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# *Microcystis aeruginosa* and microcystin detection by Polymerase Chain Reaction

# Detección de *Microcystis aeruginosa* y microcistina mediante la Reacción en Cadena de la Polimerasa

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## ABSTRACT

Cyanobacteria are photosynthetic organisms important in multiple biosphere life cycles; however, some genera such as *Microcystis* can form blooms and produce cyanotoxins that compromise water quality. This study aimed to validate the use of end-point PCR to detect cyanobacteria, the species *Microcystis aeruginosa*, and its genetic capacity to produce microcystins. Three pairs of oligonucleotides were used to obtain the sequences of the 16S rRNA gene regions of cyanobacteria and genus *Microcystis*, as well as, the *mcyA* gene associated with microcystin production. The use of end-point PCR allowed the specific detection of cyanobacteria and *Microcystis aeruginosa* MicArg in cell concentrations up to 10 times below the bloom alert limit, as well as the detection of the *mcyA* gene, both in isolated strains and in a simulated microbial community. The effectiveness of using end-point PCR for the specific detection of cyanobacteria makes it an early monitoring tool, capable of predicting the potential production of microcystins and, therefore, highlighting its usefulness for managing water quality for human consumption.

**KEY WORDS:** Cyanobacteria, cyanotoxin, *mcyA*, *Microcystis aeruginosa*.

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**RESUMEN**

Las cianobacterias son organismos fotosintéticos importantes para diversos ciclos vitales de la biósfera; sin embargo, algunos géneros como *Microcystis* tienen la capacidad de formar floraciones y producir cianotoxinas que comprometen la calidad del agua. Este estudio tuvo como objetivo validar el uso de PCR punto final para detectar cianobacterias, el género *Microcystis* y su capacidad genética para producir microcistinas. Se utilizaron tres pares de oligonucleótidos para obtener las secuencias de las regiones del gen 16S ARNr de cianobacterias y del género *Microcystis*, así como del gen *mcyA* asociado con la producción de microcistinas. El uso de PCR de punto final permitió la detección específica de cianobacterias y de *Microcystis aeruginosa* MicArg en concentraciones celulares hasta 10 veces por debajo del límite de alerta de florecimiento, así como la detección del gen *mcyA*, tanto en cepas aisladas como en una comunidad microbiana simulada. La eficacia del uso de PCR de punto final para la detección específica de cianobacterias lo convierte en una herramienta de monitoreo temprano, capaz de predecir la producción potencial de microcistinas y, por tanto, resaltar su utilidad para la gestión de la calidad del agua para consumo humano.

**KEY WORDS:** Cianobacterias, cianotoxina, *mcyA, Microcystis aeruginosa.*

## Introduction

Cyanobacteria are oxygenic photosynthetic prokaryotes with an evolutionary history dating back to 3.5 billion years (Kaushik & Balasubramanian, 2013). Although they are naturally found in rivers and water supplies, human-induced environmental changes, such as eutrophication and climate change, have accelerated their proliferation in recent decades (Thawabteh *et al*., 2023). This has led to more frequent cyanobacterial blooms, which can severely degrade water quality and pose a significant threat to both drinking and recreational water sources (Chorus *et al*., 2021). A key concern is the ability of many cyanobacterial species to produce cyanotoxins, potent toxins that can impact humans, animals, and plants. These toxins are classified based on their toxicological effects into hepatotoxins, cytotoxins, neurotoxins, and dermatoxins (Bláha *et al*., 2009; Codd *et al*., 2020).

The *Microcystis* genus, which has been reported to bloom on every continent except Antarctica, is one of the cyanobacteria capable of forming blooms and producing toxins (Zurawell

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*et al*., 2005). Some *Microcystis* species produce microcystins (MCs), toxins notorious for their toxicity to humans, animals, plants, phytoplankton, zooplankton, and fish (Campos *et al*., 2021; Lad *et al*., 2022; Ren *et al*., 2023). MCs are hepatotoxic cyclic heptapeptides that contain a shared core component, consisting of a seven amino acid sequence (Watanabe *et al*., 1996). More than 246 isoforms of MCs have been detected (Meriluoto *et al*., 2016), primarily differing in the L-amino acids at positions 2 and 4, which result in differences in toxicokinetic and toxicodynamic properties (Rinehart *et al*., 1994). These toxins can contaminate rivers and streams, posing a significant threat to drinking water supplies, as they can bypass standard water treatment systems undetected (Thawabteh *et al*., 2023). Thus, effective water management policies are essential not only for detecting cyanobacteria responsible for blooms but also for assessing the presence of cyanotoxins in water bodies. While microscopy has traditionally been used to identify and quantify cyanobacterial species, it has significant limitations, including the inability to differentiate between toxic and nontoxic strains and the need for considerable taxonomic expertise. On the other hand, molecular methods offer specificity, reliability, and speed, allowing for the detection and quantification of cyanobacteria and their toxin-encoding genes. These highly sensitive techniques enable early warning of toxic cyanobacteria, often detecting them well before visible blooms occur, making them a valuable tool for water quality monitoring (Codd *et al*., 2020; Farrer *et al*., 2015). In this regard, efforts to detect genes associated with MC production have primarily focused on the *mcyA* gene, one of ten genes within the *mcy* gene cluster responsible for MCs biosynthesis (55 kbp in total) (Beversdorf *et al*., 2015). The *mcyA* gene is specifically highlighted because it encodes a nonribosomal peptide synthetase, which plays a crucial role in synthesizing toxins (Rouhiainen *et al*., 2004). Furthermore, the abundance of *mcyA* genes has been shown to significantly correlate with MC concentrations in freshwater environments (Lee *et al*., 2020).

Therefore, this study aims to validate the use of PCR for detecting cyanobacteria, specifically of the genus *Microcystis,* and its genetic potential to produce MCs in a simulated microbial community, by amplifying the 16S rRNA gene of cyanobacteria (Valério *et al*., 2009) and genus *Microcystis* (Martins & Vasconcelos, 2011), as well as the *mcyA* gene (Sabart *et al*., 2015), to assess the effectiveness of PCR as an early warning tool for harmful cyanobacterial blooms.

## Material and Methods

### Microbial strains

The studied strains were provided by: i) Instituto de Ciencias del Mar y Limnología (ICML), Mazatlán, México (strains M132/1 and NIES-39); ii) Colección de Microalgas del Proyecto de Cultivos de Microalgas Usos Potenciales, Universidad Autónoma Metropolitana, Iztapalapa, México (SalJ 1, PAM1, and AAS1); iii) *Microcystis aeruginosa* MicArg by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina; and iv) bacterial and fungal strains from the Colección de Microorganismos Edáficos y Endófitos Nativos (COLMENA) (de los Santos-Villalobos *et al*., 2018, 2021), Cd. Obregón, México (*Bacillus cabrialesii* TE3$^{T}$,

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*Bacillus paralicheniformis* TRQ65, *Priestia megaterium* TRQ8, *Rhizopus* sp. 11, *Curvularia spicifera* 6, *Fusarium* sp. 20, *Nannochloropsis* sp. P6, *Nannochloropsis* sp. P11, and *Nannochloropsis* sp. P13). The cyanobacterial isolates were cultured to obtain biomass using BG-11 medium (SIGMA) (Rippka *et al*., 1981), nutrient broth (MCD LAB) was used for *Bacillus* and *Priestia* strains, and dextrose and potato broth (MCD LAB) for fungal strains; these cultures were incubated at 28 °C for 7, 2, and 5 days, respectively.

### DNA extraction, Polymerase Chain Reaction (PCR), and amplicon sequencing

DNA extraction was performed using the protocol described by Raeder & Broda (1985). The integrity of the extracted DNA was assessed using 1% agarose (MCD LAB) gel electrophoresis at 100 V for 1 h, and the quality was evaluated using a NanoDrop 2000C. The primer pairs for the amplification of the 16S rRNA of cyanobacteria (CYAN738F: ATACCCCWGTAGTCCTAGC; CYAN1281R: GCAATTACTAGCGATTCCTCC) (Valério *et al*., 2009), 16S rRNA of *Microcystis* (MICR 184F: GCCGCRAGGTGAAAMCTAA; MICR 431R: AATCCAAARACCTTCCTCCC) (Martins & Vasconcelos, 2011), and *mcyA* gene (mcyA-Cd-1F: AAAATTAAAAGCCGTATCAAA; mcyA-Cd-1R: AAAAGTGTTTTATTAGCGGCTCAT) (Sabart *et al*., 2015), were tested as mentioned in the following sections through end-point PCR (n=3). These PCR were carried out using the PCR Master Mix 2X PCR kit (Promega) and the following program: initial denaturation at 94 °C for 3 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing using 57 °C to 16S rRNA of cyanobacteria and *mcyA,* and 54 °C to 16S rRNA of *Microcystis* for 40 seconds, and elongation at 72 °C for 30 seconds, and a final elongation step was performed at 72 °C for 5 minutes. The detection limit of the evaluated primers was determined as described above and using 0.001, 0.01, 0.1, 1, 10, 100, and 1000 ng of DNA per reaction, corresponding to 1.39, $1.39\times10^{1}$, $1.39\times10^{2}$, $1.39\times10^{3}$, $1.39\times10^{4}$, $1.39\times10^{5}$, $1.39\times10^{6}$ cells of *Microcystis aeruginosa* MicArg/mL respectively. PCR products were visualized by electrophoresis in a 1.5% agarose gel at 100 V for 60 min.

Thus, amplicons of the 16S rRNA of cyanobacteria and genus *Microcystis*, as well as the *mcyA* gene used in this work were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and then sequenced by Sanger technology (Langebio-Cinvestav, Irapuato, Guanajuato). The sequences obtained were: i) processed using FinchTV V1.4.0; ii) aligned in CLC Sequence Viewer 8 to create a phylogenetic tree using the Neighbor-Joining construction method with 1,000 replicates (only 16S rRNA of cyanobacteria); and iii) uploaded into the GenBank database.

### Detection of cyanobacteria, *Microcystis,* and *mcyA* gene in a simulated microbial community

A simulated microbial community was formulated using all cyanobacterial, bacterial, and fungal strains mentioned above (see microbial strains section), in the presence and absence of *Microcystis aeruginosa* MicArg, carrying out end-point PCR using the evaluated primers at 30 ng of DNA for each isolate per reaction, under the amplification conditions mentioned before. Amplicons were visualized by electrophoresis in a 1.5% agarose gel at 100 V for 60 min.

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## Results and Discussion

### DNA extraction and evaluation

Electrophoresis of the extracted DNA showed a band of intact genomic DNA, as seen at the top of the lanes in Figure 1. The results of microvolume spectrophotometry showed values of 22.5-402.5 ng/µL, 1.50-2.22 (260/280), and 1.68-2.29 (260/230), which are suitable concentrations for the following evaluations (Kadri, 2020; Lucena-Aguilar *et al*., 2016).

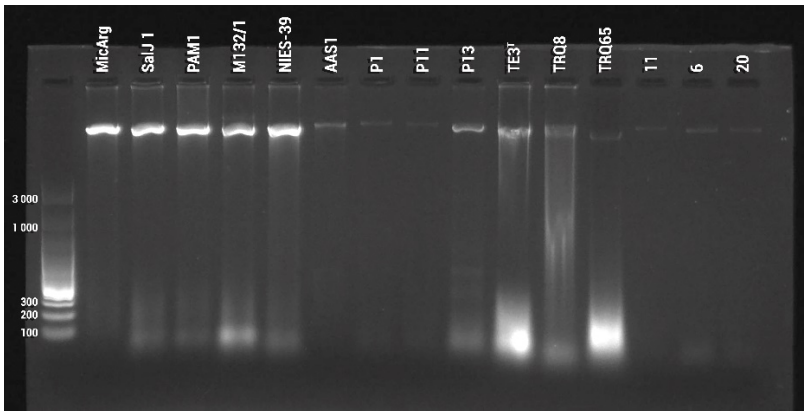


**Figure 1. DNA extraction of studied strains.**

Source: own elaboration.

### Determination of detection limit

The detection limits of the 16S rRNA gene of cyanobacteria and *Microcystis* were determined from 1 ng per reaction, which corresponds to $1.39\times10^3$ cells of *Microcystis aeruginosa* MicArg/mL (Figure 2). This detection is 1 order of magnitude below the lower alert limit for health effects published by the Environmental Protection Agency (EPA) (2024), or considering a biovolume equivalent to 0.099 $mm^3$ of *Microcystis aeruginosa* MicArg/L (Reynolds *et al*., 1981), 40 times below the vigilance level set at 4 $mm^3$ of cyanobacteria/L published by the World Health Organization (WHO) (2021). The detection of the *mcyA* gene was possible from 10 ng per reaction. This approach enables to detection of cyanobacteria and *Microcystis* at concentrations ten times lower than those considered risky and to monitor the presence of the *mcyA* gene.

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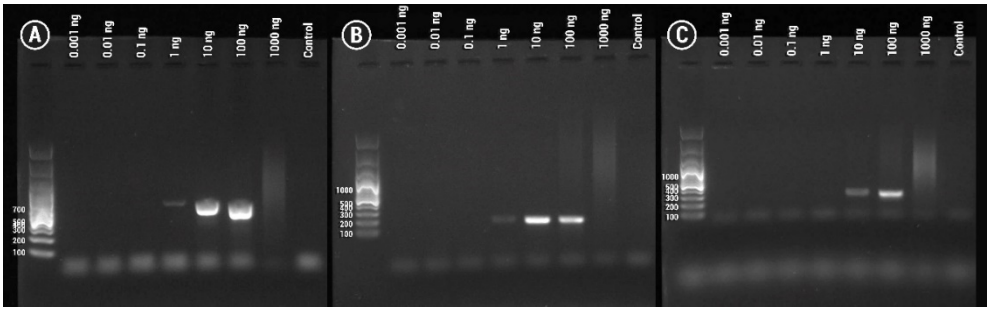


**Figure 2. The detection limit of the genes (A) 16S rRNA from cyanobacteria, (B) 16S rRNA from *Microcystis,* and (C) *mcyA*, using *Microcystis aeruginosa* MicArg DNA and their respective controls without DNA template.**

Source: own elaboration.

## 16S rRNA and mcyA sequencing of cyanobacterial strains

Amplification of the studied genes showed amplicons with the expected band sizes (Figure 3), showing 16S rRNA amplicons from cyanobacteria of ~550 bp (Valério *et al*., 2009), 16S rRNA from *Microcystis* of ~220 bp (Martins & Vasconcelos, 2011) and *mcyA* of ~290 bp (Sabart *et al*., 2015). Likewise, the sequences obtained after purification and sequencing of the amplicons were used to make the phylogenetic tree (Figure 4), and to perform BLAST® searches (Table 1).

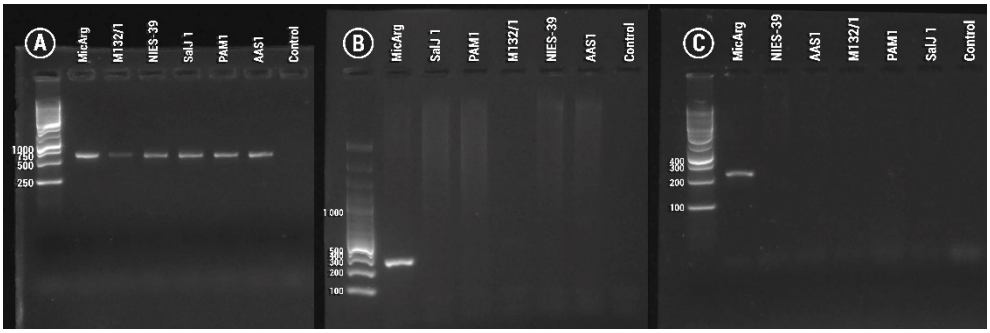


**Figure 3. Gene amplification: (A) 16S rRNA from cyanobacteria, (B) 16S rRNA from *Microcystis,* and (C) *mcyA*, with DNA from cyanobacteria isolates, and their respective controls without DNA template.**

Source: own elaboration.

The positive control, strain MicArg, was accurately identified as *Microcystis aeruginosa* through both phylogenetic analysis and National Center for Biotechnology Information (NCBI)

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database searches. Taxonomic classification of these organisms can be achieved by analyzing the hypervariable regions of the 16S rRNA gene. These regions, containing both conserved and hypervariable sequences, allow for the differentiation of phylogenetic relationships and the assignment of taxonomic groups (Shahi *et al*., 2017; Valério *et al*., 2009). Although the sequencing of a conserved gene is a good approach to the affiliation of a microorganism, polyphasic taxonomy is necessary to provide more information about the isolate and allow this task to be performed accurately (Morales-Sandoval *et al*., 2021).

Amplification of the 16S rRNA gene of *Microcystis* was obtained only with the *Microcystis aeruginosa* MicArg DNA and not with the rest of the cyanobacteria (Figure 3); This result aligns with previous taxonomic classification and confirms the accuracy of our approach (Martins & Vasconcelos, 2011).

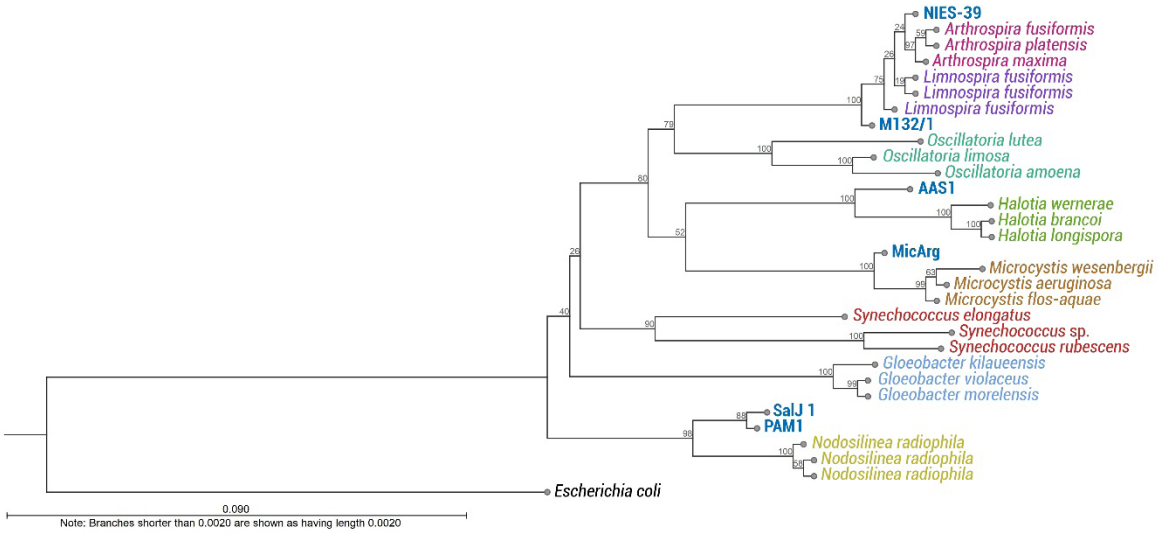


Figure 4. Phylogenetic tree constructed by the Neighbor-Joining method with 1,000 replicates, using the amplified sequence of the 16S rRNA gene with specific primers for cyanobacteria.

Source: own elaboration.

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**Table 1. Identification of 16S rRNA and *mcyA* sequences in BLAST®.**

| Gene | Strain | Affiliation | Query cover (%) | Similarity (%) | Accession number |
|---|---|---|---|---|---|
| 16S rRNA cyanobacteria | M132/1 | *Limnospira* sp. | 99 | 98.44 | PQ308697 |
| | NIES-39 | *Arthrospira* sp. | 99 | 99.81 | PQ308696 |
| | AAS1 | *Halotia* sp. | 100 | 97.34 | PQ285593 |
| | SalJ 1 | *Nodosilinea* sp. | 100 | 97.13 | PQ301223 |
| | PAM1 | *Nodosilinea* sp. | 99 | 96.83 | PQ301224 |
| 16S rRNA *Microcystis* | MicArg | *Microcystis aeruginosa* | 100 | 100.00 | PQ276908 |
| | MicArg | *Microcystis aeruginosa* | 99 | 100.00 | PQ276909 |
| *mcyA* | MicArg | *Microcystis aeruginosa* peptide synthetase (*mcyA*) gene | 100 | 99.33 | PQ303791 |

Source: own elaboration.

Furthermore, the morphological features observed under the microscope (Figure 5) strengthen the taxonomic affiliation by molecular techniques, observing the distinctive morphologies of: i) *Limnospira* (M132/1) forming trichomes of uniform width, granular protoplasm, facultative aerotropes (not observed) and width of 6-8 µm (Santos *et al*., 2023); ii) *Arthrospira* (NIES-39) being filamentous, cylindrical, and forming trichomes of 3-12 µm wide by 50-500 µm long (Wan *et al*., 2016); iii) *Halotia* (AAS1) forms trichomes with especially spherical or hemispherical cells (Genuário *et al*., 2015); iv) *Nodosilinea* (SalJ 1 and PAM1) has rounded ends, is unbranched, forms trichomes and has diffuse-looking vines (Perkerson III *et al*., 2011); and iv) *Microcystis aeruginosa* (MicArg) is spherical, forms colonies and appears agglomerated (Radkova *et al*., 2020).

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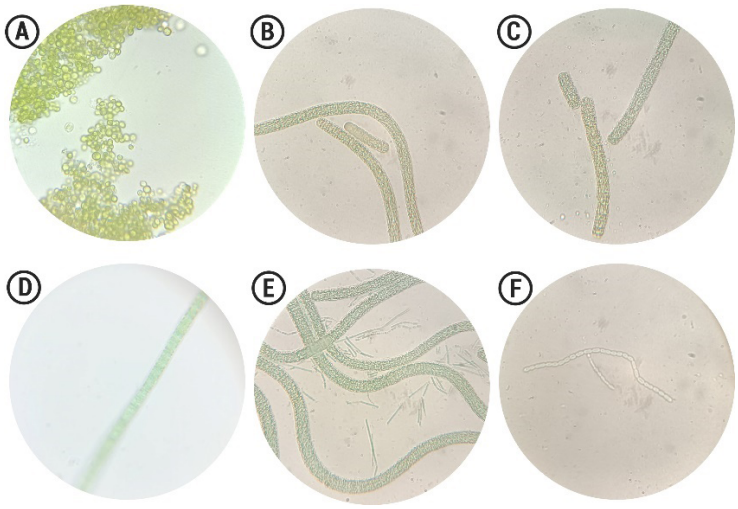


**Figure 5. Microscopic morphology observation of (A) *Microcystis aeruginosa* MicArg, (B) *Arthrospira* sp. NIES-39, (C) *Limnospira* sp. M132/1, (D) *Nodosilinea* sp. SalJ 1, (E) *Nodosilinea* sp. PAM1 and (F) *Halotia* sp. AAS1.**

Source: own elaboration.

Considering the previous taxonomic affiliation, and the known ability of only certain cyanobacterial genera (*Microcystis*, *Anabaena* now *Dolichospermum* or *Sphaerospermopsis*, *Oscillatoria* now *Planktothrix*, *Nostoc*, *Cylindrospermopsis*, *Anabaenopsis*, *Aphanocapsa*, *Aphanizomenon* and *Hapalosiphon)* to produce MCs (Beasley, 2020; Bittencourt-Oliveira *et al*., 2014), the detection of the *mcyA* gene confirms the presence of a potential MCs-producing organism and verify that there are no false positives. The detection of this gene constitutes a crucial step in predicting the production of MC in a sample, this is because the *mcyA* gene codes for the production of a Non-Ribosomal Peptide Synthetase (NRPS), which together with the enzymes produced by the *mcyA-E* and *mcyG* genes participate in 45 of the 48 reactions to give rise to the formation of MCs (Omidi *et al*., 2018; Zhou *et al*., 2021). Furthermore, it has been reported that non-toxigenic strains mainly lose this gene, inhibiting the production of MCs (Chen *et al*., 2021).

## Detection of cyanobacteria, *Microcystis*, and *mcyA* gene in a simulated microbial community

By amplifying the genes studied using DNA from a simulated microbial community, it was possible to identify *Microcystis aeruginosa* MicArg and the *mcyA* gene only when this organism was in the sample (Figure 6).

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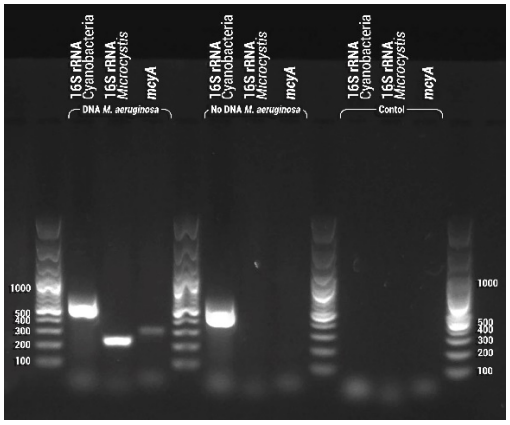


**Figure 6. Amplification of cyanobacterial and *Microcystis* 16S rRNA, and *mcyA* using DNA from a simulated microbial community and their respective controls without DNA template.**

Source: own elaboration.

The detection of *Microcystis aeruginosa* MicArg and the *mcyA* gene in a simulated microbial community, and extrapolation of similar results expected in environmental samples, suggests that there is a risk of MCs production. As demonstrated by Singh *et al*. (2015), there is a correlation between *mcy*A gene detection and MCs concentration in water samples, additionally, the presence of *mcy*A genes aligns with the detection of cyanobacteria using 16S rRNA gene analysis. Supporting this idea, subsequent studies showed a positive correlation between the number of copies of the *mcyA* gene and the concentration of MCs in water (Dong *et al*., 2016), and research by Hu *et al*. (2016) determined how the concentration of MCs in water increases as the copies of the *mcyA* gene increase over a year. This shows a trend where the presence of *Microcystis* and the *mcyA* gene is related to the production of MCs in water samples, aligning with potential environmental scenarios.

In addition, Ngwa *et al*. (2014) demonstrate the effectiveness of conventional PCR-based methods in detecting MCs-producing *Microcystis* genotypes. They applied three independent PCR assays targeting the *mcyA*, *E,* and *G* genes to rapidly and reliably detect and quantify potentially toxic *Microcystis* genotypes in water. Similarly, Valério *et al*., (2010) developed a multiplex PCR method that simultaneously amplifies multiple *mcy* gene fragments. Their method achieved high sensitivity and specificity in identifying MCs-producing cyanobacteria, regardless of their taxa. This approach, validated with both isolates and environmental samples, further supports the utility of PCR-based methods for assessing the toxigenic potential of cyanobacteria in water systems.

MCs, the most widespread cyanobacterial toxins worldwide, pose a serious threat to human health, affecting organisms from microalgae to mammals (De Figueiredo *et al*., 2004). Humans can be exposed to MCs through contaminated water, food, algal supplements, inhalation, skin contact,

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and hemodialysis (Massey *et al*., 2018). Once in the bloodstream, MCs primarily target the liver but also affect the brain, kidneys, lungs, heart, and reproductive system (Massey *et al*., 2018; Shi *et al*., 2021). Therefore, identifying genes responsible for MCs biosynthesis through molecular methods is essential for monitoring harmful cyanobacteria and toxin production (Saker *et al*., 2007). To reduce health risks, water utilities must develop sensitive, reliable detection methods across various sample types (Kaushik & Balasubramanian, 2013). PCR offers an approach to detect toxic cyanobacteria directly from environmental samples, with its high sensitivity enabling early detection of toxin-producing species before they reach harmful concentrations, thereby improving the monitoring of toxic cyanobacterial blooms (De Figueiredo *et al*., 2004; Saker *et al*., 2007).

## Conclusion

End-point PCR using specific primers enables the detection of cyanobacteria, the genus *Microcystis*, and its genetic capacity for MCs production. This confirms the value of PCR for the specific detection of harmful cyanobacteria and explores its application in environmental samples.

### Authors' contribution

Conceptualization of the work, SdlSV, RAR, MPH.; development of the methodology, RACL, MJOF.; software management, RACL.; experimental validation, RACL, MJOF.; analysis of results, RACL, MJOF, SdlSV.; Data management, RACL, SdlSV.; writing and preparation of the manuscript, RACL, MJOF, SdlSV.; writing, review, and editing, RACL, MJOF, SdlSV, RAR, MPH.; project administrator, SdlSV.; acquisition of funds, SdlSV. All authors of this manuscript have read and accepted the published version of the same.

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### Conflict of interest

The authors declare that they have no conflict of interest.

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