Chapter

Overview of PCR Methods Applied for the Identification of Freshwater Toxigenic Cyanobacteria

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Abstract

Although cyanobacteria are essential microorganisms on earth, some cyanobacteria produce toxins known as cyanotoxins, threatening humans and animals' health. Hence, it is imperative to rapidly and accurately identify those toxic cyanobacteria. Unfortunately, traditional microscopic methods have limitations for accurate identification due to the lack of discernable morphological difference between toxic and non-toxic strains within the same cyanobacterial species or genus. In contrast, their genetic profiles are inherently conserved; therefore, nucleic acid-based assays can be more reliable for precise identification. Furthermore, molecular assays can provide high throughput and significantly reduce the turnaround time of test results. Such advantages make those assays a preferred method for rapid detection and early warning of potential toxicity. Toxigenic cyanobacterial species have synthetase genes (DNAs) for toxin production, which can be excellent marker genes. Numerous molecular assays targeting cyanotoxin synthetase genes have been developed for the identification of toxigenic cyanobacteria at various taxonomic levels. Polymerase chain reaction (PCR)-based assays are the most prevailing. Among different versions of PCR assays, the real-time quantitative PCR can be utilized to quantify the genes of interest in samples, fulfilling the purpose of both taxonomic recognition and biomass estimation. Reverse transcription (RT)-PCR assays can be used to detect transcripts (i.e., mRNAs) from toxin synthetase genes, probably enhancing the predictive value of PCR detection for toxin production from observed cyanobacterial species. Nevertheless, the utility of toxin synthetase gene- or its transcript-based PCR assays for routine cyanotoxin monitoring needs to be further evaluated on a large scale.

Keywords: cyanobacteria, cyanotoxins, toxin synthetase genes, molecular techniques, polymerase chain reaction

1. Introduction

Cyanobacteria are essential microorganisms on earth as they produce oxygen and account for a large part of primary aquatic productivity. Simultaneously, some freshwater cyanobacteria can produce various toxins, named cyanotoxins, some of which are potently poisonous to humans and animals. A well-known cyanotoxicosis

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in humans was reported from Brazil in association with medical malpractice in 1996. In this incident, 126 patients in a hemodialysis unit were affected, and 60 of them died due to using microcystin-contaminated water from a local reservoir. A cyanobacterial bloom was found in that reservoir concurrently [1]. Besides, there have been reports concerning human cyanotoxin poisoning by drinking water or via injury after contacting recreational water [2]. Apart from humans, numerous animal poisoning cases have also taken place because they can reach the unprocessed natural water directly so that the risk of being poisoned becomes higher. These cases involve livestock, pets, and wildlife [3–10].

Cyanobacterial blooms occurred more frequently in recent years, which may have been attributed to the aggravating eutrophication in freshwater and global warming. As such, cyanotoxin poisoning incidents have also been increasingly reported. Nowadays, freshwater cyanobacterial blooms have broader geographical and temporal impacts on local water bodies that act as vital municipal or agricultural water supplies. With the possibility of cyanotoxin contamination, humans and animals residing in surrounding areas continue to be threatened. Therefore, testing for toxic cyanobacteria or cyanotoxins is imperative for detection and preventive measures.

Although cyanobacteria can be observed under a microscope, their toxigenicity cannot be determined by microscopy because the toxigenic cyanobacteria do not have unique morphological characteristics. Some laboratories have adopted a testing strategy that combines microscopic observation and cyanotoxin detection to indicate the existence of toxigenic cyanobacteria in samples. Although this strategy may seem reasonable and pragmatic, it needs collaboration between chemical analysts and microalgal biologists to reach an agreement on the conclusion. Furthermore, it neglects the complex phenomena of the same toxin production by different species or genera, leading to an incorrect judgment of the truly culpable toxin producers.

Cyanotoxin testing has been in place. Yet, available tests have shortcomings. For example, commercial enzyme-linked immunosorbent assays (ELISAs) have been widely employed in water testing for cyanotoxins. However, it still has issues, such as low sensitivity [11] or inaccuracy. Erroneous detection is due to the cross-reactivity of isomorphic substances with targets. False-positive results can occur in a worst-case scenario [12]. The high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC–MS) are the most accurate analytical methods and have been often employed in cyanotoxin testing [11, 13–16]. But they require exquisite instruments and complicated operations, making them not as affordable as ELISA-based testing. Aside from these limitations, chemical testing can only tell the presence and/or quantity of cyanotoxins without identifying the toxin producer(s). However, it is crucial to recognize the existence of toxigenic cyanobacteria in water bodies for monitoring and early warning of cyanotoxin poisoning incidents.

It is known that cyanotoxin synthesis is catalyzed by a string of relevant enzymes encoded by toxin synthetase genes [17–23]. Lack of essential genes for forming a toxin backbone or disruption of the enzymatic cascade toward toxin production results in the failure of toxin synthesis. Therefore, the detection of toxin synthetase genes in samples by a molecular test can disclose the presence or absence of toxigenic cyanobacteria. In this chapter, we review the application of molecular techniques, particularly PCR-based assays, for detecting toxigenic cyanobacteria in freshwater.

2. General genomic organization of toxigenic cyanobacteria

Like other bacteria, cyanobacteria often have one circular chromosome and a few plasmids that consist of the whole genome. The cyanobacterial chromosome is

a few megabases in size and contains most of the genes, while plasmids play a role in transferring DNA elements. Compared to the eukaryotic microalgae, the cyanobacterial genome is highly compressed but still contains all genes essential for aquatic and photosynthetic life. Some species even have genes that can facilitate competitive superiority in the environment. For example, gas vesicle genes in *Planktothrix* spp. encode structural proteins that can form gas vesicles, endowing the cells with more buoyancy to the water's surface to gain more sunlight (i.e., solar energy) [24]. In addition, cyanotoxins were found effective in suppressing the growth of non-toxic species so that the toxigenic cyanobacteria have more survival advantages [25].

Cyanotoxin synthetase genes often cluster together in the genome and constitute one or more operons that are transcribed in identical or opposite directions [19, 21–23, 26]. The reason for such an arrangement is likely that the transcription can be well regulated so that all pertaining genes are transcribed simultaneously. This process may ensure that all necessary enzymes/proteins are present for subsequent toxin synthesis. The whole-genome sequencing of toxic cyanobacteria to date has demonstrated only a single copy of the toxin gene cluster in the cyanobacterial genome [27–29]. The toxin synthetase genes have conserved sequences encoding conserved domains/motifs in the corresponding proteins with specific functions during toxin syntheses, such as polyketide synthesis, adenylation, and methylation. The genes are always clustered closely with whose proteins conduct successive functions in a cascade reaction. It should be reiterated that the synthetase genes are indispensable for toxin production, making them the ideal targets for molecular detection.

Cyanotoxins are traditionally named after the first identified toxin-producing genus, as in the case of microcystin (*Microcystis*), anatoxin-a (*Anabaena*), cylindrospermopsin (*Cylindrospermopsis*), and so on. However, many different genera can produce the same cyanotoxin, indicative of the fact that these intergeneric toxic species have similar genetic elements for toxin production. For example, microcystin and microcystin synthetase genes (*mcy*) are reportedly found in *Microcystis*, *Anabaena*, *Planktothrix*, and *Aphanizomenon* [30]. Nevertheless, the gene clusters are disparate between genera regarding sequences, gene numbers, constitutions, and relative loci [17, 20, 26, 31, 32]. Such a characteristic is believed to be caused by divergent evolution from the common ancestors [33] or horizontal gene transfer [34]. Therefore, PCR identification of toxigenic cyanobacteria is usually designed at the genus level, although there have been reports of detecting multiple genera producing the same cyanotoxin based on the conserved intergeneric sequences [35].

3. Cyanotoxins and toxin biosynthesis

3.1 Microcystin

Microcystin is the most common cyanotoxin implicated in human and animal poisoning incidents [36–38]. It is a hepatotoxin and thus can cause severe impairment in the liver when ingested by the casualties. The toxin is known to be produced by several genera of cyanobacteria, such as *Microcystis*, *Anabaena*, and *Planktothrix*, to name a few.

Microcystin is a cyclic heptapeptide that inhibits the eukaryotic protein phosphatase type 1 and 2A in humans and animals by forming an irreversible covalent bond to a cysteine in the catalytic domain of these enzymes. It consists of the following amino acids: D-alanine, X, D-MeAsp (D-erythro-ß-methyl-aspartic acid), Z, Adda ((2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), D-glutamic acid, and Mdha (N-methyldehydroalanine). X and Z

represent variable L amino acids. It has reportedly over 80 variants, mostly differing in amino acids at the positions X and Z [39].

Microcystin is a non-ribosomal oligopeptide, which means unlike most of the peptides and proteins, it is not synthesized by cellular ribosomes. The enzymes responsible for its synthesis contain the non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) modules as well as tailoring functional domains. All the enzymes are the protein products encoded by the microcystin synthetase genes (*mcy*) that cluster together in the genome (**Table 1**, **Figure 1**). In *Microcystis*, ten *mcy* genes (*mcyA-J*) span 55 kb near the center of its 5.8 Mb circular chromosome and form two operons (*mcyABC* and *mcyD-J*) of which the transcription proceeds in discretely opposite directions [26, 40]. The 55.4 kb *mcyA-J* gene cluster in *Anabaena* also form two operons (*mcyABC* and *mcyG-DJEFIH*), one of which the gene order differs from *Microcystis* [31]. In contrast, *Planktothrix* has a 55.6 kb *mcy* cluster including eight essential genes (*mcyABC-DEGHJ*) that form a single operon and one unique gene (*mcyT*), and the arrangement and sequence of specific domains in the gene products differ from those in other genera [17].

Per annotation of *mcy* genes, microcystin biosynthesis is initiated by McyG to covalently bind a phenylacetate precursor that is then methylated by McyJ. Next, McyD elongates the growing chain by accepting a malonyl-CoA, and McyE introduces another malonyl-CoA and further extends the backbone of microcystin. As a racemase, McyF is involved in either the supply of D-glutamate or D-MeAsp, or the peptidyl epimerization of L-glutamate. Then McyA captures an L-serine and installs it into the growing chain, and the Mdha moiety is synthesized by McyI. Following the addition of an amino acid into position X and a D-MeAsp by McyB, McyC adds the last amino acid into position Z. Finally, mature microcystin is formed by the cyclization of the linear precursor. McyH, as an ATP-binding cassette (ABC) transporter, may be accountable for the transmembrane export of

Gene	Size (bp)[1]	Encoded domain or function[2]	Existence in different genera		
			Microcystis	***Anabaena***	***Planktothrix***
mcyA	8838	NRPS, C, NMT, E	yes	yes	yes
mcyB	6318	NRPS, A, T, C	yes	yes	yes
mcyC	3876	NRPS, C, A, TE	yes	yes	yes
mcyD	11721	PKS, KS, AT, KR, DH, ACP, CM	yes	yes	yes
mcyE	10464	PKS, NRPS, KS, AT, ACP, CM, AMT	yes	yes	yes
mcyF	756	Racemase	yes	yes	no
mcyG	7896	NRPS, PKS, KS, AT, CM, DH, KR, ACP	yes	yes	yes
mcyH	1617	Transporter	yes	yes	yes
mcyI	1014	Dehydrogenase	yes	yes	no
mcyJ	837	OM	yes	yes	yes
mcyT	< 1000	TE	no	no	yes

[1]*Values are from Microcystis aeruginosa PCC7806 [26] except mcyT from Planktothrix agardhii CYA 126 [17].*
[2]*NRPS, non-ribosomal peptide synthetase; C, condensation; NMT, N-methyltransferase; E, epimerization; T, thiolation; TE, thioesterase; PKS, polyketide synthase; KS, β-ketoacyl synthase; AT, acyltransferase; KR, β-ketoacyl reductase; DH, dehydratase; ACP, acyl carrier protein; CM, C-methyltransferase; AMT, aminotransferase; OM, O-methyltransferase.*

Table 1.
Comparison of microcystin synthetase genes.

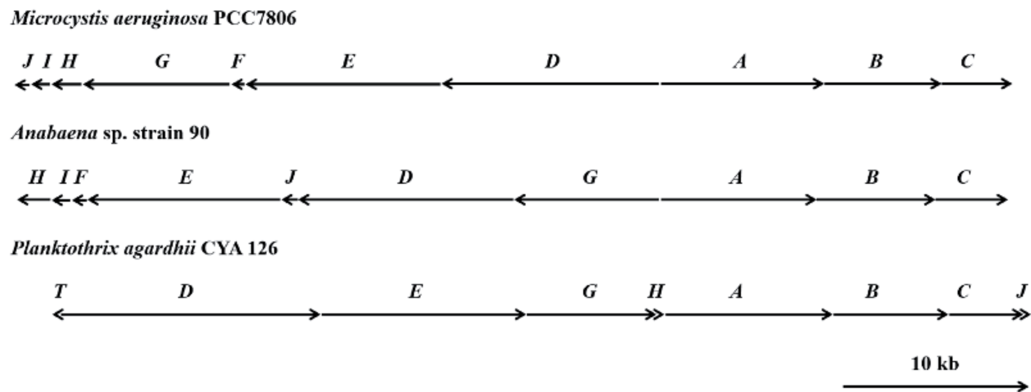

Figure 1.
The microcystin synthetase gene (mcy) clusters of Microcystis aeruginosa PCC7806, Anabaena sp. strain 90, and Planktothrix agardhii CYA 126. Each gene is indicated by its designated letter above the arrows, and genes shorter than 500 bp are drawn as an arrowhead only. The diagram only exhibits the size and position of each gene without the distances between two adjacent genes. The measuring scale arrow represents 10 kb.

microcystin. The *Planktothrix*-exclusive McyT is a putative thioesterase that may edit the synthesis by removing mis-primed amino acids from the NRPS and PKS enzymes.

3.2 Anatoxin-a

The cyanobacterial alkaloid anatoxin-a has been found in different genera, such as *Anabaena*, *Oscillatoria*, *Phormidium*, and *Cylindrospermum* [30, 41]. It is a neurotoxin that can bind to the neuronal nicotinic acetylcholine receptors and affect signal transmission between neurons and muscles as a nicotinic agonist [42]. By persistently stimulating the receptors to release signals for muscular contraction, the toxin eventually leads to respiratory arrest until victims' death in a few minutes. Homoanatoxin-a, in which a methyl group displaces a hydrogen atom at the end of the straight chain of anatoxin-a, is a natural analog of anatoxin-a and is also a potent nicotinic agonist.

Although anatoxin-a doesn't look structurally complicated, its synthesis still requires a cascade of many enzymes whose genes known as anatoxin-a synthetase genes (*ana*) have distinctive arrangements and sequences across genera like *mcy* genes (**Table 2**, **Figure 2**). For example, the toxic *Anabaena* has two clusters (20.3 kb *anaBCDEFG* and 1.7 kb *anaIAJ*) located 6.9 kb apart and transcribed in head-to-head opposite directions. The first cluster contains two operons (*anaBCDEF* and *anaG*) with a 288 bp gap between the operons [19, 32]. In contrast, the *ana* genes in *Oscillatoria* compose a ~ 23 kb cluster *anaJABCDEFGH* and a single upstream *anaI* gene transcribed in the opposite direction [32, 43]. *Cylindrospermum* has the most complicated arrangement of *ana* genes. The *anaIAHJ* and *anaBCDKEFG* are clustered together and are transcribed in oppositely separative directions. The *anaH*, however, is transcribed reversely in the small *anaIAHJ* cluster.

To start the anatoxin-a synthesis, AnaC activates and tethers the precursor proline to AnaD, which covalently combines with the proline. Then AnaB dehydrogenates the heterocyclic ring of proline to form a "C=N" double bond. AnaE introduces a carbonyl group into its connection with the heterocycle passed from AnaD. Then AnaJ catalyzes a cyclization step to form the characteristic bicyclic ring structure of anatoxin-a by connecting the heterocyclic ring with the backbone. At the same time, the growing chain is bound to the acyl carrier protein domain of AnaF. Finally, the bicyclic thioester is transferred to AnaG for chain extension

Gene	Size (bp)[1]	Encoded domain or function[2]	Existence in different genera		
			Anabaena	*Oscillatoria*	*Cylindrospermum*
anaA	750	TE	yes	yes	yes
anaB	1143	Proline-ACP oxidase	yes	yes	yes
anaC	1596	Proline adenylation	yes	yes	yes
anaD	273	Acyl carrier	yes	yes	yes
anaE	6438	PKS, KS, AT, DH, ER, KR, ACP	yes	yes	yes
anaF	5619	PKS, KS, AT, DH, KR, ACP	yes	yes	yes
anaG	4896	PKS, KS, AT, CM, ACP	yes	yes	yes
anaH	< 1000	Transposase	no	yes	yes
anaI	< 2000	Transporter	yes	yes	yes
anaJ	723	Cyclase	yes	yes	yes
anaK	<1000	Reductase	no	no	yes

[1]*Values are from Anabaena sp. Strain 37 [19] except anaH from Oscillatoria sp. PCC 6506 [32] and anaK from Cylindrospermum stagnale PCC 7417 [41].*
[2]*TE, thioesterase; ACP, acyl carrier protein; PKS, polyketide synthase; KS, β-ketoacyl synthase; AT, acyltransferase; DH, dehydratase; ER, enoylreductase; KR, ketoreductase; CM, C-methyltransferase.*

Table 2.
Comparison of anatoxin-a synthetase genes.

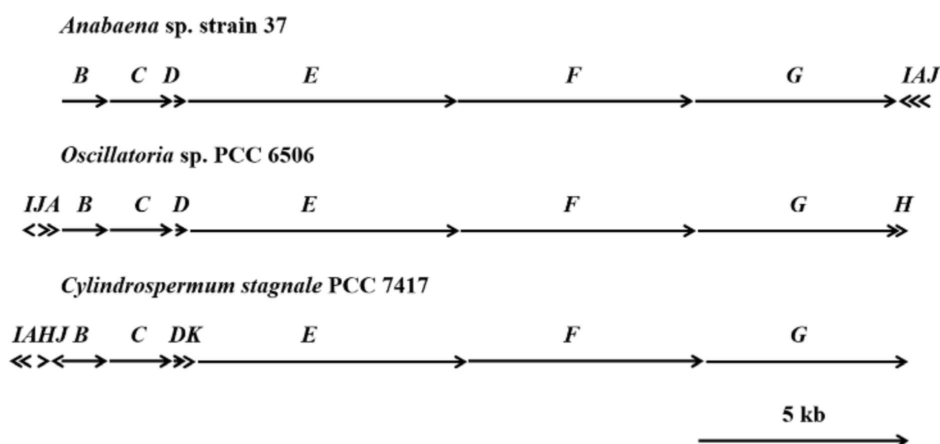

Figure 2.
The anatoxin-a synthetase gene (ana) clusters of Anabaena sp. strain 37, Oscillatoria sp. PCC 6506, and Cylindrospermum stagnale PCC 7417. Each gene is indicated by its designated letter above the arrows, and genes shorter than 250 bp are drawn as an arrowhead only. The diagram only exhibits the size and position of each gene without the distances between two adjacent genes. The measuring scale arrow represents 5 kb.

by adding an acyl group, followed by the enzymatic reaction of AnaA to break the single "SCO-C" covalent bond connecting the enzyme (AnaG) and final product for the completion and releasing of anatoxin-a. Similar to its counterpart McyH in microcystin-producing cyanobacteria, AnaI transports the toxin through the cytomembrane. The rest of the Ana proteins are not commonly shared across different genera and have their own functions. AnaH is a transposase only found in *Oscillatoria* and *Cylindrospermum* instead of *Anabaena*, implicating the toxic cyanobacteria in the former two genera were endowed with the toxin genes by intergeneric replicative transposition mechanism. *Cylindrospermum* has a unique AnaK that functions in further modification of anatoxin-a into dihydroanatoxin-a. The whole process is inferred as per the functional annotation of *ana* genes.

3.3 Cylindrospermopsin

Cylindrospermopsin can be produced by various cyanobacterial genera, such as *Cylindrospermopsis*, *Aphanizomenon*, *Anabaena*, and *Oscillatoria* [20, 30]. It is a cyclic sulfated guanidine alkaloid and can lead to cytotoxic, hepatotoxic, and neurotoxic impacts. Its molecule contains a central functional guanidino moiety, a hydroxymethyluracil ring, and a hetero tricyclic ring. The mechanism of its toxicity lies in many aspects, including the inhibition of glutathione and protein synthesis, the inhibition of cytochrome P450, and direct interaction with DNA [44–47].

Cylindrospermopsin is synthesized via a string of NRPS/PKS reactions conducted by up to over a dozen Cyr proteins (**Table 3**, **Figure 3**). The cylindrospermopsin synthetase genes (*cyr*) are clustered together but are also distinctive with respect to numbers, sequences, and organization in different genera. The toxic *Cylindrospermopsis* has the cluster *cyrDFGIKLHJABECMNO* in which *cyrK*, *cyrM*, and *cyrN* are transcribed in the opposite direction of the rest genes [22]. The *cyr* cluster in toxic *Aphanizomenon* and *Oscillatoria* is *cyrJMKIGFDCABE* and *cyrDC-orf1-cyrH-trp1-cyrKAB-trp2-cyrEJFGIN*, respectively [20, 48].

Gene	Size (bp)[1]	Encoded domain or function[2]	Existence in different genera		
			Cylindrospermopsis	*Aphanizomenon*	*Oscillatoria*
cyrA	1176	AMT	yes	yes	yes
cyrB	8754	NRPS, PKS, PCP, KS, AT, DH, MT, KR, ACP	yes	yes	yes
cyrC	5005	PKS, KS, AT, KR, ACP	yes	yes	yes
cyrD	5631	PKS, KS, AT, DH, KR, ACP	yes	yes	yes
cyrE	5667	PKS, KS, AT, DH, KR, ACP	yes	yes	yes
cyrF	4074	PKS, KS, AT, ACP	yes	yes	yes
cyrG	1437	Uracil ring formation	yes	yes	yes
cyrH	1431	Uracil ring formation	yes	yes	yes
cyrI	831	Hydroxylation	yes	yes	yes
cyrJ	780	Sulfotransferase	yes	yes	yes
cyrK	1398	Exporter	yes	yes	yes
cyrL	750	Transposase	yes	no	no
cyrM	318	Transposase	yes	no	no
cyrN	600	Adenylylsulfate kinase	yes	no	yes
cyrO	1548	Regulator	yes	no	yes
orf1	152	ATP-grasp protein	no	no	yes
trp1	404	Transposase	no	no	yes
trp2	299	Transposase	no	no	yes

[1]*Values are from Cylindrospermopsis raciborskii AWT205 [22] except orf1, trp1, and trp2 from Oscillatoria sp. Strain PCC 6506 [20].*
[2]*AMT, aminotransferase; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase; PCP, peptidyl carrier protein; AT, acyltransferase; DH, dehydratase; MT, methyl transferase; KR, ketoreductase; ACP, acyl carrier protein; KS, β-ketoacyl synthase.*

Table 3.
Comparison of cylindrospermopsin synthetase genes.

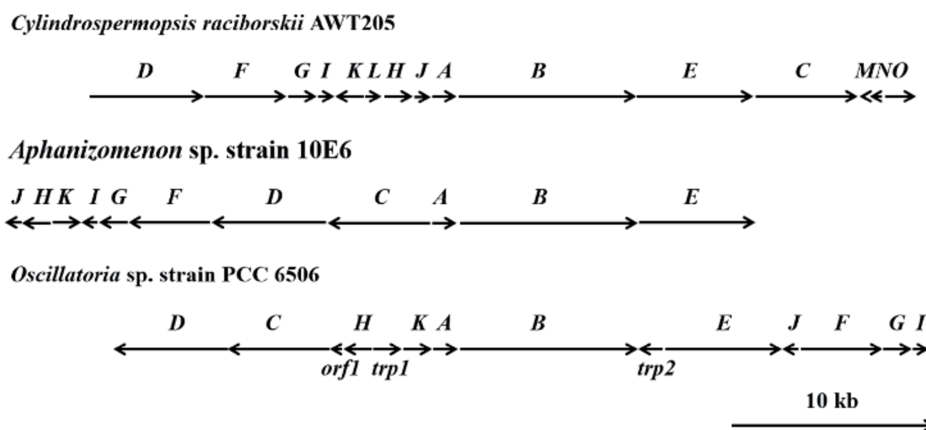

Figure 3.
The cylindrospermopsin synthetase gene (cyr) clusters of Cylindrospermopsis raciborskii AWT205, Aphanizomenon sp. strain 10E6, and Oscillatoria sp. strain PCC 6506. Each gene is indicated by its designated letter above the arrows, and genes shorter than 500 bp are drawn as an arrowhead only. The diagram only exhibits the size and position of each gene without the distances between two adjacent genes. The measuring scale arrow represents 10 kb.

As *cyr* genes have been annotated with exact biological functions, the cylindrospermopsin biosynthesis is inferred as follows. CyrA conducts the transamidination of an L-arginine to a glycine to form a guanidinoacetate. Then the product is activated by CyrB, and the first ring of the tricyclic structure is formed in the end. CyrC elongates the growing chain by an acetate via activation of a malonyl-CoA and its condensation with the chain. CyrD and CyrE catalyze the formation of the second and third rings of the tricyclic rings. CyrF accepts and extends the growing chain by adding an acetate. Next, CyrG and CyrH carry out the formation of the uracil ring, and CyrJ and CyrN together catalyze the sulfation at the hydroxyl group in the hetero tricyclic ring. At last, CyrI completes cylindrospermopsin synthesis by introducing a hydroxyl group to the carbon atom between the rings. In addition, CyrK is an exporter and resembles the function of McyH and AnaI. CyrO has diverse regulatory and signal transduction roles. The transposases CyrL and CyrM are exclusively found in *Cylindrospermopsis*. In comparison, *trp1* and *trp2* genes encode unique transposases in *Oscillatoria*, and the *orf1* gene, that is unique in *Oscillatoria*, codes for an ATP-grasp protein.

3.4 Nodularin

Nodularin is a cyclic pentapeptide and has the identical chemical structure as microcystin except the lack of D-alanine and the amino acid at position X. The mechanism of its toxicity is the same as microcystin's, i.e., inhibiting the eukaryotic protein phosphatase catalytic subunit type 1 and 2A and leading to severe liver damage. Different from the three aforementioned cyanotoxins, nodularin is solely found in *Nodularia* and synthesized by the nodularin synthetase gene (*nda*) cluster that contains two operons, *ndaAB* and *ndaDEFGHI*, transcribed in opposite directions (**Table 4**, **Figure 4**) [23]. Because of the remarkable resemblance with *mcy* clusters with a few missing regions and genes, *nda* genes could be regarded as the degenerative *mcy* genes.

Nodularin synthesis is conducted putatively according to the annotated functions of each Nda protein. NdaC activates the starter unit as phenylalanine or phenylacetate, and then NdaE catalyzes the transfer of a methyl group to the growing chain. NdaD is involved in two further polyketide extension steps, and NdaF facilitates the final round of polyketide extension and the biosynthesis of

Gene	Size (bp)[1]	Encoded domain or function[2]
ndaA	2607	NRPS, A, NM, PCP, C
ndaB	1299	C, A, PCP, TE
ndaC	2640	NRPS, PKS, A, PCP, KS, AT, CM, KR, ACP
ndaD	3872	PKS, KS, AT, CM, DH, KR, ACP
ndaE	927	OM
ndaF	3475	PKS, NRPS, KS, AT, CM, ACP, AMT, C, A, PCP
ndaG	235	Racemase
ndaH	341	D-3-phosphoglycerate dehydrogenase
ndaI	601	ABC transporter

[1]Values are from Nodularia spumigena strain NSOR10 [23].
[2]NRPS, non-ribosomal peptide synthetase; A, adenylation; NM, N-methyltransferase; PCP, peptidyl carrier protein; C, condensation; TE, thioesterase; PKS, polyketide synthase; KS, ketosynthase; AT, acyltransferase; CM, C-methyltransferase; KR, ketoreductase; ACP, acyl carrier protein; DH, dehydratase; OM, O-methyltransferase; AMT, aminotransferase.

Table 4.
Comparison of nodularin synthetase genes.

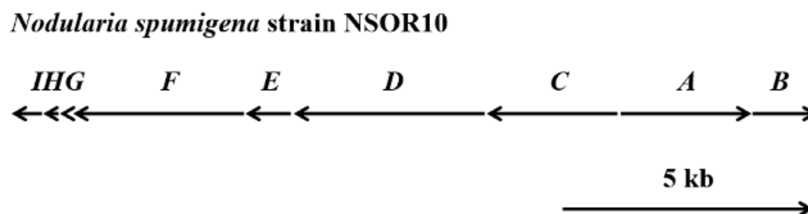

Figure 4.
The nodularin synthetase gene (nda) clusters of Nodularia spumigena strain NSOR10. Each gene is indicated by its designated letter above the arrows, and genes shorter than 250 bp are drawn as an arrowhead only. The diagram only exhibits the size and position of each gene without the distances between two adjacent genes. The measuring scale arrow represents 5 kb.

Adda. Next, epimerization of L-glutamic acid is catalyzed by NdaG, followed by the peptide condensation carried out by NdaA and NdaB. During the condensation, NdaH participates in the conversion of N-methyl-L-threonine (MeThr) to N-methyldehydrobutyrine (MeDhb) with a cofactor nicotinamide adenine dinucleotide (NADH). Finally, the mature peptide chain is cyclized by NdaB and released from the enzyme-substrate complex. As an ABC-transporter, NdaI is responsible for the transmembrane transportation of nodularin for extracellular excretion.

4. PCR detection of toxic cyanobacteria

PCR-based assays have been most commonly utilized in molecular identification studies because the assays are able to recognize targets accurately. The assays incorporate oligonucleotide primers explicitly designed for complementary sequences of the target gene(s). Two types of PCR methods have been used: conventional gel-based PCR and real-time PCR. In general, the real-time PCR has higher sensitivity (i.e., detect a low amount of the target) than the conventional PCR. The real-time PCR also offers better specificity than the conventional PCR since it uses an additional oligonucleotide known as a probe, which is complementary to sequences between primer-binding sequences.

Furthermore, the real-time PCR allows estimating the number of the intended target in samples when performed with standards with a known copy number of the target sequences. This procedure is referred to as quantitative real-time PCR (qPCR). In addition, reverse transcription (RT)-PCR or RT-qPCR platforms have been utilized for specifically detecting transcripts (i.e., mRNAs) from the target genes of cyanobacteria. Typically, PCR can be completed within one or two hours, much shorter than the traditional analytical methods and microscopy mentioned above.

4.1 Microcystin-producing cyanobacteria

The molecular identification of microcystin-producing cyanobacteria has been conducted using nearly all *mcy* genes; nonetheless, most studies have selected *mcyA*, *B*, *D*, and *E* as the target genes for *Microcystis*. Tillett et al. designed PCR primers from the *N*-methyltransferase domain of *mcyA* gene of *Microcystis*, evaluated those primers on 37 *Microcystis* strains with and without toxin production and found the molecular outcomes were significantly in concordance with the toxicity of each strain [49]. Kurmayer et al. designed *Microcystis*-specific primers based on *mcyB* gene and observed that the proportion of toxic cells in the overall *Microcystis* population correlated positively with the size of *Microcystis* colonies [50]. Using *mcyD* as an indicator, Kaebernick et al. reported that light had a positive effect on the transcriptional response of *mcy* gene cluster in *Microcystis* over specific threshold intensities [51].

Although most publications have been concerned about toxic/toxigenic *Microcystis*, there are reports of identifying other toxigenic genera with their *mcy* genes. Toxic *Planktothrix* in a French lake was identified and quantified by qPCR using primers devised in the condensation and adenylation domains of *mcyA* gene but was accounted for only 54% of the variation in microcystin levels [52]. Mbedi et al. reported the use of a highly variable region in *mcyE* encoding the adenylation domain to design primers specific to *Planktothrix*, which were validated by 46 *Planktothrix* strains in a conventional PCR assay [53]. Vaitomaa et al. developed two *mcyE*-based qPCR assays specific to *Anabaena* and *Microcystis*, respectively, and utilized them to investigate the two toxic genera in two Finnish lakes. They concluded the microcystin concentrations correlated positively with the sum of *Anabaena* and *Microcystis mcyE* gene copy numbers [54]. Ngwa et al. also applied *mcyE*-based primers to specific detection of *Planktothrix* and *Microcystis*, respectively, using the qPCR and RT-qPCR assays and found a significant positive correlation between microcystin concentrations and abundances of *mcyE* genes rather than transcripts from the *mcyE* genes [55].

The rest of the *mcy* genes have been less often used for molecular detection in comparison to the four genes above. Yuan et al. developed a conventional PCR method for detecting toxigenic *Microcystis* based on *mcyC*. They demonstrated a good correlation between the presence of this gene and microcystin in water samples from farm ponds [56]. Mbedi et al. and Ouahid et al. used *mcyG* with a few other *mcy* genes as the targets for the recognition of toxigenic *Planktothrix* and *Microcystis*, respectively. They performed a multiplex PCR using the primers targeting *mcyG* and *mcyD* on field colonies and showed the same outcome as lab cultures, suggesting that simultaneous amplification of several gene regions was feasible [53, 57]. *mcyJ* has been used for the quantification of microcystin-producing *Microcystis* genotypes via qPCR in ecological investigations in China and Korea. Interestingly, Zhang et al. reported a weak correlation of gene numbers versus toxin concentrations, whereas Joung et al. found a strong correlation [58–60]. Although *mcyT* was chosen in the specific detection of toxigenic *Planktothrix* due to its uniqueness,

the gene was also found in non-toxigenic strains in the same study, negating its candidacy for specific molecular detection of toxigenic *Planktothrix* [53]. No reports of using *mcyF* and *mcyI* as a target for a molecular assay have been made yet. These genes are not common in all *mcy* clusters disclosed to date, thus not good candidates for the detection purpose to cover a wide range of microcystin-producing cyanobacteria. As *mcyH* codes for a transporter that is not necessary for microcystin biosynthesis, the gene has not been generally considered as a suitable target for the identification of toxigenic cyanobacteria.

With increased bioinformatic data related to *mcy* genes in multiple cyanobacterial genera, the molecular identification of microcystin-producing cyanobacteria has proceeded to multi-genera detection. Conserved domains in *mcy* genes provide adequate genetic information for searching out consensus sequences across different toxic genera for multi-generic molecular identification. Hisbergues et al. designed a pair of primers from the condensation domain of *mcyA* that could facilitate the detection of microcystin-producing cyanobacteria including *Anabaena*, *Microcystis*, and *Planktothrix* by PCR, and the toxin producer could be recognized at the genus level by combining PCR with the restriction fragment length polymorphism assay [61]. Hautala et al. found consensus sequences in *mcyB* among *Anabaena*, *Microcystis*, and *Planktothrix*, devised specific primers and genus-specific probes for qPCR assays and demonstrated the positive correlation between gene copy numbers and toxin concentrations [62]. On the contrary, Ye et al. employed these primers in a survey of the cyanobacterial population producing microcystin to assess their dynamics and concentrations in a lake in China and found no correlation between gene copies and toxin concentrations [63]. Beversdorf et al. selected *mcyE* and *mcyA* as the genes of interest for designing primers specific for *Microcystis*, *Planktothrix*, and *Anabaena* in their qPCR assay and concluded *mcy* genes were not a good indicator of microcystin in the environment [35].

There are a few unidentified open reading frames (ORFs) flanking the *mcy* cluster, which may have relevant functions in microcystin synthesis, such as the *dnaN* and *uma* in *Microcystis* [26]. Nevertheless, these ORFs have not been used as targets for detecting the toxigenic cyanobacteria, probably due to their undetermined roles. The only example is that Tillet et al. obtained the corresponding amplicons by a PCR using *uma1* primers from 20 *Microcystis* strains and found the physical distances in the genome were consistent between *uma1* and *mcyC* across all strains [49].

4.2 Anatoxin-a-producing cyanobacteria

The *ana* genes have been widely used for genus-specific detection of anatoxin-a producing cyanobacteria via PCR. For *Anabaena*, Legrand et al. set up a nested-PCR assay based on *anaC* gene for detecting the toxigenic planktonic *Dolichospermum* (previously known as *Anabaena*) and averted possible none-amplification in a few anatoxin-a-producing strains due to less stringent specificity [64]. For *Aphanizomenon*, Ballot et al. confirmed the discovery of an anatoxin-a-producing strain in a German lake by detecting the production of the toxin from and the existence of *anaF* in the cyanobacteria using a PCR with the primers designed out of the gene [65]. For *Oscillatoria*, the *anaG* region encoding the methylation domain was amplified and sequenced for the strains producing anatoxin-a and homoanatoxin-a isolated from a French river [66]. For *Phormidium*, Wood et al. detected *anaF* gene in 20 strains isolated from two rivers in New Zealand tested positive for anatoxin-a, homoanatoxin-a, dihydroanatoxin-a, or dihydrohomoanatoxin-a, disclosing a total agreement between the presence of toxins and the existence of genes [67]. However, such an agreement was not observed in all toxigenic strains when Rantala-Ylinen

et al. chose *anaC* as the gene of interest and designed genus-specific primers for *Oscillatoria* and *Anabaena*, respectively [19].

Like *mcy* gene cluster, *ana* has also been used for multi-generic detection for anatoxin-a-producing cyanobacteria by PCR. For instance, Rantala-Ylinen et al. designed *anaC* primers specific for three toxigenic genera, *Anabaena*, *Oscillatoria*, and *Aphanizomenon*. They revealed the presence of both *Anabaena* and *Oscillatoria* as potential anatoxin-a producers in Finnish freshwaters and the Baltic Sea [19].

4.3 Cylindrospermopsin-producing cyanobacteria

Molecular detection of cylindrospermopsin-producing cyanobacteria has been mostly reported for *Cylindrospermopsis*. Burford et al. established a qPCR method based on the *cyrA* gene to detect the toxigenic *Cylindrospermopsis* in field blooms in an Australian reservoir. They found the increase in cell quotas (i.e., toxin amount per cell) of cylindrospermopsin correlated with the increase in the proportion of *cyrA*/16S rDNA in the blooms [68]. Moreira et al. directly applied the K18/M4 primer set that Fergusson et al. [69] designed based on *cyrC* to evaluate toxigenic *Cylindrospermopsis* abundance and toxicological potential by qPCR in a lake in Portugal. They found only one out of ten samples were positive for *cyrC* and cylindrospermopsin [70]. Another pair of *cyrC* primers cyl2/cyl14 was designed to recognize toxigenic *Cylindrospermopsis* via PCR by Wilson et al. [71]. That primer set was utilized by Marbun et al. in the on-site monitoring of the toxic cyanobacteria in reservoirs in Taiwan using qPCR [72]. The authors revealed good accordance between cylindrospermopsin concentrations and toxigenic *Cylindrospermopsis* cell numbers.

Multi-generic detection of cylindrospermopsin-producing cyanobacteria was reported as well. Campo et al. found that *cyrJ* is the gene suitable for designing primers and probes and established a Taqman qPCR assay for specific detection of toxigenic *Aphanizomenon* and *Cylindrospermopsis*. The presence of the *cyrJ* gene in cyanobacteria was in concordance with the toxin production, as revealed by testing 11 experimental strains [73]. Fergusson et al. designed primers out of *cyrC* regions encoding polyketide synthase and peptide synthetase and combined them into a multiplex PCR. The PCR was able to identify the cylindrospermopsin-producing *Cylindrospermopsis*, *Anabaena*, and *Aphanizomenon*. In their study, the complete matching of positive/negative detection of gene versus toxin was shown by testing of 39 related strains [69].

There are also a few ORFs flanking the *cyr* cluster that encode proteins related to cylindrospermopsin synthesis, as shown with microcystin. Nevertheless, these ORFs have not been evaluated in detecting the toxic cyanobacteria due to their unidentified or unnecessary roles in toxin biosynthesis.

4.4 Nodularin-producing cyanobacteria

Since *nda* gene clusters are only found in *Nodularia*, all molecular identification studies were developed for this genus. Kruger et al. devised 11 pairs of primers for all nine *nda* genes in a comparative PCR study with toxigenic and non-toxigenic *Nodularia* strains and discovered that the lack of toxicity was caused by the absence of all the *nda* genes [74]. Koskenniemi et al. aimed at *ndaF* gene for primer design and set up a qPCR method for the detection of toxigenic *Nodularia* spp. in the Baltic Sea. A significant positive correlation was found between *ndaF* gene copy numbers and nodularin concentrations, referring to a relatively constant toxin production [75]. To investigate the expression of *nda* genes in a bloom-forming *Nodularia* strain

from the Baltic Sea, Jonasson et al. designed nine pairs of primers for the nine *nda* genes and used them in an RT-qPCR assay. They observed that all genes were continuously expressed during growth. Still, the intracellular and extracellular nodularin concentrations did not vary significantly in contrast to the shifts in gene expression, indicating unknown regulatory mechanisms acting on the enzyme activity level and regulating the biosynthesis and/or the maturation of nodularin [76].

5. Other cyanotoxins and PCR detection of the toxic cyanobacteria

Apart from the four most commonly reported cyanotoxins mentioned above, there are a few other cyanotoxins, such as saxitoxin, lyngbyatoxin, guanitoxin, β-N-methylamino-L-alanin (BMAA), aplysiatoxin, and lipopolysaccharide [18, 77, 78]. Hitherto, only the gene clusters for the biosynthesis of saxitoxin and lyngbyatoxin have been characterized.

Saxitoxin belongs to the group of carbamate alkaloid toxins composed of a tetrahydropurine group and two guanidinium moieties [79] and can also be produced by marine phytoplankton [80]. It can cause paralytic shellfish poisoning syndrome and afflict human health via bioaccumulation. At least 30 clustered saxitoxin synthesis genes (*sxt*) have been reported to be involved in the biosynthesis of saxitoxin, which might be the most complicated within all known cyanotoxins [81]. Saxitoxin production has been found in multiple cyanobacteria genera, such as *Cylindrospermopsis*, *Anabaena*, *Aphanizomenon*, and *Lyngbya*, putatively due to frequent horizontal gene transfer [21, 34].

The *sxtA* gene of *Anabaena* was used as the template for primer designing in a qPCR assay. Still, the amplicon was also produced from three other saxitoxin-producing genera, demonstrating its multi-generic detection capacity [82]. The study also revealed that the saxitoxin concentrations correlated positively with *stx* gene copy numbers, indicating the latter can be used as a measure of potential toxigenicity in *Anabaena* and other cyanobacterial blooms. Al-Tebrineh et al. employed the primers by aligning *sxtA* and *cyrA* genes from four genera in both conventional PCR and qPCR for a field survey along an Australian river [83]. They found cyanobacteria with the genes were widespread and massive in the surveyed areas. The authors also suggested that the molecular method may be used as a proxy for bloom risk assessment due to the positive correlation between concentrations of each cyanotoxin and respective toxin gene copy numbers.

Lyngbyatoxin is characterized as a potent skin irritant produced by *Lyngbya* which has been found in estuarine and coastal waters in tropical and subtropical regions [18]. Its biosynthesis reportedly involves four lyngbyatoxin synthetase genes (*ltxA-D*). However, molecular detection of lyngbyatoxin-producing cyanobacteria using these genes has not been documented in the public domain to date.

No literature regarding molecular detection of cyanobacteria producing the rest of the toxins mentioned above could be searched. It is most likely because there are few reports as to the molecular mechanisms of their biosynthesis. Nevertheless, it is worthwhile to briefly introduce guanitoxin, previously known as anatoxin-a(S), to emphasize its difference from anatoxin-a. Guanitoxin was recently renamed due to its structural and toxicological disparities from anatoxin-a [77]. It is a guanidino organophosphate neurotoxin that irreversibly inhibits acetylcholinesterase's active site, leading to excess acetylcholine, which causes severe salvation and chromodacryorrhea, so-called "bloody tears" before respiratory arrest [84]. Up to now, it was only found in planktonic *Dolichospermum* that was previously designated as *Anabaena*.

6. Perspectives

As various cyanobacterial genera can produce the same cyanotoxin, the development of toxigenic cyanobacteria identification needs to be multi-generic detection. Furthermore, as many genes for different toxins have sequences for the same conserved domains, designing PCR methods for all the cyanobacteria producing multiple toxins would be ideal.

Although most publications have focused on the selected cyanotoxins and their producers, more attention should be paid to other cyanotoxins and producers due to their potential of posing a significant threat to animal and human health. However, many cyanotoxin-producing cyanobacteria still lack bioinformation for the synthesis-related genes (e.g., guanitoxin), and it is thereby urgent to make further exploration to enrich the gene pools and their sequences so that a much more comprehensive understanding of the molecular mechanisms and the development of nucleic acid-based identification methods can be facilitated.

With the technical advance in PCR, researchers have been able to develop multiplex PCR methods in which many cyanotoxin biosynthesis genes can be detected simultaneously. For example, Ouahid et al. devised a multiplex PCR assay to detect six *mcy* genes (*mcyA-E*, *G*) at the same time for the typing of toxigenic *Microcystis* [85]. Rasmussen et al. developed a duplex qPCR for the detection of toxigenic *Cylindrospermopsis* using *cyrA*, *cyrB*, and *cyrC* genes [86]. Al-Tebrineh et al. established a quadruplex qPCR assay that could concurrently detect *mcyE*, *ndaF*, *cyrA*, and *sxtA* genes for most of the toxin-producing genera in a single reaction [87] and successfully employed the assay in an investigation of a cyanobacterial bloom that occurred along a river in Australia [83]. The invention of multiplex PCR assays has enhanced the throughput significantly for toxigenic cyanobacteria detection and can provide great aid to large-scale ecological surveys.

Cyanobacteria with cyanotoxin synthetase genes in their genome are clearly equipped with the ability of toxin production. However, transcription of toxin biosynthesis genes is triggered by various environmental factors [88–90]; hence, toxin production is not consistently ongoing. It means the presence of genes itself may not always translate into the appearance of toxins unless they are inter- or extra-cellularly accumulated and detectable. Furthermore, the significant positive correlation between gene copies and toxin levels is still controversial, as described in this chapter and another review [91]. Instead, the presence of mRNA transcripts from cyanotoxin synthase genes may be more closely associated with toxin production. Consequently, cDNA detection is justifiable to indicate an ongoing toxin synthesis, which is more critical and useful for monitoring the toxin-producing cyanobacteria. For this purpose, genes located at the end of operons should be good candidates for two reasons. One, primers designed from those genes can be directly used in cDNA testing like other genes because cyanobacteria lack introns. Two, the appearance of those genes in cDNA form signifies the successful cascade transcription of the clustered genes, gearing up all pertinent proteins for toxin synthesis. For example, *mcyC* and *mcyJ* located at the 3′ terminals of each operon in toxigenic *Microcystis* would be more useful in this respect than *mcyA*, *B*, *D*, and *E* which are more often used. However, it should be noted that a significant positive correlation might not exist between cDNA copies and toxin levels, likely due to the incomplete transcription or complex regulation of transcription and the elusive fate of toxins [55].

Although qPCR is preferred due to its many advantages, conventional PCR should also be considered for assessing the presence or absence of toxigenic cyanobacteria in water samples, as previously reported [49, 56]. In addition, the simplicity and cheaper operation may make conventional PCRs a more cost-effective tool for molecular detection of toxigenic cyanobacteria in comparison to qPCRs.

Besides PCR-based assays, there are other molecular technologies applicable to the identification and/or characterization of toxigenic cyanobacteria. A noteworthy method is the next-generation sequencing (NGS) technology. The technology has been widely used to identify previously unrecognized agents, non-culturable microorganisms, and/or variants because of its advanced and hypothesis-free sequencing ability [92] and has been applied to cyanobacteria research. Although most NGS studies have been investigations of taxonomic diversities using representative cyanobacterial genetic markers such as 16S rDNA [93, 94], the potential toxigenicity of cyanobacteria can be disclosed by sequencing the pooled libraries of toxin biosynthesis associated genes. Casero et al. revealed the existence of multiple toxigenic taxa in a summer bloom in a Spanish reservoir using *mcyE*, *anaF*, and *stxI* genes, and the relative abundance of toxigenic cyanobacteria in those populations correlated with the respective toxin concentrations [94]. Another method worthwhile to mention is DNA microarray/chip technology which has also been employed in the identification of toxigenic cyanobacteria. For example, four microcystin-producing genera were detected by a genus-specific DNA chip assay based on *mcyE* gene by Rantala et al. [95]. These high-throughput technologies can serve the purpose of molecular identification of toxigenic cyanobacteria in conjunction with PCR.

7. Conclusions

Nowadays, freshwater cyanobacterial blooms are seen more frequently than ever before because of increased eutrophication of their habitats and climate changes (e.g., global warming), which are utterly favorable to the overgrowth of cyanobacteria. Even though toxic cyanobacterial species are not always the mere culprit for these ecological disasters, they are often the dominant organisms and cause more destructive consequences because they can produce potent cyanotoxins into the water. There is no doubt that the toxic freshwater cyanobacteria pose a grave threat to human and animal health, agricultural production, tourism, to name a few. Hence, advancing techniques and technologies for rapid and reliable identification and monitoring of toxic cyanobacteria is an inevitable mission for healthcare, economy, and environmental conservation. To date, molecular assays, especially PCR-based tests, have been employed in toxic cyanobacterial identification, but their utilization should be further expanded into large-scale and long-term detection tasks and routine monitoring programs for not only the acute poisoning incidents but also the chronic impacts and preventative measures.

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Conflict of interest

The authors declare no conflict of interest.

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