canberra, Australia, pp 108

Rinehart KL, Harada KI, Namikoshi M, Chen C, Harvis CA, Munro MHG, Blunt JW, Mulligan PE, Beasley VR, Dahlem AM, Carmichael WW (1988) Nodu-

larin, microcystin, and the configuration of Adda. J Am Chem Soc 110:8557–8558

Rinehart KL, Namikoshi M, Choi BW (1994) Structure and biosynthesis of toxins from blue–green alga (cyanobacteria). J Appl Phycol 6:159–176

Rinta–Kanto JM, Ouellette AJA, Boyer GL, Twiss MR, Bridgeman TB, Wilhelm SW (2005) Quantification of toxic *Microcystis spp* during the 2003 and 2004 blooms in Western Lake Erie using quantitative real–time PCR. Environ Sci Technol 39:4198–4205

Rouhiainen L, Paulin L, Suomalainen S, Hyytiäinen H, Buikema W, Haselkorn R, Sivonen K (2000) Genes encoding synthetases of cyclic depsipeptides, anabaenopeptilides, in *Anabaena* strain 90. Molecular Microbiol 37(1):156–167

Rouhiainen L, Vakkilainen T, Lumbye B, Siemer, Buikema W, Haselkorn R, Sivonen K (2004) Genes coding for hepatotoxic heptapeptides (microcystins) in the cyanobacterium *Anabaena* strain 90. Appl Environ Microbiol 70(2):686–692

Rudi K, Skulberg OM, Kulberg R, Jakobsen KS (2000) Application of sequence–specific labelled 16S rRNA gene oligonucleotide probes for genetic profiling of cyanobacterial abundance and diversity by array hybridisation. Appl Environ Microbiol 66:4004–4011

Schembri MA, Neilan BA, Saint CP (2001) Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii*. Environ Toxicol 16:413–421

Simon WJ, Hall JJ, Suzuki I, Murata N, Slabas AR (2002) Proteomic study of the soluble proteins from the unicellular cyanobacterium *Synechocystis sp*. PCC6803. using automated matrix–assisted laser desorption/ionisation–time of flight peptide mass fingerprinting. Proteomics 2:1735–1742

Sivonen K (2000) Chapter 26, Freshwater cyanobacterial neurotoxins: ecobiology, chemistry and detection. In: Seafood and Freshwater Toxins. L. M. Botana (Edn) Marcel Dekker, Inc, New York, USA, pp 567–582

Sivonen K, Jones G (1999) Cyanobacterial toxins. In: Toxic Cyanobacteria in Water: a Guide to Public Health Significance, Monitoring and Management. I Chorus and J Bertram (Edn) The World Health Organization. ISBN 0–419–23930–8. E and FN Spon, London, UK, pp 41–111

Sivonen K, Kononen K, Carmichael WW, Dahlem AM, Rinehart K, Kiviranta J, Niemelä SI (1989) Occurrence of the hepatotoxic cyanobacterium *Nodularia spumigena* in the Baltic Sea and the structure of the toxin. Appl Environ Microbiol 55(8):1990–1995

Sivonen K, Namikoshi M, Evans WR, Färdig M, Carmichael WW, Rinehart KL (1992) Three new microcystins, cyclic heptapeptide hepatotoxins, from *Nostoc sp* strain 152. Chem Res Toxicol 5(4):464–469

Surakka A, Sihvonen LM, Lehtimäki JM, Wahlsten M, Vuorela P, Sivonen K (2005) Benthic cyanobacteria from the Baltic Sea contain cytotoxic *Anabaena, Nodularia* and *Nostoc* strains and an apoptosis inducing *Phormidium* strain. Environ Toxicol 20:285–292

Tillett D, Dittmann E, Erhard M, von Döhren H, Börner T, Neilan BA (2000) Structural organization of microcystin biosynthesis in *Microcystis aeruginosa*

PCC 7806: an integrated peptide–polyketide synthase system. Chem Biol 7:753–764

Tillett D, Parker DL, Neilan BA (2001) Detection of toxigenity by a probe for the microcystin synthetase A gene (*mcyA*) of the cyanobacterial genus Microcystis: comparison of toxicities with 16S rRNA and phycocyanin operon (phycocyanin intergenic spacer) phylogenies. Appl Environ Microbiol 67**:**2810–2818

Vaitomaa J, Rantala A, Halinen K, Rouhiainen L, Tallberg P, Mokelke L, Sivonen K (2003) Quantitative real–time–PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. Appl Environ Microbiol 69(12):7289–7297

Vezie C, Brient L, Sivonen K, Betru G, Lefeuvre GC, Salkinoja–Salonen M (1998) Variation of microcystin content of cyanobacterial blooms and isolated strains in Lake Grand–Lieu (France). Microbial Ecology 35:126–135

Via–Ordorika L, Fastner J, Kurmayer R, Hisbergues M, Dittmann E, Komarek J, Erhard M, Chorus I (2004) Distribution of microcystin–producing and non–microcystin–producing *Microcystis sp* in European freshwater bodies: detection of microcystins and microcystin genes in individual colonies. System Appl Microbiol 27:592–602

Welker M, Christiansen G, von Döhren H (2004) Diversity of coexisting *Planktothrix* (cyanobacteria) chemotypes deduced by mass spectral analysis of microystins and other oligopeptides. Arch Microbiol 182(4):288–98

Wintzingerode VF, Göbel UB, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR–based rRNA analysis. FEMS Microbiol Rev 21:213–229