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RESEARCH PAPER

The first report of the production of anatoxin-a by Bolivian terrestrial cyanobacteria

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Abstract

Cyanobacterial harmful algal blooms (CyanoHABs) are observed in many regions worldwide with increasing frequency. The massive development of cyanobacteria is a severe problem for the water environment due to negative changes in water parameters, the introduction of toxic metabolites (cyanotoxins) into the water, and the resulting disruption of ecological relations in the ecosystem. Knowledge regarding CyanoHABs in aquatic reservoirs is increasing. However, information about cyanobacteria development in other, untypical habitats like deserts, open soils, or polar regions is still insufficient. Similarly, data regarding the distribution of cyanotoxins are extensive for some regions (for example, in Europe or North America), whereas, in other localities, such as those in South America, the data are scarce. In this paper, we investigated if phototrophic microbial communities collected from open soil (La Paz department, Sud Yungas province, Bolivia) contained cyanobacteria described as cyanotoxin producers. We performed qualitative and quantitative analysis of typical cyanotoxins found in aquatic reservoirs – anatoxin-a (ATX-a), cylindrospermopsin (CYN), and microcystin-LR (MC-LR). The obtained results showed a relatively high biodiversity of the studied microbial phototrophic community, which consists of several cyanobacterial and algal genera. Analyses of cyanotoxins showed that CYN and MC-LR were not present in the studied samples. However, despite the lack of cyanobacteria described as ATX-a producers, high-performance liquid chromatography (HPLC) chromatograms were revealed, and mass spectrometry (MS) spectra confirmed the presence of the toxin in the studied material. The results presented in this paper are, to the best of our knowledge, the first confirmation of the presence of ATX-a in open soil habitats, as well as the first record of cyanotoxin occurrence in Bolivia. The identification of anatoxin-producing cyanobacteria in open soil environments presents a novel finding that necessitates further work to elucidate their prevalence, abundance, and associated potential hazards, as well as the taxonomic classification of the specific cyanobacterial species able for anatoxin synthesis within these soil habitats. Future studies should focus on the distribution of cyanotoxins in cyanobacterial communities in untypical habitats and in localities for which, to date, the information on cyanotoxin occurrence is not currently available.

Keywords

cyanotoxins; anatoxin-a; cylindrospermopsin; microcystin-LR; Bolivia; soil surface

1. Introduction

Massive occurrences of harmful cyanobacterial algal blooms (CyanoHABs) have been observed in many regions all over the world worldwide in recent decades (Huisman et al., 2018; Sukenik & Kaplan, 2021). Some negative consequences of CyanoHABs in the aquatic environment are similar to those observed for other typical blooms caused by green algae. A large amount of cyanobacterial biomass increases the water turbidity and changes the light intensity in water bodies. Additionally, a high metabolic rate of blooms during their most intensive development stage leads to anoxia. The decrease in oxygen concentration directly contributes to the decline of the biodiversity of both plants and animals in a whole reservoir. Moreover, many cyanobacterial cells and the compounds they release cause an unpleasant appearance and odor in the reservoir (Al-Ghelani et al., 2005; Christensen et al., 2019; Visser et al., 2016). The most crucial feature of CyanoHABs is that the bloom-forming cyanobacteria produce and introduce into the water toxic secondary metabolites called cyanotoxins, which are one of the most bioactive compounds present in the natural environment (Adamski et al., 2021; Antoniou et al., 2005; Corbel et al., 2014; Filatova et al., 2020; Huertas & Mallén-Ponce, 2021).

Recently, one of the most studied cyanotoxins are cytotoxic alkaloid cylindrospemopsin (CYN) (Figure 1A), hepatotoxic oligopeptide microcystine-LR (MC-LR) (Figure 1B), and neurotoxic alkaloid anatoxin-a (ATX-a) (Figure 1C). The impact of cyanotoxins on living cells is broad and affects many processes in various organs (Adamski et al., 2014; Campos et al., 2021; Corbel et al., 2014; de La Cruz et al., 2013; Jaiswal et al., 2008; Malta et al., 2022).

Among the toxins produced by cyanobacteria, CYN has the broadest range of action and impairs several metabolic pathways in both animal and plant cells (Adamski et al., 2014; de La Cruz et al., 2013; Moreira et al., 2013). For the first time, it was isolated from *Raphidiopsis raciborskii* (former *Cylindrospermopsis raciborskii*) after an incident of hepatoenteritis in humans on Palm Island in Queensland (Australia) (Hawkins et al., 1985). Since then, several freshwater species of cyanobacteria occurring on all continents have been described as CYN producers (Adamski et al., 2020; de La Cruz et al., 2013).

The main mechanism of MC-LR action is the inhibition of protein phosphatases type 1 and 2A (PP1 and PP2A) in hepatocyte cells (Eriksson et al., 1990; Lone et al., 2015). To date, one incident of human poisoning by MC-LR was confirmed and refers to the death of 60 patients after hemodialysis with ineffectively purified water from a reservoir in Caruaru (Brazil) that was covered by cyanobacteria bloom (Pouria et al., 1998). The first species described as an MC-LR producer was *Microcystis aeruginosa*, and later, the ability to synthesize it was also confirmed for other cyanobacteria species (Botes et al., 1982; Sivonen, 2009). Similarly to CYN, MC-LR distribution is worldwide (Dittmann & Wiegand, 2006; Svirčev et al., 2019; Via-Ordorika et al., 2004).

ATX-a easily connects to the receptors that in neuromuscular systems, in extreme cases, leads to suffocation (Sivonen, 1996, 2009). Initially, the source of ATX-a was *Dolichospermum flos-aquae* (former *Anabaena flos-aquae*). Nowadays, several freshwater species of cyanobacteria that occur all over the world are known as ATX-a producers (Codd et al., 2005; Humbert, 2009). To date, there is no confirmed case of human poisoning by this toxin. However, the data obtained for animals showed that the toxic effects of ATX-a could develop extremely rapidly, and therefore, it was called a *very fast death factor* (VFDF) (Moore & Puschner, 2012; Tufariello et al., 1984).

Due to climate changes and the increasing introduction of agricultural and communal pollution into water bodies, cyanobacterial blooms occur in regions where they were not common before. The information about formation and development, as well as the factors that influence the CyanoHABs dynamic in aquatic reservoirs, are relatively plentiful (Barros et al., 2019; Li et al., 2018; Massey et al., 2022; Merel et al., 2013). However, the knowledge about the presence of potentially toxic cyanobacteria in other ecosystems is not yet fully explored. Moreover, in some regions, studies on cyanotoxins occurrence and studies on the occurrence of cyanotoxins, even in the typical aquatic environment, are not performed, unfortunately. For example, cyanotoxin in South America, data for cyanotoxin distribution are available only for Brazil, Argentina, and Uruguay; in South America, data for cyanotoxin distribution are available only for Brazil, Argentina, Uruguay, and Chile (Svirčev et al., 2019).

Cyanotoxins have been reported in extreme environments such as alkaline lakes, hot deserts, hot springs, hypersaline environments, and polar regions (both Antarctic and Arctic) (for review, see Cirés et al., 2017), but their fate in those habitats is still uncomplete. The studies on cyanotoxins found in unusual habitats show that there is a correlation between dominant types of toxins and those observed in typical aquatic environments, but their concentration could be different. Different is often microbial community presence in atypical habitats, and in some cases, none of the studied cyanobacteria collected in nature have been confirmed as capable of synthesizing toxins in laboratory cultures (Chrapusta et al., 2015; Khomutovska et al., 2020; Metcalf et al., 2012).

Due to the scarcity of data that would allow us to properly assess the risk of cyanotoxins for organisms living in untypical habitats in the natural environment and potentially also for humans, it seems to be very important to broaden the knowledge about cyanobacteria in those localities. Moreover, the data for cyanotoxin occurrence in regions where its monitoring has not been performed to date should be updated. Therefore, the main aim of this paper is to investigate if the terrestrial phototrophic microbial community, being a part of the Bolivian solid crust, may contain cyanobacteria known as cyanotoxin producers and the implementation of qualitative and quantitative analysis of cyanotoxins commonly found in natural aquatic environments: anatoxin-a, cylindrospermopsin, and microcystin-LR.

2. Material and methods

2.1. Sampling and material conservation

Samples of biological crust, including cyanobacterial communities and lichens, growing on open mineral soil and rocks near a road in the montane Yungas forest were collected during a lichenological expedition to Bolivia from a single sampling site (Figure 2). The location was recorded in the

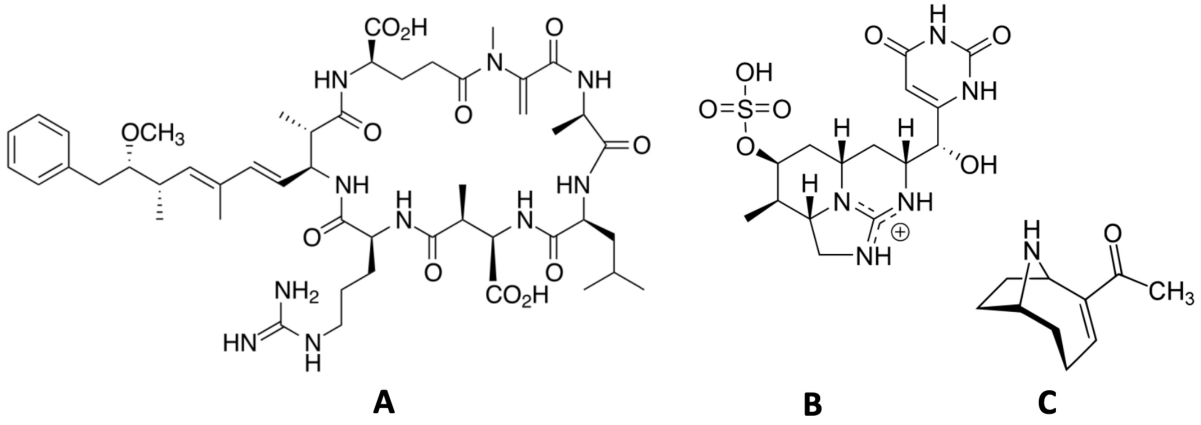

Figure 1 Chemical structures of (**A**) cylindrospermopsin; (**B**) microcystin-LR; (**C**) anatoxin-a.

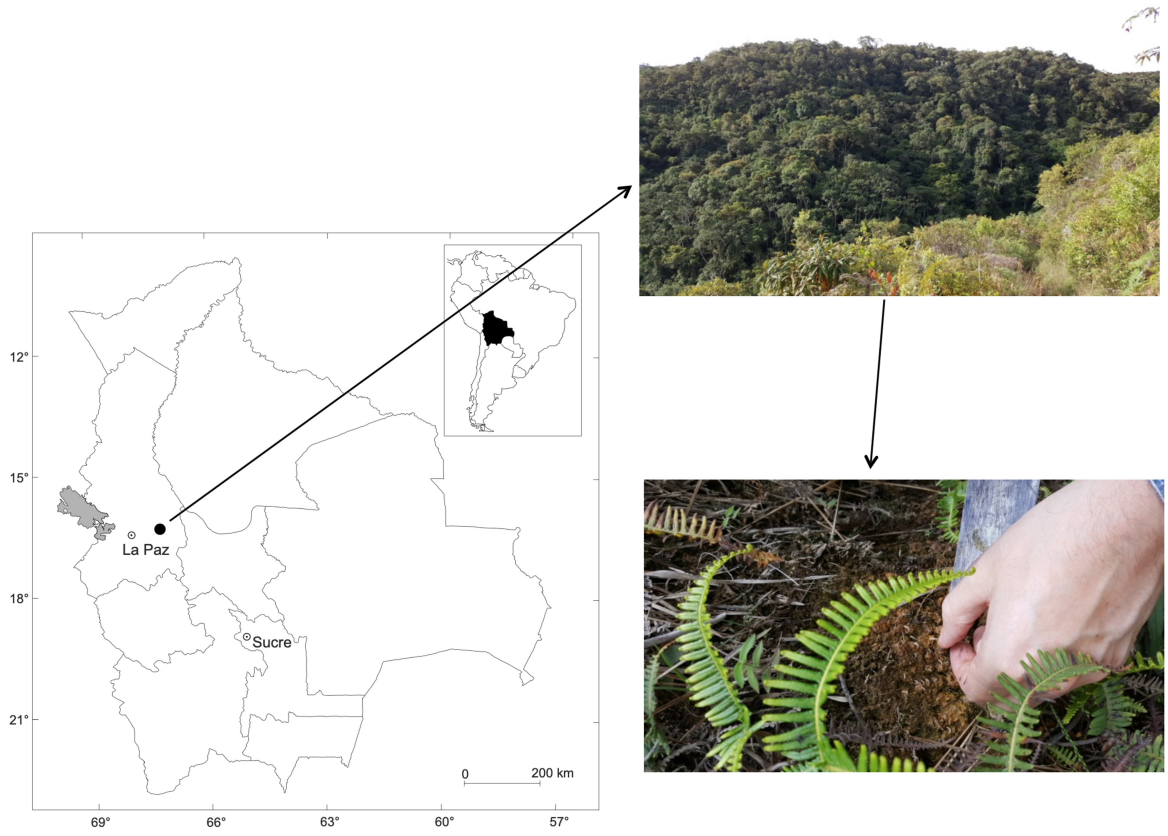

Figure 2 Geographic locations and photos of sampling sites.

La Paz department, Sud Yungas province, close to Reserva Ecológica de Apa Apa, locality of Sanani, near Chulumani, 16°20′39.70″S, 67°29′54.32″W; 2,423 m, Yungas montane forest, 23 January 2020. Dry specimens and/or slides are deposited at KRAM (Krakow) and LPB (La Paz) herbaria. Fragments of soil and rocks with cyanobacteria were probed by sterile spatulas, air-dried, sealed in paper bags, and kept in the darkness. After two weeks, the samples of soil and rocks were placed into sterile flasks with BG11 medium (Stanier et al., 1971) and cultivated in a phytotron at 22 ± 1 °C with 80 ± 5% humidity and 25 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) under a 12 h light/12 h dark photoperiod (lamps AQUAEL 18 W PLANT). All cultures were shaken once a day.

2.2. Taxonomic analysis

After one month of cultivation, the samples of phototrophic microbial communities that developed in the medium were subjected to taxonomic analysis. Cyanobacteria and algae were identified under a Nikon Eclipse 600 light microscope via standard phycological techniques. Micrographs were taken with a Nikon Coolpix 995 camera. For genus and species identification, references were made to published keys (Ettl &

Gärtner, 1995; Hindák, 2008; Komárek, 2013; Komárek & Anagnostidis, 2000, 2005).

2.3. Isolation of cyanotoxins

Cyanotoxins were extracted from the lyophilized cells and purified using the method described by Meriluoto and Codd (2005), with modifications as follows: the cultures were filtered using GF/C glass fiber filters after one month (Whatman, UK), and cellular material was immediately frozen at −20 °C, then cyclically thawed and frozen to destroy cell membranes and walls. In the next step, the material was further lyophilized, and its dry weight was determined. Then, the biomass was treated with 100% methanol and shaken in darkness until the discoloration of the filters. The supernatant was collected and evaporated until a dry residue was obtained. The extract was reconstituted on ice with Milli-Q water and centrifuged at 10,000 × g for 5 min. The obtained supernatant was subjected to further purification and concentration by a solid phase extraction (SPE). Afterward, prepared samples were filtered using syringe filters (0.22 µm, Merck, Germany) and injected into High Performance Liquid Chromatography (HPLC).

2.4. Analytical determination

To determine the presence and concentration of studied cyanotoxins, all samples were analyzed on a Shimadzu Nexera-I LC-2040C 3D Plus Ultra High Performance Liquid Chromatograph (UHPLC). The gradient mobile phase consisted of water/acetonitrile (both acidified with 0.05% trifluoroacetic acid), where the organic phase increased from 2% to 90% over 15 min at a flow rate of 0.75 mL min^{-1}. Samples were separated on a Gemini® NX-C18 Column (110 Å, 3.0 µm, 150 mm × 4.6 mm) maintained at 40 °C. The autosampler cooler temperature was 4 °C, and the PDA cell temperature was 40 °C. Toxins were identified by comparing the retention time, and UV-spectra determined for commercial standards and quantified by absorbance at 228, 239, and 261 nm for ATX-a, MC-LR, and CYN, respectively. A multilevel calibration curve was obtained using commercial standards (from 0.01 to 10.00 µg mL^{-1}). The presence of toxins in samples was confirmed by using an ultra-performance liquid chromatography tandem-mass spectrometer (UPLC-MS/MS) coupled with a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). The chromatographic separations were performed using the Acquity UPLC BEH (bridged ethyl hybrid) C18 column (2.1 × 100 mm, 1.7 µm) equipped with the Acquity UPLC BEH C18 VanGuard precolumn (2.1 × 5 mm, 1.7 µm). The column was maintained at 40 °C and eluted under the following conditions: 100% of eluent A over 2 min, then a gradient elution from 100% to 30% of eluent A over 10 min, at a flow rate of 0.3 mL min^{-1}. Eluent A was water/formic acid (0.1%, v/v), and eluent B was acetonitrile/formic acid (0.1%, v/v). Chromatograms were made using a Waters eλ PDA detector. Spectra were analyzed in the 200–700 nm range with 1.2 nm resolution and a sampling rate of 20 points/s. The Mass Spectrometry (MS) detection settings of the Waters TQD mass spectrometer were as follows: source temperature 150 °C, desolvation temperature 350 °C, desolvation gas flow rate 600 L h^{-1}, cone gas flow 100 L h^{-1}, capillary potential 3.00 kV, cone potential 20 V. Nitrogen was used as both the nebulizing and drying gas. The data were obtained in a scan mode ranging from 50 to 1,000 m/z in 0.5 s intervals. Collision-activated dissociations (CAD) analyses were conducted with an energy of 30 eV, and all the fragmentations were observed in the source. Consequently, the ion spectra were obtained by scanning from 30 to 500 m/z. The data acquisition software was MassLynx V 4.1 (Waters).

2.5. Chemicals

All reagents were of MS/MS, HPLC or analytical grade and were obtained from Merck Millipore (Bedford, MA, USA).

2.6. Statistical analysis

All data were expressed as means ± standard deviation (SD) of three independent replicates.

3. Results and discussion

The analysis of phototrophic microbial communities inhabiting the studied soil showed relatively high biodiversity. Dominant taxa belonging to cyanobacteria were represented by: *Fischerella* sp. (Bornet at Flahault) Gomont, *Nostoc sphaericum* Vaucher ex Bornet at Flahault, *Symploca* sp. Kützing ex Gomont, and *Hassallia bouteillei* Bornet & Flahault (Figure 3). CYN is synthesized by several freshwater species belonging to *Anabaena*, *Aphanizomenon*, *Dolichospermum*, *Lyngbya*, *Microseria*, *Oscillatoria*, *Phormidium*, *Raphidiopsis* and *Umezakia* (Adamski et al., 2020; de La Cruz et al., 2013; Moreira et al., 2013). None of these genera were identified in the studied samples. Among genera of cyanobacteria able to MC-LR production are *Anabaena*, *Anabaenopsis*, *Aphanizomenon*, *Arthrospira*, *Chroococcus*, *Fischerella*, *Microcystis*, *Nodularia*, *Nostoc*, *Oscillatoria*, *Planktothrix* and others (Fiore et al., 2009; Metcalf & Codd, 2012). Taxa identified at studied sites contain one representative from the genus *Nostoc* – *Nostoc sphaericum*, but this species is not known as a MC-LR producer. Cyanobacteria from the genus *Fischerella* were also identified. Species synthesized ATX-a belong to *Anabaena*, *Aphanizomenon*, *Arthrospira*, *Cylindrospermum*, *Microcystis*, *Oscillatoria*, *Phormidium*, *Planktothrix* and *Tychonema* genera (Metcalf & Codd, 2012; Shams et al., 2015). None of them were marked in the collected samples.

Results of HPLC and MS analysis showed that CYN and MC-LR were not present in the studied samples. However, despite the lack of cyanobacteria described as ATX-a producers, HPLC chromatograms revealed and MS spectra confirmed the presence of the toxin in the studied material (Figure 4).

The average ATX-a content was 1.69 ± 1.42 µg g^{-1} DW, which was significantly lower than values obtained from samples collected from tropical aquatic reservoirs (4.4 × 10^3 µg g^{-1} DW) (Chorus & Bartram, 1999). Probably the ratio of ATX-a synthesis in the phototrophic microbial community from studied rocks is lower than in water reservoirs covered by blooms. Cyanobacteria that develop rapidly and create the CyanoHABs are usually the dominant microorganisms occurring in reservoirs during the bloom. In the studied habitat the cyanobacteria were co-existing with other microorganisms. We identified five taxa belonging to green algae and diatoms: *Chlorella* sp. 1 and sp. 2., *Klebsormidium subtile* (Kützing)

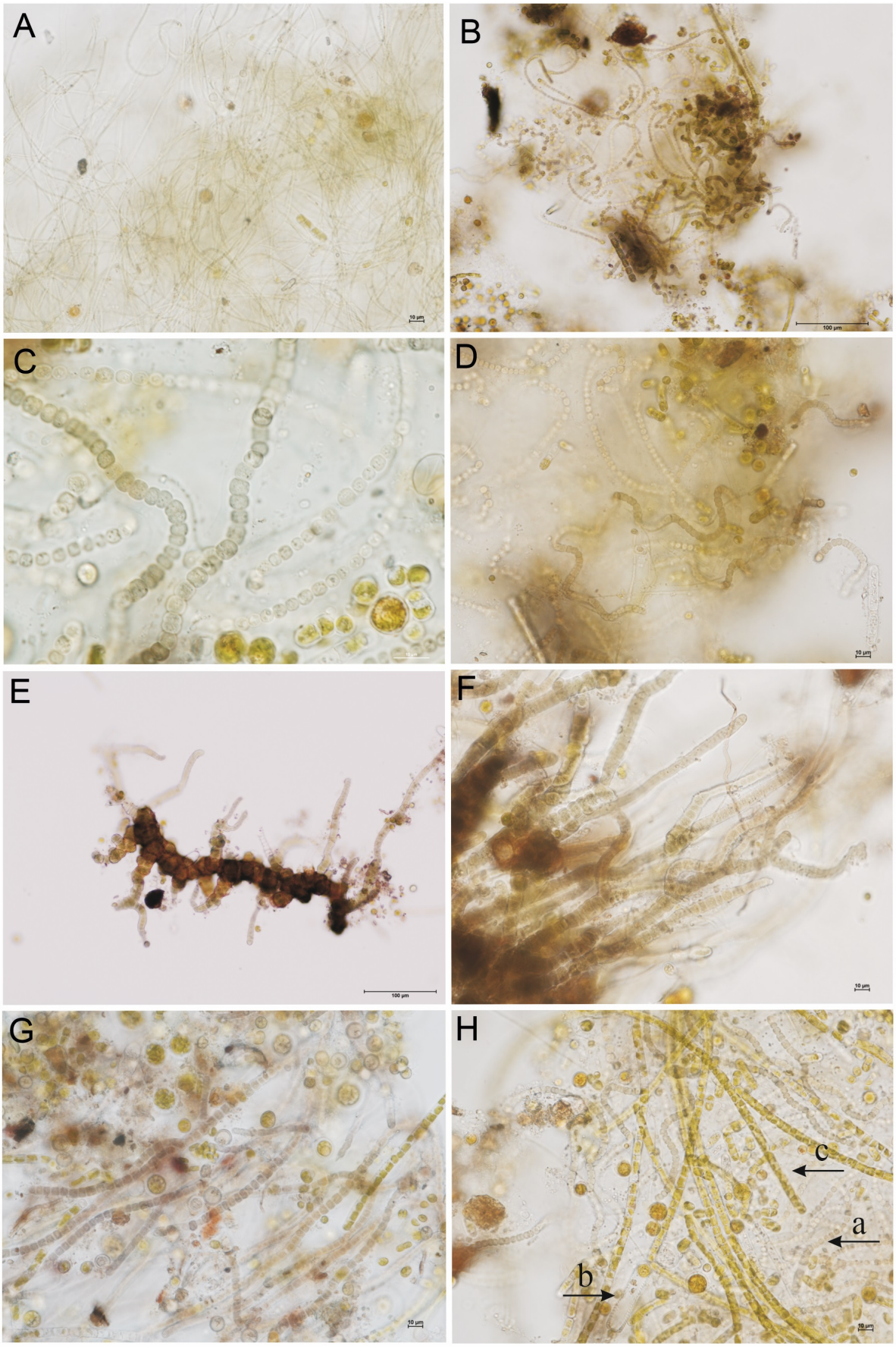

Figure 3 Cyanobacteria marked on soil surface located in Bolivia. (**A**) *Symploca* sp.; (**B–D**), (**Ha**) *Nostoc sphaericum*; (**E–F**) *Fischerella* sp.; (**G**) *Hassallia bouteillei*; and algae (**Hb**) *Pinnularia* sp.; (**Hc**) *Ulothrix tenerrima*.

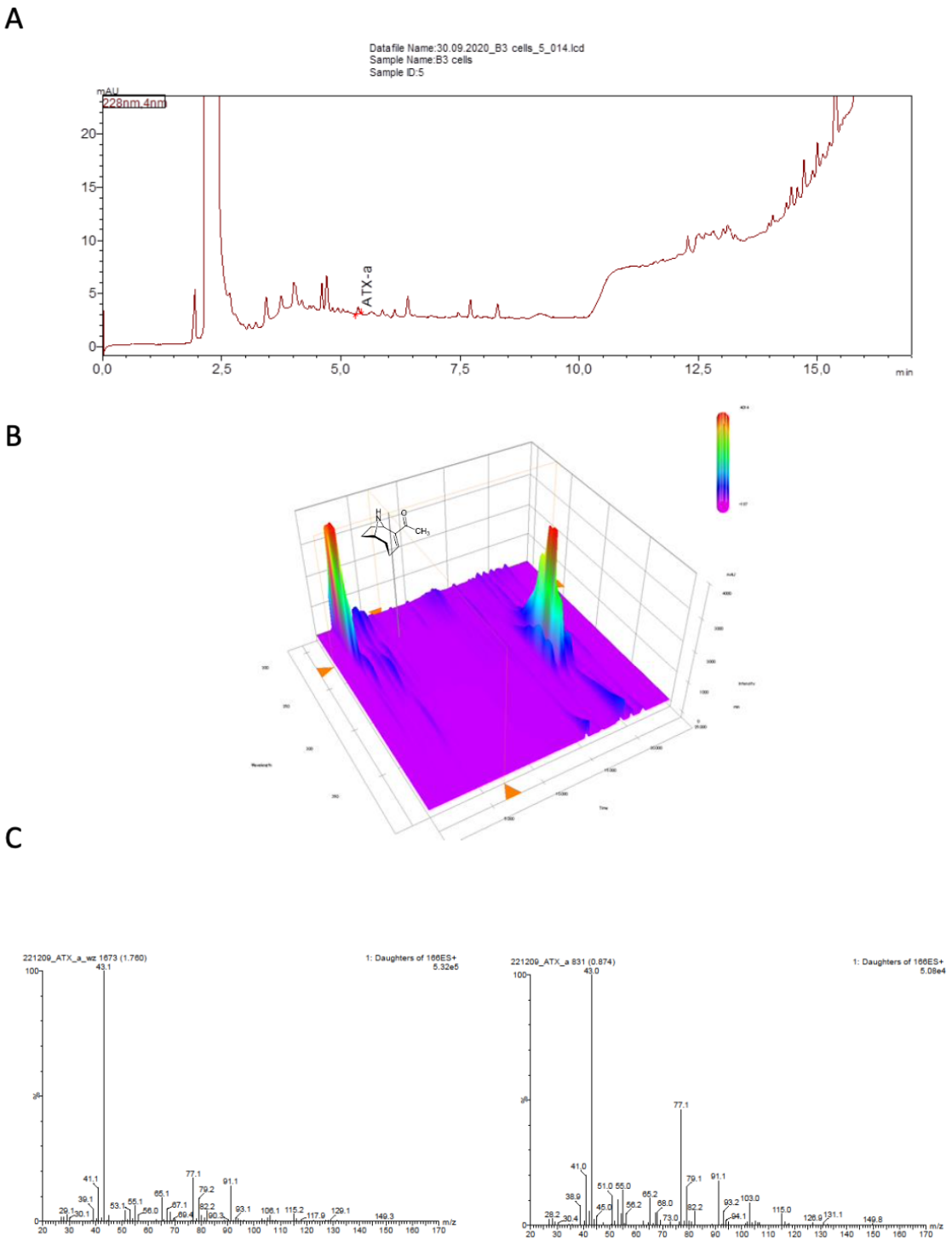

Figure 4 Chromatogram spectrum of extract containng anatoxin-a (**A**); 3D chromatogram spectrum of extract with anatoxin-a (**B**); fragmentation mass spectra of isolated anatoxin-a (right) and commercial standard (left) (**C**).

Mikhailuk, Glaser, Holzinger & Karsten, *Ulothrix tenerrima* (Kützing) Kützing and *Pinnularia* sp. Ehrenberg, respectively (Figure 5). The interactions like competition or allelopathy could probably impact both, the growth rate of cyanobacteria and the ATX-a production ratio. Teneva et al. (2012) studied a potential bioactivity of two *Nostoc* species (*Nostoc linckia* and *Nostoc punctiforme*) and showed that both produced compounds cytotoxic to vertebrates, as well as ATX-a and MC-LR. Perhaps *N. sphaericum* identified in this study could be the source of ATX-a detected in studied samples. Kurmayer (2012) found that *Nostoc* sp. Strain 152 produces a maximal amount of MC under stress conditions. Potentially, the conditions occurring in the studied Bolivian habitat could be periodically demanding for *N. sphaericum*, and the production of ATX-a could be the cyanobacteria cellular response.

Undertaking a more in-depth study of ATX-a occurrence in the studied sites, including monocultures of isolated cyanobac-

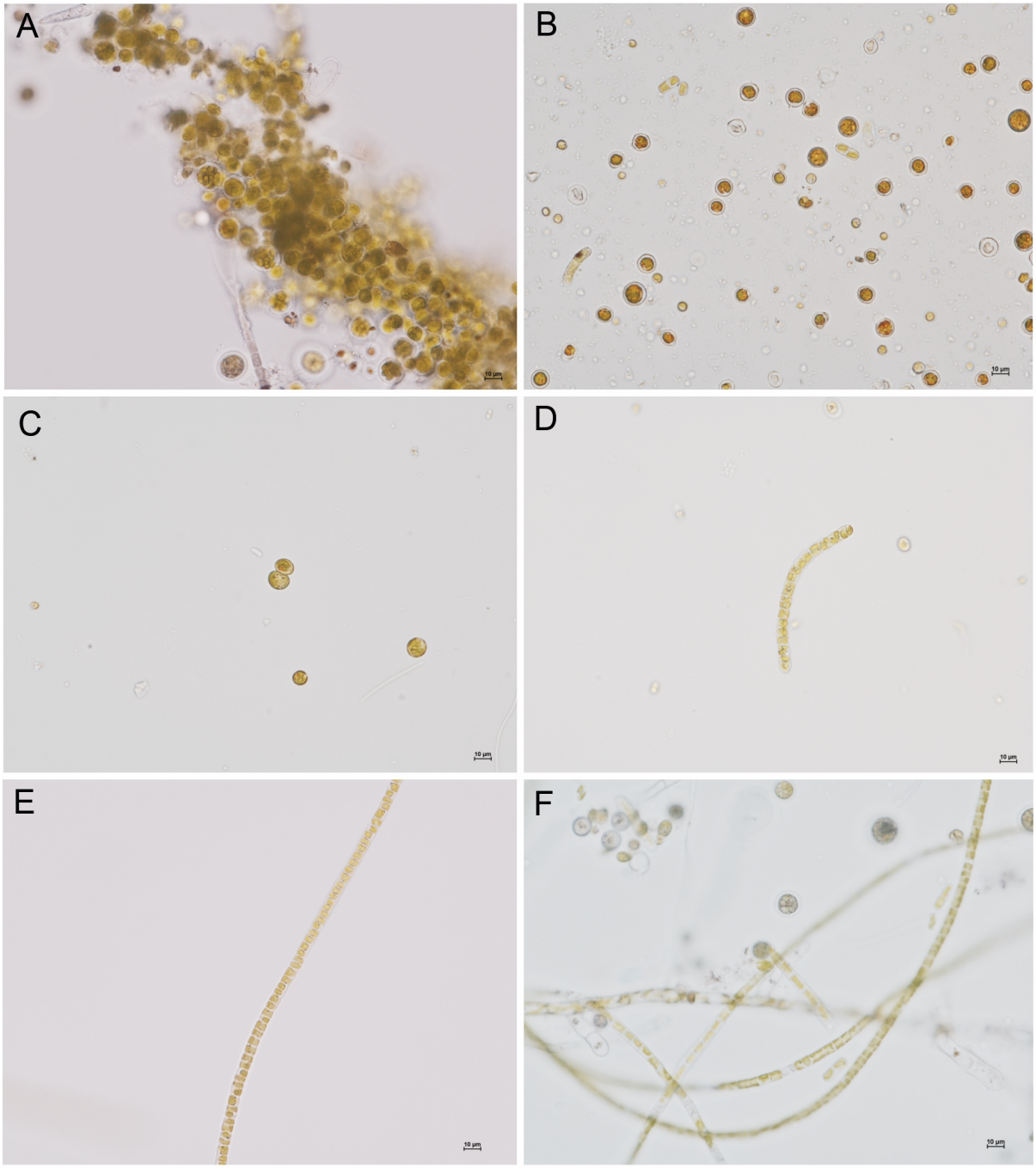

Figure 5 Algae marked on soil surface located in Bolivia. (**A**) *Chlorella* sp. 1; (**B–C**) *Chlorella* sp. 2; (**D**) *Klebsormidium subtile*; (**E–F**) *Ulothrix tenerrima*.

teria and accurate determination of the toxin concentration, could provide an answer to which of the identified organisms is the ATX-a producer. However, it should be mentioned that the reasons for cyanotoxin synthesis are not fully elucidated. Therefore the rate of their production or the amount of compounds could be different in microbial communities compared to monoculture kept in laboratory conditions.

Microcystins (MCs) are the cyanotoxins that are found most frequently in all temperature zones (Bartram et al., 1999; Metcalf et al., 2012; Svirčev et al., 2019). Our results did not show any presence of MC-LR or other structurally similar compounds that potentially could be variants of this toxin. Perhaps, the MCs producers did not occur in studied sites or did not regrow from dormant cells under laboratory conditions. The lack of MC-LR traces can also potentially result from the open soil surface conditions and co-existence of other microorganisms that may favor the toxin's decomposition. For example, a temperature above 21 °C under alkaline conditions or 40 °C at acidic pH results in MC-LR breakdown (Harada et al., 1996). Additionally, to this date, there is a broad knowledge about bacteria degrading microcystins (for review, see Massey & Yang, 2020). To confirm the absence or possible presence of MCs in the studied habitats, more detailed tests like rapid analyses of MCs concentration from fresh material

or thorough examination of abiotic factors should be performed.

Similarly to MCs, CYN is also often detected during CyanoHABs all over the world (de La Cruz et al., 2013; Moreira et al., 2013). The reasons why it was not present in the studied samples could be similar to the ones described above for MC-LR. It was demonstrated that abiotic factors like alkaline pH or UV light led to CYN decomposition (Adamski et al., 2020; Adamski et al., 2016a, 2016b; Chiswell et al., 1999). The important issue seems to be the stability of CYN, which could potentially be introduced to the microbial communities present in open soil. Unfortunately, the fate of CYN in the natural environment is still not fully understood.

In contrast to the considerable information about MC-LR and CYN producers and their ecotoxicology in freshwater reservoirs, the data about these cyanobacteria from other habitats are scarce. Thus far, there is only some information about the MC-LR-producing cyanobacteria living in arctic biocrusts or deserts (Chrapusta et al., 2015; Kleinteich et al., 2013; Metcalf et al., 2012; Wood et al., 2008). Even though the MC-LR and CYN should be treated as compounds typically occurring in the aquatic environment, considering their potential impact on the environment and other organisms, the research about their distribution in other habitats should be intensified. Potentially very useful in this issue could be genetic methods that allow the detection of genes responsible for MC-LR and CYN production. It seems to be interesting and important for cyanotoxin monitoring and management systems.

The information about the distribution of cyanotoxins in South America is very limited. To date, the cyanotoxins have been found only in a few countries on the continent, and the most distributed toxin is MC-LR, whereas ATX-a is found only in Brazil. None of the typical cyanotoxins have been identified from Bolivia so far (Svirčev et al., 2019).

However, in recent years, new reports have started to appear from areas where cyanotoxins were thought to be rare or absent. For example, John et al. (2019) reported the presence of ATX-a in Australia for the first time and brought up an important topic of regular updating of the monitoring strategies in a shifting global distribution of cyanotoxins. It seems to be especially important in the context of cyanobacterial invasive alien species colonizing new territories (Sukenik et al., 2012; Wilk-Woźniak et al., 2016). Sukenik et al. (2012) described a high invasion potential of Nostocales species to subtropical and temperate freshwater lakes. The information about reverse colonization – to tropical zones from subtropical and temperate zones is unfortunately absent.

In this study, we report the presence of ATX-a in a open soil habitat developed in tropical mountain forests in Bolivia. To our best knowledge, it is the first report of ATX-a detected in such habitat, as well as the first record of the toxin occurrence in Bolivia. The presented data show that the ecotoxicology of cyanotoxins is still incomplete. The identification of anatoxin-producing cyanobacteria in open soil environments presents a novel finding that necessitates further work to elucidate their prevalence, abundance, and associated potential hazards. Moreover, endeavors should be undertaken to ascertain the taxonomic classification of the specific cyanobacterial species able for anatoxin synthesis within these soil habitats. It is important to emphasize that in addition to ATX-a, cyanobacteria naturally produce its congeners, which also exhibit a high level of bioactivity. Research focusing on the qualitative and quantitative analysis of these compounds in natural habitats should also be conducted in parallel with studies dedicated to ATX-a (Puddick et al., 2021; Wood et al., 2018).

Future studies should focus on the distribution of cyanotoxins in cyanobacterial communities at untypical habitats in the context of its ecotoxicology. The important issue is also broadening the knowledge about cyanotoxins occurrence in South America, especially in the eastern part of the continent.

4. Conclusions

The key outcomes of this study are that (i) cyanobacteria living on Bolivian open soil surfaces can produce ATX-a; (ii) this is the first report of ATX-a presence in phototrophic microbial community that developed on soil and rocks habitat; (iii) this is the first report of cyanotoxin occurrence in Bolivia; (iv) the future study should focus on cyanotoxins presence in unusual habitats and determining cyanobacterial species capable of synthesizing toxins in such habitats; (v) future study should also focus on the distribution of cyanotoxins in South America, especially in the Andes, and their evolution in the context of climate change.

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