

Toxic cyanobacteria: the evolving molecular toolbox

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Toxic cyanobacteria are a diverse and widely distributed group of organisms that can contaminate natural and man-made bodies of water. Anthropogenic eutrophication can exacerbate the risks, allowing toxic cyanobacteria to grow unchecked and resulting in harmful algal blooms with potentially serious economic and health-related impacts. Predicting bloom events is an important goal of monitoring programs and is of fundamental interest to those examining the ecology of aquatic ecosystems. While microscopic identification and toxin analysis have traditionally been employed for monitoring purposes, molecular biological methods may provide rapid and sensitive diagnoses for the presence of toxic and toxigenic cyanobacteria, and are useful for general ecological studies. The molecular toolbox of ecologists and resource managers is evolving rapidly. Current techniques and their applications will help bring about a better understanding of the ecology of these events.

Front Ecol Environ 2003; 1(7): 359–366

Harmful algae include eukaryotes and prokaryotes that persist in both freshwater and marine systems. They range from organisms that form red and brown tides to cyanobacteria that turn lakes and ponds green (Figure 1) and red. The toxins (termed “cyanotoxins”) that these organisms produce are usually secondary metabolites, and are more numerically diverse than the organisms that produce them. Here we will focus on the use of molecular methods for detecting toxic cyanobacteria, as in-depth discussions of marine harmful algae, cyanobacterial ecology, cyanotoxin production, toxicology, and water management issues related to toxic cyanobacteria (Watanabe *et al.* 1996; Paerl and Millie 1996; Chorus and Bartram 1999; Whitton and Potts 2000; Carmichael 2001; Chorus 2001; Hoagland *et al.* 2002; Landsberg 2002) are beyond the scope of this review.

The global distribution of toxic cyanobacteria attests to their ecological success, which at times causes problems for humans. Communities often use surface water and reservoirs for potable water. When these sources are subject to algal blooms, nontoxic taste and odor compounds that may be released by the cyanobacteria can compromise water

quality. When the organism in question produces a toxin, the threat can be considerable. Although the health effects of exposure to low levels of cyanotoxins remain unknown, chronic exposure to toxic cyanobacteria in water sources over a long period of time could be harmful (Chorus and Bartram 1999; Carmichael 2001; Chorus 2001).

The detection of toxic cyanobacteria and their cyanotoxins is therefore fundamental for sound water management. Traditionally, microscopic identification of cyanobacteria has been used alone or in combination with direct analysis for toxins. The morphological discrimination between toxic and nontoxic cyanobacteria can be difficult, as some genera contain both toxic and nontoxic members. Some cyanobacteria are known to be toxic, some may be genetically capable of producing toxins (toxigenic) but do not under all conditions, and some do not produce toxins at all. DNA-based detection methods have become popular because of their potential specificity (targeting genes involved in toxin biosynthesis), sensitivity, and speed. This review highlights some of these advances

In a nutshell:

- Many cyanobacteria produce toxins, which can poison waterways
- While algal toxins in marine systems receive much attention, cyanobacteria are a growing threat to freshwater systems
- Molecular methods are increasingly popular for detecting toxic cyanobacteria, as well as for identifying the conditions that affect toxin production
- Characterizing microbial community structure and function is critical to the management of our shared water resources

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Figure 1. Scum-forming cyanobacterial blooms (left) in Chautauqua Lake, NY, and (right) in the north basin of Lake Winnipeg, Canada. This *Aphanizomenon* (right) bloom tested positive for microcystins (H Kling, pers comm).

and the use of molecular tools to detect, identify, and study toxic cyanobacterial ecology.

■ Cyanotoxins and other secondary metabolites

The structural diversity of known cyanotoxins includes many variants of alkaloids and cyclic peptides. A variety of cyanobacteria produce one or more cyanotoxins (Table 1). The two most common types of cyanotoxins are the microcystins and nodularins, of which numerous structural variants have been characterized (Rinehart *et al.* 1994; Carmichael 1997; Sivonen and Jones 1999). These cyclic peptides exert their toxic effects by inhibiting certain protein phosphatases. The cyclic peptides and some of the alkaloids are produced non-ribosomally by large enzyme complexes. Other non-ribosomal peptides from bacteria and fungi are well known, and include enterobactin, cyclosporin-a, and erythromycin.

■ Ecological roles for cyanotoxins?

Can the production of cyanotoxins confer an ecological advantage? Their biosynthesis is an energetically demanding process, and the functions of cyanotoxins are unclear. While “cyanotoxin” is a fitting term for these molecules, their toxic properties may have nothing to do with their functions.

Understanding conditions that regulate cyanotoxin production may shed light on potential functions. Conditions investigated include varying nutrients (eg phosphorus, nitrogen, and iron), light, and temperature. The literature contains an abundance of such studies, which at times conflict (see Watanabe *et al.* 1996; Chorus and Bartram 1999; Kaebernick and Neilan 2001; Chorus 2001). Most of the efforts to understand the regulation of toxin production have relied on analyses of the toxins themselves, which have yielded many useful studies. However, if cellular processes exist that make the toxin difficult to identify and quantify (for example, if the cyanotoxin also exists in a modified form), then analysis may miss this pool of the total “toxin”. Using a variety of molecular methods to monitor the expression of genes and proteins involved in cyanotoxin biosynthesis is particularly appealing as a way to help uncover the ecological relevance of cyanotoxins. In this review we will focus on *Microcystis* and its cyanotoxin, microcystin.

Table 1. Cyanotoxins and their producers. A range of structural variants has been identified for the various toxins. For each genus listed, toxic and nontoxic strains are known to exist. For details, see Rinehart *et al.* (1994), Sivonen and Jones (1999), and Li *et al.* (2001)

Cyanotoxin	Known toxin producers
Hepatotoxins	
Microcystins	Microcystis, Planktothrix, Nostoc, Anabaena, Anabaenopsis
Nodularins	Nodularia
Cylindrospermopsis	Cylindrospermopsis, Aphanizomenon, Umezakia, Raphidiopsis
Neurotoxins	
Anatoxin-a	Anabaena, Planktothrix, Aphanizomenon
Anatoxin-a(S)	Anabaena
Saxitoxins	Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Planktothrix
Dermatotoxins	
Lyngbyatoxin-a	Lyngbya
Aplysiatoxins	Lyngbya, Schizothrix, Planktothrix

■ Toxin biosynthesis genes offer molecular targets

Microcystin biosynthesis genes (*mcy* genes) have been completely sequenced from strains of *Microcystis* (Nishizawa *et al.* 1999; Tillett *et al.* 2000; Nishizawa *et al.* 2000) and one strain of *Planktothrix* (Christiansen *et al.* 2003). Knowing these sequences allows us to design oligonucleotide probes for the specific detection of these genes. While some *Microcystis* species are not known to produce toxins, all *Microcystis* strains containing the microcystin genes should be viewed as potential microcystin producers (ie toxigenic).

■ Toxins, genes, and proteins

In order to gain a complete picture of toxin production in response to various environmental conditions and ecological situations, we need quantitative and qualitative information on regulation at the gene, mRNA, and protein levels, as well as toxin quantification. Recent transcriptional analysis of *mcyB* and *mcyD* genes has revealed differential expression in response to light quality and quantity, growth phase, and chemical stressors (Kaebernick *et al.* 2000). For both genes, increased light levels resulted in greater expression and red light was more effective than white light; chemical stressors such as sodium chloride or methylviologen decreased expression of *mcyB*. Molecular methods were also used to reveal the existence and response to light of multiple, alternative *mcy* messages (Kaebernick *et al.* 2002).

In an intriguing study, Dittmann *et al.* (2001) found that a microcystin deficient mutant (*-mcyB*) of *Microcystis* exhibited altered gene and protein expression as compared to wildtype. The proteins identified are similar to *Rhizobium* Rhi proteins, whose levels are controlled by a quorum-sensing regulator (Rodelas *et al.* 1999). This link between microcystin biosynthesis and potential quorum-regulated genes, combined with the existence of a puta-

tive microcystin transporter protein (Tillett *et al.* 2000), supports the idea that microcystin might function in sensing (Dittmann *et al.* 2001).

Allelopathic interactions (when chemicals produced by one species inhibit growth in another species) between *Microcystis* and the dinoflagellate *Peridinium*, which naturally co-occur, were recently identified (Vardi *et al.* 2002; Sukenik *et al.* 2002). Using antibodies against *mcyB*, it was revealed that spent *Peridinium* growth media caused an increase in detectable *mcyB* protein, concomitant with the death of the majority of the *Microcystis* cells (Vardi *et al.* 2002).

These molecular studies, which elucidate the regulation of genes involved in or linked to microcystin biosynthesis, are adding to our understanding of the regulation, physiology, and ecology of microcystin. In addition to illuminating bacteria's ecological significance, delineating what regulates toxin production may be of great value for predicting, preparing for, or preventing harmful algal events.

■ Molecular ecology and microbial diversity

Molecular biology techniques often used for detecting and identifying microbial assemblages in the environment are shown in Figure 2. While we focus on aquatic habitats, these techniques are also applied to samples obtained from a variety of environments, including soil, ice, cyanobacterial mats, and even dental biofilm.

Molecular methods for studying microbial diversity and activity have gained widespread popularity and have revolutionized microbiology, in large part because they can produce data without the need to cultivate the organisms in question. Many of the methods involve the amplification of genes, using the polymerase chain reaction (PCR) technique (Saiki *et al.* 1988). PCR uses primers, complimentary to portions of the gene of interest, as starting points for DNA replication by a thermostable DNA polymerase. (The nucleotide sequences of the primers are designed for the desired specificity.) If done properly, the result is the exponential amplification of the genetic fragment of interest. Gel electrophoresis and staining can then be used to separate and visualize the PCR products.

The choice of which genes to amplify depends on the questions one wants to ask. Carl Woese led a phylogenetic revolution by employing ribosomal RNA (rRNA) gene sequences, thereby revealing a phylogenetic tree in which life is structured within three domains: the Eucarya, Bacteria, and Archaea (Woese 1987). He argued that nucleotide mutation rates in rRNA genes were slow and constant enough that these genes can be used as molecular timers. Since then, the use of gene sequences to define, discover, and otherwise understand evolutionary relationships has become common (Pace 1997; Woese 2000). For prokaryotes, the 16S rRNA gene is the most widely used for identification. It has become routine to isolate DNA from complex environmental samples and, using PCR, to amplify the mixture of 16S rRNA genes using various "uni-

versal" primer sets which, for example, target appropriately conserved regions of the gene. Much can be done with this basic starting platform, including assessing diversity by using gene fingerprinting methods, analyzing the sequences of the amplified genes, and quantifying the number of genes in the sample. Sequence analysis has become increasingly easy owing to the falling cost of sequencing and the increasing availability of bioinformatics resources and free databases containing tremendous numbers of sequences.

Bioinformatics is the mathematical analysis of the information stored in both the structure and function of nucleic acids and proteins. Simply put, it is the use of computer programs to analyze nucleic acid and protein sequences to reveal identity, similarity, structure, function, etc. For example, a sequence can be submitted for Basic Local Alignment Search Tool (BLAST) analysis, which queries a database for similar sequences and returns the best matches (Altschul *et al.* 1997). BLAST can be used to predict the phylogeny of a genetic element, and therefore the organism it came from. The DNA sequence can also be aligned to other sequences corresponding to the same gene, allowing a phylogeny (a "family tree") to be constructed.

■ Molecular detection of toxic cyanobacteria

The number of publications using molecular methods to detect toxic cyanobacteria is rapidly increasing. Before the sequences of the microcystin biosynthesis gene cluster were published, DNA-based methods for the detection and phylogenetic analysis of toxic cyanobacteria were investigated (Neilan 1995; Neilan 1996; Rudi *et al.* 1998a; Rudi *et al.* 1998b; Neilan *et al.* 1999). Recently sequenced microcystin biosynthesis genes provide very specific molecular targets (Nishizawa *et al.* 1999; Tillett *et al.* 2000; Nishizawa *et al.* 2000). These sequences are being used throughout the world for the design and construction of primer sets for PCR-based toxin gene detection (Schatz *et al.* 2000; Baker

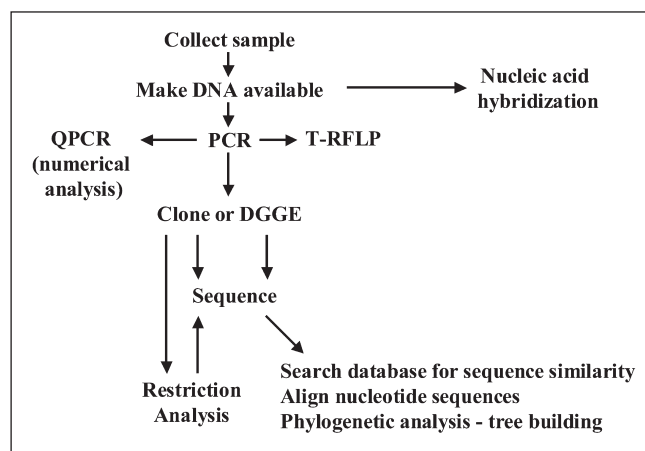


Figure 2. Some common DNA-based approaches for microbial detection, genetic fingerprinting, and identification: denaturing gradient gel electrophoresis (DGGE), polymerase chain reaction (PCR), quantitative PCR (QPCR), and terminal restriction fragment length polymorphism (T-RFLP).

et al. 2001; Tillett *et al.* 2001; Nonneman and Zimba 2002; Pan *et al.* 2002; Baker *et al.* 2002; Kurmayer *et al.* 2002). This approach is appealing as an early warning diagnostic, and is very sensitive because of the amplification achieved by PCR. Following the sequencing and publishing of genes involved in other cyanotoxin biosynthetic pathways, molecular methods will be developed to detect these genes. Potential identification of genes involved in the biosynthesis of the toxins nodularin (Moffitt and Neilan 2001) and cylindrospermopsin (Schembri *et al.* 2001) have already been reported.

■ A multilevel, multiplex approach

We are interested in the analysis of cyanobacterial community structure and the detection of *Microcystis*, discriminating between toxic and nontoxic varieties (Figures 3). The use of various phylogenetic probes provides us with this differentiating specificity. We are currently investigating three levels of desired sensitivity in an attempt to understand the dynamics of cyanobacterial communities that contain *Microcystis*:

- (1) Rapid detection of toxic and nontoxic *Microcystis*
- (2) Qualitative evaluation of the community (fingerprinting and clone library analysis)
- (3) Quantitative analysis of the cyanobacterial community structure by determining the relative abundance ratio of cyanobacteria to total *Microcystis* to toxic *Microcystis*.

We collect microorganisms from water using filtration, and extract DNA immediately or from frozen filters, allowing us to analyze it (Figure 2). In this paper we illustrate the applications of some of these methods by presenting data from a pond in Knoxville, TN, in the summer and fall of 2002. We first chose this pond because of the green color of the water, usually a first indication of bloom problems.

Light micrographs and corresponding chlorophyll autofluorescence micrographs of several cyanobacterial strains are shown in Figure 3a–3l. Morphology cannot always distinguish toxic strains from nontoxic strains. In Figure 3m–3s, representative experiments are shown to illustrate the PCR-based detection of cyanobacteria (CYA), *Microcystis* (MIC), and microcystin biosynthesis genes (*mcyB* and *mcyD*) PCR products. CYA products are generated for all cultures, while MIC generates products only from *Microcystis* cultures. Amplicons (the resulting products of a PCR amplification) from *mcyB* and *mcyD* are produced only in the *Microcystis* cultures reported to produce microcystin. While this *Planktothrix* strain (Figure 3f, l, r) is described as toxic, it is not known to produce microcystins. Using these methods, we have conducted PCR on over 20 cyanobacterial cultures, and the results demonstrate a high degree of specificity. By using the same primers as other research groups (Urbach *et al.* 1992; Neilan *et al.* 1997; Kaebernick *et al.* 2000; Nonneman and Zimba 2002), it will be possible to make a

more direct comparison of data generated from various sites by different researchers on different continents.

All the samples from the Knoxville pond tested positive for cyanobacteria, *Microcystis*, and at least one toxin gene (Figure 3s), showing that toxic *Microcystis* was present throughout the summer and into the fall, the latest sampling point. In most environmental samples we have analyzed, the relative intensity of MIC, *mcyB*, and *mcyD* bands are not approximately equal, as is seen for cultures containing only one algal species (compare Figure 3p and 3q to 3s). The differential amplification of environmental samples may indicate the presence of both nontoxic and toxic *Microcystis*. Also, complex DNA extracted from environmental samples may contain compounds that interfere with some or all of the PCR reactions. While all of the data in Figure 3r are reproducible in terms of the presence of PCR products, the relative intensity of some of these products varies from experiment to experiment.

While microscopy cannot always distinguish between toxic and nontoxic *Microcystis*, this relatively quick PCR approach provides the required resolving power by targeting the toxin genes directly. Sample collection, DNA extraction, and PCR analysis can be accomplished in 12 hours. DNA sequence analysis of the bands can be undertaken subsequently, to confirm identity.

When enough data are available, the specificity of molecular probes used for routine monitoring can be tailored to suit different geographical regions. We will be able to use a more specific set of probes to target specific toxic organisms during routine monitoring of water bodies of interest. A more comprehensive set of probes can be employed from time to time, or during a bloom event, to assess the presence of any new toxic organisms. As improved probes or detection methods become available, frozen samples or extracted DNA can be probed years later.

■ Cyanobacterial community analysis

PCR provides a rapid approach (Figure 3) for the detection of toxic *Microcystis*, and is potentially a very valuable tool for understanding the geographical and temporal distribution of the organism. It can also be used in the analysis of the cyanobacterial communities with and without *Microcystis*, allowing us to assess the relationships within communities that influence its presence and/or proliferation. Comparing cyanobacterial communities from a single water body over time may reveal a temporal fluctuation of *Microcystis* that correlates with community structure changes. Both fingerprinting and quantitative approaches are being developed for this purpose.

■ Genetic fingerprinting

Two commonly used genetic fingerprinting methods that allow comparisons of microbial communities are denaturing gradient gel electrophoresis (not discussed here) and terminal restriction fragment length polymorphism (T-RFLP) analysis

(Avaniss-Aghajani *et al.* 1994; Liu *et al.* 1997). For the latter, PCR is carried out using one or both primers labeled with a fluorophore (a detectable fluorescent tag). The DNA from the resulting PCR band is made up of a mixture of various DNA sequences from different organisms. A restriction enzyme is then used to cut the DNA at specific positions within certain sequences. When restriction fragments from a mixed microbial community PCR reaction are separated by electrophoresis, and the fluorophore is monitored, a series of different sized peaks (representing the labeled terminal fragments) can be seen. As an illustration, Figure 4 shows T-RFLP data comparing the Knoxville pond to Sandusky Bay (Lake Erie). The data in blue and green are from the two ends of the DNA molecule. The number and sizes (in base pairs) of the DNA fragments illustrates the diversity in the communities.

■ The molecular librarian

To identify members of a cyanobacterial community, clone libraries can be constructed and analyzed. In this approach, mixed community DNA is amplified using PCR with the same primers as for T-RFLP, but without the fluorophore. For sequence analysis, the different cyanobacterial sequences in the single PCR band must be physically separated. To accomplish this the PCR products are used to generate clonal libraries, with each clone representing a unique PCR product. After sequencing, the data can be used to infer the phylogeny and identity of the organism originally present in the water sample. A picture of the cyanobacterial community begins to emerge as the identities of the organisms are revealed. While this technique does not ensure the identification of all the cyanobacteria present, it does give an indication of the community structure, and is used frequently for microbial community analysis.

For the Knoxville pond, clone libraries were generated from two samples. A number of clones from each sample were analyzed by restriction fragment length polymorphism (RFLP) mapping, to identify unique clones. Like T-

RFLP, this procedure yields different sized fragments from sequences that are appropriately different. Each unique pattern of bands on a gel is defined as an individual operational taxonomic unit (OTU). As an example of some of the restriction patterns we observed, Figure 5 shows a portion of an agarose gel within which seven of the digested clones were subjected to gel electrophoresis. For these seven clones, there are six unique patterns. For the 77 clones analyzed, 18 different OTUs were identified

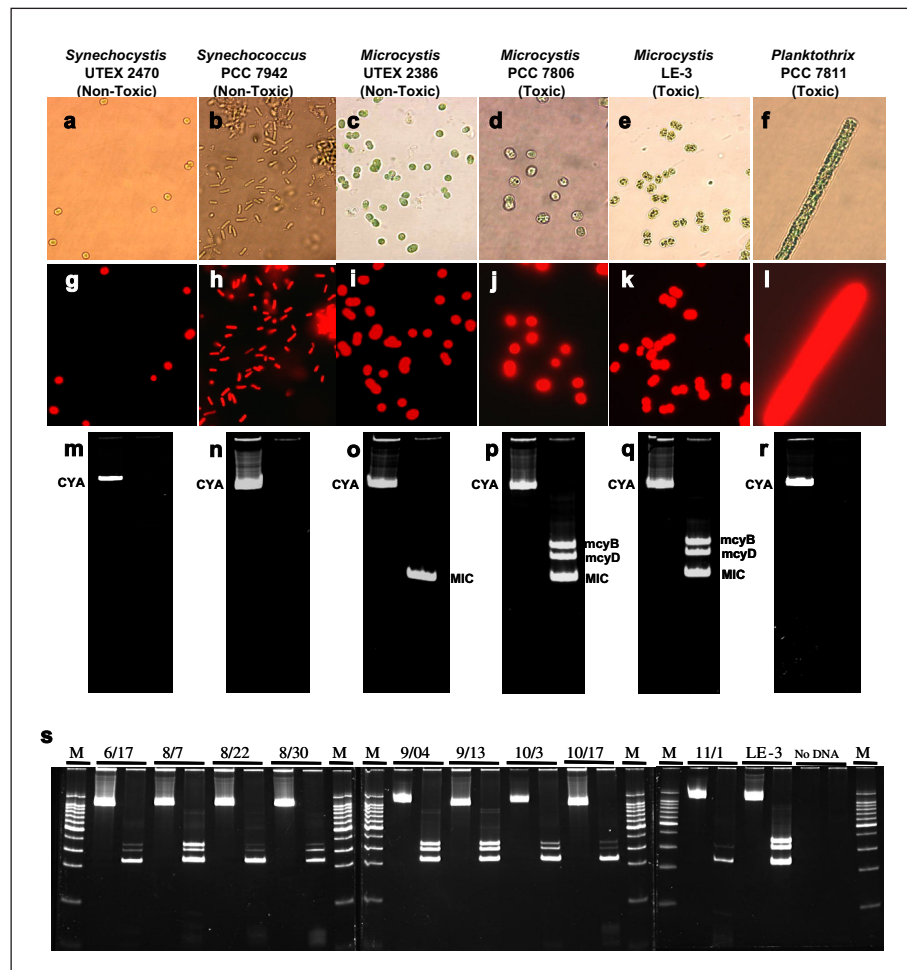


Figure 3. Microscopic and PCR methods for detecting and identifying toxic and nontoxic cyanobacteria. (a–f) Detection of *Synechocystis* and *Synechococcus* (both nontoxic), nontoxic *Microcystis*, two toxic strains of *Microcystis*, and toxic *Planktothrix* using light microscopy, (g–l) autofluorescence microscopy and (m–r) PCR. (s) Samples from a local pond tested positive for toxic *Microcystis* using PCR and gel electrophoresis. In (s), LE-3 (from Lake Erie) was used as the positive control and water was used as the negative control. The presence or absence of appropriate sized PCR bands for cyanobacterial 16S rRNA genes (CYA), *Microcystis* 16S rRNA genes (MIC), and toxin genes *mcyD* and *mcyB* demonstrates the selectivity of the different primers (Urbach *et al.* 1992; Neilan *et al.* 1997; Kaebnick *et al.* 2000; Nonneman and Zimba 2002). Although toxic, the *Planktothrix* PCC 7811 is not known to produce microcystins. The M in panel (s) denotes 100 base pair DNA markers. The same PCR conditions were applied for the cyanobacterial cultures (m–r) and the environmental samples (s), except that lysed whole cell material was used for the cultures, and genomic DNA was used for the environmental samples. Cyanobacterial cultures originated from the Pasteur Culture Collection of Cyanobacteria, the Culture Collection of Algae at the University of Texas, or from Lake Erie (Brittain *et al.* 2000).

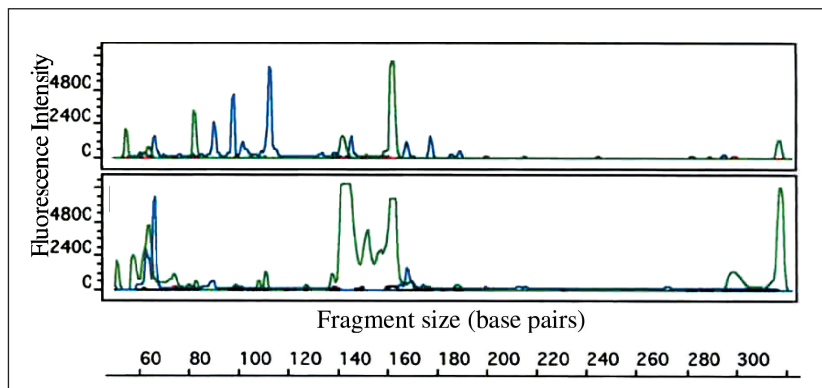


Figure 4. T-RFLP analysis of 16S rRNA PCR fragments from environmental water samples. Data from (top) Sandusky Bay, Lake Erie, July 2002 and (bottom) the Knoxville pond, August 2002 illustrate this profiling technique for oxygenic photosynthetic plankton. Blue and green denote forward and reverse primers, respectively.

(Figure 6 inset), of which OTU 1 was the most frequently observed. Sequences were generated from representative clones and aligned (based on sequence similarity) with sequences from known cyanobacterial cultures and database entries, to generate a phylogenetic tree (Figure 6). The Knoxville pond sequences were also searched using BLAST. Sequence similarities to known organisms that are greater than 90% are indicated by the genus (Figure 6). The primers used target 16S rRNA genes from cyanobacteria (CYA) (Figure 3), but also amplify 16S rRNA genes from eukaryotic algae chloroplasts, as well as some heterotrophic bacteria.

Although all the pond samples tested positive for *Microcystis* 16S rRNA genes and *Microcystis* toxin genes (Figure 3s), *Microcystis* sequences were not obtained from the clone libraries using the CYA primers. Clone library analysis and T-RFLP give qualitative pictures concerning the community makeup. For the relative number of *Microcystis* to total cyanobacteria, we use quantitative, real-time PCR (Higuchi *et al.* 1993) based on the Taq exonuclease approach (Holland *et al.* 1991). Using primer/probe sets with differing specificities (as in Figure 3), we can quantify the number of total cyanobacteria, *Microcystis*, and toxin genes.

Our preliminary results indicate that abundance of *Microcystis* as compared to total cyanobacteria is 0.1–2.1% for all of the Knoxville pond samples. This approach enables us to understand the abundance of *Microcystis*, both toxic and nontoxic, compared to the total cyanobacterial community. Less than 1.3% of the analyzed CYA clone libraries should contain *Microcystis* sequences (from the quantitation data), so it is not surprising that our sequenced CYA clones do not contain *Microcystis* sequences. This shows that the common and useful technique of clone library generation and analysis can miss less abundant organisms. We sequenced clones from *Microcystis* 16S rRNA and toxin gene libraries, and all of the sequences were 98–100% identical to the expected genes, confirming that the appro-

priately sized PCR bands are indeed from the targeted genes, and that toxic *Microcystis* are present. Thus, combining complementary quantitative and qualitative approaches using different specificity primers, we are able to evaluate the communities at many different levels.

■ Conclusions and future directions

Here we have illustrated the applications of some PCR-based methods for toxic cyanobacterial detection and identification. Speed, price, ease of use, specificity, and detection limits are essential factors in the application of these techniques for routine water monitoring. These tools have the potential to reveal temporal dynamics and successions for impor-

tant organisms that are present in small numbers and may be overlooked by microscope. The detection approaches discussed here (Figure 3) are a component of a newly funded monitoring effort of the lower Great Lakes (Monitoring and Event Response for Harmful Algal Blooms – Lower Great Lakes), where toxic cyanobacteria and cyanotoxins are of increasing concern. Microscopic identification of toxic and nontoxic algae is an important component of water monitoring and ecosystem characterization, and is an integral component of any harmful algae monitoring program.

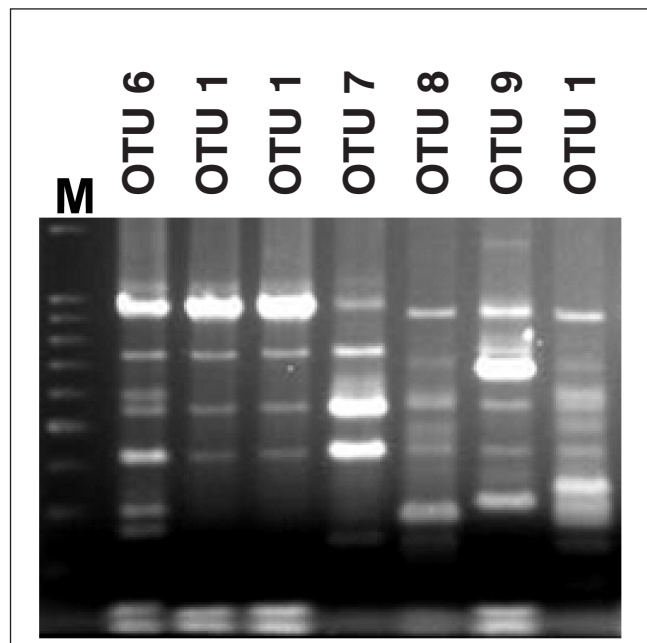


Figure 5. Restriction analysis of 16S rRNA genes from cloned planktonic organisms. Cyanobacterial-like 16S rRNA genes from environmental clones were amplified, digested with the restriction enzyme *DdeI*, then subjected to electrophoresis on an agarose gel and stained with ethidium bromide. “M” denotes DNA standards (100 bp ladder). Of the seven clones depicted, there are six unique operational taxonomic units (OTUs), as determined by the different banding patterns.

Development of these techniques will continue and will include the numerous organisms associated with harmful algal blooms. Environmental factors that affect toxin production are of key importance for understanding the ecology of toxic cyanobacteria, as well as predicting toxic events. To that end, it is essential to monitor toxin gene expression and the abundance of toxin biosynthesis proteins, in response to a wide range of physical, chemical, and biotic factors. The integration of functional genomics, proteomics, metabolomics, and toxin analysis from laboratory and field studies will afford great insight into the ecology of toxic cyanobacteria.

Acknowledgements

We thank Melanie Eldridge, Sara Handy, and Leo Poorvin. This article includes research supported by funds from the American Water Works Association Research Foundation (AWWA #2818), the National Oceanic and Atmospheric Administration Coastal Ocean Program (MERHAB-LGL, #NA-160 P2788), and the National Science Foundation (DEB-0129118).

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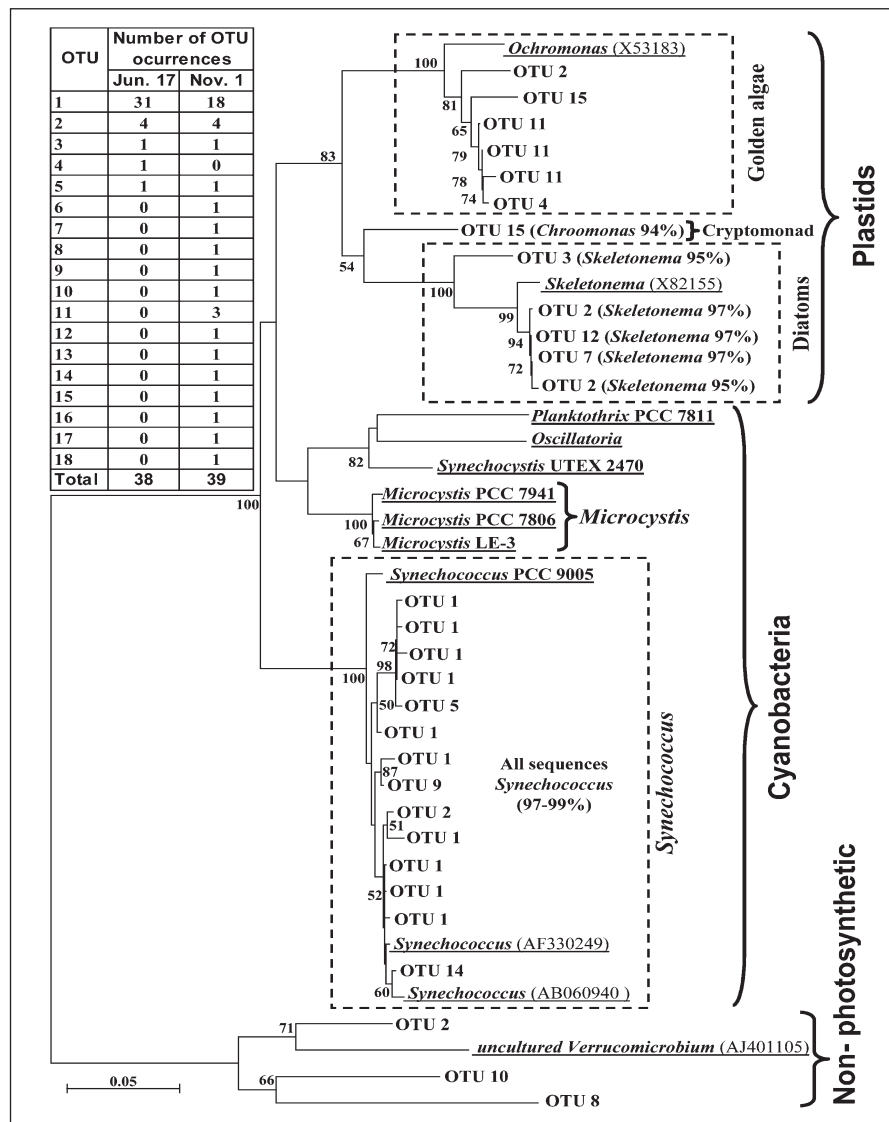


Figure 6. Phylogenetic tree created using partial 16S rRNA gene sequences from environmental clone libraries, cultures, and sequence database entries. Of the 77 environmental clones, 29 were sequenced, and the assigned OTUs from restriction analysis of the clones are denoted. The inset lists the number of times the individual OTUs were found from each clone library (June 17 and November 1, 2002). Sequences from cultures and database entries are underlined, and database entries have the accession number in parentheses. BLAST results are shown only if the search yielded a sequence over 90% similar to known organisms. Sequences were aligned using PileUp software, then manually edited. Neighbor-joining analysis was then conducted using the Mega2 program (Kumar et al. 2001). The numbers at the branches represent bootstrap values greater than 50% from 5000 replications. Scale bar indicates number of substitutions per site.

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