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Cyanobacteria and Cyanotoxins in Mediterranean Reservoirs: Ecological Variability, Risks and Implications for Water Quality Assessment

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ABSTRACT

The increasing demand for freshwater, in combination with climate change and pollution, compromises the quality and quantity of water resources. These pressures have intensified cyanobacterial blooms, including toxic events that threaten human health and aquatic ecosystems. This study complements the objectives of the Water Framework Directive by addressing the current lack of systematic data on toxic cyanobacteria and cyanotoxins. Therefore, the occurrence of toxic cyanobacteria in Mediterranean reservoirs was revisited, and the findings were complemented by case studies of two Portuguese reservoirs. Phytoplankton community composition and toxin concentrations were analysed, and cyanobacterial strains were screened for toxin-producing genes. The data gathered from literature revealed widespread cyanobacterial blooms across Spain, Portugal and Italy, with cell densities frequently exceeding the WHO medium-risk threshold (20,000 cells/mL) and occasionally reaching the very high-risk level (1000,000 cells/mL). Microcystins were the most frequently detected toxins, often occurring above the WHO guideline (1 μg/L), whereas saxitoxin, anatoxin and cylindrospermopsin were detected less frequently. In Portugal, the Aguieira reservoir exhibited recurrent blooms and high phytoplankton biomass under eutrophic conditions, while the Rabagão reservoir showed no relevant cyanobacterial proliferation. Molecular analyses confirmed the presence of the mcyA gene in *Microcystis aeruginosa* isolated from Aguieira, indicating its potential for microcystin production, whereas *Aphanizomenon flos-aquae* lacked detectable toxin biosynthesis genes. Overall, these findings highlight the ecological variability of cyanobacteria in Mediterranean reservoirs and emphasize the importance of integrating toxin monitoring and molecular tools into water quality assessments to strengthen management strategies and safeguard public health.

1 | Introduction

Water is a vital natural resource, historically considered inexhaustible, but it is now nearing a critical threshold where human consumption could exceed the rate at which it is naturally replenished (Pinto et al. 2023). Anthropogenic pressures, particularly urbanization, agriculture and industrial activities, are the major drivers of water degradation (Portela et al. 2024; Pinto et al. 2025a). Despite these factors, the combined effects of nutrient influx, particularly nitrogen and

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phosphorus, along with rising global temperatures, promote a higher level of water degradation with the occurrence of eutrophication processes (Benredjem et al. 2022). Moreover, when this process is associated with cyanobacterial blooms in inland waters, the prevalence of toxic blooms may occur, making the water unsuitable for anthropic purposes (e.g., irrigation and consumption) (Cordeiro et al. 2020; Haida et al. 2024).

Cyanobacteria are among the most abundant microorganisms on earth, thriving across diverse aquatic and terrestrial habitats (Benredjem et al. 2022). These photosynthetic prokaryotes possess gas vesicles that facilitate vertical movement in stratified waters, enabling access to sunlight and nutrient-rich shaded regions in the epilimnion and hypolimnion. Their ability to produce oxygen and convert CO_2 into biomass through photosynthesis provides a distinct ecological advantage. Additionally, some cyanobacteria fix atmospheric nitrogen via specialized heterocysts, allowing survival in nitrogen-limited environments (Cordeiro et al. 2020). Cyanobacterial blooms, characterized by the excessive growth of these organisms in lentic ecosystems, disrupt local biota and pose serious health risks through the release of harmful cyanotoxins (Moreira et al. 2020; Hinojosa et al. 2023). Cyanobacterial blooms are a global environmental concern, with estimates indicating that over 75% of these events lead to the production of a wide range of toxic metabolites, collectively referred to as cyanotoxins (Haida et al. 2022). Based on their mode of action, cyanotoxins are classified into hepatotoxins (e.g., microcystins and nodularins), cytotoxins (e.g., cylindrospermopsin), neurotoxins (e.g., anatoxins and saxitoxins) and dermal toxins (e.g., aplysiatoxin and lyngbyatoxin) (Moreira et al. 2022). These compounds have been linked to severe biological damage in a wide range of organisms, raising concerns for ecosystem integrity and human health (Cordeiro et al. 2020; Moreira et al. 2020; Stroski et al. 2024).

Microcystins (MCs) are the most frequently toxin detected, especially during bloom events. Produced by genera such as *Microcystis*, *Anabaena* and *Planktothrix*, MCs are hepatotoxins with over 270 known variants, with microcystin-LR (MC-LR) being the most toxic and widespread (Bouaïcha et al. 2019; Haida et al. 2024). These compounds can cause disrupt antioxidant and detoxification enzyme activity and DNA damage in zooplankton and fish (Pham and Utsumi 2018; Passos et al. 2022) and can induce tissue damage (e.g., liver, intestines and gills) in bivalves and fish (Pham and Utsumi 2018). Nodularins (NODs), structurally similar to MCs, are mainly produced by *Nodularia spumigena* and *Nodularia sphaerocarpa*. Although less studied, NODs exhibit similar toxic effects and are considered hazardous to human and animal health, causing allergic reactions, gastrointestinal disorders, and liver damage in amphibians, reptiles and fish; at cellular level, they induce effects such as DNA damage and lipid peroxidation in all aquatic organisms (Yilmaz et al. 2022; Melaram et al. 2024). Another important cyanotoxin is cylindrospermopsin (CYN), a cytotoxin produced by various cyanobacteria genera including *Raphidiopsis*, *Anabaena* and *Oscillatoria* (Hinojosa et al. 2023). CYN inhibits protein and glutathione synthesis, increases oxidative stress in all aquatic organisms (Chen et al. 2021) and can lead to apoptosis and DNA damage (e.g., liver, heart, gastrointestinal tract and immune and nervous systems) in amphibians, reptiles and fish (Buratti et al. 2017).

Anatoxin-a (ATX) is a neurotoxin produced by several genera, such as *Anabaena* and *Planktothrix* (Yilmaz et al. 2022). Although it can reach high concentrations during blooms, it degrades quickly in aquatic environments (Welker and Von Döhren 2006; Funari and Testai 2008). ATX mimics acetylcholine, disrupting neuromuscular function, and has been linked to DNA damage and developmental effects in fish (Buratti et al. 2017; Christensen and Khan 2020). Lastly, saxitoxins (STX), a group of over 30 neurotoxic alkaloids, are produced by cyanobacteria such as *Cylindrospermopsis* and *Aphanizomenon* (Trainer and Hardy 2015). Originally associated with marine environments, they are increasingly detected in freshwater systems as described by Trainer and Hardy (2015). STX disrupts neuronal and muscular electrical signalling by blocking sodium, calcium and potassium channels in zooplankton, crustaceans and fish (Buratti et al. 2017).

Over the past few decades, the intensity and frequency of toxic cyanoblooms and the presence of cyanotoxins have increased worldwide, posing significant challenges to freshwater bodies, namely, reservoirs (Codd et al. 2005; Huisman et al. 2018; Chorus and Welker 2021). As a result, organisms inhabiting or relying on contaminated waters for drinking or feeding are chronically exposed to cyanotoxins. Such exposure can disrupt the development and reproduction of aquatic invertebrates (e.g., amphibians and fish) and, in severe cases, may even result in the death of aquatic and terrestrial species (Mehinto et al. 2021). Furthermore, humans are frequently exposed to cyanotoxin-contaminated water, whether through recreational activities or through its use in crop irrigation (Haida et al. 2024). Regarding the effects in the human population, the World Health Organization (WHO) has established maximum allowable concentrations of 1.0 μg/L for total microcystins (MCs) and 3.0 μg/L for total saxitoxins (SXTs) in drinking water (Passos et al. 2022). For cylindrospermopsins, national regulations have set different limits, such as 1.0 μg/L in Australia and New Zealand and 15 μg/L in Brazil (Moreira et al. 2022). However, from an environmental and water policy perspective in Europe, these compounds have not yet been integrated into water quality assessments, as they are not included in the current European Water Framework Directive (European Union 2000) or any of the five associated Watch Lists (Carvalho et al. 2015; Napierska et al. 2018; Gomez Cortes et al. 2020, 2022, 2025).

Into the context of current global environmental pressures, synthesizing knowledge on the dynamics of cyanobacteria and cyanotoxins occurrence is increasingly critical. Continuous monitoring efforts are demanded in Portugal for improving knowledge of future national and international regulations (Moreira et al. 2022). Based on this, the present study aims (i) to conduct a review focused on the historical occurrence of cyanobacteria and cyanotoxins in a European countries context; and (ii) to evaluate the cyanobacteria impacts on water quality in two Portuguese reservoirs (Rabagão and Aguieira). This assessment seeks to strengthen the WFD approach, particularly the evaluation based on the phytoplankton element, which

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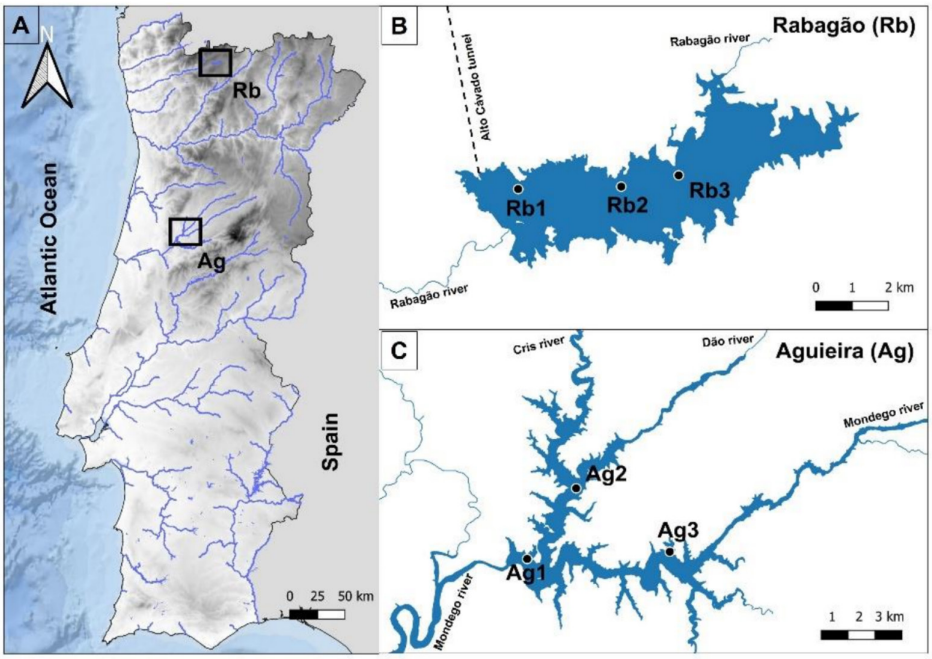


FIGURE 1 | Location of the two study areas: (A) in the context of the country Portugal; (B) sampling sites locations in Rabagão reservoir (Rb1: 41°44′52.9″ N, 7°51′03.0″ W; Rb2: 41°44′54.8″ N, 7°48′60.0″ W; and Rb3: 41°45′04.9″ N, 7°47′51.4″ W); (C) sampling sites locations in Aguieira reservoir (Ag1: 40°20′32.7″ N, 8°11′18.4″ W; Ag2: 40°21′59.2″ N, 8°10′01.3″ W; and Ag3: 40°20′41.6″ N, 8°07′34.0″ W).

currently overlooks the presence of toxic cyanobacteria and does not include cyanotoxins in its list of targeted compounds.

2 | Materials and Methods

2.1 | Review of Cyanobacteria and Cyanotoxins Quantifications

2.1.1 | Literature Review Criteria

A literature review was conducted in the Scopus database to identify primary research articles focused on the quantification of cyanobacteria and cyanotoxins in Europe reservoirs. Scopus was selected due to its broad multidisciplinary coverage and frequent use in ecological and environmental research (Portela et al. 2024). The following search terms were used in the Scopus database: 'reservoir' AND ('Portugal' OR 'Spain' OR 'Greece' OR 'Italy' OR 'France' OR 'Cyprus' OR 'Romania') AND ('cyanobacteria' OR 'neurotoxin' OR 'anatoxin' OR 'homoanatoxin' OR 'saxitoxin' OR 'hepatotoxin' OR 'microcystin' OR 'nodularin' OR 'cylindrospermopsin' OR 'dermatotoxin' OR 'lipopolysaccharide').

The literature review was limited to articles published in English between 1993 (the year of the earliest identified publication) and 2024. The resulting articles were screened based on the following inclusion criteria: (i) availability of the full text; (ii) study location, retaining only studies conducted in countries belonging to the WFD Mediterranean Geographical Intercalibration Group; (iii) system type: only studies carried out in reservoirs were included; (iv) inclusion of water quality parameters as explicit variables, excluding literature reviews and non-empirical studies; and (v) clear quantification of measured parameters, excluding cases that refer only to presence/absence.

All the articles that met the eligibility criteria and reported quantifications of cyanobacteria and cyanotoxins were categorized based on the type of toxin: microcystins, anatoxins, saxitoxins, cylindrospermopsins and nodularins.

We used the dataset compiled from the literature review to construct Sankey diagrams—a type of flow diagram that visually represents relationships within a system, where the width of the lines corresponds to the magnitude of the flow. These diagrams were used to illustrate the frequency of studies conducted in each country, as well as the specific focus and frequency of studies on cyanobacteria and cyanotoxins. The diagrams were generated using the free online tool SankeyMATIC (https://sankeymatic.com/build/).

2.2 | Case of Study in Portugal

2.2.1 | Study Areas

The Rabagão reservoir, also known as Pisões, is located in the municipality of Montalegre, northern Portugal (Figure 1). This reservoir is located on the Rabagão River and receives inflows from the Alto Cávado reservoir (Pinto et al. 2025a). It was built in 1964 and is one of the largest reservoirs in Portugal, covering a total area of approximately 2000 ha and extending over 10 km (Pinto et al. 2025b). The main purpose of this reservoir is hydroelectric power generation, as well as water supply, supporting trout farming and recreational activities (Almeida et al. 2020). Additionally, the region has become a popular tourist destination, offering natural

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landscapes and opportunities for leisure activities. Regarding land use and land cover within the hydrographic basin, forests are predominant, followed by agricultural and agro-forestry areas. A trout farming aquaculture facility represents the only recorded punctual qualitative pressure affecting this reservoir (Guimarães et al. 2025). Rabagão reservoir showed a good ecological potential, with general physical, chemical and biological elements within the reference values according to the three WFD monitoring cycles (Agência Portuguesa do Ambiente 2022a).

Aguieira reservoir is located in the Coimbra and Viseu district, centre of Portugal (Figure 1), and has been operating since 1981 with the purpose of energy production, consumption, irrigation, water storage and recreational activities (Rodrigues, Pinto, Formigo, and Antunes 2021). The Aguieira reservoir was formed by the confluence of the Dão, Cris and Mondego rivers and is situated within the hydrographic basin of Mondego river (Pinto et al. 2025a). The surrounding landscape is mainly used for forestry, intensive agriculture and industrial activities, like food, textile, wood and cork industries (Rodrigues, Pinto, Martins, et al. 2021; Guimarães et al. 2025). Furthermore, a higher number of pressures were identified, including numerous underground catchments ($n = 137$), mainly for agricultural purposes, substantial discharges from WWTPs and recurrent wildfire events (Guimarães et al. 2025). This reservoir was included in the WFD intercalibration study (Pinto, Rodrigues, and Antunes 2021), and according to the three WFD monitoring cycles, this water body presents a poor or moderate ecological potential, mainly due to the high nutrient concentrations and poor quality of the phytoplankton element (Pinto, Rodrigues, Lage, et al. 2021; Agência Portuguesa do Ambiente 2022b).

2.2.2 | Rabagão and Aguieira Phytoplankton Historical Data

A historical survey of the composition of the phytoplankton community was carried out, specifically the Algae Group Index (AGI) and the cyanobacteria composition (mm^3/L) namely the *Aphanizomenon* sp. and *Microcystis* sp. The data were obtained by EDP, S.A., under the concession contract and made available by Agência Portuguesa do Ambiente, I.P. Data were also collected by our team from the Rabagão reservoir in 2014 (Almeida et al. 2020) and from the Aguieira reservoir as part of the ReDEFine project in 2018–2019 (POCI-01-0145-FEDER-029368; Pinto, Rodrigues, and Antunes 2021).

2.2.3 | Collection of Phytoplankton and Water Samples

Throughout 2023, sub-superficial water samples (1 L) were collected to analyse the phytoplankton community following the national protocol (Instituto da Água 2009), which requires three summer sampling campaigns at a minimum 3-week intervals (Agência Portuguesa do Ambiente 2023), supplemented by an additional sampling campaign in each of the seasons (winter, spring, autumn; Pinto et al. 2025b).

The analysis and characterization of the phytoplankton community was carried out in accordance with the legal guidelines (Instituto da Água 2009; Agência Portuguesa do Ambiente 2023), including the determination of the Algae Group Index (AGI) composition and the cyanobacteria biovolume (mm^3/L) (for more details, see Pinto, Rodrigues, and Antunes 2021).

Additionally, 1-L subsurface water samples were collected for cyanotoxins analysis. For homogenization and to conduct the cell lysis, the water samples were sonicated in ultrasound, and the cell concentration/cleaning was carried out in a dual system of C-18 solid phase cartridges and activated carbon. The cyanotoxin research was carried out using the liquid chromatography technique coupled with a mass spectrometry detector in the Waters MicroMass system. The method used determines the presence/absence of anatoxin, saxitoxin, cylindrospermopsin, microcystins (LR, RR, YR, LY, LA and LF) and nodularin using specific standards of Anatoxin-a (CRM-03-ANA, Lot 16-001, 99% purity), Saxitoxin (CRM-00-STX, Lot 16-001, 99% purity), Microcystin-LR (CRM-00-MC-LR, Lot 19-001, 96% purity), Microcystin-RR (CRM-00-MC-RR, Lot 19-001, 96% purity), Microcystin-YR (CRM-00-MC-YR, Lot 19-001, 96% purity) and Cylindrospermopsin (CRM-03-CYN, Lot 16-001, 99% purity) supplied by Cifga (Lugo, Spain).

2.2.4 | Cyanobacteria Isolation and Culture

Cyanobacteria were isolated using a micromanipulation technique, followed by streak plating on a 1.5% agar culture medium. The plates were regularly examined with a Carl Zeiss 37081 inverted microscope (Carl Zeiss, Gottingen, Germany). Isolated strains were transferred to fresh plates and also cultivated in liquid medium. The cyanobacteria were cultivated and maintained in Z8 medium under a 14 h^L:10 h^D light cycle at a temperature of $20°C \pm 2°C$. To prevent the growth of eukaryotic microorganisms, cycloheximide (Sigma, Dorse, UK) was added to the culture medium at a concentration of 100 μg/mL during the initial isolation steps (Rippka 1988).

2.2.5 | Morphological Characterization

Morphological identification followed the classical taxonomy outlined by Komárek and Anagnostidis (1998) and Komárek (2013). Observations included the formation of colonies or filaments, the arrangement of the filaments, shape and size of vegetative cells, the presence and position of heterocytes and akinetes, the isolates' colour and chromatic adaptation capability (CCA), the number of trichomes per filament, the presence or absence of sheaths, constrictions at cross-walls and necridic cells. Measurements were conducted on 3-week-old cultures, with each measurable characteristic assessed 20 times in various positions on the slide preparation.

Morphological characterization of cyanobacterial isolates was conducted during the exponential growth phase (2- to 3-week-old cultures) using a Motic Panthera Series Microscope

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TABLE 1 | Primer sets used in the molecular screening.

Gene	Primer name	Sequence (5′-3′)	Annealing temperature	Amplicon size (bp)	Reference
16S	CYA359F	GGGGAATYTTCCGCAATGGG	55°C	~400	(Nübel et al. 1997)
	CYA781R	GACTACTGGGGTATCTAATCCCATT			
	1494R	TACGGCTACCTTGTTACGAC	56°C	~1100	(Neilan et al. 1997)
	27F	AGAGTTTGATCCTGGCTCAG	56°C	~2000	(Neilan et al. 1997)
	23S30R	CTTCGCCTCTGTGTGCCTAGGT	57°C		(Lepère et al. 2000)
Microcystin	mcyA1F	AAAATTAAAAGCCGTATCAAA	52°C	~300	(Rantala et al. 2004)
	mcyA1R	AAAAGTGTTTTATTAGCGGCTCAT			
Anatoxin	anaC-genF	TCTGGTATTCAGTCCCCTCTAT	54°C	~166	(Rantala-Ylinen et al. 2011)
	anaC-genR	CCCAATAGCCTGTCATCAA			
Saxitoxin	sxtI 682F	GGATCTCAAAGAAGATGGCA	52°C	~196	(Ballot et al. 2010)
	sxtI 877R	GCCAAACGCAGTACCACTT			
Cylindrospermosin	cynsulF	ACTTCTCTCCTTTCCCTATC	50°C	~278	(Mihali et al. 2008)
	cylnamR	GAGTGAAAATGCGTAGAACTTG			

(Motic China Group Co. Ltd.) equipped with a HD digital camera. Microphotographs of each isolate were captured and analysed, and morphometric measurements were performed using Motic Image plus 3.1. software at 40× and 100× magnifications.

2.2.6 | DNA Extraction, PCR Amplification and Sequencing

Cyanobacteria strains were characterized for the first time after 15–20 days of cultivation. Genomic DNA was then extracted using the Genomic DNA Mini Kit (Invitrogen, USA), according to the manufacturer's instructions for Gram-negative bacteria. To obtain the complete sequence of 16S rRNA gene, PCR amplification was performed using the oligonucleotide primers 27F (Lane 1991) and 23S30R (Lepère et al. 2000) (Table 1). PCR reactions were performed in a final volume of 20 μL containing 1× Green GoTaq Flexi Buffer, 2.5-mM $MgCl_2$, 125.0 mM of each deoxynucleotide triphosphate, 1.0 μM of each primer, 0.5 U of GoTaq Flexi DNA Polymerase (Promega, USA), 10 mg/mL of bovine serum albumin (BSA) and 10–30 ng of template DNA, on a Veriti Dx Thermal Cycler (Invitrogen, USA). The PCR conditions were the following: initial denaturation at 94°C for 5 min, followed by 10 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s and extension at 72°C for 2 min, followed by 25 cycles of denaturation at 92°C for 45 s, annealing at 54°C for 45 s and extension at 72°C for 2 min with a final elongation step at 72°C for 7 min. The PCR reactions were performed in duplicate. PCR products were separated by 1.5% agarose gel stained with SYBR safe (Invitrogen, USA), and DNA fragments with the expected size were excised and purified using NZYGelpure (NzyTech, Genes and Enzymes, Lisbon, Portugal) following the manufacturer's instructions. Since the sequences were obtained by direct sequencing of purified amplicons, internal primers CYA359F, CYA781R and 1494R (Table 1) were used to improve the quality of sequences. The sequencing was performed at GATC Biotech (Germany), and the nucleotide sequences obtained were manually inspected for quality and assembled using the Geneious 11.1.5 software (Biomatters Ltd., New Zealand). The obtained consensus sequences were submitted to the GenBank database of the National Center for Biotechnology Information (NCBI) (GenBank, http://www.ncbi.nlm.nih.gov) for alignment employing the Basic Local Alignment Search Tool (BLASTn) algorithm of nucleotides in the 16S rRNA sequences database to determine their phylogenetic affiliations. For the toxin alignment, the BLASTx tool was used to make sure the gene encoded for the protein.

2.2.7 | PCR Amplification for the Detection of Toxin Production

PCR amplification was used to target toxin-synthesis-related genes, including those encoding microcystin (mcyA), saxitoxin (sxtI), anatoxin (anaC) and cylindrospermopsin (cyrJ) using specific primer sets (listed in Table 1). All PCR reactions were performed using the GoTaq DNA polymerase (Promega, Madison, WI, USA) in a volume of 20 μL containing 1× Green GoTaq Flexi Buffer, 2.5-mM $MgCl_2$, 125.0 mM of each deoxynucleotide triphosphate, 1.0 μM of each primer, 0.5 U of GoTaq Flexi DNA Polymerase (Promega, USA), 10 mg/mL of bovine serum albumin (BSA) and 10–30 ng of template DNA on a Veriti Dx Thermal Cycler (Invitrogen, USA).

Microcystins mcyA PCR amplification was performed using the primer pair mcyA1F/mcyA1R with the following PCR conditions: an initial denaturation at 92°C for 2 min and 35 cycles at 92°C for 20 s, 52°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 5 min (Table 1). A *Microcystis aeruginosa* microcystins-producing strain LEGE 00063 was used as a positive control. Anatoxin-a PCR amplification was carried out using the primer pairs anaC-genF/anaC-genR, and the PCR conditions were as follows: an initial denaturation at 94°C for 2 min and 35 cycles at 94°C for 30 s, 54°C for 30 s, extension at 72°C for

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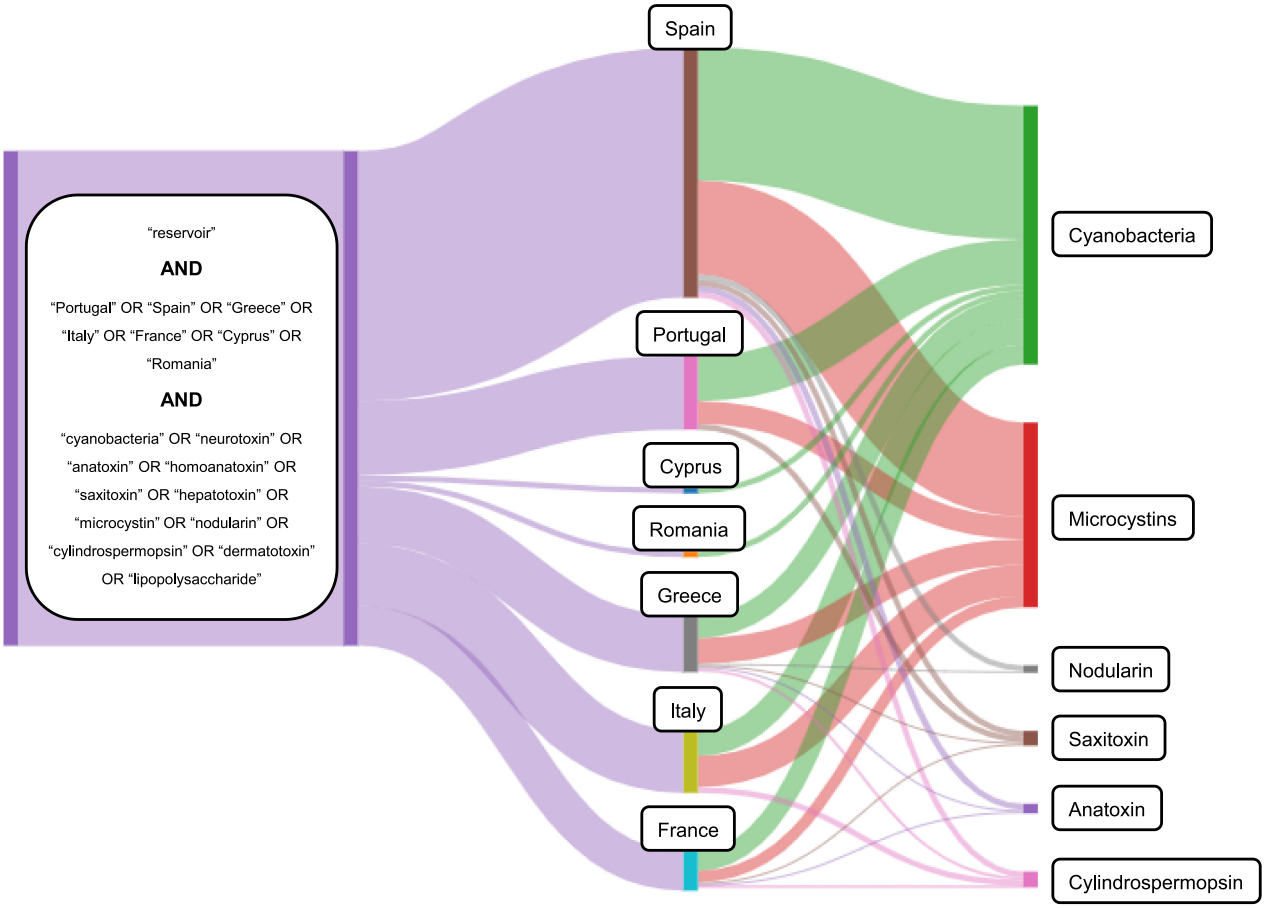


FIGURE 2 | Sankey diagram of the frequency of studies conducted in European countries (1993–2024), focusing on cyanobacteria and cyanotoxins. The thickness of the connecting lines represents the number of articles found for each category in the literature.

30 s and final extension at 72°C for 5 min. A *Dolichospermum* sp. anatoxin-a-producing strain LEGE X-002 was used as a positive control. Gene stxI involved in saxitoxin biosynthesis was amplified using the primer pair sxtI682F/sxtI877R and PCR conditions were as follows: an initial denaturation at 94°C for 3 min and 35 cycles at 94°C for 20 s, 52°C for 20 s, extension at 72°C for 1 min and final extension at 72°C for 7 min. A *Chrysosporum gracile* strain LMECYA 040 was used as a positive control. Cylindrospermopsins PCR amplification was carried out using the primer pairs cynsulfF/cylnamR (cyrJ) (Table 1) with the following PCR conditions: an initial denaturation at 95°C for 3 min and 35 cycles at 95°C for 45 s, 50°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 3 min (Table 1). *Cylindrospermopsis raciborskii* cylindrospermopsin-producing strain LEGE 97047 was used as a positive control. All PCR amplifications were carried out in a Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA), and all the PCRs had a holding at 10°C.

2.2.8 | Phylogenetic Analysis

The phylogenetic analyses were conducted with maximum likelihood (ML) and Bayesian inference (BI) analyses. For that, we aligned the 16S rRNA gene sequences of our isolates with sequences of cyanobacterial reference strains and additional sequences retrieved from GenBank (NCBI) by BLAST. These included genera from the orders Nostocales, Chroococcales, Chroococcidiopsidales and Nodosilineales. GTR + G + I evolutionary model was selected by MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura et al. 2021), according to the AIC criterium. The robustness of ML tree was estimated by bootstrap percentages, using 1000 replications using IQ-Tree online version v1.6.12 (Trifinopoulos et al. 2016). The BI tree was constructed in two independent runs, with four chains each, for 5×10^6 generations, burn-in fraction set to 0.25, sample frequency 1000, using MrBayes (Ronquist et al. 2012) in Cipres Gateway (Miller et al. 2010). The processing and visualization of these trees were made using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

3 | Results and Discussion

The literature review database initially identified 237 articles. After screening based on the defined criteria, 87 studies were selected that focus on reservoirs and quantify cyanobacteria

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and/or cyanotoxin concentrations within countries belonging to the WFD Mediterranean Geographical Intercalibration Group (Figure 2). Among these, Spain accounted for the largest number of studies (44), followed by Portugal (13), Italy (11), Greece (10) and France (7). Only one study was found for each of the remaining countries (Figure 2).

The World Health Organization (2003) established that a risk level to human health should be assigned according to the cell concentration: low risk (< 20,000 cells/mL), medium risk (20,000–100,000 cells/mL), high risk (100,000–1,000,000 cells/mL) and very high risk (> 1,000,000 cells/mL). Across the conducted reviewed studies, cyanobacteria concentrations were reported in 66 cases (Figure 2), expressed as biomass (cell/mL or mm^3/m^3) or as percentage composition.

The only study identified in Cyprus, conducted at the Kouris Reservoir by Tzoraki et al. (2017), reported a cyanobacterial concentration of 1000 cells/mL corresponding to the WHO low-risk level. According to the authors, the reservoir is classified as oligotrophic to mesotrophic, and during summer, the water temperature can reach 27°C, a condition that may favour the growth of *Aphanizomenon flos-aquae*, *Microcystis flos-aquae* and *Microcystis viridis* strains identified in the reservoir. Regarding Greece, Italy, France and Spain, several studies have reported cyanobacterial concentrations in reservoirs. In Greece, the Polyphytos reservoir, used for hydroelectric power generation, irrigation and drinking water supply, recorded a maximum of 378,000 cells/mL of *Microcystis aeruginosa* (Chrisostomou et al. 2009) corresponding to the WHO high-risk category. In Italy, Zuccarello et al. (2021) reported a bloom of *Microcystis* sp. in a Sicilian reservoir during the summer of 2017, with a concentration reaching 100 million cells/mL, far exceeding the WHO very high-risk threshold. In France, Pitois et al. (2018) monitored 10 reservoirs, analysing 192 integrated water samples collected monthly from June to October in 2007, 2008 and 2010. Results showed that 12% of samples contained fewer than 1000 cells/mL, 41% exceeded 20,000 cells/mL (WHO medium risk) and 24% surpassed 100,000 cells/mL (WHO high risk). The highest density, 3,320,500 cells/mL, was recorded in 2007, placing it in the WHO very high-risk category. In Spain, Medina-Cobo et al. (2014) analysed 23 reservoirs during summer months over multiple years, reporting an average concentration of 1,018,974 cells/mL and a maximum of 9,933,031 cells/mL, both within the WHO very high-risk category.

In Portugal, several investigations have reported cyanobacterial blooms in reservoirs. In the Torrão Reservoir, Saker et al. (2007) reported a peak concentration of 415,700 cells/mL of cyanobacteria (including *Microcystis* sp.), which corresponds to a high-risk level according to WHO guidelines. Similarly, Bellém et al. (2013) detected a *Microcystis aeruginosa* bloom in the Boavista Reservoir (south of Portugal with Mediterranean climate conditions) in 2007, with a concentration of 1,270,173 cells/mL, classified as very high risk. The same authors also reported a bloom of *Aphanizomenon* sp. in the Enxoé Reservoir (south of Portugal with Mediterranean climate conditions), reaching 817,530 cells/mL, again falling under the high-risk category. In the same region of Portugal, Valério et al. (2008) documented a maximum cyanobacterial concentration of 13,677,222 cells/mL in the Alvito reservoir, far exceeding the WHO very high-risk threshold.

Overall, nearly all countries included in this review reported concerning levels of cyanobacteria, underscoring the importance for continued monitoring and effective water management. Building a historical record and improving knowledge on the presence of these organisms are essential for accurately assessing the ecological status of water bodies.

Among the cyanotoxins analysed, microcystins were the most frequently investigated, with 52 studies reporting their presence (Figure 2). While some studies quantified only total microcystins, others provided a more detailed analysis by distinguishing between individual microcystin variants. The highest reported concentration of microcystins was 9893.14 μg/L in the San Juan Reservoir, Spain, coincident with a *Microcystis* spp. bloom episode (Lezcano et al. 2018). The other cyanotoxins analysed were reported less frequently: saxitoxin (1.2 μg/L) by Gkelis and Zaoutsos (2014) in the Kerkini reservoir, Greece; anatoxin (0.46 μg/L) by Pitois et al. (2018) in France; and cylindrospermopsin (12.1 μg/L) by Gkelis et al. (2017) in Karla reservoir, Greece. Nodularin toxin was mentioned in a single study; however, it was not detected in any quantitative analysis (Kaloudis et al. 2013). Although there are no regulatory values for cyanotoxins, the World Health Organization (1998) established a provisional guide value for microcystins (1 μg/L) in water, for human consumption. This value was adopted by the WHO as a parametric guideline and has been incorporated into the legislation of several countries, including Spain, France and Portugal (Ministério do Ambiente 2007). The World Health Organization (1998) also defines 0.7 μg/L as a guideline value for cylindrospermopsin; however, unlike microcystins, this value has not yet been included into the legislation of any country.

Others guide values for the cyanotoxins analysed in this work are established in different countries. For instance, the guideline value used for microcystins is 1.3 μg/L in Australia (NHMRC 2011) and 1.6 μg/L for adults in the EUA (EPA 2015a). For cylindrospermopsin, the Australian guideline is 1.0 μg/L (NHMRC 2011), while the EUA health advisory for adults is 3.0 μg/L (EPA 2015b), and in Brazil, a value of 15 μg/L is used in technical studies and guidance documents (Ministério da Saúde 2021). For other cyanotoxins in Australia, the guideline values are 1.0 μg/L for anatoxin-a and 3.0 μg/L for saxitoxins (NHMRC 2011). In the Mediterranean region, none of the countries have yet incorporated these guideline values into their legislation. Nevertheless, the concentrations reported in the reviewed studies underscore their critical importance. Of the 52 studies that quantified microcystins, 42 (80%) reported levels exceeding the WHO recommended limit for Mediterranean countries (1 μg/L). For anatoxin-a, two of the four studies (50%) reported concentrations above 1 μg/L, the guideline value recommended in Australia. In contrast, all four studies quantifying saxitoxin reported concentrations below the recommended threshold of 3 μg/L. Cylindrospermopsin was quantified in five studies, with four (80%) reporting concentrations above the WHO-recommended limit of 0.7 μg/L.

In Portugal, the temporal variation of the Algae Group Index biovolume in the Rabagão (Figure 3A) and Aguieira (Figure 3B)

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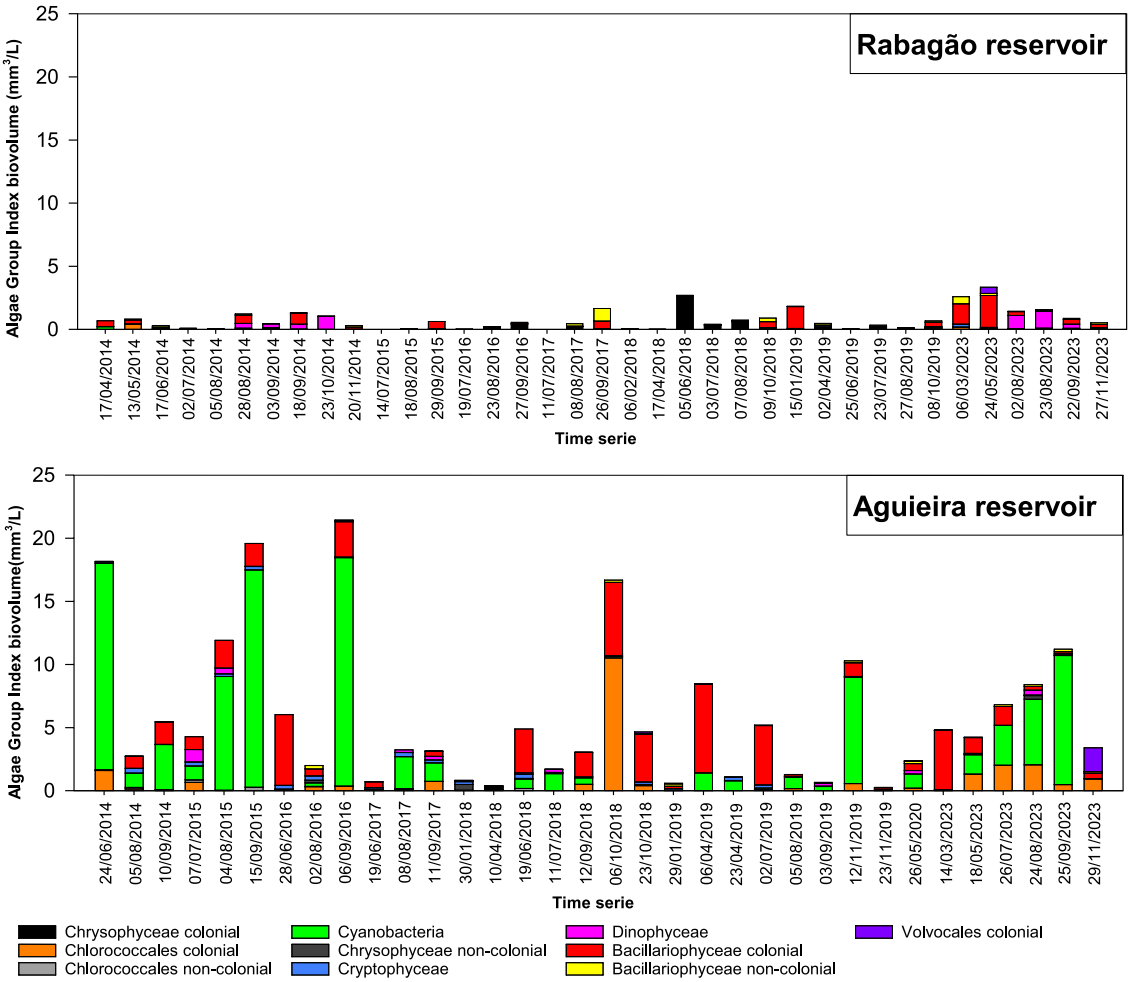


FIGURE 3 | Time series of the Algae Group Index biovolume (mm^3/L) in the two reservoirs studied.

reservoirs showed several differences. In the Rabagão reservoir, the phytoplankton biomass remained at very low levels throughout the entire analysed period (2014–2023), rarely exceeding 1 mm^3/L, and Bacillariophyceae colonial (e.g., *Aulacoseira* sp.), and Chrysophyceae colonial (e.g., *Dinobryon* sp.) were the most representative *taxa*. Indeed, the Rabagão reservoir has been classified as mesotrophic based on the chlorophyll *a* concentration (Instituto da Água 2011). Water bodies with this classification typically exhibit low to moderate primary productivity and do not experience large-scale phytoplankton proliferations. Moreover, the Rabagão reservoir is generally characterized by low concentrations of nutrients, particularly phosphorus and nitrogen, rarely exceeding the 0.04 and 1 mg/L, respectively, corresponding to the threshold levels for achieving the good potential status (SNIRH: Sistema Nacional de Informação de Recursos Hídricos, n.d.; Almeida et al. 2020; Pinto et al. 2025b; Pinto et al. 2025a). Furthermore, cyanobacteria are generally underrepresented in this reservoir (Figure 3A), with *Merismopedia* sp. being the most representative genus within this group. Although *Merismopedia* typically is not considered a problematic cyanobacterium, its presence in large quantities may indicate an environmental imbalance (Yongo et al. 2023).

In contrast, the Aguieira reservoir exhibited greater phytoplankton biomass variability, with values exceeding 20 mm^3/L, in several periods (Figure 3B). Cyanobacteria (e.g., *Aphanizomenon* sp. *Microcystis* sp., *Pseudanabaena* sp. and *Woronichinia* sp.) and Bacillariophyceae colonial (e.g., *Asterionella* sp. *Aulacoseira* sp. and *Fragilaria* sp.) were the most representative groups (and genera). These results suggest more favourable environmental conditions (e.g., nutrient input) for the development of specific groups such as cyanobacteria. The Aguieira reservoir is classified as a eutrophic ecosystem with a long history of cyanobacteria blooms and decrease of water quality (Vasconcelos et al. 1996; Geraldes et al. 2016; Pinto, Rodrigues, and Antunes 2021; Pinto, Calisto, et al. 2021; Rodrigues et al. 2023; Portela et al. 2024). This poor water quality is driven by excessive nutrient concentrations, particularly total phosphorus and nitrogen, which combined with contaminant inputs, promoting frequent cyanobacterial blooms, and the occurrence of cyanotoxins (Vasconcelos et al. 2011; Pinto et al. 2025a, 2025b). Indeed, high nutrient concentrations have been reported in this reservoir over the last decades. Pinto, Rodrigues, and Antunes (2021), in 2018, observed a phosphorus concentration of 0.22 mg/L, a concentration much higher than the reference value (0.04 mg/L) for good ecological potential. In addition, considering the historical record of values quantified in this reservoir (SNIRH: Sistema Nacional de Informação de Recursos Hídricos n.d., Pinto et al. 2025a), it is possible to observe strong temporal variation, as well as high peaks in phosphorus and nitrogen nutrient concentrations, namely at summer and autumn.

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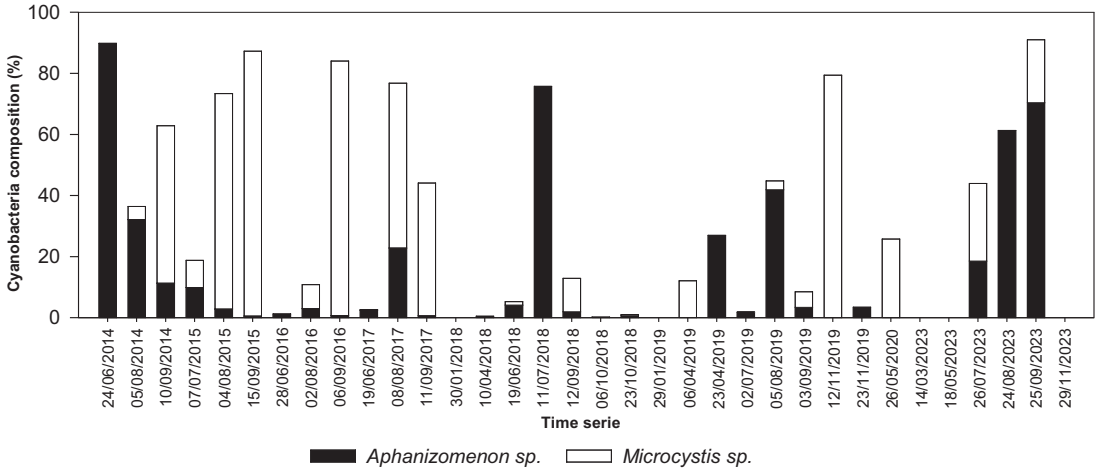


FIGURE 4 | Time series of *Aphanizomenon* sp. and *Microcystis* sp. abundance (%) of the Cyanobacteria group in the Aguieira reservoir.

In the context of cyanobacteria community, it is particularly noteworthy that in the time series analysed in the present work, *Aphanizomenon* sp. and *Microcystis* sp. are present in high proportions, in the Aguieira Reservoir (Figure 4). For example, in September of 2023 (Figure 4), a cell concentration of *Aphanizomenon* and *Microcystis* of 87,867 and 36,450 cells/mL, respectively, was observed. Furthermore, during the same period, Pinto et al. (2025a) reported total phosphorus and total nitrogen concentration of 0.042 and 0.24 mg/L, respectively. These findings reinforce the association between elevated nutrient levels and high cyanobacteria concentrations in this reservoir. According to WHO guidelines, both species individually fall within the moderate health risk category. However, because the WHO classification is based on the total cyanobacterial cell concentration, their combined abundance exceeds the high-risk threshold of 100,000 cells/mL. Consequently, the data seem to indicate that this water body periodically experiences conditions that are less favourable to human health and to the ecosystem. Furthermore, these two genera have been consistently observed (Pinto, Rodrigues, and Antunes 2021; Pinto, Calisto, et al. 2021; Pinto et al. 2025a, 2025b), and Vasconcelos et al. (2011) had already highlighted the issue of high *Microcystis aeruginosa* concentrations since 1994. In the spring of 2018, Pinto, Rodrigues, and Antunes (2021) and Pinto, Calisto, et al. (2021) observed a *Microcystis aeruginosa* bloom, with a concentration of 7,164,000 cells/mL, exceeding the WHO high-risk threshold. High concentrations of cyanobacteria pose significant ecological risks, including the deterioration of water quality, disruptions in aquatic food webs and, in severe cases, mass fish mortality (Cordeiro et al. 2020; Moreira et al. 2020; Stroski et al. 2024). Their ecological impact is particularly concerning because several genera are well-documented for forming harmful algal blooms (HABs) and producing cyanotoxins (Figueiredo et al. 2006; Vasconcelos et al. 2011). Despite these impacts, *taxa* capable of producing cyanotoxins are not currently considered in ecological potential assessment or legislations, including the Water Framework Directive (WFD) for heavily modified water bodies such as reservoirs. Given their significant role in water quality degradation and ecosystem disturbances, continuous monitoring and the integration of cyanobacterial blooms into ecological status assessments are crucial for developing effective water management strategies.

In this study, two cyanobacterial strains, *Microcystis aeruginosa* LEGE 231512 and *Aphanizomenon flos-aquae* LEGE 231513, were isolated from Aguieira reservoir in central Portugal (Table 2 and Figure 5) and subjected to polyphasic characterization. This included morphological analysis, 16S rRNA gene sequencing for taxonomic identification and molecular screening for cyanotoxin biosynthesis genes. The strain LEGE 231512 is confirmed as *Microcystis aeruginosa* (Figure 5a,b). Morphologically, the strain fits in the description of this species, presenting spherical or irregular colonies with densely arranged cells (Figure 5a,b). The cells are spherical with numerous aerotopes (include measurements) (Table 2). The mucilage is diffluent and colourless (Figure 5a,b) (Komárek and Anagnostidis 1998). The strain LEGE 231513 is confirmed as *Aphanizomenon flos-aquae* (Figure 5a,b). Morphologically, the strain fits in the description of this species, presenting parallel-arranged, cylindrical and straight trichomes, which can be slightly constricted at the cross-walls analysis (Figure 5c,d). The cells are cylindrical, isodiametric or longer than wide, with rounded ends and numerous aerotopes (Komárek 2013).

The resulting maximum likelihood tree showed strong support for the taxonomic placement of both isolates from Aguieira reservoir within their respective genera (Figure 6). Strain LEGE 231512 clustered within a highly supported clade (bootstrap value = 100%) comprising *Microcystis aeruginosa* reference sequences, including NIES1535 and NIES90. This confirms the identification of *M. aeruginosa*, a globally distributed cyanobacterium also recorded recurrently in the Mediterranean region (Figures 2 and 4). Moreover, this cyanobacterium is frequently associated with harmful algal blooms (HABs) with significant impacts to the freshwater ecosystems (Otsuka et al. 2000; Humbert et al. 2013). Similarly, LEGE 231513 grouped within a robust clade (bootstrap = 100%) containing *Aphanizomenon flos-aquae* reference sequences, such as strain 1tu37s13, confirming its identity. This species is also widely reported in freshwater ecosystems, namely, reservoirs in the Mediterranean region (Figures 2 and 4), with known bloom-forming capacity and links to cyanotoxin production (Sivonen and Jones 1999). These results underscore the relevance of applying an integrative taxonomic approach that combines classical morphological observation with molecular tools, particularly 16S rRNA gene

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TABLE 2 | Summary classification of morphological traits of the isolated cyanobacteria strains from the Aguieira reservoir in 2023.

Strain name	*Microcystis aeruginosa* LEGE 231512	*Aphanizomenon flos-aquae* LEGE 231513
Family	Microcystaceae	Aphanizomenonaceae
Genus	*Microcystis*	*Aphanizomenon*
Morphological description	Spherical or irregular colonies with densely arranged cells (Figure 5a,b)	Parallel arranged, cylindrical and straight trichomes, slightly constricted at cross-walls (Figure 5c,d)
Isolator	Ivo Pinto (orcid: 0000-0002-6454-079X)	Ivo Pinto (orcid: 0000-0002-6454-079X)
Sample description	Water sample	Water sample
Sampling site	Albufeira da Aguieira	Albufeira da Aguieira
Country	Portugal	Portugal
GPS coordinates	40°20′32.7″ N, 8°11′18.4″ W	40°20′32.7″ N, 8°11′18.4″ W
Habitat	Lentic freshwater ecosystem	Lentic freshwater ecosystem
Culture type	Non-axenic, unicyanobacterial	Non-axenic, unicyanobacterial
Temp (°C)	19	19
Medium	Z8 (Kótai 1972)	Z8 (Kótai 1972)
Accession number	PX928730	PX928731

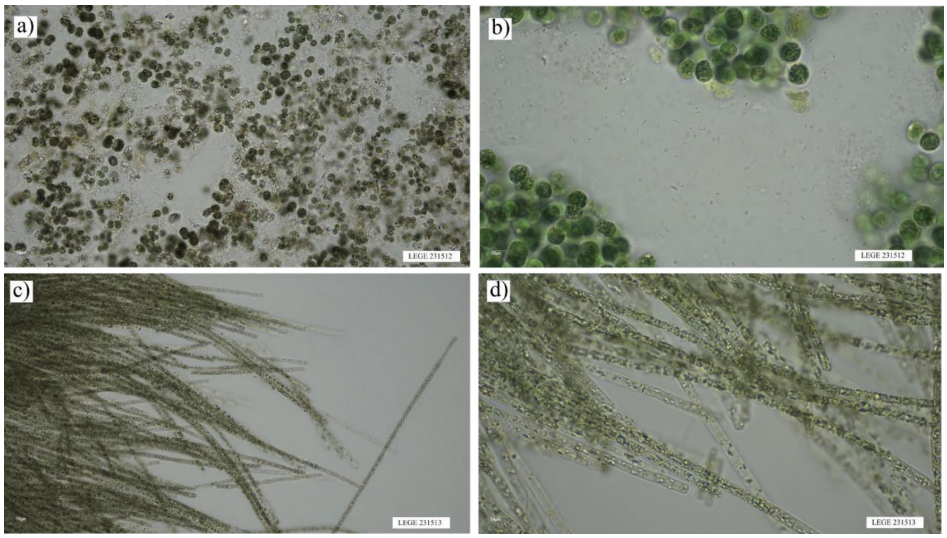


FIGURE 5 | Microphotographs of the isolated cyanobacterial strains *Microcystis aeruginosa* LEGE 231512 40× (a) and 100× (b) and *Aphanizomenon flos-aquae* LEGE 231513 40× (c) and 100× (d).

sequencing. Although this strategy is more demanding in terms of time and cost, its advantages clearly outweigh the drawbacks: morphological traits alone can be misleading due to phenotypic plasticity and environmental influence, while molecular markers such as the 16S rRNA gene may lack resolution to discriminate among closely related *taxa*. By combining both approaches, misidentifications are minimized, cryptic diversity can be revealed and the overall robustness of taxonomic assignments is enhanced. A polyphasic strategy is especially valuable for cyanobacteria, a group characterized by considerable morphological plasticity that can hinder accurate species identification when using microscopy alone. Recent studies have reinforced the utility of this approach for describing new taxa (de Oliveira et al. 2024; Scotta Hentschke et al. 2024), especially in temperate regions such as Portugal, where multiple novel genera have been recently identified from LEGE-CC using 16S rRNA and ITS region analyses.

Genera of cyanobacteria are commonly responsible for harmful algal blooms (HABs) and are known for their potential to produce cyanotoxins, including microcystins, anatoxins, saxitoxins and cylindrospermopsins (Codd et al. 2005; Chorus and Welker 2021). Molecular screening for toxin biosynthesis genes of species collected in the Aguieira reservoir targeted the mcy (microcystin), ana (anatoxin), sxt (saxitoxin) and cyr (cylindrospermopsin) gene clusters. The results revealed that *M. aeruginosa*

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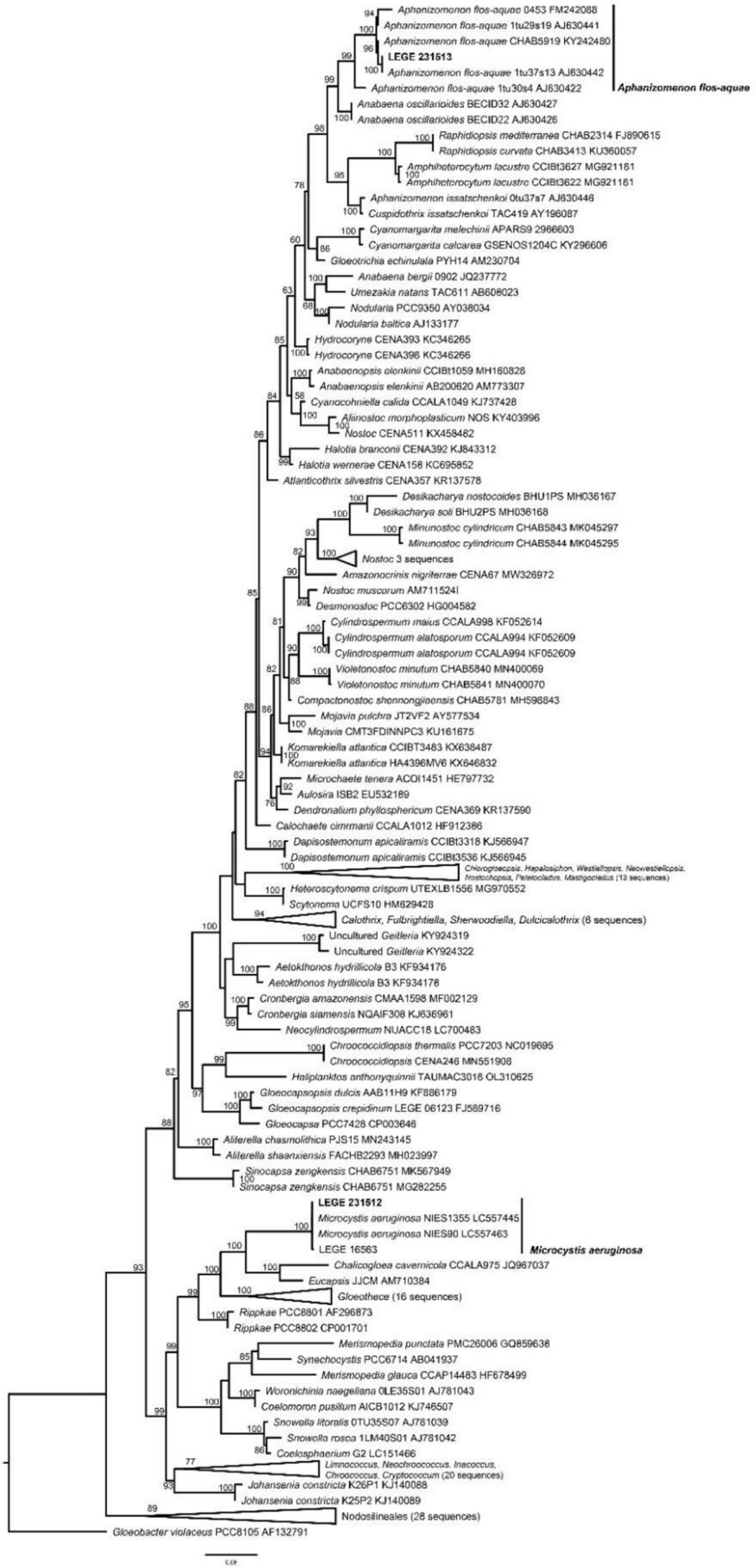


FIGURE 6 | Legend on next page.

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FIGURE 6 | Maximum likelihood phylogenetic tree based 16S rRNA gene sequences of cyanobacteria. *Gloeobacter violaceus* PCC 8105 was used as outgroup. The strains used in this work are indicated in bold with the LEGE code.

LEGE 231512 harboured the mcyA gene, indicating the genetic potential for microcystin production. In contrast, *A. flos-aquae* LEGE 231513 tested negative for all four gene clusters, suggesting that this strain lacks the genetic machinery required for the biosynthesis of the cyanotoxins evaluated. The detection of the mcyA gene in *M. aeruginosa* confirms its well-known potential for toxin production and highlights the importance of ongoing monitoring of this genus in freshwater systems, as sites where *M. aeruginosa* harbouring mcyA are strongly associated with microcystin contamination (Vasconcelos et al. 2011; Lezcano et al. 2018). On the other hand, the absence of toxin biosynthesis genes in *A. flos-aquae* suggests a non-toxigenic profile for this particular strain (Kaebernick and Neilan 2001). However, a study by Ferreira et al. (2001) in the Crestuma-Lever reservoir, Portugal, isolated *A. flos-aquae* and detected both the presence and production of saxitoxin. This finding reinforces the understanding that not all strains possess toxin-producing genes and highlights the importance of identifying strains with toxigenic potential.

The characterization of the cyanobacterial strains studied here contributes to the growing knowledge of cyanobacterial diversity and highlights the importance of laboratory collections, such as the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC). Both LEGE 231512 and LEGE 231513 are now deposited in this collection and are available for future research and biotechnological applications. From an ecological and monitoring perspective, the identification of toxigenic strains is relevant for water quality assessments and management strategies, as it enables early-warning systems, targeted water treatment approaches and informed decisions regarding recreational use, irrigation and public health protection.

4 | Conclusion

This study reinforces concerns about the proliferation of cyanobacteria and cyanotoxins occurrence in freshwater ecosystems, particularly in a Mediterranean context, where escalating pressure such as nutrient enrichment and warming temperatures are increasingly prevalent. By integrating literature reviews, field data and molecular analyses, we demonstrated that many water bodies, including Portuguese reservoirs, are vulnerable to cyanobacterial blooms, some of which reach concentrations posing high or very high health risks, according to WHO guidelines. The isolation and characterization of *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* strains from Aguieira reservoir provided further insight into the toxigenic variability among cyanobacteria. While the *M. aeruginosa* strain carried the gene responsible for microcystin biosynthesis, the *A. flos-aquae* strain seems to lack the toxin-related gene clusters screened, reinforcing the importance of molecular tools to complement morphological identification. Given the ecological relevance and public health risks associated with toxic cyanobacterial blooms, it is essential that monitoring programs under the Water Framework Directive evolve to include cyanobacteria with toxin-producing potential, as well as cyanotoxins, into their assessment criteria. Overall, this study enhances our understanding of cyanobacterial diversity and associated risks in Portuguese reservoirs and underscores the importance of integrating molecular, ecological and regulatory approaches to ensure effective water quality management.

Author Contributions

Conceptualization: I.P., R.S. and S.C.A.; methodology: I.P. and J.M.; formal analysis: I.P., R.S., G.S.H. and J.M.; investigation: I.P., R.S. and J.M.; resources: V.V. and S.C.A.; data curation: I.P., R.S. and G.S.H.; writing – original draft preparation: I.P. and G.S.H.; writing – review and editing: I.P., R.S., G.S.H., V.V., L.A. and S.C.A.; supervision: L.A. and S.C.A.; funding acquisition: V.V. and S.C.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data are contained within the article.

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