

The genetic basis of toxin production in Cyanobacteria

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Abstract

The increasing incidence of mass developments of Cyanobacteria in fresh- and brackish water is a matter of growing concern due to the production of toxins that threaten human and livestock health. The toxins that are produced by freshwater Cyanobacteria comprise hepatotoxins (cyclic peptides such as microcystins and nodularin, as well as alkaloids such as cylindrospermopsin) and neurotoxins (alkaloids such as anatoxin-a, anatoxin-a(S) and saxitoxins). The variation in toxicity between and within species of Cyanobacteria has been recognised for a long time. However, the toxic and non-toxic genotypes within a species cannot be discriminated under the microscope, which has been a major obstacle in identifying those factors that influence toxin production both in the laboratory and in the field. During the last decade, major advances were achieved due to the elucidation and functional characterisation of genes, such as the gene cluster encoding the synthesis of the hepatotoxic heptapeptide, microcystin. Genetic techniques, in particular, have been used to explore (i) the genetic basis, biosynthesis pathways, and physiological regulation of toxin (microcystin) production, (ii) gene loss processes resulting in a patchy distribution of the microcystin synthetase gene cluster among genera and species, as well as (iii) the distribution and abundance of the microcystin genes in the environment. In recent years, experience in detecting microcystin genes directly in the field has increased enormously and robust protocols for the extraction of DNA and the subsequent detection of genes by PCR (polymerase chain reaction)-based methods are now available. Due to the high sensitivity of PCR, it is possible to detect toxic genotypes long before a toxic cyanobacterial bloom may occur. Consequently, waterbodies that are at risk of toxic bloom formation can be identified early on in the growing season along with environmental factors that can potentially influence the abundance of toxin producing genotypes.

Keywords: Microcystin; nodularin; cylindrospermopsin; saxitoxin; physiological regulation; evolution; environmental regulation; real-time PCR; monitoring.

Introduction

Cyanobacteria (blue-green algae) constitute the most

primitive and ancient organisms with oxygen-generating photosynthesis on earth. They occur in both aquatic and terrestrial habitats, dominate extreme environments such

as ultraoligotrophic oceans or the soil crusts of semi-arid areas, and in freshwater systems they frequently dominate and form surface blooms due to a number of adaptations rendering them competitively superior to other phytoplankton (Dokulil & Teubner, 2000). The increasing incidence of mass developments of Cyanobacteria is a matter of growing concern, however, due to the production of toxins (so-called 'cyanotoxins') that threaten humans and livestock (Chorus & Bartram, 1999; Chorus et al., 2000; Carmichael, 2001).

For many years cyanotoxins have been classified according to their symptoms as observed in humans and vertebrates: hepatotoxins (such as microcystins, nodularin and cylindrospermopsin), neurotoxins (such as anatoxin-a, anatoxin-a(S) and saxitoxins) and irritant-dermal toxins (Carmichael, 2001). The human exposure routes include drinking water, dialysis, recreational activities (Chorus et al., 2000) and the transfer of the toxins via the food chain, i.e. through fish, mussels and larger crustaceans (Ibelings & Chorus, 2007). To help in the assessment of health and environmental risk, in 1997 the World Health Organization (WHO) published a first guideline on the tolerable concentration of the hepatotoxic microcystin-LR in drinking water (Chorus & Bartram, 1999). Most of the naturally occurring chemicals for which the guideline values were derived are inorganic. The only organic chemical is microcystin-LR (WHO 2006).

The first chemical structures of the toxins that are produced by Cyanobacteria were derived in the period 1970–1990: Devlin et al. (1977) described the structure of the neurotoxin anatoxin-a and Botes et al. (1984) were the first to describe the structure of the hepatotoxin microcystin-LA. Subsequently, the number of fully characterised toxin variants greatly increased (Carmichael, 1992; Rinehart et al., 1994; Sivonen & Jones, 1999; Harada et al., 2004). In parallel, the molecular mechanisms of toxification were elucidated (MacKintosh et al., 1990; Carmichael, 1994; Goldberg et al., 1995; Kuiper-Goodman et al., 1999). Later progress was achieved in the detection and quantification of cyanobacterial toxins in the environment (Codd et al., 1989; Codd et al., 1994;

Harada et al., 1996) as well as in the global distribution and occurrence of toxic Cyanobacteria (Sivonen & Jones, 1999).

Since 1996, the identification of the genes that are involved in toxin synthesis, such as microcystin synthesis in Cyanobacteria, opened a new avenue of research (Meißner et al., 1996; Dittmann et al., 1997). While it had been known for a long time that toxic and non-toxic strains coexist in surface water (Carmichael & Gorham, 1981), these could not be distinguished under the microscope. It was now possible, however, to investigate the genetic basis of this phenomenon and techniques now allow the detection of toxic genotypes. It is our aim to review the available information on the genetic basis of toxin production and how genetic techniques have been used to elucidate

1. the regulation of toxin production,
2. gene loss processes contributing to the patchy distribution of the genes involved in toxin production, and
3. the distribution and abundance of the genes encoding toxin production in the environment.

Since the *mcy* gene cluster encoding the synthesis of the toxic heptapeptide microcystin was the first that was characterised, most results up to date have been obtained on this group of hepatotoxins. However, analogous applications may arise for other toxins in the near future. It is beyond the present scope to review all the studies that have been conducted on the detection and regulation of the genes that are involved in toxin production. Instead, the reader is referred to recent reviews (Ouellette & Wilhelm, 2003; Dittmann & Börner, 2005; Neilan et al., 2008; Pearson & Neilan, 2008; Sivonen, 2008; Sivonen & Börner, 2008). In this review, we focus on the hepatotoxins microcystin, nodularin and cylindrospermopsin, and the neurotoxin saxitoxin.

The genetic basis of microcystin and nodularin production

Microcystins are produced by the planktonic freshwater genera *Microcystis*, *Anabaena*, *Anabaenopsis*, *Planktothrix* and *Nostoc* (Sivonen & Jones, 1999). Microcystin production has also been documented, however, in a broad range of

terrestrial genera (Honkanen et al., 1995), for example in *Hapalosiphon* (Prinsep et al., 1992), and recently in the fresh- and brackish water genera *Arthrospira*, *Oscillatoria*, *Phormidium*, *Plectonema*, *Pseudanabaena*, *Synechococcus* and *Synechocystis* (Sivonen & Börner, 2008). In contrast, the closely related nodularin has been characterised from the brackish water species *Nodularia spumigena* only, while in the marine sponge, *Theonella swinhoei*, a nodularin analogue called motuporin has been found (Sivonen & Jones, 1999).

Microcystins are cyclic heptapeptides and share the common structure cyclo (- D-Ala⁽¹⁾ - X⁽²⁾ - D-MAsp⁽³⁾ - Z⁽⁴⁾ - Adda⁽⁵⁾ - D-Glu⁽⁶⁾ - Mdha⁽⁷⁾), where X and Z are variable L-amino acids (e.g. microcystin (MC)-LR refers to leucine and arginine in the variable positions), D-MAsp is D-erythro- β -iso-aspartic acid, Adda is (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and Mdha is N-methyl-dehydroalanine (Carmichael et al., 1988). Considerable structural variation has been reported most frequently in positions 2, 4, and 7 of the molecule and more than 65 structural variants have been characterised (molecular weight between 909 da and 1115 da) either from field samples or from isolated strains (Diehnelt et al., 2006). Nodularin (824 da) and motuporin (812 da) are both pentapeptides containing N-methyl-dehydrobutyrine (Mdhb) instead of Mdha⁽⁷⁾, and lack D-Ala⁽¹⁾ and X⁽²⁾ when compared with microcystin. Nodularin differs from motuporin due to the substitution of L-Arg⁽⁴⁾ by L-Val⁽⁴⁾ (Fig. 1).

Microcystins and nodularins are synthesised by the thiotemplate mechanism characteristic for non-ribosomal peptide synthesis (NRPS), polyketide synthesis (PKS) and fatty acid synthesis (Fischbach & Walsh, 2006). The microcystin (*mcy*) gene cluster contains peptide synthetases, polyketide synthases and tailoring enzymes that are

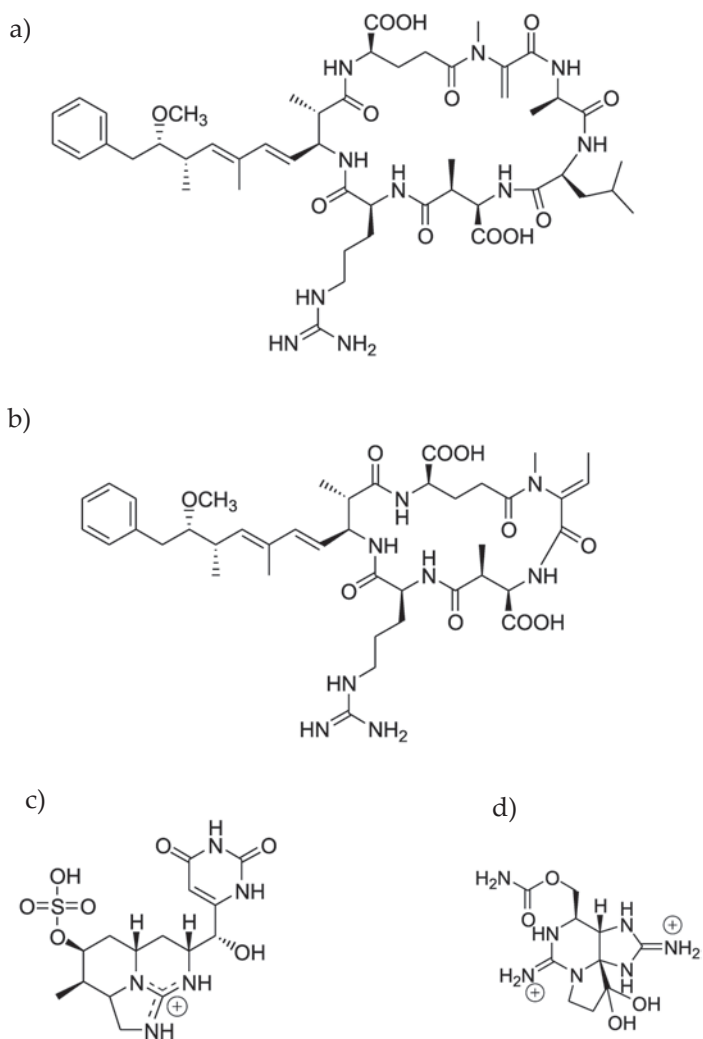


Fig. 1. Chemical structures of the cyanotoxins for which the genetic basis has been identified: the cyclic hepatotoxic peptides microcystin (a) and nodularin (b), the hepatotoxic alkaloid cylindrospermopsin (c) and the tricyclic neurotoxic alkaloid saxitoxin (d).

encoded by ten (*Microcystis*, *Anabaena*) or nine (*Planktothrix*, *Nodularia*) genes (Fig. 2, Tillett et al., 2000). Each module of the multifunctional peptide synthetase contains specific functional domains for activation (aminoacyl adenylation domain) and thioesterification (peptide carrier domain) of the amino acid substrate and for the elongation (condensation domain) of the growing peptide (Tillett et al., 2000). Up to date, the *mcy* gene clusters from *Microcystis*, *Planktothrix*, *Anabaena* (Nishizawa et al., 1999, 2000; Tillett et

al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004), and the closely related nodularin (*nda*) synthetase gene cluster from *Nodularia* (Moffitt & Neilan, 2004) have been sequenced.

Functional proof for the putative involvement of *mcy* genes in microcystin synthesis has been obtained by insertional inactivation in *Microcystis aeruginosa* PCC7806 (Dittmann et al., 1997), i.e. by inserting foreign DNA into a specific gene to inactivate it. The *mcy* gene knock-out mutant was totally impaired in microcystin production in comparison with the wild type strain, and by this approach it could be demonstrated that one gene cluster is encoding the production of various structural variants (Dittmann et al., 1997). In these experiments, the transformation construct contains a selection marker that is flanked by homologous sequences on both 5' and 3' ends, and is introduced into the cell by conjugation or other techniques (Thiel, 1994). In general, however, very few Cyanobacteria are amenable to genetic manipulation (Flores et al., 2008), with *Planktothrix agardhii* being one of the rare cases of

toxin-producing Cyanobacteria that have been genetically manipulated repeatedly (Christiansen et al., 2003; Ishida et al., 2007; Philmus et al., 2008). Other species amenable to genetic manipulation include the red-pigmented *P. rubescens*; for example, in our laboratory the insertional inactivation of the *mcyD* gene of the *P. rubescens* strain No21/2 resulted in the complete inactivation of microcystin synthesis when compared with the wild type (Fig. 3).

The genetic basis of cylindrospermopsin production

The structure of cylindrospermopsin was first described from *Cylindrospermopsis* (Ohtani et al., 1992). Later on it was described from *Umezakia natans* isolated from a lake in Japan as well as *Aphanizomenon ovalisporum*, *Aphanizomenon flos-aquae*, *Anabaena bergii* and *Raphidiopsis curvata* (Sivonen & Börner, 2008).

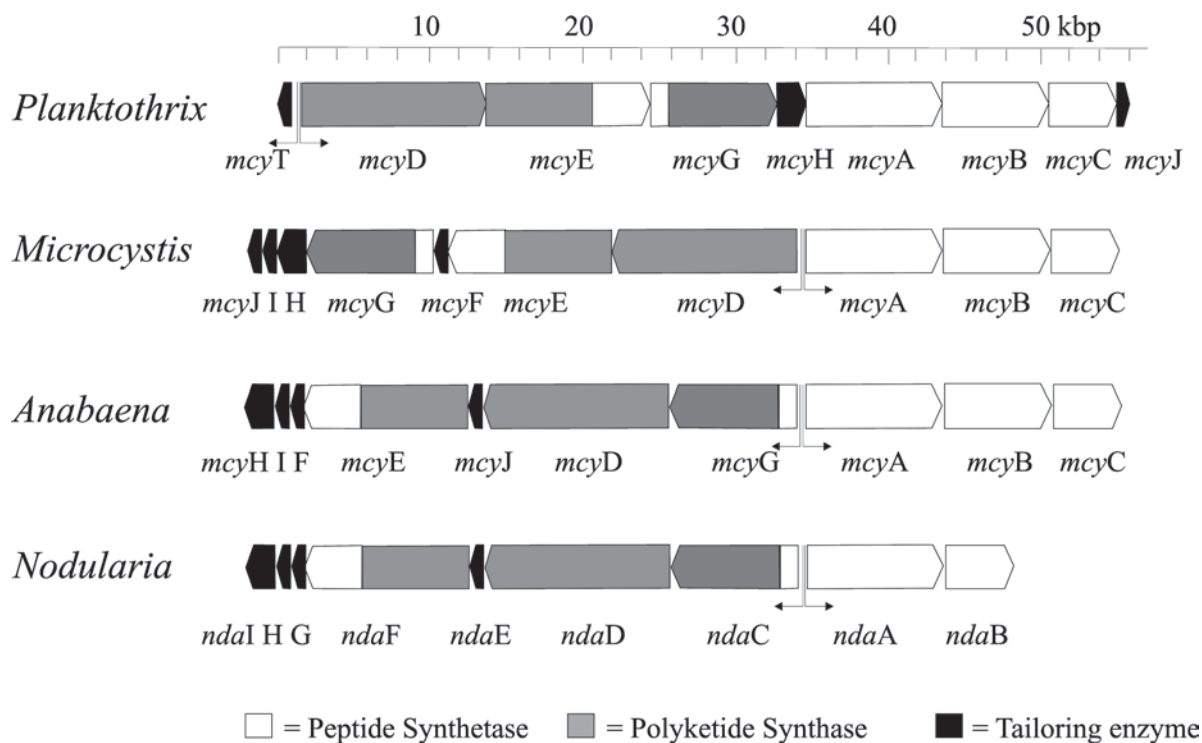


Fig. 2. Scheme of the structural organisation of the microcystin (*mcy*) gene clusters from *Microcystis* (Nishizawa et al., 1999, 2000; Tillett et al., 2000), *Planktothrix* (Christiansen et al., 2003) and *Anabaena* (Rouhiainen et al., 2004) and of the nodularin (*nda*) synthetase gene cluster from *Nodularia* (Moffitt & Neilan, 2004). Arrows indicate the direction of transcription, in which the (bi)-directional promoter sites have been indicated. The scale indicates the length of the gene clusters in kilo base pairs (kbp).

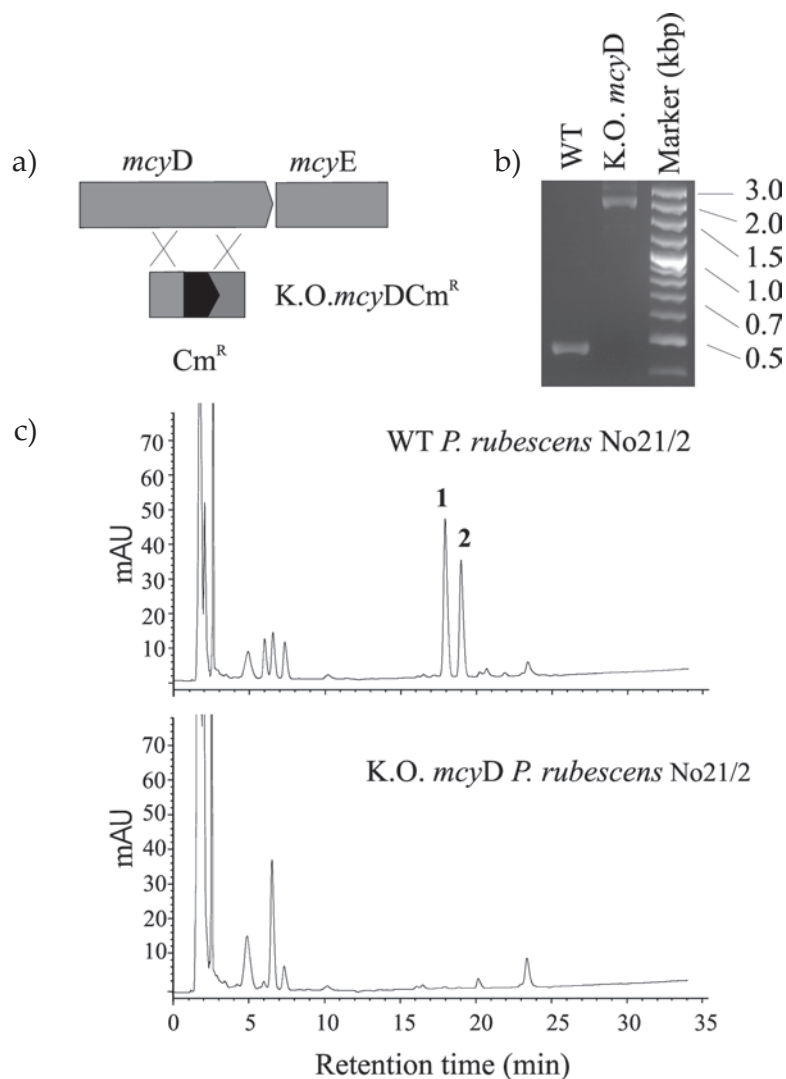


Fig. 3. (a) Inactivation of the *mcyD* gene through insertional inactivation via homologous recombination. The transformation construct (*K.O.mcyDCm^R*) contains a selection marker (*Cm^R*, 1900 bp, in black) that is flanked by homologous sequences on both 5' and 3' ends. (b) Testing the full segregation of the *mcyD* mutation in *P. rubescens* strain No21/2 by PCR using primers specific to the flanking region of the construct inserted into *mcyD* (sequence position 10036–10521 of Access. No AJ441056, Christiansen et al., 2003, 2008). While the wildtype (WT) gives the calculated PCR product of 500 bp, the K.O. (knock out) *mcyD* mutant gives only an amplicon of 500 bp + 1900 bp (*Cm^R* fragment) = 2400 bp. (c) Analysis of the aqueous-methanolic extracts of WT *P. rubescens* strain No21/2 (upper chromatogram) and K.O. *mcyD* mutant deficient in microcystin synthesis (lower chromatogram), by high performance liquid chromatography (Kurmayer et al., 2004). 1, 2 represent microcystin-HtyR (17.9 min) and microcystin-LR (19.0 min), respectively.

Cylindrospermopsin is a cyclic guanidine alkaloid hepatotoxin (molecular weight 415 da). In contrast to the microcystins, the structural variability is much lower, i.e.

three variants of the cylindrospermopsin molecule have been described (Sivonen & Börner, 2008). Inspection of the structure of cylindrospermopsin suggested a polyketide origin of the alkaloid (Fig. 1, Burgoyne et al., 2000). Indeed, isotope-labelled precursor feeding experiments revealed the incorporation of five acetate units with guanidinoacetic acid serving as the starter unit (Burgoyne et al., 2000). A screening of 13 *Cylindrospermopsis* strains revealed that the presence of PKS and NRPS genes was linked to cylindrospermopsin production (Schembri et al., 2001). Subsequently, Shalev-Alon et al. (2002) identified an amidinotransferase in *Aphanizomenon ovalisporum* that has been suggested to transfer the amidino group from L-arginine to glycine in the starter unit. Most recently the putative cylindrospermopsin biosynthesis gene cluster encoding an amidinotransferase (*CyrA*), one mixed PKS/NRPS module (*CyrB*), four PKS modules (*CyrC*–*CyrF*) and additional tailoring enzymes has been described and a pathway of cylindrospermopsin synthesis has been proposed (Kellmann et al., 2006; Mihali et al., 2008). Biochemical proof for the role of the *cyr* gene cluster in cylindrospermopsin biosynthesis is lacking. However, the screening of 17 *Cylindrospermopsis* strains revealed that *cyrJ*, encoding a sulphotransferase putatively catalysing the sulphation of the C-12

atom, was present only in cylindrospermopsin-producing strains indicating the involvement of the *cyr* gene cluster in cylindrospermopsin synthesis (Mihali et al., 2008).

The genetic basis of saxitoxin production

Saxitoxins, better known as paralytic shellfish poisons (PSPs), are produced by certain genera of marine dinoflagellates (Landsberg 2002) and Cyanobacteria. Among Cyanobacteria, saxitoxins have been found in *Aphanizomenon flos-aquae*, *Aphanizomenon* sp., *Aphanizomenon issatschenkoi*, *Anabaena circinalis*, *Cylindrospermopsis raciborskii*, *Planktothrix* sp. and benthic *Lyngbya wollei* (Sivonen & Börner, 2008). A study on the distribution of PSPs among 234 diverse isolates of Australian freshwater and marine microalgae revealed five species containing PSPs, including only one freshwater cyanobacterium (*Anabaena circinalis*) (Negri et al., 2003).

Saxitoxins (Fig. 1, molecular weight 299 da) are a group of alkaloid tricyclic compounds that are either non-sulphated (saxitoxins and neosaxitoxin), single sulphated (gonyautoxins), or doubly sulphated (C-toxins) resulting in more than 20 structural analogues (Shimizu, 1993; Sivonen & Jones, 1999; Kellmann & Neilan, 2007). Isotope-labelled precursor feeding experiments suggested that the skeleton of the tricyclic ring system is formed by the Claisen-type condensation of acetate on the α -carbon of arginine with the loss of the carboxyl group of arginine and subsequent amidation and cyclisation (Shimizu et al., 1984; Shimizu, 1993). The side-chain carbon is derived from methionine via S-adenosylmethionine (SAM). This has been confirmed by in vitro experiments (Kellmann & Neilan, 2007). Recently, a candidate saxitoxin (*sxt*) biosynthesis gene cluster comprising 31 open reading frames has been described from *Cylindrospermopsis raciborskii* (Kellmann et al., 2008). Comparative sequence analysis of the *sxtA* gene revealed the occurrence of four catalytic domains, SxtA1–SxtA4, forming a new type of polyketide synthase which can putatively execute the methylation of acetate and a Claisen condensation reaction between propionate and arginine. Biochemical proof for the role of this gene cluster in saxitoxin biosynthesis is lacking. In the absence of suitable tools for genetic transformation, the functions of the open reading frames (ORFs) were bioinformatically inferred and this prediction was

combined with the liquid chromatography-tandem mass spectrometry analysis of the biosynthetic intermediates.

Regulation of toxin production

It is generally accepted that the toxins that are produced by Cyanobacteria are secondary metabolites (Carmichael, 1992), i.e. those compounds that are not used by the organism for its primary metabolism. During the last two decades, the regulation of toxin net production has been addressed by a number of studies that quantified the effects of various environmental conditions on the toxin content for individual strains in the laboratory, e.g. temperature, irradiance, macronutrients (nitrate, ammonium, phosphate), trace elements (iron and others), salinity, CO₂ and pH (Sivonen & Jones, 1999). The toxins concerned commonly included were the microcystins, nodularin and anatoxin-a. In the majority of these studies, the different environmental factors were indeed found to induce changes in the toxin content, but usually by a factor of no more than three to four. In a few cases (Rapala et al., 1997) up to a 30-fold variation under different temperature conditions was reported when the growth of the strains was poor. In consequence, Orr & Jones (1998) suggested microcystin production to be coupled linearly to the cell division of the organism and concluded that – although microcystin is a secondary metabolite – it instead displays the attributes of essential intracellular nitrogenous compounds in Cyanobacteria. This correlation does not necessarily imply that toxin synthesis is causally related to the cell division cycle (Kosol et al., 2009). According to the Orr & Jones theory, however, the influence of all the possible environmental factors is indirect, through their effect on cell division and growth, whereas the direct effects on microcystin biosynthesis are of a relatively minor importance.

In parallel to the elucidation of the *mcy* gene cluster, the initiations and regulation of its transcription were also investigated. Firstly, for the *mcy* gene cluster of *Microcystis*, the promoter region was found to be bi-directional with two different transcription initiation sites under high and low light conditions (Kaebernick et al., 2002). In addition,

the promoter region was reported to contain binding regions for transcription factors, such as a ferric uptake regulator (Fur) that usually represses gene expression in the presence of Fe²⁺ (Martin-Luna et al., 2006a). Indeed the Fur protein was shown to bind the promoter region in vitro, by protein-DNA gel retardation assays (Martin-Luna et al., 2006b). Notably, an in vivo inter-relationship between the extracellular iron availability and *mcy* transcription rate has been reported recently (Sevilla et al., 2008).

Nishizawa et al. (1999) and Kaebernick et al. (2000) emphasised direct effects of irradiance, i.e. high light vs. low light or dark conditions, on the transcription of the *mcy* genes encoding microcystin biosynthesis in *Microcystis*. Most importantly, an increased transcription of *mcy* genes under high light (68 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and red light was documented while the transcription rates became reduced under low light (16 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and dark conditions (Kaebernick et al., 2000). Blue light and artificial stress factors also reduced the *mcy* transcription, however it is important to note that *mcy* transcription never ceased. The same positive relationship between *mcy* transcription rates and irradiance was observed for *Planktothrix agardhii* (Tonk et al., 2005), although the *mcy* transcription rates decreased at the highest light conditions (> 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Tonk et al. (2005) were the first to report a positive relationship between the *mcyA* transcript level and the total microcystin net production rate as determined under continuous culture conditions. When studying the influence of irradiance and temperature, the interrelationship between both factors on the *mcy* transcript level was observed (Kim et al., 2005). Taken together, the transcriptional results confirm

the view that the synthesis of microcystin is related to cell division and growth, although the amount of microcystin that is produced is regulated in response to environmental factors such as light (Wiedner et al., 2003; Tonk et al., 2005) and macronutrients (Lee et al., 2000; Long et al., 2001).

Evolution of toxin genes

The high similarity of the *mcy* synthesis gene cluster between genera is remarkable (Fig. 2). Except for the tailoring enzymes *mcyI* (a 2-hydroxy-acid hydrogenase, Pearson et al., 2007), *mcyF* (an aspartate racemase, Sielaff et al., 2003) and *mcyT* (a type II thioesterase, Christiansen et al., 2008), all other genes *mcy*ABCDEFGHIJ are always present. The horizontal transfer of the total or partial *mcy* gene cluster has been suggested, not least because of the patchy distribution of the *mcy* gene cluster among the strains and genera, but also because elements that are putatively involved in horizontal transfer such as transposases have been identified at the downstream 3' end of the gene clusters (Tillett et al., 2000; Tillett et al., 2001; Moffitt & Neilan, 2004). A type IV pilus system that has been shown to be involved in DNA uptake in many bacteria has been described in *Microcystis* and it has been suggested that this system may allow for the receiving of the *mcy* gene cluster via lateral transfer (Nakasugi et al., 2007).

Other evidence, however, supports the role of vertical inheritance and gene loss processes. Surprisingly, the phylogenetic tree calculated from the housekeeping genes 16S rDNA and *rpoC1* showed congruency with the phylogenetic tree as calculated from the genes *mcyA*, *mcyD*

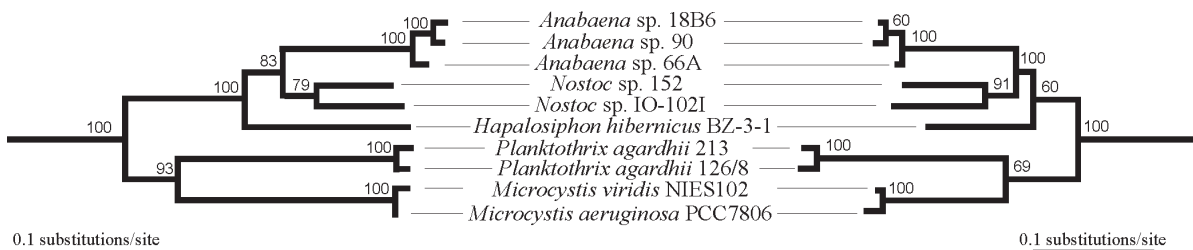


Fig. 4. Phylogenetic congruence between housekeeping and microcystin synthetase genes. Congruence between the topology as revealed from housekeeping genes of the producer organism (16S rRNA, *rpoC1*, *rpoB*, *tufA*, *rbcL*) on the left and the microcystin synthetase genes (*mcyD*, *mcyE*, and *mcyG*) on the right. Bootstrap values above 50% from 1000 replicates are given at the nodes. Redrawn from Fewer et al. (2007). Copyright © BioMed Central [BMC Evolutionary Biology, 7, 183].

and *mcyE* of the microcystin synthetase gene cluster for all genera (Rantala et al., 2004; Fewer et al., 2007, Fig. 4). Phylogenetic trees that are derived from different genes are congruent only if reproduction is strictly clonal and vertical inheritance is of major importance when compared with intra- or extrachromosomal genetic recombination (Maynard Smith et al., 2000). Consequently, several deletion events of the *mcy* gene cluster must be invoked in order to explain the patchy distribution of the *mcy* genes among the different genera of the Cyanobacteria that produce microcystin today: section I (*sensu* Rippka et al., 1979, including unicellular species such as *Microcystis*, *Synechocystis*, *Synechococcus* etc), section III (including filamentous species such as *Planktothrix* (*Oscillatoria*), *Phormidium* etc) and sections IV, V (including filamentous organisation and the formation of heterocysts, *Anabaena*, *Nodularia*, *Nostoc* and *Hapalosiphon*). The hypothesis that Cyanobacteria share a common microcystin-producing ancestor implies that potentially all Cyanobacteria are able to produce microcystin and indeed the number of cyanobacterial genera discovered to produce microcystin continues to increase (Sivonen & Börner, 2008). Rantala et al. (2004) further concluded that a common ancestor of a cyanobacterium containing the *mcy* gene cluster must have existed before the mesoproterozoic (1000–1600 million years),

which was considerably before the time of the emergence of eukaryotic organisms potentially feeding on Cyanobacteria.

Other reports on the occurrence of the inactive *mcy* genotypes of *Planktothrix* spp. (Kurmayer et al., 2004) can be taken to support the *mcy* gene loss hypothesis, as can the discovery of relatively large deletions and insertions of transposable elements affecting *mcy* genes (Christiansen et al., 2006). According to Rantala et al. (2004), the patchy distribution of *mcy* genes among the strains of a single species is also the result of the loss processes of the *mcy* gene cluster, although these loss processes must have occurred over a much shorter evolutionary timescale. We recently screened a larger number of *Planktothrix* strains that lack the *mcy* gene cluster for the presence of remnants of the *mcy* genes, which are indicative of a putative gene loss event (Christiansen et al., 2008). To minimise the chance that the same *Planktothrix* population has been sampled repeatedly, 25 non-toxic strains isolated from eight European countries were selected. Notably, in all 25 non-toxic strains the flanking regions of the *mcy* gene clusters occurred with *mcy* remnants flanked by identical copies of a transposable element. The majority of those non-toxic strains still contain *mcyT*, a type II thioesterase that was shown to be involved in microcystin synthesis (Fig. 5, Christiansen et al., 2008). These results provide clear evidence against the

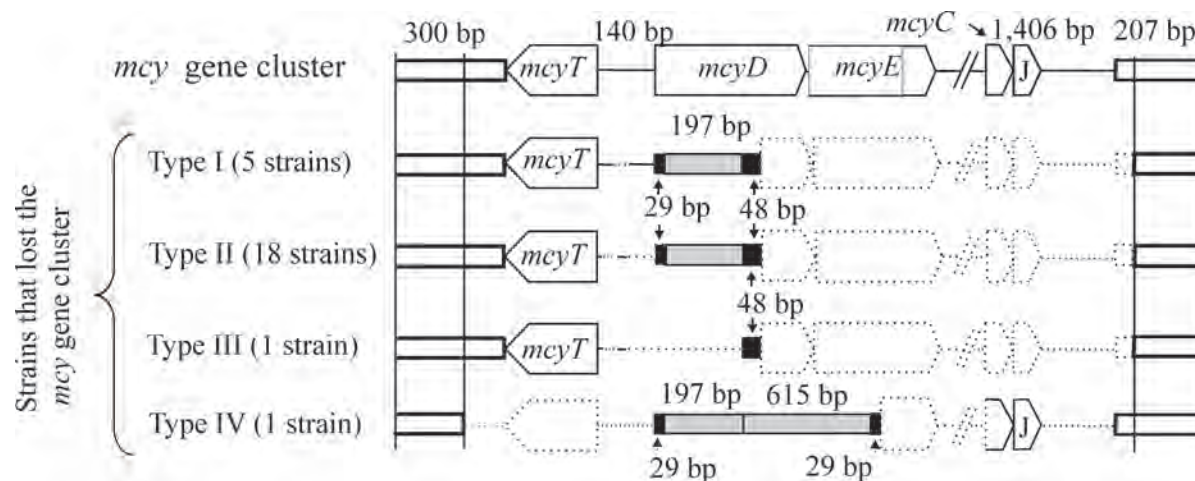


Fig. 5. Schematic view of the *mcy* operon remnants and flanking regions in strains of *Planktothrix* that lost the *mcy* gene cluster. The four types (I–IV) of gene cluster deletion events are shown. Vertical straight lines enclose the identical 5' and 3' ends. The grey regions represent the remnants of insertion elements (197 bp) containing short terminal inverted repeats (IR in black) that probably caused the inactivation of the *mcy* gene cluster and subsequently its deletion. The dotted lines indicate the deleted areas. From Christiansen et al. (2008). Copyright © Oxford University Press [Molecular Biology and Evolution, 25, 1695–1704].

role of horizontal transfer of the *mcy* gene cluster causing its patchy distribution among the strains of *Planktothrix agardhii* and *Planktothrix rubescens*. The localisation and characterisation of the remnants of the *mcy* gene cluster, however, provide the potential to explore the mechanism and frequency of *mcy* gene loss in other Cyanobacteria.

As an alternative to the direct comparison of the *mcy* genes, the sequencing of multiple housekeeping loci has been applied to explore the distribution of *mcy* genes among the clades of strains of cyanobacterial species. In general, the sequences from multiple housekeeping loci from a larger number of strains are concatenated to construct a dendrogram (Maynard-Smith et al., 2000). Usually the genetic variation on seven gene loci (involved in primary metabolism) is recorded, while the ability to resolve genotypic clusters also requires large numbers of strains (Hanage et al., 2006). For *Microcystis aeruginosa* it could be shown that within 164 strains the distribution of microcystin production shows a clonal dependence, i.e. microcystin production occurred in two clades only, excluding a recent horizontal transfer of the *mcy* gene cluster between the different phylogenetic branches (Tanabe et al., 2007). This conclusion has been confirmed independently by a partial comparison of the flanking regions of the *mcy* gene cluster among nine toxic and seven non-toxic strains of *M. aeruginosa* (Tooming-Klunderud et al., 2008). These results indicate that there is also no reason to conclude a frequent horizontal transfer of the complete *mcy* gene cluster between strains of *M. aeruginosa*.

On the other hand, the homologous recombination of shorter gene fragments (< 1000 bp (base pairs)) contributed significantly to the incongruent dendrograms obtained from specific enzyme domains as a part of the *mcy* gene cluster. For example, Tanabe et al. (2004) demonstrated the occurrence of intragenic and intergenic recombination events for the first adenylation domain containing the N-methyltransferase located in *mcyA* of *Microcystis*, but did not find the recombination of genetic regions for the O-methyltransferase of *mcyJ*, the adenylation domain of *mcyG*, and a dehydratase located in *mcyD*. Similarly, frequent recombination and gene duplication events were observed within the adenylation domains of the

mcyB and *mcyC* genes in *M. aeruginosa* (Mikalsen et al., 2003; Fewer et al., 2007; Tooming-Klunderud et al., 2008) and *Planktothrix* spp. (Kurmayer & Gumpenberger, 2006), while the adjacent condensation domains seem to be much less affected by genetic recombination (Fewer et al., 2007). In summary, these results imply that while horizontal gene exchange contributes to the mosaic structure as seen for the adenylation domains of the *mcyA* and *mcyB* genes, it cannot account for the sporadic distribution of the *mcy* gene cluster within the genera of *Microcystis* and *Planktothrix*.

Abundance and distribution of toxic genotypes in the environment

It has been suggested that specific environmental parameters may influence the microcystin concentration in surface water through their overall effects on the rate of increase or decline in cell numbers of a specific culture or strain rather than by individual and specific influences on the microcystin biosynthetic pathways (Orr & Jones, 1998). In order to identify those environmental factors influencing the abundance of toxin-producing genotypes, PCR (polymerase chain reaction) assays specified to amplify both the total population of a species as well as part of the *mcy* gene cluster have been designed (Kurmayer et al., 2003, 2004; Rantala et al., 2006; Hotto et al., 2007). Rantala et al. (2006) analysed the distribution of *mcy* genes in relation to environmental factors, e.g. macronutrients, lake size, phytoplankton biomass and secchi depth, among 70 lakes in Finland. In the majority of the samples, *mcyE* genotypes of the three genera *Anabaena*, *Planktothrix*, *Microcystis* occurred. In contrast to oligotrophic and mesotrophic systems, in which one or two genera of *mcyE* genotypes were usually present, a co-occurrence of *mcyE* genotypes of all three genera was found in eutrophic and hypertrophic systems. It is unclear as to whether this positive relationship between *mcyE* occurrence and trophic status is due to selective factors that favour *mcyE* genotype occurrence or other factors not directly related to the *mcyE* gene.

The sensitivity of PCR, in general, allows for the detection of *mcy* genes in single filaments or colonies

of Cyanobacteria that are directly isolated from the environment (Kurmayer et al., 2002; Kurmayer et al., 2004). Isolating single filaments/colonies of Cyanobacteria and describing the genetic structure of cyanobacterial populations has become an accepted technique (Barker et al., 2000; Hayes et al., 2002; Kurmayer et al., 2004; Lodders et al., 2005). Testing of colonies/filaments of Cyanobacteria in parallel with species identification according to morphological criteria allows the distribution of specific genotypes among specific morphotypes of a cyanobacterial species to be investigated. For example, it has been shown that specific morphotypes of *Microcystis* differ significantly in the proportion containing *mcy* genes (Kurmayer et al., 2002; Via Odorika et al., 2004). Typically, morphotypes assigned to *M. aeruginosa* have a high proportion of *mcy* genotypes (> 70 %) while the proportion of *mcy* genotypes among other morphotypes is much lower: *M. ichthyoblabe* (< 20 %) and *M. wesenbergii* (0 %). Consequently, the variation in abundance of those morphotypes results in significant differences in microcystin net production, this occurring both along a gradient in the *Microcystis* colony size (Kurmayer et al., 2003) and during the seasonal increase and decrease of the total *Microcystis* population (Park et al., 1993; Harada et al., 2001).

In order to directly quantify the toxin genes in a given volume of water, quantitative real-time PCR assays were developed (Kurmayer & Kutzenberger, 2003; Vaitomaa et al., 2003; Rinta-Kanto et al., 2005; Koskenniemi et al., 2007). In particular, the Taq nuclease assay (TNA) has been introduced, which is based on

1. the quantification of the total population of a specific cyanobacterium by a TNA targeted to a housekeeping gene (i.e. the intergenic spacer region within the phycocyanin operon) and;
2. another TNA targeted to the *mcy*-containing subpopulation (Kurmayer & Kutzenberger, 2003).

This principle has been applied to control for uncertainties in quantifying toxic genotypes; for example, due to a physiological variation of the genome copy number (Kurmayer & Kutzenberger, 2003). Although there are limitations to the accuracy of this technique in estimating genotype proportions, relating to the semi-logarithmic calibration curves, the real-time PCR technique is the only quantitative technique available.

A recent interlaboratory comparison of TNAs between three different real-time PCR instruments (ABI7300, GeneAmp5700, ABI7500) revealed that

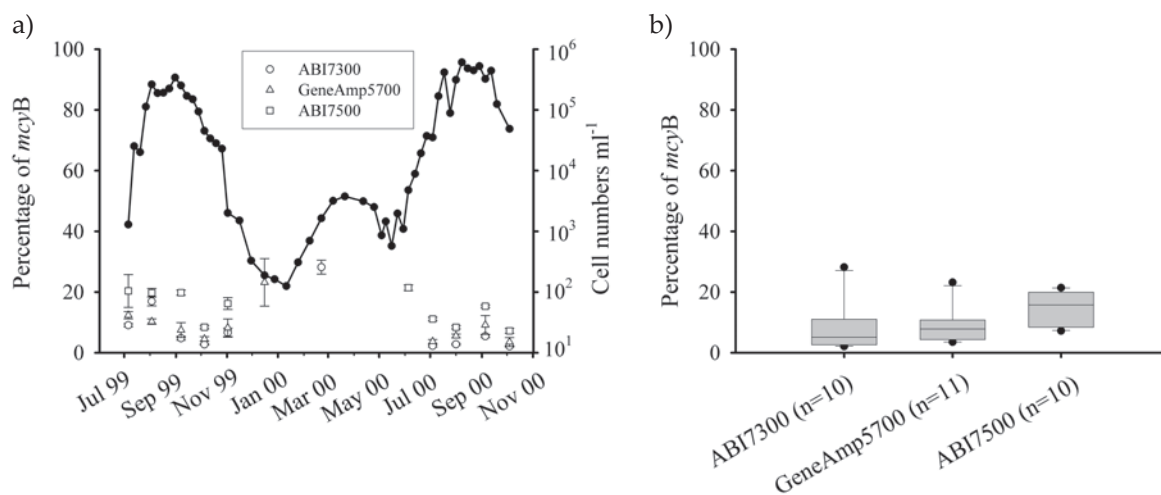


Fig. 6. (a) Proportion of *Microcystis mcyB* genotypes in hypertrophic Lake Wannsee (Berlin, Germany) from July 1999 to October 2000 determined via the Taq Nuclease assay using three instruments (ABI7300, GeneAmp5700, ABI7500), mean (\pm SE). The cell number determined in the microscope is shown by the black dots and the solid line (y-axis on the right). (b) Box-plots showing the median and the 5 % and 95 % percentiles during the same period. From Schober et al. (2007). Copyright © Elsevier [Journal of Microbiological Methods, 69, 122-128].

1. all of the research groups were able to follow the variation in *mcy* genotype proportion, both within the mixtures of strains in the laboratory as well as in field samples, and;
2. the proportions of *mcy* genotypes were overestimated or underestimated by 0–72 % and 0–50 %, respectively (Schober et al., 2007).

However, the averaged *mcy* genotype proportions showed correspondence between the three instruments resulting in the same conclusion, i.e. that the proportion of *mcy*-containing genotypes in a population of *Microcystis* in Lake Wannsee (Berlin, Germany) was low (10–20% of the population, Fig. 6). Yoshida et al. (2007) also reported a low percentage, but continuous occurrence, of the *mcyA* genotype in Lake Mikata, Japan (between 0.5 % and 35 %). Similarly, in the much larger southern and western basin of Lake Erie, the proportion of *Microcystis mcuD* genotypes that was calculated from the estimated gene copies and cell abundances was found to be relatively low: 0.4 % to 32 % (mean 8.2 % \pm SE 2.7 %) based on *Microcystis* 16S rDNA gene copies, and 0.3 % to 136 % (mean 29 % \pm 11 %) based on *Microcystis* cell numbers (Table 5 in Rinta-Kanto et al., 2005). The relatively low proportion of *mcy* genotypes in *Microcystis* that can be observed across the Northern

hemisphere implies that only a small part of the *Microcystis* population is of relevance for the microcystin net production in lake water (Kurmayer et al., 2003; Hotta et al., 2008). The relatively low proportion of *mcy* genotypes in populations in general could be explained if the *mcy* gene cluster loss events, as described in the above section, occurred not relatively recently but a long time ago during the evolution from a common microcystin-producing ancestor of the Cyanobacteria that is assigned to *Microcystis* today. Indeed, for the green-pigmented *Planktothrix agardhii* occurring in shallow lakes throughout Europe, we estimated that the loss of the *mcy* gene cluster took place at least 3.6 million years ago (Christiansen et al., 2008).

On a seasonal scale, the *mcy* genotype proportion in the *Microcystis* population in Lake Wannsee was found to vary within weeks of sampling (minimum 1 % to maximum 38 %), but the average *mcy* proportions from June 1999 to October 2000 were found to be fairly constant (mean \pm 95% C.L., 11.4 % \pm 2.6 %, n = 46) (Kurmayer & Kutzenberger, 2003). No seasonal differences in *mcy* genotype proportion were detected (Fig. 7a). Accordingly, the *mcy* genotype number was found to increase in parallel to the total population as estimated from another independent TNA via the phycocyanin gene (Fig. 7b).

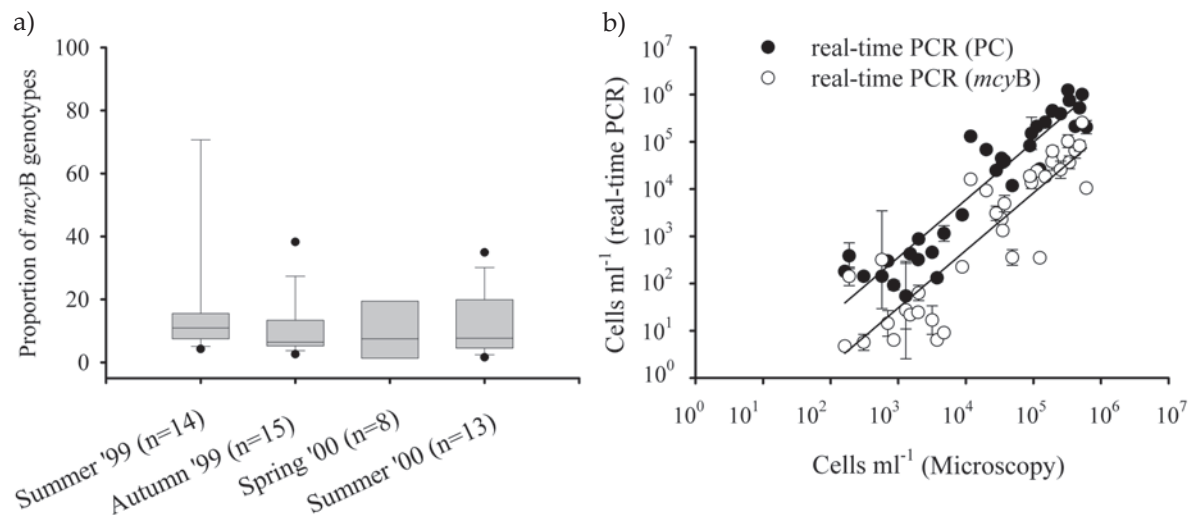


Fig. 7. (a) Proportion of *Microcystis mcuB* genotypes during different seasonal growth periods in Lake Wannsee from June 1999 to October 2000 (box-plots showing the median and the 5 % and 95 % percentiles). (b) Comparison between the cell number determined under the microscope (x-axis) and that determined via Taq Nuclease assay for the phycocyanin gene PC (black) and *mcuB* (white) of the same population (mean \pm 1SE). From Kurmayer & Kutzenberger (2003). Copyright © American Society for Microbiology [Applied and Environmental Microbiology, 69, 6723–6730, 2003].

This implies that the abundance of *mcy* genotypes mainly depended on the increase of the total population over a range in population density from 10^2 to 5×10^5 cells ml^{-1} (Kurmayer & Kutzenberger, 2003). At present, the factors causing the variation in *mcy* genotype proportion in the short-term are poorly investigated, although it has been suggested that cyanophages may select phage resistant genotypes and indirectly affect a shift between *mcy*-containing and *mcy*-free genotypes in *Microcystis* (Yoshida et al., 2008). Theoretical models, however, have predicted that these phage-mediated dynamics do not lead to the complete extinction of a particular genotype but instead to an oscillating abundance of phage resistant and less phage resistant clones (Thingstad & Lignell, 1997). Notably, the same genotype of *Microcystis aeruginosa* strain PCC7806 was re-isolated 36 years after its isolation from Braakman reservoir (The Netherlands) in 1972 (Guljamow et al., 2007). In Lake Steinsfjorden (Norway), the same microcystin-producing strains of *Planktothrix* were isolated over a period of 33 years (Rohrlack et al., 2008). The co-existence of microcystin-producing and non-microcystin producing strains over decades again implies that the evolution of microcystin synthesis was relatively slow.

Application of genetic methods in monitoring

The potential consequences of cyanobacterial toxins to users of recreational waters and in drinking water mean that those charged with the management of waters for recreational use or water supply require efficient methods to detect the toxins and their producers. Several efficient and sensitive chemical analytical methods and bioassays have been developed to detect toxins, which are currently applied during routine monitoring. For example, immunoassays such as ELISA (Enzyme Linked Immunosorbent Assay) are able to estimate very low toxin concentrations (ng L^{-1}) within minutes to hours (Lawton & Edwards, 2008; Sivonen, 2008). The sampling effort, including the sampling preparation, travel time, and sample extraction is still the most limiting factor for the timely identification of a potential health risk, this being particularly relevant for

authorities with large monitoring programmes. In such cases, surveillance protocols comprising initial microscopic analysis of the phytoplankton followed by toxin analysis triggered only above a specified threshold cell density, have proved to be an efficient and effective way of monitoring cyanotoxin risks (Watzin et al., 2006).

Monitoring for toxin producers must detect all Cyanobacteria that are known to produce a specific toxin. For the *mcy* gene cluster, primers have been derived from conserved gene regions located within *mcyA* and *mcyE* that are able to detect the microcystin and nodularin genes of all microcystin/nodularin-producing genera (Hisbergues et al., 2003; Rantala et al., 2004; Jungblut & Neilan, 2006). Similarly, gene probes for the detection of cylindrospermopsin producers have been developed (Schembri et al., 2001; Kellmann et al., 2006). In general, this approach consists of three mandatory steps (Fig. 8):

1. DNA extraction from phytoplankton collected on filters or single colonies/filaments that have been isolated under the microscope (Tillett & Neilan, 2000; Schober & Kurmayer, 2006; Srivastava et al., 2007);
2. (real-time) PCR amplification of genes indicative of toxin production;
3. detection of PCR products using either agarose gel electrophoresis, fluorescence (via real-time PCR), or the ligation detection reaction (LDR) linked to a DNA microarray (Rantala et al., 2008).

Such an approach could be adopted in parallel with routine microscopic inspection of phytoplankton, the DNA being extracted from filters or pellets and then used as a template in PCR targeted to amplify toxin genes as described above. In order to check for the presence and quality of the DNA, each sample is analysed for the phycocyanin gene (PC-ITS region, Neilan et al., 1995) simultaneously, which is used as a positive control (Kurmayer et al., 2002). Alternatively, a multiplex PCR technique that is able to simultaneously detect the presence of microcystin-producing Cyanobacteria and also *Microcystis* during one PCR has been proposed (Saker et al., 2007). A multiplex PCR assay has also been developed to rapidly identify cylindros-

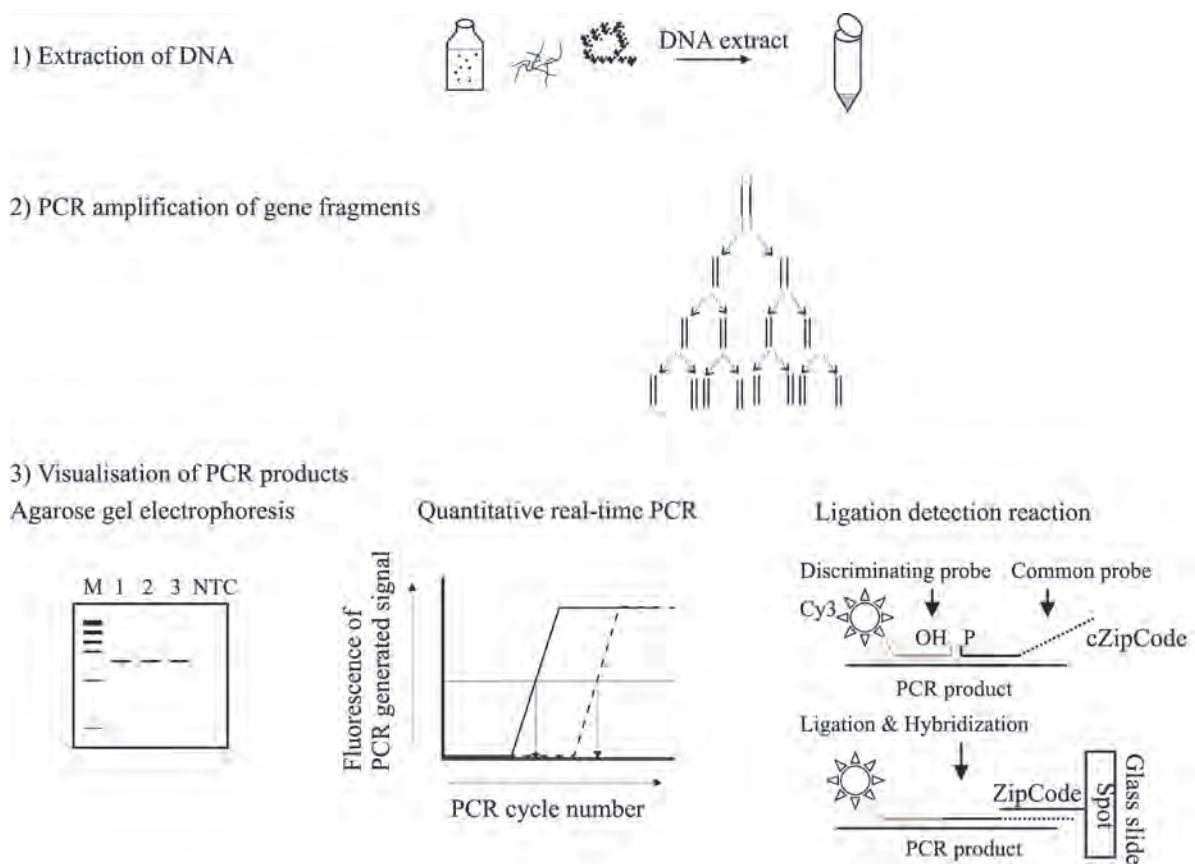


Fig. 8. Work flow of a genetic analysis of water samples or isolated colonies of Cyanobacteria to aid risk assessment: 1) extraction of the DNA from phytoplankton and benthic algae collected on filters or single cyanobacterial filaments or colonies isolated under the microscope; 2) PCR amplification using short oligonucleotides (primers) targeted to gene fragments indicative of toxin production; 3) visualisation of PCR products by means of DNA staining and agarose gel separation, quantitative real-time PCR, or the ligation detection reaction (LDR) coupled to a DNA chip (NTC, nontemplate control).

permopsin-producing Cyanobacteria, specifically *Cylindrospermopsis raciborskii* (Fergusson & Saint, 2003).

Due to the high sensitivity of PCR based methods, there is the potential to detect toxic genotypes in water a long time before the occurrence of a cyanobacterial bloom and detectable toxin concentrations. 'Cryptic' toxin-producing Cyanobacteria, for example those not forming a visible surface bloom or those growing on the sediments of lakes and rivers, could also be identified by PCR based methods. Consequently, waterbodies or habitats at risk of toxic bloom formation could be identified early on in the growing season. Although genetic methods are only able to indicate the potential of toxin synthesis, this early warning could result in more efficient surveillance

of waterbodies, with monitoring effort being focused on those waterbodies that have been found to have both toxin-producing genotypes and a high risk of cyanobacterial bloom formation. A few studies have indeed shown a significant relationship between the abundance of specific toxin genotypes and the concentration of the respective toxin: in one, the concentration of nodularin was found to be related to the *ndaF* gene copy number (Koskeniemi et al., 2007) and in another, the concentration of microcystin was found to be linearly related to the concentration of the *mcyD* genotype in *Microcystis* (Hotto et al., 2008). More investigations are needed, however, to confirm the significance of the relationship

between genotype numbers and the respective toxin concentration in recreational water and in drinking water.

As an alternative to the manual set-up of PCR, the automation of genetic methods, including DNA isolation from field samples and the quantitative detection of genotypes via real-time PCR, might be possible in the near future. Recently, Rasmussen et al. (2008) developed a real-time PCR assay, detecting *Cylindrospermopsis* and three genes encoding cylindrospermopsin production, that was optimised both on a fixed and a portable device. Their results showed that the reliable limit of detection for the assay was 100 copies per reaction or 1000 cells ml⁻¹. Fully automated real-time PCR systems, providing results within 3 h, are available for monitoring concentrations of pathogens such as *Legionella* and *Salmonella* in the environment. If these techniques were also applicable to Cyanobacteria in the field, results could be transferred directly from the sampling site to surveillance authorities and reduce the time and effort required for sample processing.

Conclusions

The recent finding that microcystin synthesis evolved during ancient times and has not been influenced by horizontal gene transfer events recently but has a clonal dependence, has important implications for environmental studies, water management and risk assessment. One implication is that geographic patterns in microcystin production may occur due to linkage disequilibrium (predominantly clonal growth). Linkage disequilibrium has already been observed among microcystin/nodularin-producing Cyanobacteria both across distances of hundreds of kilometres (Barker et al., 2000; Tanabe et al., 2007; Christiansen et al., 2008) as well as over short distances of a few kilometres (Kurmayer & Gumpenberger, 2006).

Since the evolution of the microcystin synthesis gene clusters appears to be relatively slow, it follows that the same *mcy* genotype can occur within a single waterbody for many years. Possible seasonal shifts in *mcy*-containing and *mcy*-free genotypes among populations of Cyanobacteria may occur, however they are not necessarily related directly to microcystin production (Christiansen

et al., 2008; Yoshida et al., 2008). It is unlikely that in a single habitat a population of a cyanobacterial species changes from microcystin-producing to completely non microcystin-producing and vice versa during seasonal development within short periods (months) of time.

Although knowledge concerning the application of genetic techniques to cyanotoxins has increased enormously during the last decade, the use of these techniques for environmental studies, water management and risk assessment is still in its infancy. Since it is the prerequisite for all PCR based methods that the respective biosynthetic genes are known, the elucidation of gene clusters encoding the synthesis pathways of cyanotoxins other than microcystin will provide the basis for widening the genetic approach. The development of automated and field-applicable methods will contribute to a more simplified integration of genetic tools into routine monitoring programmes.

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