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RESEARCH ARTICLE

Cyanotoxin profiling of seasonal spring cyanobacteria *Nostoc fuscescens* by LC-HRMS

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Abstract

Cyanotoxins, the metabolites synthesized by cyanobacteria, have a significant impact on ecosystems and are important in pharmaceutics. Cyanotoxins are of diverse chemical nature, including peptides, alkaloids, etc. and are found in various aquatic and terrestrial habitats. We investigated the presence of cyanotoxins in the cyanobacteria *Nostoc fuscescens* from a seasonal water spring situated in the northern Western Ghats, India. We identified cyanotoxins in *N. fuscescens* blooms using LC-HRMS analysis. We detected the presence of four cyanotoxins: Microcystin, Nodularin, Cylindrospermosin, and Anatoxin. Additionally, we also identified four variants of Microcystin. The results revealed that *N. fuscescens* contains different cyanotoxins with multiple structural variants. These results highlight the potential of cyanobacteria on wet rocks in seasonal spring to produce cyanotoxins of diverse chemical nature, that can be explored further for bioprospection.

Keywords: Water spring; Cyanotoxins; *Nostoc fuscescens*; Microcystin.

1. Introduction

Cyanobacteria, or blue-green algae, are among the oldest autotrophic life forms with cosmopolitan distribution and can be found in different habitats, including Antarctic lakes and thermal springs. Under certain conditions like eutrophication, cyanobacteria massively bloom in response to increased temperature and nutrients such as phosphorus. Cyanobacterial blooms have long-term ecological and economic consequences (Huisman et al., 2018). The expansion of cyanobacterial blooms has a significant impact on water quality, biodiversity, and ecosystem functioning (Sukenik et al., 2015; Amorim and Moura, 2021). For example, cyanobacteria increase phytoplankton diversity and richness while decreasing zooplankton diversity (Amorim and Moura, 2021). The toxins produced by cyanobacteria can have unbearable consequences on human health and aquatic fauna (Zanchett and Oliveira-Filho, 2013; Otero and Silva, 2022). Additionally, cyanobacteria have been of great interest due to their potential to produce cyanotoxins of diverse chemical nature. Cyanotoxins, secondary metabolites produced by cyanobacteria, play a significant role in their physiology, especially during environmental stress (Kaebernick and Neilan, 2001; Downing et al., 2015).

Although they are harmful chemicals by nature, cyanotoxins are becoming pharmaceutically important compounds as many of them have anticancer, antimicrobial properties, and other biocidal properties (Dias et al., 2015; Vijayakumar and Menakha, 2015; Ricciardelli et al., 2023). Cyanotoxins are categorized based on their functional or toxicological properties, such as hepatotoxins,

Table 1. Details of the tests performed to detect the presence of various chemicals.

Phytoconstituents	Method	Presence/ absence
Glycosides		
I) cardiac glycosides	Keller-kiliani test	Present
Ii) anthraquinon glycosides	Borntrager's test	Absent
Iii) saponin glycosides	Olive oil method	Absent
Iv) coumarin glycosides	Sodium hydroxide method	Present
V) cyanogenetic glycosides	Picrate-impregnated paper	Absent
Alkaloids	Dragendorff"s test, mayer's test, hager's test	Present
Flavonoids	Alkaline reagent test and shinod's test	Present
Tannins and phenolic compounds	Ferric chloride test and lead tetra acetic acid test	Present
Steroids	Chloroform and h2so4 test	Absent
Proteins	Biuret test and ninhydrin test	Absent
Carbohydrates	Molish test and benedict's test	Present

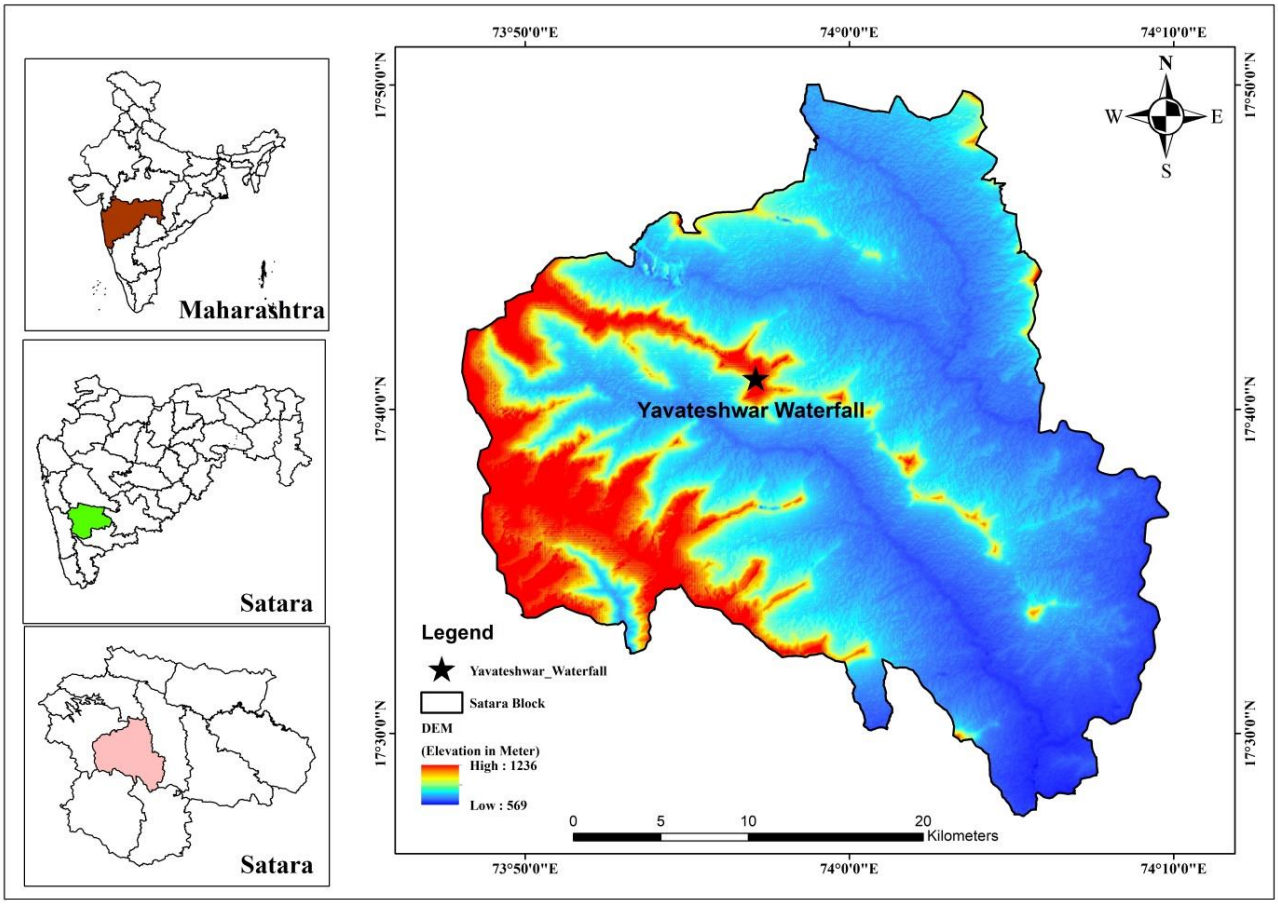

Figure 1. Map showing the geospatial location of collection site, Yevateshwar waterfall in Satara district, Maharashtra, India (A) and seasonal waterfall (B)

neurotoxins, cytotoxins, etc. (Chorus and Welker, 2021a). Cyanotoxins are of diverse chemical natures including peptides, alkaloids, lipopolysaccharides etc. (Vijayakumar and Menakha, 2015; Ricciardelli et al., 2023). Microcystins, one of the major hepatotoxins are one of the major group of cyanotoxins with more than 250 structural variants (Bouaïcha et al., 2019; Chorus and Welker, 2021b). Another hepatotoxic cyanotoxin, Nodularins are monocyclic peptides have low structural diversity (Melaram et al., 2024; Catherine et al., 2016). Alkaloid cyanotoxins such as Anatoxin A and Cylindrospermopsin have toxic effects on nervous system and skin (Chorus and Welker, 2021b).

Detection and quantification of cyanotoxins are challenging as many of them have diverse structural variants and their standards are not available (Kaushik and Balasubramanian, 2013; Moreira et al., 2014). Novel variants of cyanotoxins are being described in the literature (Kust et al., 2018; Johansson et al., 2019). Moreover, many cyanobacterial species from different habitats (such as terrestrial) are yet to be investigated for the presence of toxins. Previous studies were mainly focused on the detection of cyanotoxins from aquatic (marine and freshwater) ecosystems (Filatova et al., 2020; Zhang et al., 2023). However, terrestrial habitats such as rocks in seasonal springs are largely neglected (Dulić et al., 2022). In the present study, we investigated the presence of cyanotoxins in the cyanobacteria *Nostoc fuscescens* from the wet rock surfaces in the seasonal water spring in the northern Western Ghats of India. We detected the presence of cyanotoxins and identified them using LC-HRMS analysis.

2. Material and method

2.1. Collection and identification of Nostoc Species

Greenish-brown biofilms were collected in the post-monsoon season (September - October) from the moist rocks near the waterfall situated in the hills around Yavteshwer village (Satara, Maharashtra, India; Figure 1). The biofilms were collected in the beakers using a sterile knife and brought to the laboratory. The collected samples were observed under the microscope (Olympus CX41) for morphological characterization and purity (Figure 2). Pure colonies were isolated and cultured by using BG11 media (Rippka et al., 1979). Identification was done following the keys by (Komárek, 2013).

2.2. Preparation of extract

The freshly collected samples were washed under tap water and cleaned thoroughly to remove soil particles and debris, if any. Then the samples were dried under the shed for two weeks (Figure 1 and Figure 2) and ground into powder using a grinder. The powder was then sieved through a 0.2 mm strainer. Five grams of the powder was 50 ml methanol and kept in the shaker at 60 rpm/min and 25 °C temperature. After 24 h the extract was filtered through Whatman filter paper no.3. The filtrate was dried under reduced pressure using a rotary evaporator (Tripathi et al., 1996). The dried extract was used for Cyanotoxin detection.

2.3. Phytochemical analysis of algal extract

The qualitative phytochemical analysis of crude extract was performed following the standard methods described by (Brain and Turner, 1975; Evans, 2009; Kokate, 2014). The presence of Glycosides, alkaloids, flavonoids, fats and oil, carbohydrates, proteins, steroids, Tannins and Phenolic compounds was determined (Table 1).

2.4. Thin Layer Chromatography

Dried extract (in the form of spots) was applied on a silica-coated glass plate and developed using ethyl acetate as a solvent. After spot development, the plate was dried at room temperature for 5 min to evaporate the remaining solvent. The spots were visualized under UV light. The plate was then placed in the chamber with iodine vapour and the positions of the spots were marked. The spots were

Table 2. Cynotoxins with their fragment ions detected in the algal extract.

	M/Z	Fragment assignment	Reference
Anatoxin-A	166.08	M+H	(Zervou et al., 2017)
	130.1	M-NH_4OH	
Cylindrospermopsin	415.41	M+H	(Kokociński et al., 2009)
	365.1	M-H_2SO	
	381.07	M-H_2S	
Microcystin-LR	917.59	M-135	(Benke et al., 2015)
	599.44	Agr-Adda-Glu+H	
Microcystin-WA	475.32	Ala-Trp-MaSp-Z+NH_4	(Puddick et al., 2013)
Microcystin-NfKA	665.26	Adda-Glu-Mdha-Ala-X+H	(Puddick et al., 2013)
Microcystin- HphR	1057.44	Asp^3, $ADMAdda^5$	(Kaasalainen et al., 2012)
Nodularin	839.5	M+H	(Meriluoto et al., 2016)
	775.53	M-OMe	
	791.51	M-NH_2-C=NH-NH_3	

Figure 2. (A) Habitat explored for the Collection of *Nostoc fuscescens*, (B) *Nostoc fuscescens* in natural habitat, (C) Microscopic details of *N. fuscescens* (100x), and (D) Drying of *N. fuscescens* bloom for preparation of methanolic extract.

scraped off, dissolved in 2 ml methanol and used for HR-MS analysis.

2.5. High-Resolution Mass Spectrum (HRMS) analysis

HRMS analysis was performed by direct infusion of 20 µl aliquots into the electrospray ionization (ESI) chamber at the rate of 0.120 min^{-1}. The mass spectra of the sample were recorded on a Bruker impact HD Q-TOF spectrometer (Bruker Daltonics, Billerica, MA, USA). The parameters of the mass spectrum were as follows: Source type- ESI, Focus- Active, Scan- 50-1500m/z, ion polarity- Positive, Set capillary-4500V, Set end plate offset- -500V, Set charging voltage- 2000V, Set nebulizer- 1.7 Bar, Set dry heater- 200 °C, and Set dry gas 7.0 L/min. The mass was confirmed by m/z ratio of the sample with the reference standards. Four cyanotoxins were identified by comparing their m/z ratio with their standard.

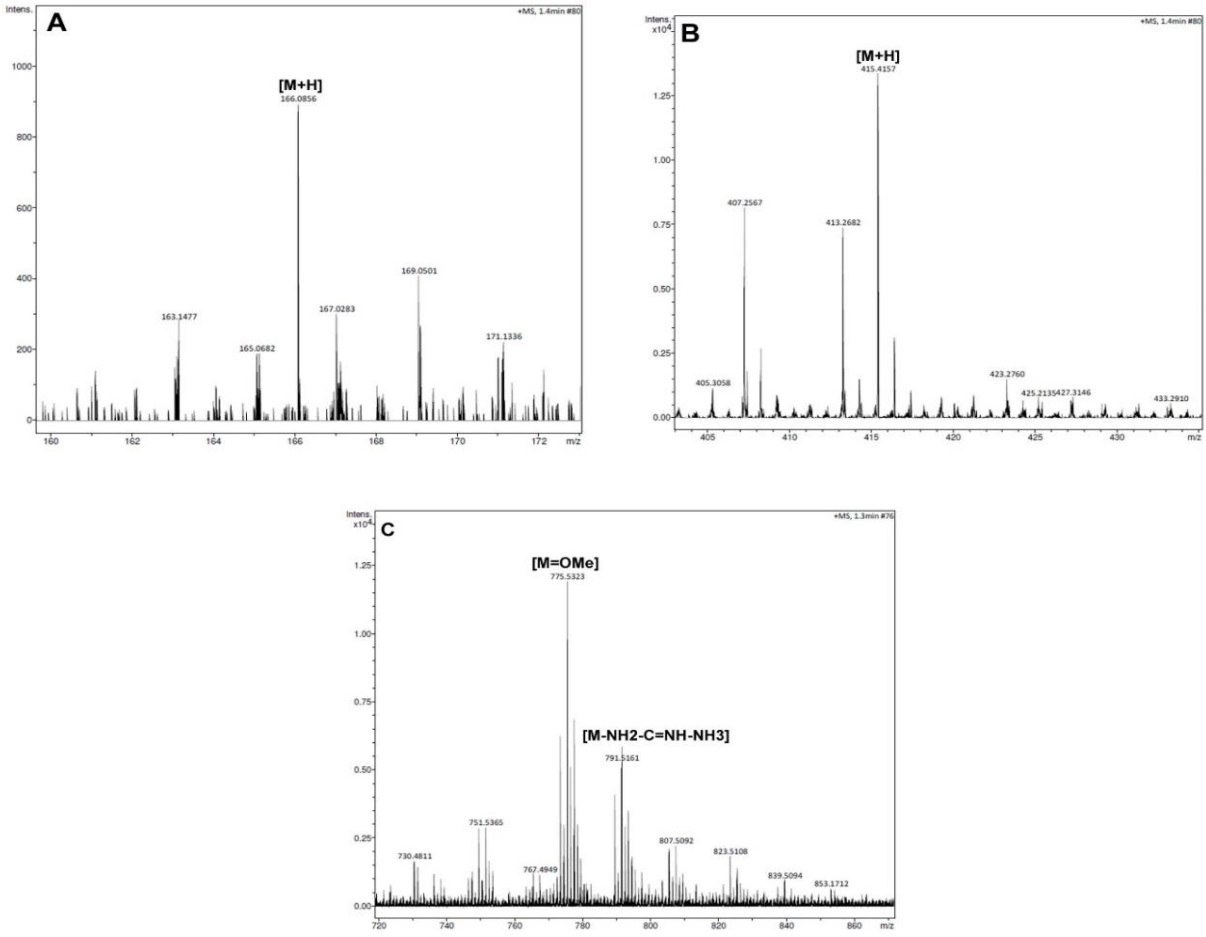

Figure 3. LC-HRMS Spectrogram showing chromatographic and mass spectrometric analysis of (A) Anatoxin-A, (B) Cylindrospermopsin, and (C) Nodularin

3. Results

Preliminary analysis revealed the presence of alkaloids, flavonoids, fats, proteins, and different glycosides in the methanolic extract of *N. fuscescens* (Table 1). LC-HRMS analysis by direct infusion experiment provided fragmentation curves for selected precursor ions. Based on the literature and LC-HRMS analysis molecular structures were attributed to the most intense fragments. The LC-HRMS analysis confirmed the presence of four cyanotoxins Anatoxin-a (ANA), Cylindrospermopsin (CYN), Microcystins (MCs) and Nodularin (NOD). Anatoxin-a forms $[M+H]^+$ precursor ion at m/z 166.08 with fragment ion m/z 130.10 which is the most intense fragment ion (Table 2; Figure 3). Cylindrospermopsin (CYN) forms $[M+H]^+$ precursor ion at m/z 416.41 with two product ions which are m/z 365.10 and m/z 381.07 (Table 2; Figure 3). Nodularin (NOD) forms $[M+H]^+$ precursor ion at m/z 839.50 with two fragment ions which are m/z 791 and m/z 775.53 (Figure 3). A total of four variants of microcystin were identified; Microcystin-LR at m/z 917.59 (m-135) with fragment ion 599.44 this fragment ion is associated with Adda moiety (Figure 4). Microcystin-WA at m/z 475.32 (Ala-Trp-MaSp-Z+NH_4), Microcystin-NfKA at m/z 665.26 (Adda-Glu-Mdha-Ala-X+H), and Microcystin- HphR at m/z 1057.44 (Table 2; Figure 4).

4. Discussion

In the present study, we reported four cyanotoxins, Microcystin, Nodularin, Cylindrospermocin, and Anatoxin in the cyanobacteria from the wet rock surface in the seasonal water spring. Microcystin is one of the most common toxins in cyanobacteria from diverse habitats including hypersaline water, soil, hot springs, etc. (Chorus and Welker, 2021b). Microcystin was also detected in the *Nostoc* strains which are symbionts of lichen (Oksanen et al., 2004). Nodularin is reported from seawater, brackish water, and coastal freshwater lakes (Bolch et al., 1999; Akcaalan et al., 2009). Similar to Microcystin, nodularin is also reported from the Nostoc symbiont of lichen (Gehringer et al., 2012). Among cyanotoxins detected in the present study, all of them are reported in *Nostoc* species (Ghassempour et al., 2005; Kinnear, 2010; Chorus and Welker, 2021b). Cylindrospermopsin were detected from freshwater and brackish water cyanobacteria (Kinnear, 2010; Rzymski and Poniedziałek, 2014). Anatoxin was reported from freshwater cyanobacteria (Christensen and Khan, 2020) including *Nostoc carneum* species (Ghassempour et al., 2005). These observations imply that compared to other cyanobacteria *N. fuscescens* produces diverse cyanotoxins. The present study for the first time reports the presence of four cyanotoxins from the wet rocks surface in the seasonal spring.

In the present study, we detected four variants of Microcystin. Previous studies have reported 2-3 variants of Microcystin in a single species of cyanobacteria (Bouaïcha et al., 2019; Chorus and Welker, 2021b). Among Microcystin variants, MC-LA is relatively abundant in different species of cyanobacteria (Bouaïcha et al., 2019; Chorus and Welker, 2021a). In the present study, we could detect three rare variants of Microcystin MC-WA, MC-NfkA, and MC-HphR. All these Microcystin variants were reported from freshwater cyanobacteria (Puddick et al., 2013; Bouaïcha et al., 2019).

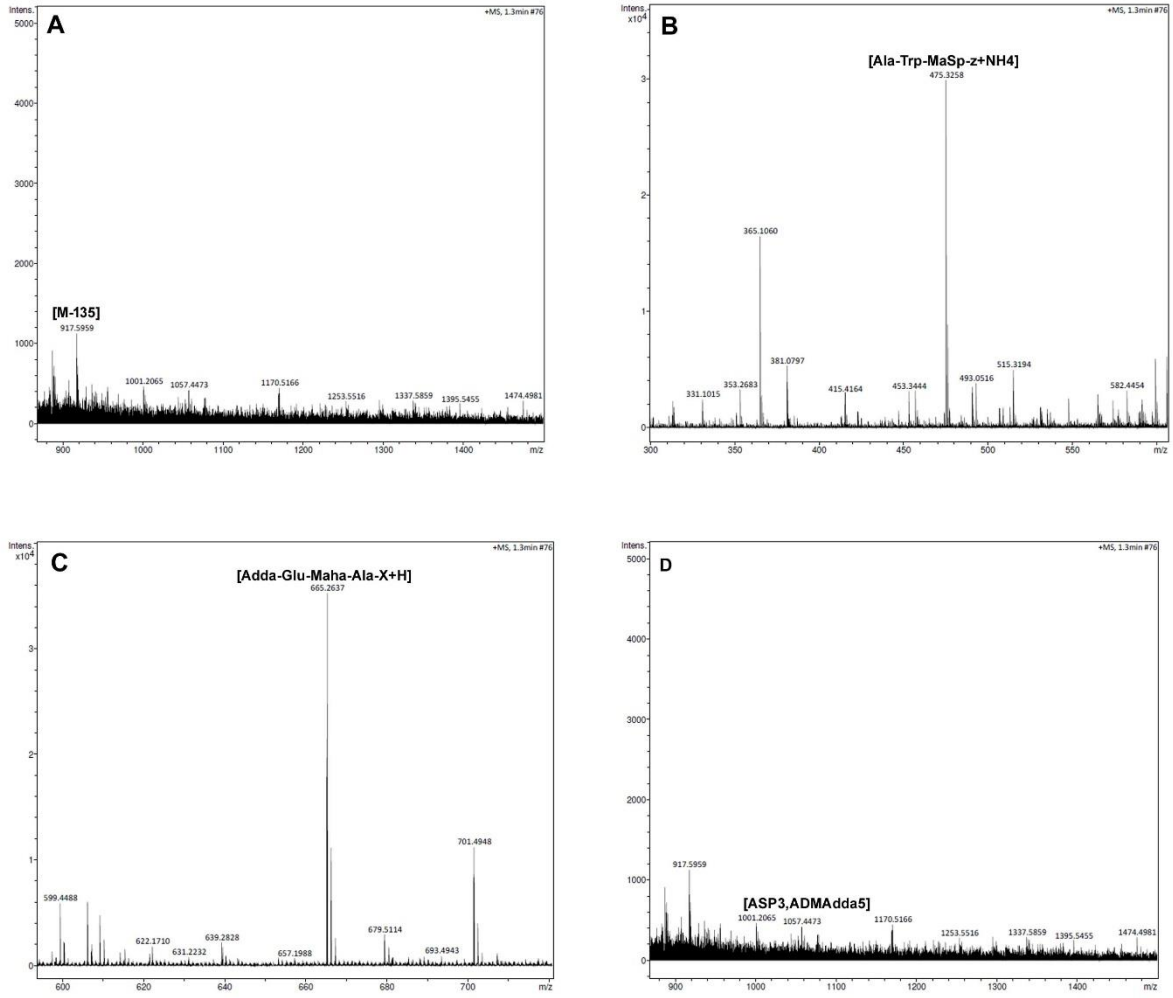

Figure 4. LC-HRMS Spectrogram showing chromatographic and mass spectrometric analysis of Microcystin variants (A) MC-LR, (B) MC-WA, (C) MC-MfKA, and (D) MC-MphR

Cyanobacterial toxins evolved as a cellular defence mechanism in response to resource competition and maintain physiological homeostasis (Holland and Kinnear, 2013). The detection of toxins in the cyanobacteria from diverse habitats (including marine, freshwater and terrestrial) (Gómez-Leyva et al., 2024; Jablonska et al., 2024; Sahu et al., 2024) highlights their importance in physiological functioning. In the present study, we detected cyanotoxins in *N. fuscescens* from wet rocks around the seasonal water springs. Additionally, the spring investigated in the present study remains dry for at least six months. The presence of diverse cyanotoxins in *N. fuscescens* suggests their possible role in maintaining physiological balance under changing environmental conditions (Holland and Kinnear, 2013).

Detection of cyanotoxins has limitations due to the unavailability of standards as quantitative methods such as LC-MS/MS rely on analytical standards for accurate quantification. Therefore, diverse chemical methods have been employed to identify cyanotoxins and their variants in environmental samples. Cyanotoxins can be identified using LC-MS or LC-MS/MS method even when standards are not available (Kaushik and Balasubramanian, 2013; Moreira et al., 2014). Liquid chromatography (LC) coupled with HRMS is increasingly used for quantitative and qualitative analysis of cyanotoxins (Panda et al., 2022; Sundaravadivelu et al., 2022). In the present study, we detected diverse cyanotoxins using LC-HRMS analysis.

Currently, cyanotoxins of diverse chemical nature are being increasingly detected in freshwater and pose a significant threat to humans and wildlife (Stewart et al., 2008; Zanchett and Oliveira-Filho, 2013; Ash and Patterson, 2022). The changing environment due to global warming is facilitating cyanobacterial growth which may have adverse effects on not only aquatic fauna but also terrestrial animals (Paul, 2008; Visser et al., 2016). Seasonal water springs in the Western Ghats drain flow to the water reservoirs which are the major source for urban population and agriculture. Therefore, detection and identification of cyanotoxins from diverse habitats using advanced methods is necessary. The present study highlights the necessity of cyanotoxin detection in neglected habitats.

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Author's contributions

Both authors were involved in designed the study, analysis of the results and writing of the manuscript. SG collected samples and analyzed chemical constituents.

Conflict of Interest

Authors have no conflict of interest to declare.

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