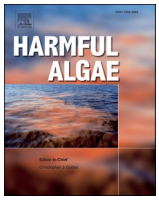
Harmful Algae 142 (2025) 102785

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal



A new quantitative PCR assay for detection of potentially anatoxin-producing cyanobacteria

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ARTICLE INFO

Keywords:
Cyanotoxins
Water management
Early warning
Risk assessment
qPCR
ana gene cluster
anaC gene

ABSTRACT

Anatoxins (ATX) are globally occurring toxins produced by some species of cyanobacteria in aquatic habitats. They can cause acute poisoning in animals, leading to muscle paralysis and respiratory failure, and might also pose a long-term health risk to humans. Thanks to advances in molecular methods and genomic knowledge, it is now possible to rapidly detect and quantify the genes associated with cyanotoxin production for most major groups of cyanotoxins except ATX. The aim of this study was to develop and validate a new quantitative PCR (qPCR) assay for general detection of all potential ATX producers in the environment. After specificity testing in silico and in vitro with 16 cyanobacterial strains (endpoint PCR, amplicon sequencing and qPCR), two assays targeting the *anaC* gene were thoroughly validated for linearity, amplification efficiency, sensitivity, dynamic range, inter-assay and intra-assay variability, and the influence of background DNA. The assays were then applied to 144 environmental samples of plankton and biofilm from lakes and rivers whose ATX content had previously been measured by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Amplification efficiency of the two designed assays was between 94 % and 103 %, and the limits of quantification and detection were up to, but mostly below, 322 and 32 cells/mL, respectively. Both assays showed better or equal specificity in cyanobacterial cultures than currently available PCR assays and were able to predict the presence of ATX detected by LC-MS/MS in most environmental samples (83 % in plankton and 52–62 % in biofilm). A higher number of discrepancies between qPCR and LC-MS/MS results in biofilm than in plankton samples indicates limited knowledge and sparse genomic data on benthic cyanobacteria. These assays are the first published general qPCR assays targeting all ATX producers and could provide water managers with a rapid and cost-effective risk assessment to better protect human and animal health.

1. Introduction

Anatoxins (ATX), potent neurotoxins produced by certain cyanobacteria, have been detected worldwide except in Antarctica (Du et al., 2019), and yet they remain understudied in comparison to other cyanotoxins such as microcystins. They bind to nicotinic receptors, cause depolarization of the cell membrane and essentially lead to muscle paralysis and respiratory failure (Wonnacott and Gallagher, 2006). Due to their acute toxicity, a number of poisonings in animals, mostly dogs, have been recorded in recent decades (reviewed in Backer et al., 2013; Testai et al., 2016; Ash and Patterson, 2022). There has been no reported human illness associated with ATX to date, but there is a potential risk to human health, which has been analysed in detail by Testai et al. (2016). An effective assay to detect all ATX producers could significantly reduce the risk to human and animal health in vulnerable areas. With the advances in molecular genetic methods, a PCR-based assay would be a time- and cost-efficient option, which has already been shown for other cyanotoxins (Pacheco et al., 2016). Quantitative PCR (qPCR) has the further advantage that it provides quantitative data, results can be tracked in real time and no downstream analysis is required.

While qPCR assays have been developed for most major groups of cyanotoxins (e.g., Al-Tebrineh et al., 2010, for saxitoxins; Al-Tebrineh et al., 2011, for microcystins; Campo et al., 2013, for cylindrospermopsins), no general assay has been published for detection of ATX producers. To our knowledge, the only published qPCR assay targets only one genus, *Microcoleus/Phormidium* (Kelly et al., 2018). Some general and genus-specific endpoint PCR assays have also been developed (Cadel-Six et al., 2009; Ballot et al., 2010; Rantala-Ylinen et al.,

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https://doi.org/10.1016/j.hal.2024.102785
Received 1 June 2024; Received in revised form 27 November 2024; Accepted 3 December 2024
Available online 15 December 2024

2011; Sabart et al.; 2015), but some have proven to be either too specific or not specific enough (Legrand et al., 2016). To overcome this problem, Legrand et al. (2016) proposed nested PCR, which significantly improved the results, but they still pointed out that improvements in primer design are needed. In addition, none of the listed endpoint PCR assays can be directly applied to qPCR as the latter method requires a shorter target fragment.

Due to the lack of nucleotide data, the general assays mentioned above were mostly designed based on *Dolichospermum/Anabaena* and *Kamptonema/Oscillatoria* sequences only. The *ana* gene cluster was first described in 2009 (Mejean et al., 2009) and consists of ten genes (*anaA–anaJ*). However, it is unclear whether the *anaI, anaJ* and *anaH* genes are present in all ATX-producing strains (Ballot et al., 2018), therefore only the partial gene cluster *anaA–anaG* was considered in this study. To date, it has been sequenced in one *Dolichospermum* strain (Rantala-Ylinen et al., 2011), one *Kamptonema/Oscillatoria* strain (Mejean et al., 2009) and five *Cuspidothrix/Aphanizomenon* strains (Jiang et al., 2015; Ballot et al., 2018). However, with the increasing number of whole genome sequences, it is possible to identify putative gene clusters in other cyanobacterial genera based on nucleotide or amino acid homology.

In this study, we aimed to develop a new qPCR assay for the detection of all potential ATX producers in environmental samples. To this end, we extracted all available data from nucleotide databases to expand the set of ATX-producing strains for the assay design. Using this information, we designed two new degenerated primer pairs targeting parts of the *anaC* gene, tested them in silico and in vitro with endpoint PCR and qPCR on cyanobacterial cultures and environmental samples, and confirmed the target products by sequencing. We propose two qPCR assays (anaC-441 and anaC-1039) suitable for the general detection and quantification of all ATX producers in environmental samples based on the presence of *anaC* gene, and discuss their strengths and limitations.

2. Methods

In general, the assay design in our study followed the workflow proposed by Bustin and Huggett (2017). First, all available genomic data were retrieved from databases and conserved regions were identified. Six assays were designed, which were first tested in silico for their predicted specificity and annealing temperatures. Then, their specificity was evaluated in vitro by endpoint PCR on cyanobacterial cultures together with four previously published assays for comparison, and the obtained PCR products were sequenced for confirmation. The newly developed assays were then adapted for qPCR application, and their sensitivity, linearity, dynamic range, amplification efficiency, inter-assay and intra-assay variability, and the influence of background DNA were tested. Finally, performance of the assays was tested on 144 environmental samples from plankton and biofilm, and the results were compared with the ATX concentrations determined in previous studies from the same samples (Salmaso et al., 2022; Jablonska et al., in prep).

2.1. Target identification, primer design and in silico check

Ana gene clusters from *Oscillatoria* sp. PCC 6506 (FJ477836.2; Mejean et al., 2009) and *Anabaena* sp. 37 (JF803645.1; Rantala-Ylinen et al., 2011), were used as references. The genera originate from two distinct clades on a phylogenetic tree (Walter et al., 2017), so they cover a large genetic diversity in the gene cluster.

1. Other cyanobacterial strains with gene clusters of high similarity were identified by (i) nucleotide BLAST (Altschul et al., 1990) of the entire reference gene cluster (*Anabaena* sp. 37) against the NCBI RefSeq Genome Database (O'Leary et al., 2016), and (ii) translated nucleotide blastn of the individual reference genes *anaA–anaG* (*Anabaena* sp. 37) against the NCBI Nucleotide Collection (Sayers et al., 2022).
2. Tentative *ana* gene clusters were determined in the identified genomes (Table 1) based on homology with the reference genes at the protein level and colocalization, using the shell programming language and Artemis 18.0.3. First, all contigs were merged into a pseudomolecule, then all coding sequences were extracted and translated into amino acid sequences. If the genome was not annotated, the coding sequences were annotated based on homology using prokka 1.14.5 from https://github.com/tseemann/prokka. Finally, the reference amino acid sequences were blasted against the reference.
3. Conservation of the *anaA–anaG* genes was assessed at the protein level between the newly identified clusters and the two reference clusters.
4. Gene organization of the identified gene clusters was assessed using GeneGraphics (Harrison et al., 2018).
5. Nucleotide sequences of individual genes *anaA–anaG* were extracted from all gene clusters, and aligned with SeaView using clustalo algorithm, and genes *anaB* and *anaC* were selected as potential target regions.
6. Four conserved regions were identified within the *anaC* target gene and five conserved regions in *anaB* gene, and different primer combinations were designed according to the following criteria: (i) primer sequences are mostly conserved between all genomes ($\leq$ two mismatches), (ii) each primer is at least 15 nucleotides long, (iii) the expected product size is between 70 and 200 bp to be suitable for qPCR amplification, and (iv) there is no insertion or deletion within the region to be amplified.
7. Five primer pairs (two from *anaC* region and three from *anaB* region) were evaluated in silico: specificity was checked with Primer-BLAST (Ye et al., 2012) and annealing temperatures were calculated. At this point, assays anaB-862 (F 5' AAGCAATTTGGTCAAGCVATTGG 3', R 5' AYTGAACCCAAGCTTCRCTAAT 3') and anaB-772 (F 5' ATGGAGTGGGARCGRGGA 3', R 5' CCAATBGCTTGACCAAATTGCTT 3') were excluded due to poor results (see 3.1 Target selection within *ana* gene cluster).
8. Primers were extended as needed to achieve comparable melting temperatures (Tm) of both primers, and degenerated to ensure 100 % alignment with all available strains.
9. Three primer pairs were initially analysed in vitro. At this point, assay anaB-454 (F 5′ GATATCTATAGYCTCAAGACAACRGC 3′, R 5′ GGTYACATAGTGTTTGTRTCCGTT 3′) was excluded due to poor results (see 3.1 Target selection within *ana* gene cluster). Two primer pairs anaC-441 and anaC-1039 targeting *anaC* gene (Fig. 1; Table 3) were therefore selected for further validation.

Table 1
Cyanobacterial strains that were used for development of new qPCR assays. All strains contain the *ana* gene cluster encoding for anatoxin production, and the corresponding genomes or gene clusters are listed.

Strain	NCBI GenBank/Genome accession number
Anabaena sp. 37	JF803645.1
Anabaena sp. AL93	GCA_001672085.1
Anabaena sp. UHCC 0054	GCA_017355725.1
Aphanizomenon flos-aquae FACHB-1040	GCA_014696815.1
Dolichospermum flos-aquae UHCC 0037	GCA_009712125.1
Dolichospermum sp. WA102	GCF_001277295.1
Cuspidothrix issatschenkoi CAWBG02	LT984883.1
Cuspidothrix issatschenkoi CHABD3	KM245025.1
Cuspidothrix issatschenkoi CHARLIE-1	GCA_002934005.1
Cuspidothrix issatschenkoi LBRI48	KM245023.1
Cuspidothrix issatschenkoi NIVA-CYA 711	LT984882.1
Cuspidothrix issatschenkoi RM-6	KM245024.1
Cylindrospermum stagnale PCC 7417	GCA_000317535.1
Kamptonema formosum PCC 6407	GCA_000332155.1
Kamptonema sp. PCC 6506	FJ477836.2
Nostocales cyanobacterium HT-58–2	GCA_002163975.1

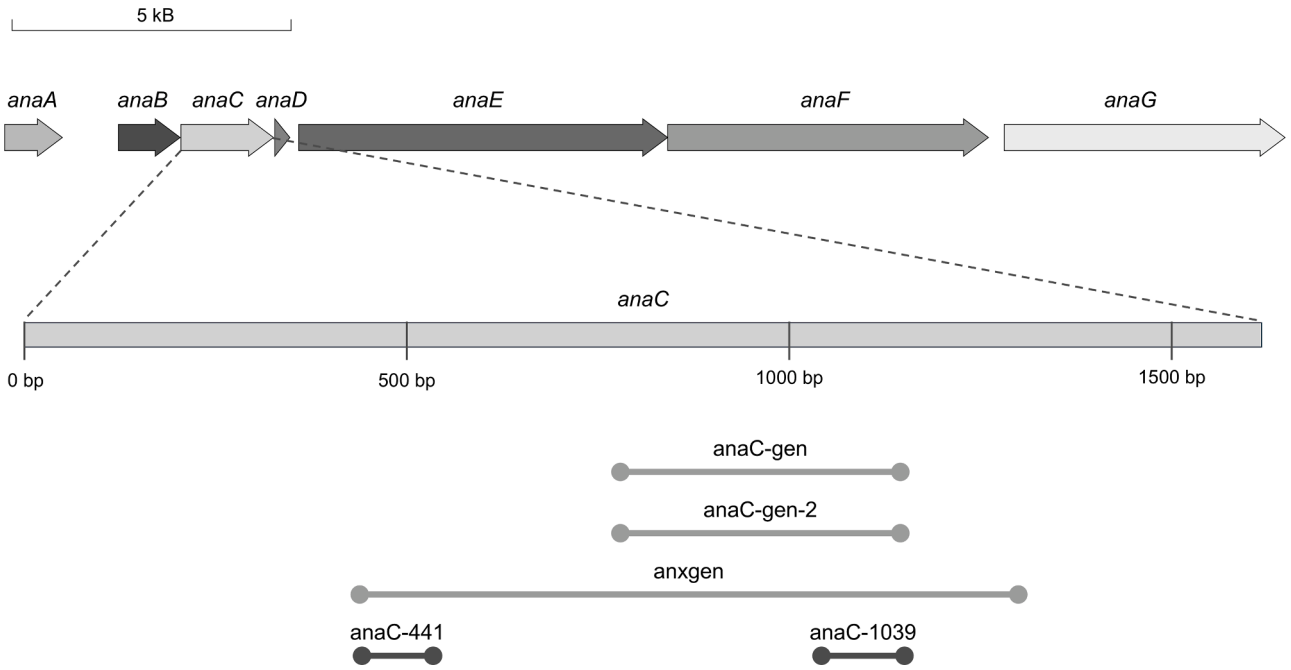

Fig. 1. Partial *ana* gene cluster encoding anatoxin production (top), *anaC* gene (middle) and target regions (bottom) of the five (q)PCR assays targeting *anaC* used in this study, shown on *Kamptonema* sp. PCC 6506. The full length of the *anaC* gene is 1617 bp. The assays anaC-441 and anaC-1039 (black) were developed in this study, the other assays (grey) originate from previous studies.

2.2. Specificity testing with cyanobacterial strains

2.2.1. Cyanobacterial cultures

For the assay validation, 16 cyanobacterial strains were obtained from different culture collections (Table 2). Six main genera of ATX producers were selected (*Cuspidothrix/Aphanizomenon, Cylindrospermum, Dolichospermum/Anabaena, Kamptonema/Oscillatoria, Microcoleus/Phormidium*, and *Tychonema*), with each genus represented by one or two confirmed ATX-producing strains, and one non-toxic strain as a negative control. The strains of the genus *Microcoleus/Phormidium* are benthic, and all other strains are planktic. Moreover, the strains were carefully selected to produce different variants of ATX (ATX-a, dihydroATX-a, and homoATX-a). Toxicity data were obtained from the corresponding culture collections or published literature as indicated in Table 2. Cultures of five selected ATX-producing strains (*Cuspidothrix* issatschenkoi NIVA-CYA 711, *Cylindrospermum* stagnale PCC 7417, *Anabaena* sp. UHCC 0054, *Kamptonema* sp. PCC 6506, and *Tychonema bourrellyi* NIVA-CYA 96/3) were examined microscopically to determine cell concentrations with Nikon Eclipse 80i light microscope equipped with Nikon DS-Fi1 camera. No strain of the genus *Microcoleus/Phormidium* was included, becaasuse due to the benthic form of these strains, the cell count would not be comparable to that of the planktic strains. Depending on the culture density, either Nageotte or Bürker Türk counting chambers were used. Since all strains were filamentous, the lengths of three filaments (adding up to 24–130 cells) were first measured (400–1000-fold magnification), the numbers of cells counted, and the average cell lengths calculated. Then, all filaments within selected counting fields were measured with software NIS-Elements AR 3.2 (100–200-fold magnification), and cell numbers were calculated (at least 490 cells for each strain). The cell counts were converted into cell concentrations, taking into account the surface area and the volume of the culture examined.

2.2.2. DNA extraction and quality control

DNA was extracted from cyanobacterial cultures by filtering them through GF/C filters with a pore size of 1.2 μm (Whatman, Cytiva, USA) and extracting DNA from the filters with DNeasy PowerWater Kit (Qiagen, Germany). DNA concentration and purity were assessed spectrophotometrically using a DS-11 FX spectrophotometer fluorometer (DeNovix Inc., USA). Additionally, all samples were checked for sufficient DNA quality by amplifying PC-IGS region (specific for cyanobacteria) with PCR using primers PCβF/PCαR (Table 3).

2.2.3. Endpoint PCR

First, the PC-IGS region (specific for cyanobacteria) was amplified using primers PCβF/PCαR (Table 3) to check for sufficient DNA quality. Then, a literature review was carried out, identifying all assays developed to date for detection of potential ATX producers. Genus-specific primers were not considered, as they are not suitable for a general assay. Seven assays (five pre-designed and two newly designed in this study; Fig. 1; Table 3) were tested on 16 cyanobacterial cultures (additionally, anaB-454 designed in this study was only tested via qPCR and is not listed in Table 3; see 2.1 Target identification, primer design and in silico check).

Reactions were prepared in 0.2 mL 8-strip PCR tubes (Brand GMBH), consisting of 1 × DreamTaq PCR buffer with 2 mM $MgCl_2$ (Thermo Scientific) and additional 1 mM $MgCl_2$, dNTP mix and primers as described below, 0.25 U DreamTaq polymerase, 1 μL of undiluted DNA template and nuclease-free water to a final volume of 10 μL. Concentrations of dNTP mix and each primer were as follows: (i) anaC-gen and anxgen: 0.2 mM dNTP mix and 0.5 μM of each primer; (ii) atxoaf/atxar: 0.25 mM dNTP mix and 0.5 μM of each primer; (iii) anaC-gen-2: 0.2 mM dNTP mix and 0.4 μM of each primer; (iv) antx: 0.2 mM dNTP mix and 0.2 μM of each primer; and (v) PCβF/PCαR, anaC-441 and anaC-1039: 0.3 mM dNTP mix and 0.5 μM of each primer..

PCR amplification was performed in a GeneAmp PCR system 9700 (Applied Biosystems, ThermoFisher Scientific, USA), using the following cycling conditions: (i) anaC-gen and anxgen: 2 min at 94°C, 35 cycles including 30 s at 94°C, 30 s at the annealing temperature, and 30 s at 72°C, followed by 5 min at 72°C; (ii) anaC-gen-2: 2 min at 94°C, 35 cycles including 1 min at 94°C, 1 min at the annealing temperature, and 1 min at 72°C, followed by 5 min at 72°C; (iii) atxoaf/atxar: 4 min at 94°C, 30 cycles including 10 s at 94°C, 20 s at the annealing temperature, and 1 min at 72°C, followed by 5 min at 72°C; (iv) antx: 2 min at

Table 2
Cyanobacterial strains used for the validation of the PCR and qPCR assays and the results obtained with each assay. The anaC-441 and anaC-1039 assays have been developed in this study, and other assays are from previous studies. + amplicon of expected size, - no amplicon, (+) very weak amplicon (unclear result).

Genus	No.	Strain	Origin	Source	Toxin production	Reference for toxicity	anaC-gen[1]	anaC-gen-2[2]	anxgen[1]	antx[3]	atx[4]	anaC-441[5]	anaC-1039[5]
Aphanizomenon/ Cuspidothrix	1	CAWBG02	New Zealand	CICCM	ATX-a	Wood et al., 2007	-	+	+	-	+	+ / +	+ / +
	2	NIVA-CYA 711	Germany	NORCCA	ATX-a	Ballot et al., 2010	+	+	+	-	+	+ / +	+ / +
	3	NIES-81	Japan	NIES	non-toxic	culture source	-	-	-	-	-	- / -	- / -
Cylindrospermum	4	PCC 7417	Sweden	PCC	dihydroATX-a	Mejean et al., 2016	+	+	+	+	+	+ / +	+ / +
	5	PMC 238.04	France	MNHN	non-toxic	culture source	-	-	-	-	(+)	- / -	- / +
Dolichospermum/ Anabaena	6	NIES-1669[6]	Japan	NIES	ATX-a	culture source	-	-	-	-	-	- / -	- / +
	7	UHCC 0054	Finland	UHCC	ATX-a	culture source	+	+	-	+	+	+ / +	+ / +
	8	NIES-1668	Japan	NIES	non-toxic	culture source	-	-	-	-	(+)	- / -	- / +
Kamptonema/ Oscillatoria	9	PCC 6506	unknown	PCC	ATX-a, homoATX-a	Araoz et al., 2005	+	+	+	+	+	+ / +	+ / +
	10	No697[6]	Austria	ILIM	ATX-a	culture source	-	-	-	-	-	- / +	- / -
	11	UHCC 0018	Finland	UHCC	non-toxic	culture source	-	+	(+)	-	+	- / +	- / +
Microcoleus/ Phormidium	12	PMC 240.04	France	MNHN	ATX-a	Gugger et al., 2005	+	+	+	+	+	+ / +	+ / +
	13	CAWBG24	New Zealand	CICCM	non-toxic	culture source	-	+	-	-	-	- / +	- / +
Tychonema	14	FEMGT323	Italy	FEM	ATX-a	culture source	+	+	+	+	+	+ / +	+ / +
	15	NIVA-CYA 96/3	Norway	NORCCA	ATX-a	Shams et al., 2015	+	+	+	+	+	+ / +	+ / +
	16	NIVA-CYA 69	Norway	NORCCA	non-toxic	culture source	+	+	-	-	+	- / +	- / +
Number of strains yielding expected endpoint PCR results based on known toxin production							12	11	12	12	10	14 / 12	14 / 9
% of strains yielding expected results							75	69	75	75	63	88 / 75	88 / 56

[1] Rantala-Ylinen et al., 2011,
[2] Sabart et al., 2015,
[3] Cadel-Six et al., 2009,
[4] Ballot et al., 2010,
[5] this study; results are given as endpoint PCR / qPCR,
[6] note that in these cultures, *anaC* gene concentration was low (see Supplementary File S1), which might be the reason for negative results. Culture sources: CICCM – Cawthron Institute's Culture Collection of Microalgae, New Zealand; NORCCA – Norwegian Culture Collection of Algae, Norway; NIES – National Institute for Environmental Studies, Japan; PCC – Pasteur Culture Collection of Cyanobacteria, France; MNHN – Muséum national d'Histoire naturelle, France; UHCC – University of Helsinki Culture Collection, Finland; ILIM – Research Department for Limnology, University of Innsbruck, Austria; FEM – Fondazione Edmund Mach, Italy.

Table 3
PCR and qPCR assays used in this study. All assays were tested with endpoint PCR, while the two assays designed in this study (anaC-441, anaC-1039) were used also for qPCR.

Assay	Target region	Sequence F 5–3	Sequence R 5–3	Amplicon size [bp]	Annealing T	Reference
PCβF/PCαR	PC-IGS	GGCTGCTTGTTT ACGCGACA	CCAGTACCACCA GCAACTAA	500	50°C	Neilan et al., 1995
anaC-gen	*anaC*	TCTGGTATTCAGT CCCCTCTAT	CCCAATAGCCTGT CATCAA	366	58°C	Rantala-Ylinen et al., 2011
anaC-gen-2	*anaC*	TCTGGTATTCA GTMCCCTCYAT	CCCAATARCCTGT CATCAA	366	56°C	Sabart et al., 2015
anxgen	*anaC*	ATGGTCAGAGG TTTTACAAG	CGACTCTTAATCAT GCGATC	861	52°C	Rantala-Ylinen et al., 2011
antx	*anaF*	CGCAAATCGAT GCTCACTTA	CCACTGGCTCCAT CTTGATT	412	55°C	Cadel-Six et al., 2009
atxoaf/atxar	*anaF*	TCGGAAGCGCG ATCGCAAATCG	GCTTCCTGAGAA GGTCCGCTAG	461	55°C	Ballot et al., 2010
anaC-441	*anaC*	GTCAGAGGTTTT ACAAGCTCCTTG	GTYGARCCAGATGTA TAAAGAATATAAGC	93	56°C	This study
anaC-1039	*anaC*	GCYAACACTGAA GTTTTTGTCTT	AAATCCCAATARCCT GTCATCAA	110	56°C	This study

95°C, 40 cycles including 30 s at 95°C, 45 at the annealing temperature, and 1 min at 72°C, followed by 5 min at 72°C; (v) PCβF/PCαR, anaC-441 and anaC-1039: 2 min at 95°C, 35 cycles including 30 s at 95°C, 30 s at the annealing temperature, and 30 s at 72°C, followed by 5 min at 72°C. The specific annealing temperatures are given in Table 3. The described reaction mixtures and cycling conditions for assays from previous studies (anaC-gen, anxgen, anaC-gen-2, atxoaf/atxar, and antx) are the same as in the original studies (Table 3).

All PCR products were stained with ethidium bromide, loaded onto an agarose gel (0.8 % or 3 %), run at 100–120 V for 60–90 min, and visualized with UVP ChemStudio PLUS Imaging System (Analytik Jena, Germany).

2.2.4. Amplicon sequencing

Selected PCR products of expected size (from six cultures, one from each genus) were sequenced using newly designed assays anaC-441 and anaC-1039. Additionally, three cultures with unexpected results (i.e. presumably non-toxic strains positive for *anaC*) were attempted for sequencing to identify the region that was amplifed.

PCR reactions were prepared in 0.2 mL 8-strip PCR tubes (Brand GMBH), consisting of 1 × DreamTaq PCR buffer containing 2 mM $MgCl_2$ (Thermo Scientific) and additional 1 mM $MgCl_2$, 0.3 mM dNTP mix, 0.5 μM of each primer, 0.5 U DreamTaq polymerase, 2 μL of undiluted DNA template and nuclease-free water to a final volume of 20 μL. Since the amplicons were too short to be sequenced directly (≤ 110 bp), M13 tail sequences were added to the 5′ end of each primer (M13F(-21): 5′-TGTAAAACGACGGCCAGT-3′; M13R(-26): 5′-CAGGAAACAGCTATGAC-3′). In this way, the target amplicons were elongated to 128 and 145 bp (anaC-441 and anaC-1039, respectively). PCR amplifications were performed in a GeneAmp PCR system 9700 (Applied Biosystems, ThermoFisher Scientific) under the same cycling conditions as described in 2.2.3 Endpoint PCR. PCR products were stained with ethidium bromide, loaded onto an agarose gel (3 %), run at 100–120 V for 60–165 min, and visualized with UVP ChemStudio PLUS Imaging System (Analytik Jena).

The products were purified by MinElute PCR Purification Kit (Qiagen). The DNA concentration and purity of the purified PCR products were determined sprectrophotometrically by a DS-11 FX spectrophotometer fluorometer (DeNovix Inc.) and the products were sequenced in the forward and reverse directions with primers M13F(-21) and M13R (26) using Sanger sequencing (Eurofins Genomics, Germany). The resulting sequences were analysed with BioEdit; they were mapped to an *anaC* reference gene of *Anabaena* sp. 37 (Table 1), primer regions were removed, consensus sequences of the target region were retrieved and blasted against NCBI Nucleotide Collection.

2.4. qPCR assay optimization and validation

The newly designed assays anaC-441, anaC-1039 and anaB-454 were tested via qPCR with SYBR Green chemistry. First, all 16 cyanobacterial cultures (Table 2) were tested in 10^{-1} and 10^{-2} template dilutions to test for specificity (anaB-454 was only tested up to this point due to poor results; see 3.1 Target selection within *ana* gene cluster). Then, different primer concentrations (0.3 μM, 0.6 μM and 0.9 μM) were tested for each assay to choose the optimal concentration. Standard curves were then generated using dilution series (10^{-1} to 10^{-8} dilution in 10-fold increments) of selected ATX-producing strains: *Cuspidothrix issatschenkoi* NIVA-CYA 711, *Cylindrospermum stagnale* PCC 7417, *Anabaena* sp. UHCC 0054, *Kamptonema* sp. PCC 6506, and *Tychonema bourrellyi* NIVA-CYA 96/3 to test linearity, amplification efficiency, sensitivity (limit of detection [LOD]), limit of quantification [LOQ]), and dynamic range. All qPCR reactions were performed in three technical replicates (aliquots of the same DNA template were analyzed in separate wells within the same qPCR run). Amplification efficiency was calculated from standard curves using the formula $10^{(-1/slope)}$-1. LOD was defined as a cell concentration at which at least 2/3 of the technical replicates gave a positive result. LOQ was defined as a cell concentration at which all three technical replicates gave a positive result, the quantification cycle (Cq value) was at the lower end of the linear curve, and the coefficient of variation (CV) of the Cq values of the technical replicates did not exceed 2 %. Amplification was performed on qPCR cycler Applied Biosystems 7900HT (ThermoFisher Scientific). Reactions were performed in three technical replicates in a final volume of 10 μL, consisting of 5 μL SYBR Green PCR Master Mix (Applied Biosystems, ThermoFisher Scientific), 0.9 μM (anaC-441 assay) or 0.6 μM (anaC-1039 and anaB-454 assays) of each primer, and 2 μL of DNA template. Reactions were performed in clear 384-well PCR plates (Thermo Scientific, ThermoFisher Scientific), covered with MicroAmp™ optical adhesive film (Applied Biosystems, ThermoFisher Scientific). The temperature profile was 10 min at 95°C (ramp rate 100 %), followed by 45 cycles of 15 s at 95°C (ramp rate 100 %) and 1 min at 60°C (ramp rate 100 %), and a dissociation stage with initial denaturation for 15 s at 95°C (ramp rate 100 %), followed by 15 s at 60°C (ramp rate 100 %) and a gradual increase up to 95°C (ramp rate 2 %). Each experiment included a negative control (nuclease-free water). Standard curves were generated in R (v. 4.2.2) by making a linear model between cell concentration (log10-transformed) and Cq values and extracting linear equations. Plots were generated with packages ggplot2, scales, and ggpmisc.

Inter-assay variability was evaluated in five cyanobacterial cultures (Table 4) using three technical replicates analysed in parallel, and CV between cell equivalent concentrations was calculated for each replicate. Intra-assay variability (repeatability) was tested on five cyanobacterial cultures and five environmental samples by repeating the reaction three times under the same conditions with the same template. Cell equivalent concentrations were calculated from the average Cq values (three technical replicates) for each repetition of the experiment, and CV values were calculated. The influence of background DNA was tested on the cultures *Anabaena* sp. UHCC 0054 and *Cuspidothrix issatschenkoi* NIVA-CYA 711 by spiking the DNA with DNA from closely related non-toxic strains (*Dolichospermum flos-aquae* NIES-1668 and *Aphanizomenon flos-aquae* NIES-81, respectively) in equal volume, and repeating the dilution series.

2.5. Application of the assays on environmental samples

To test the assay performance in environmental samples, 144 samples of plankton (65) and biofilm (79) from 27 lakes and two rivers (mainly from Slovenia, but also from other European countries) (Supplementary File S2) were obtained from previous projects (Jablonska et al., in prep.; Salmaso et al., 2022). Prepared DNA from previous projects was used; DNA from plankton samples has been extracted with DNEasy Sterivex PowerWater kit (Qiagen) (Salmaso et al., 2022) or DNEasy PowerWater kit (Qiagen) (Jablonska et al., in prep.), and DNA from biofilm samples has been extracted with NucleoSpin Soil kit (Macherey-Nagel, Germany) (Salmaso et al., 2022; Jablonska et al., in prep.). All these samples have shown measurable DNA concentrations (> 1 ng/μL) in previous projects, and have been successfully used for PCR, qPCR and NGS applications (Salmaso et al., 2022; Jablonska et al., 2024; Jablonska et al., in prep.). The presence of ATX has been previously confirmed in 36 of these samples by LC-MS/MS (between 1.1 and 1206.0 ng/L in plankton samples, and between 19.6 and 7782.2 ng/g dry weight in biofilm samples). No ATX has been detected in the remaining 108 samples (Jablonska et al., in prep) and they were included as negative controls. LC-MS/MS analyses have been carried out using the method described in Cerasino and Salmaso (2020), targeting ATX-a and homoATX-a.

Environmental samples were analyzed with both new assays by qPCR in three technical replicates, using 10-fold diluted template DNA. The same protocols were used as for DNA from cyanobacterial cultures. The qPCR data were analyzed in Sequence Detection System (SDS) v2.4.1 (Applied Biosystems, ThermoFisher Scientific) and subsequent calculations were performed using Microsoft Excel 2402. The Cq threshold was manually set to 0.3 for all samples and was the same as for

Table 4
Amplification efficiency (e), linearity (R^2) within the linear dynamic range, limit of detection (LOD; cells/mL / cells/reaction), and limit of quantification (LOQ; cells/mL / cells/reaction) for the two qPCR assays (anaC-441, anaC-1039) tested on five cyanobacterial cultures in 10^{-1} to 10^{-8} dilution in 10-fold increments, each in three technical replicates.

	anaC-441				anaC-1039			
Strain	e	R^2	LOD	LOQ	e	R^2	LOD	LOQ
Cuspidothrix NIVA-CYA 711	94 %	0.9986	16.35 / 5.89	163.53 / 58.87	97 %	0.9969	1.64 / 0.59	163.53 / 58.87
Cylindrospermum PCC 7417	96 %	0.9995	1.26 / 0.33	12.64 / 3.29	98 %	0.9992	1.26 / 0.33	12.64 / 3.29
Anabaena UHCC 0054	102 %	0.9994	0.35 / 0.08	35.20 / 7.74	97 %	0.9984	0.35 / 0.08	35.20 / 7.74
Kamptonema PCC 6506	96 %	0.9999	32.17 / 12.87	321.73 / 128.69	98 %	0.9996	32.17 / 12.87	32.17 / 12.87
Tychonema NIVA-CYA 96/3	103 %	0.9983	12.97 / 4.67	129.72 / 46.7	96 %	0.9981	1.30 / 0.47	129.72 / 46.7

the samples used to generate a standard curve to allow direct comparison. The baseline was set automatically by the software (SDS v2.4.1). The Tm values of the environmental samples were compared with those of the cyanobacterial cultures (75.5–78.2°C for the anaC-441 assay and 80.2–81.6°C for the anaC-1039 assay), and technical replicates with Tm outside the expected range were classified as negative. Samples were considered positive if at least 2/3 of the technical replicates were positive (amplification curve was observed and Tm matched the expected values). The average Cq values were calculated from the positive technical replicates for each sample and compared with the previously determined LOD and LOQ values. The concentration of cell equivalents in a given sample was considered quantifiable if the average Cq value did not exceed the LOQ value (30 for both assays). For quantifiable samples, the equations obtained from a standard curve were used to calculate the cell equivalent concentration. If the average Cq value exceeded the LOD value (38 and 37 for the assays anaC-441 and anaC-1039, respectively), the sample was considered negative. Repeatability was tested on five selected environmental samples (P-22–05-HO, P-22–06-PO, SI-P-018, SI-B-01, and CH-B-05; Supplementary File S2) by repeating the reaction three times under the same conditions using the same template.

3. Results

3.1. Target selection within ana gene cluster

The amino acid conservation of the individual translated genes within the *ana* gene cluster (*anaA*–*anaG*) ranged from 75.5 % to 100.0 % (Supplementary File S3). Three distinct clades were formed: *Kamptonema, Dolichospermum/Anabaena* and *Aphanizomenon*, and *Cuspidothrix*. In general, *anaD* was the most and *anaA* the least conserved gene, but conservation was fairly similar for all genes. Greater differences were observed in the organization of the gene cluster, where four different patterns were seen: (i) *Dolichospermum/Anabaena* and *Aphanizomenon*, (ii) *Cuspidothrix* and *Cylindrospermum*, (iii) *Kamptonema/Oscillatoria*, and (iv) unidentified Nostocales (Supplementary File S3).

While there were conserved regions in all genes that were suitable for primer design, the genes *anaC* and *anaB* were identified as the most appropriate target, because they encode proteins involved in specific steps of ATX production (proline adenylation and proline dehydrogenase, respectively [Rantala-Ylinen et al., 2011]). Targeting other genes could increase the possibility of detecting other polyketide synthases.

In silico analyses with Primer-BLAST were performed for two primer pairs from the *anaC* gene and three primer pairs from the *anaB* gene with appropriate predicted melting temperature (56–62°C and 56–64°C, respectively). Both *anaC* primers (Table 3) were predicted to have good specificity, i.e. anaC-441 had no non-target hits, while anaC-1039 showed potential amplification in one archaea and three eukaryota sequences, with the expected product lengths differing significantly from those of the target amplicon (539 bp and 85 bp, respectively, vs. 110 bp) and at least three mismatches in the primer regions. Two primer pairs from the *anaB* gene (anaB-862, anaB-772) showed predicted cross-reactivity with L-prolyl-[peptidyl-carrier protein] dehydrogenase from *Planktothrix tepida* PCC 9214 and *Planktothrix pseudagardhii* No.713, which is identical to some proteins from the acyl-CoA dehydrogenase family that also contains the *anaB* protein. The third *anaB* primer pair anaB-454 had no predicted amplification in non-target organisms, and was therefore evaluated in vitro on 16 cyanobacterial cultures alongside the two *anaC* primer pairs, but continuously showed amplification in all non-toxic cultures so it was not tested further. Therefore, the *anaC* gene was selected as the most appropriate target for this study.

3.2. Specificity of the qPCR assays in cyanobacterial strains

Of the five existing assays, the assays anaC-gen and anxgen targeting *anaC*, and antx targeting *anaF* (Table 3) gave the best results, producing amplification in seven or six out of ten toxic strains, and no amplification in five or six out of six non-toxic strains, so that in total 12 out of 16 strains gave the expected results (Table 2). This proportion was higher for both newly designed *anaC* assays (anaC-441, anaC-1039) with endpoint PCR (14 out of 16 strains giving the expected results), while the proportion with qPCR was slightly lower than with endpoint PCR due to positive results in presumably non-toxic cultures (see below). In other existing *anaC* and *anaF* assays, the proportion of strains with expected endpoint PCR results was lower (between nine and 11 strains out of 16).

In vitro analyses with qPCR (Table 2) showed that the assays anaC-441 and anaC-1039 were both positive in nine out of ten toxic strains. Unexpectedly, the anaC-441 assay was negative in *Dolichospermum flos-aquae* NIES-1669, which presumably produces ATX-a. PCR amplification of PC-IGS was successful in DNA from this strain, demonstrating that the DNA was of sufficient quality and quantity to perform molecular analyses, but the very weak amplification produced by the anaC-1039 assay (Supplementary File S1) and the lack of amplification with all assays with endpoint PCR indicate a very low concentration of the target gene. The same goes for *Kamptonema* sp. No697, which gave negative results with the anaC-1039 assay (only one positive technical replicate out of three) and very low amplification with anaC-441 (Supplementary File S1). On the other hand, the assays were also positive in three out of six and five out of six presumably non-toxic strains (anaC-441 and anaC-1039, respectively). ATXs were not detected in any of these samples by LC-MS/MS. However, the Cq values of these samples were relatively high (>32 and >27, respectively) compared to toxic strains (16–26 and 14–22, respectively), Tm values with anaC-441 differed slightly from those of the toxic cultures from the same genera (Supplementary File S1), and no amplification occurred with endpoint PCR using the same primers, suggesting either a very low target concentration or mismatches in the target region.

The Tm values obtained for the anaC-441 assay ranged from 75.5°C to 78.2°C with a clear distinction between (i) *Cuspidothrix/Aphanizomenon* and *Kamptonema/Oscillatoria* (around 76°C), and (ii) *Cylindrospermum, Dolichospermum/Anabaena* and *Microcystis* (around

77.5°C). In the anaC-1039 assay, Tm values for all strains were between 80.2°C and 81.6°C. All samples showed clear single peaks on the melting curve plots with anaC-441, while with anaC-1039, some strains (all *Microcoleus* and *Tychonema* strains, *Kamptonema* sp. PCC 6506, *Dolichospermum flos-aquae* NIES-1669) showed asymmetric and wide peaks with shoulders towards the left, which could indicate non-specific amplification, primer-dimer formation or other anomalies.

When PCR amplification was attempted for sequencing, all six toxic cultures resulted in a single band on the gel with the anaC-441 assay, while two products of anaC-1039 also exhibited fragments of shorter length (Fig. 2). For both assays, all six amplicons were successfully sequenced, the amplicons obtained were 40–41 bp and 64 bp long for assays anaC-441 and anaC-1039, respectively, after removal of the primers, and their identity was confirmed as expected (Supplementary File S4). Sequencing was also attempted in the three non-toxic cyanobacterial cultures that unexpectedly produced qPCR amplification with both assays (Table 2). PCR amplification with the anaC-441 assay produced single bands of expected length in two samples, and anaC-1039 produced bands of expected length together with shorter fragments in all three samples. Only the amplicon from *Planktothrix agardhii* UHCC 0018 was successfully sequenced with the anaC-441 assay and showed 98 % identity with different *Anabaena* sp. strains (Supplementary File S4), while the other amplicons most likely represented non-target amplification products.

3.3. qPCR assay validation

Amplification efficiency derived from five cyanobacterial cultures (Table 4) was between 94 % and 103 % and did not differ significantly between strains or assays. Linear dynamic range was from four to six 10-fold dilutions (Fig. 3). The anaC-441 assay was able to quantify *anaC* genes in samples with at least up to 322 cells/mL (corresponding to 129 cells per reaction), and anaC-1039 at least up to 164 cells/mL (corresponding to 59 cells per reaction), while both assays were able to detect genes in samples with at least up to 32 cells/mL (corresponding to 13 cells per reaction). However, in the majority of cultures, the LOQ and LOD were lower than that (Table 4).

Based on the standard curves, LOD cut-off values were set to Cq = 38 and Cq = 37 corresponding to 0.13 and 0.16 cells per reaction for anaC-441 and anaC-1039 assays, respectively, and LOQ cut-off value was set to Cq = 30 corresponding to 18–27 cells per reaction for both assays. *Cuspidothrix issatschenkoi* NIVA-CYA 711 was selected as the reference culture for qPCR quantification of environmental samples due to the highest linearity and a wide linear range (Fig. 3). Linear equations were derived from the standard curve: y = 34.9321 - 3.4734x and y = 34.2868 - 3.3904x were used for the assays anaC-441 and anaC-1039, respectively, where y = Cq value and x = log concentration of cell equivalents per uL of DNA (Fig. 3).

For inter-assay variability and intra-assay variability (repeatability), CV between cell equivalent concentrations from different technical replicates or from different experiment repetitions are given in Table 5. The influence of background DNA within the linear range (Fig. 3) is shown as a ratio between calculated cell equivalent concentrations in the absence and presence of background DNA (Table 5). For inter-assay variability and background influence, which were both tested on a range of DNA concentrations, it was evident that the variability was mostly higher at both edges compared to the middle of the linear range. In the middle range (samples corresponding to 100–100.000 cell equivalents/uL DNA), intra-assay variability was mainly low (CV < 10 %), and ratios between the absence and presence of background DNA were between 0.9 and 1.2, suggesting under- or overestimation of cell equivalent concentrations for no more than 10 % or 20 %, respectively, due to background DNA.

3.4. Performance in environmental samples

In the environmental samples, performance of the assays differed between plankton and biofilm samples. Both false negative (*anaC* not detected by qPCR but ATX detected by LC-MS/MS) and false positive results (*anaC* detected by qPCR but ATX not detected by LC-MS/MS) occurred more frequently in biofilm samples than in plankton samples (Table 6, non-shaded areas). In particular, in plankton samples, qPCR gave positive results in ten (83 %) and ten (83 %) of 12 samples containing ATX, while in biofilm it was 11 (52 %) and 13 (62 %) of 21 samples containing ATX for the assays anaC-441 and anaC-1039, respectively. On the other hand, qPCR results for the assays anaC-441 and anaC-1039 were positive in 16 (30 %) and ten (19 %) out of 53 plankton samples with no detected ATX, and in 22 (38 %) and 19 (33 %) out of 58 biofilm samples with no detected ATX, respectively (Table 6, Supplementary File S2). Results of the two assays matched in most cases, i.e. in 86 % of plankton and in 86 % of biofilm samples (Supplementary File S2). Therefore, if the results of the combination of both assays were considered instead of individual assays, i.e. a sample was considered positive only if both qPCR assays gave positive results, the results did not

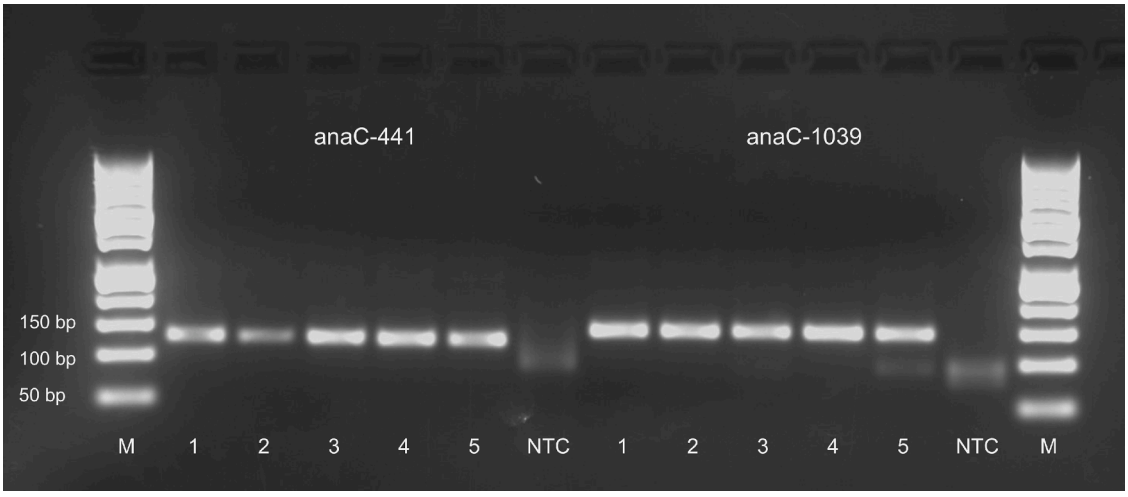

Fig. 2. Examples of PCR amplification products obtained with the assays anaC-441 (left) and anaC-1039 (right) from anatoxin-producing cyanobacterial cultures visualized on a 3 % agarose gel stained with ethidium bromide. Samples: 1 – *Cuspidothrix issatschenkoi* NIVA-CYA 711, 2 – *Cylindrospermum stagnale* PCC 7417, 3 – *Anabaena* sp. UHCC 0054, 4 – *Kamptonema* sp. PCC 6506, 5 – *Tychonema bourrellyi* NIVA-CYA 96/3. NTC – no template control, M – size marker GeneRuler 50 bp DNA ladder. Expected amplicon sizes: anaC-441 – 128 bp, anaC-1039 – 145 bp (M13 tails included in primers).

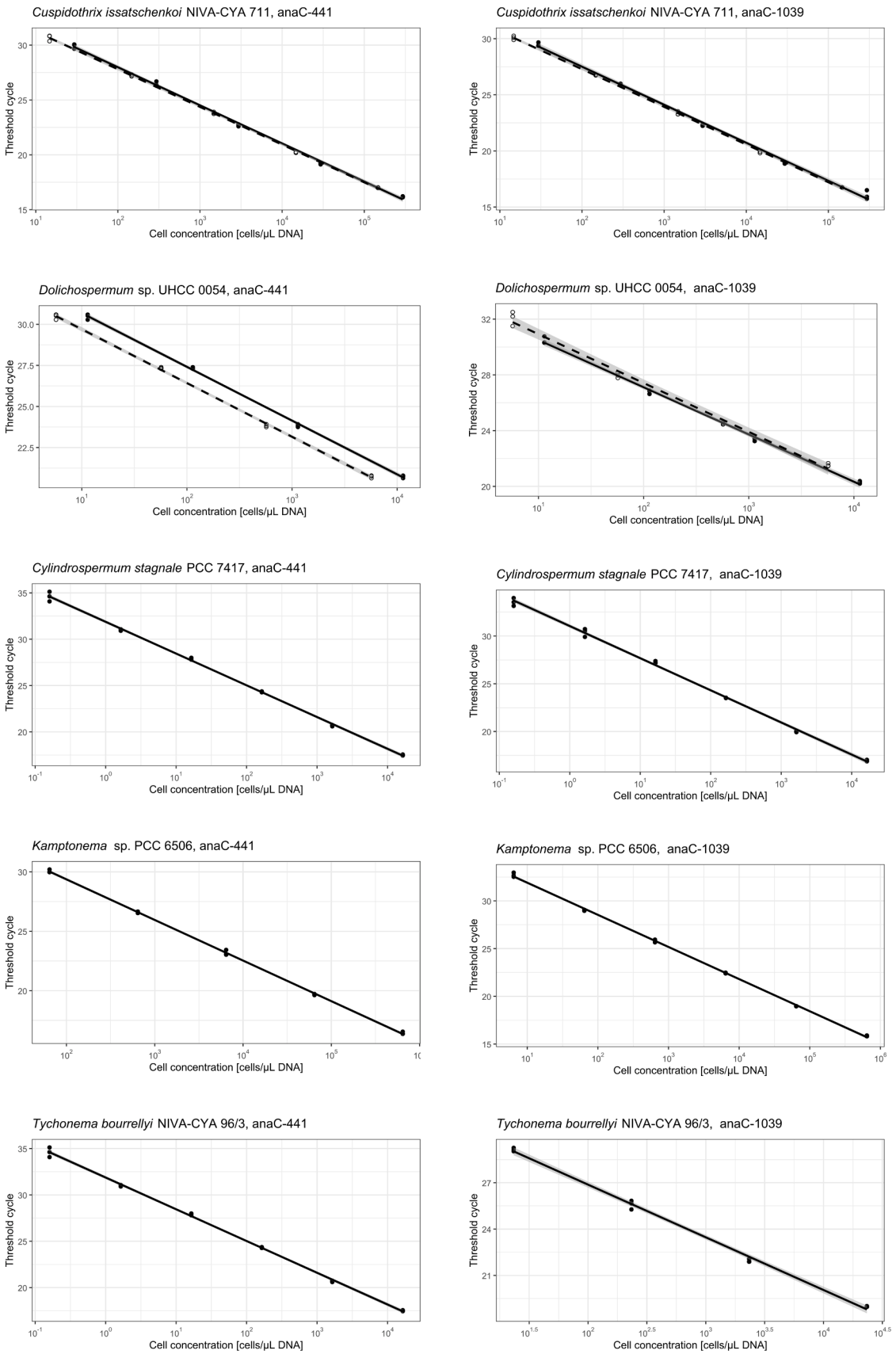

Fig. 3. Standard curves derived from different cyanobacterial strains for the assays anaC-441 (left) and anaC-1039 (right). Solid line – without the presence of background DNA, dashed line – with the presence of background DNA (tested for *Cuspidothrix* and *Dolichospermum* strains only). The dots represent three technical replicates for every dilution (aliquots of the same DNA template were analyzed in separate wells within the same qPCR run), the line represents the linear regression curve, and the gray area represents the 95 % confidence interval of the estimated regression lines.

Table 5
Different types of variability in the two newly designed qPCR assays anaC-441 and anaC-1039. Inter-assay variability was tested on five cyanobacterial cultures, intra-assay variability on five cyanobacterial cultures and five environmental samples, and background influence on two cyanobacterial cultures. CV – coefficient of variation, ce – cell equivalents, conc. – concentration, Me – median.

Variability	Sample type	Parameter	anaC-441	anaC-1039
Inter-assay variability	cultures	CV between ce conc. in technical replicates [%]	0.7–14.8 (Me 5.2)	0.2 –30.6 (Me 6.6)
Intra-assay variability (repeatability)	cultures	CV between ce conc. in experiment repetitions [%]	1.2–13.8 (Me 10.8)	3.9–34.1 (Me 14.5)
	environmental samples	CV between ce conc. in experiment repetitions [%]	3.9–20.5 (Me 13.2)	4.0–21.0 (Me 13.5)
Background influence	cultures	ratio of ce conc. with/without background	0.5–1.2 (Me 1.0)	0.8–1.5 (Me 1.1)

Table 6
Comparison of *anaC* gene fragments detected by qPCR (assays anaC-441, anaC-1039 and a combination of both) and ATX detected by LC-MS/MS from plankton and biofilm samples. In the combined results, a sample was considered positive only if both qPCR assays produced positive results. The numbers indicate the total number of samples in each category, the percentages indicate the proportion of samples in each category compared to the total number of plankton/biofilm samples, and ± indicate positive or negative results obtained by each method. Numbers of samples where the results of both methods matched are shaded.

Assay	Plankton				Biofilm			
		LC-MS/MS (ATX)				LC-MS/MS (ATX)		
			+	-			+	-
anaC-441	**qPCR**	+	10 (15 %)	13 (20 %)	**qPCR**	+	11 (14 %)	22 (28 %)
		-	2 (3 %)	40 (62 %)		-	10 (13 %)	36 (46 %)
		LC-MS/MS (ATX)				**LC-MS/MS (ATX)**		
			+	-			+	-
anaC-1039	**qPCR**	+	10 (15 %)	8 (12 %)	**qPCR**	+	13 (16 %)	17 (22 %)
		-	2 (3 %)	45 (69 %)		-	8 (10 %)	41 (52 %)
		LC-MS/MS (ATX)				**LC-MS/MS (ATX)**		
			+	-			+	-
Combined	**qPCR**	+	10 (15 %)	6 (9 %)	**qPCR**	+	10 (13 %)	16 (20 %)
		-	2 (3 %)	47 (72 %)		-	11 (14 %)	42 (53 %)

improve considerably, although the proportion of false positive results decreased slightly (Table 6). Calculated cell equivalent concentration from qPCR could not be related to ATX concentrations, as majority of the environmental samples had Cq values above the LOQ (Supplementary File S2).

4. Discussion

A good qPCR assay should not form primer-dimers, have high amplification efficiency and a linear standard curve with a wide dynamic range, be specific, sensitive and robust, i.e. perform reliably even under less than optimal conditions, and have good inter-assay and intra-assay repeatability (Bustin and Huggett, 2017). In this study, we developed and evaluated two new qPCR assays targeting *anaC* gene in ATX-producing strains of cyanobacteria. Both assays showed good and comparable amplification efficiencies, linearity and sensitivity (Table 4), wide dynamic ranges (Fig. 3) and good repeatability (Table 5). The two assays differed most in terms of specificity.

4.1. The risk of false positive results

Some results indicated that the anaC-1039 assay does not have satisfactory specificity, i.e. asymmetric peaks on the melting curve, double bands on the gel after PCR amplification, and positive results for some presumably non-toxic cyanobacterial strains (Table 2). The anaC-441 assay performed better in terms of specificity in cyanobacterial cultures, which was higher than most existing *anaC* and *anaF* assays. However, the experiments with environmental samples showed that the anaC-441 assay produced more presumably false positive results (positive by qPCR and negative by LC-MS/MS) than anaC-1039 (Table 6). Therefore, we can assume that the false positive results observed in cyanobacterial cultures do not necessarily translate to environmental samples. This could be related to the fact that (i) the Cq values of the non-toxic strains were significantly higher than those of the toxic strains (Supplementary File S1) and (ii) the environmental concentrations of potentially toxic cells are generally much lower than the concentrations in laboratory cultures.

The risk of non-specific primer annealing and false positive qPCR results could be caused by primer degeneration in combination with SYBR Green chemistry. In this study, we wanted to develop a cost-effective option suitable for wide application in water monitoring and risk assessment and therefore decided to use SYBR Green chemistry based on a fluorescent dye instead of more costly options, e.g. TaqMan hydrolysis probes. Additionally, the available *anaC* genes do not contain enough conserved regions to design a probe in proximity to the two primers. Furthermore, most primers had to be degenerated by one or two bases to enable the detection of all ATX producers from different genera. Other possible explanations for false positive results could be that (i) the detected strains are capable of producing ATX but did not produce it at the time of sampling, (ii) they produced it at concentrations too low to be detected by LC-MS/MS, (iii) degradation products of ATX were present that could not be detected by our LC-MS/MS method, or (iv) the strains contain some parts of the *ana* gene cluster but have lost other parts making the cluster inactive, as has been shown for the *mcy* gene cluster (Christiansen et al., 2008). This could possibly be the case for the *Planktothrix agardhii* UHCC 0018 culture, which was successfully sequenced with the anaC-441 primers and the identity of the target amplicon was confirmed, even though the strain is presumably non-toxic. However, the cause of false positive results may only be determined by cloning and sequencing amplicons from environmental samples (which might be difficult due to low concentrations of target genes), sequencing the entire *ana* gene clusters or targeting other *ana* genes (if suitable assays were available), and reassessing the toxicity of the culture and environmental samples in question, which is beyond the scope of this study.

The proportion of false positive results decreased slightly when a combination of both qPCR assays was considered (Table 6), but this was not a considerable improvement. However, in cases where a reliable molecular assay cannot be designed yet due to limited genomic data, usage of a combination of two or more assays might still be beneficial. In this study, both assays targeted the same gene (*anaC*), but the performance may potentially be further improved if different genes were targeted in parallel. For example, a combination of *anaC* (proline adenylation protein) and *anaF* (polyketide synthase) was used by Salmaso et al. (2016) on isolated strains and the results of the two markers matched; however, Legrand et al. (2016) used environmental samples and showed that was not always the case. A multi-marker approach might also reduce the risk of false positive results due to detection of the remaining parts of otherwise inactive *ana* gene clusters. However, each new assay has to be carefully validated on its own before attempting such coupling to ensure only the target region is amplified.

4.2. The risk of false negative results

On the other hand, false negative qPCR results can also be an issue if the target region is highly variable among the target strains and the assay is therefore too specific (e.g. as shown by Legrand et al., 2016, for some *anaC* and *anaF* PCR assays). The designed assays showed negative results in two cyanobacterial cultures that presumably produce ATX (*Dolichospermum flos-aquae* NIES-1669 and *Kamptonema* sp. No697). It is known that cyanotoxin production can change over time in laboratory cultures, e.g. as a result of inactivation of genetic clusters (e.g. Christiansen et al., 2008) or changes in gene expression due to environmental conditions (Neilan et al., 2013). This could possibly be the reason for the negative qPCR results, but seems unlikely, as the cultures were processed immediately after obtaining them from the culture collections, which ensure the supply of toxic strains. However, all cyanobacterial strains should be tested for ATX production by LC-MS/MS or another reliable method soon after receiving the strains to clarify these results.

In environmental samples, the proportion of false negative results (detected by LC-MS/MS but *anaC* genes not detected by qPCR) was small (Table 6), which shows that the assays can detect the majority of ATX-producing strains in the studied waterbodies. As most of the samples not detected by qPCR originated from biofilms, this may indicate that the assays are unable to detect all strains of benthic cyanobacteria, which are generally less well studied (Wood et al., 2020) and for which there is little genomic data (Salmaso et al., 2022). As benthic strains are important producers of ATX (Wood et al., 2020), more data from *ana* gene clusters from benthic strains are essential to design an all-inclusive molecular assay in the future. The variability of the *anaC* region between different ATX producers is also illustrated by the variability of Tm values obtained with the anaC-441 assay, with a clear difference between two groups: (i) *Cuspidothrix/Aphanizomenon* and *Kamptonema/Oscillatoria*, and (ii) *Cylindrospermum, Dolichospermum/Anabaena* and *Microcystis*.

Furthermore, there is a slight possibility of a carry-over effect during LC-MS/MS; during extraction and analysis, a sample processed immediately after a positive sample can potentially be contaminated, especially if the previous sample has a high cyanotoxin content. This could potentially explain the presence of low levels of ATX in samples in which no *anaC* genes were detected. In fact, most of the false negative samples (seven out of nine for each assay) had ATX concentrations that were very close to the LOQ of LC-MS/MS. However, the risk of carry-over effect is very low, as procedural blank was used in the analysis, and all equipment was thoroughly washed between samples. In addition, one of the false negative samples samples (B-21–08-GR; Supplementary File S2) had a putative detection of dihydroATX, for which there are no commercial standards available, so we do not have a validated method for this variant. The presence of dihydroATX is very likely, but cannot be confirmed. It should be noted that ATX concentrations were rather low not only in the false negative samples but also in most of the other environmental samples used in this study (Supplementary File S2). On the other hand, the inconsistency between qPCR and LC-MS/MS in these samples could also be caused by unsuccessful qPCR amplification in samples with very low ATX concentrations, or by the presence of sequences that require different qPCR conditions. Additionally, there could be an influence of qPCR amplification inhibition, which was not assessed in this study and could vary between plankton and biofilm samples due to the different DNA extraction methods. However, all the samples used in this study have been successfully used before for qPCR applications with other assays, along with many other plankton and biofilm samples, which were sampled in the same way and from which DNA was extracted using the same protocols (Jablonska et al., 2024; Jablonska et al., in prep.).

4.3. Potential ATX producers in environmental samples

This is the first general qPCR assay for ATX producers, i.e. it can detect all potentially toxic strains, in contrast to species-specific assays. To our knowledge, the only published qPCR assay for the detection of ATX producers was developed by Kelly et al. (2018) and is species-specific, targeting only *Microcoleus/Phormidium*. In this study, ATX and/or related *anaC* genes were frequently found in both plankton and biofilm samples (Supplementary File S2). This agrees with various previous studies in Europe, which illustrate the wide distribution of ATX producers in both habitat types (reviewed in Mantzouki et al., 2018; Wood et al., 2020). A number of reported animal poisonings with ATX were caused by benthic species (e.g. Gugger et al., 2005; Faassen et al., 2012; Bauer et al., 2020), providing another reason to put further efforts into expanding our knowledge on benthic cyanobacterial species, which are often overlooked on the account of planktic species.

To get insight into potential ATX producers in the environment, we related the results of this study to the results of two parallel studies, in which we performed next-generation sequencing (NGS) of 16S rRNA gene on the same samples (Salmaso et al., 2024; Jablonska et al., in prep). The results showed that the most common potential ATX producers (based on the number of samples with these taxa) in plankton samples belong to the genus *Cuspidothrix/Aphanizomenon*, and in biofilm samples to *Kamptonema/Oscillatoria* closely followed by *Microcoleus/Phormidium*. Besides, genera *Tychonema* and *Dolichospermum/Anabaena* were also observed in both sample types. We also examined the dominant taxa in false negative samples to see which ATX-producing taxa might be overlooked by the proposed qPCR assays. However, there was no clear pattern in the dominant ATX-producing taxa in false negative samples, but the sample pool was small (nine samples). It should be mentioned that benthic species are taxonomically more difficult to assign by NGS than planktic species due to incomplete reference databases, as shown in a previous study (Salmaso et al., 2022). Therefore, it is possible that certain benthic producers that could not be identified taxonomically are present in false negative biofilm samples, which constitute a majority of the false negative samples (Table 5).

Sixteen different potentially ATX-producing strains detected in the environmental samples (Salmaso et al., 2024; Jablonska et al., in prep) show a wide taxonomic diversity of potential ATX producers in the environment. While species-specific assays are useful for certain applications, e.g. in systems where several ATX-producing species co-occur and quantification of a single species is preferred, general assays such as those proposed in this study play a very important role. They can be widely used for risk assessment in waters with unknown cyanobacterial profiles and in situations where taxonomic classification is not relevant, but potential toxin production is.

5. Conclusions

Here we propose two new qPCR assays suitable for early detection of potential ATX-producing cyanobacteria in environmental samples, which are also the first published assays targeting all potential ATX producers. Admittedly, some cross-reactivity with non-toxic strains was detected, which could be due to remaining parts of the inactive gene cluster for ATX production. However, the screening of environmental samples showed that in most cases the presence of ATX and target genes matched. A non-negligible proportion of mismatches still indicates that our knowledge of ATX producers, especially in benthic communities, is limited. As shown by several cases of animal poisoning by ATX, water managers could greatly benefit from the inclusion of an early warning system in monitoring activities, allowing rapid risk assessment for human and animal health. Here, the strengths and weaknesses of the two proposed assay are discussed in detail so that the appropriate choice can be made. The results show that both assays are suitable for rapid risk assessment, although they should be complemented by other methods in case of doubt. Further validation would be required to make these assays reliable options for diagnostic procedures, but at this stage they are well-suited for research purposes and rapid screening of water bodies.

This study suggests that further research should focus on (i) expanding genomic databases of ATX producers, especially benthic

strains, (ii) deepening our knowledge of *ana* gene cluster, e.g. investigating the likelihood of inactive gene clusters with residues of *ana* genes, and (iii) developing analytical methods and standards for the detection of all ATX variants and their degradation products.

CRediT authorship contribution statement

Maša Jablonska: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Conceptualization. **Tina Eleršek:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Slovenian Research and Innovation Agency [grant numbers P1–0245, J2–4428, and Slovenian young researchers' program, grant 10040146 (MZ)] and the Slovenian Ministry of Defence [agreement 848–23/2018–4]. The authors are grateful to Maruša Kerenčič and Irma Zeljković Vitas for help with laboratory work, Tomaž Accetto from the University of Ljubljana for help with genomic data analysis, Polona Kogovšek from the National Institute of Biology for help with molecular data interpretation, Leonardo Cerasino from the Fondazione Edmund Mach for providing and helping interpret the LC-MS/MS data, Rainer Kurmayer from the University of Innsbruck for providing the *Kamptonema* strain No697, which was isolated by him from a swimming pond in Lower Austria in 2007 (Mistelbach) after a dog poisoning, Nico Salmaso from the Fondazione Edmund Mach for providing the *Tychonema* strain FEMGT323, the partners from the Interreg Alpine Space, Eco-AlpsWater project [grant number ASP569] for permission to use the selected samples obtained during the project, and two anonymous reviewers for helpful comments to an earlier version of this manuscript. The graphical abstract was created with BioRender.com.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2024.102785.

Data availability

Data will be made available on request.

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