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Cyanotoxin production and phylogeny of benthic cyanobacterial strains isolated from the northeast of Brazil

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ABSTRACT

Most of the knowledge about cyanobacteria toxin production is traditionally associated with planktonic cyanobacterial blooms. However, some studies have been showing that benthic cyanobacteria can produce cyanotoxins. According to this, we aimed to evaluate the production of microcystins and saxitoxins in benthic cyanobacteria isolated from aquatic ecosystems in the Northeast of Brazil and to use a polyphasic approach for their identification. Forty-five clonal strains were isolated from rivers and water supply reservoirs, and identified using morphological and molecular phylogenetic characteristics. In order to evaluate the toxins production, the strains were screened for genes involved in the biosynthesis of microcystins and saxitoxins, positive results were confirmed and cyanotoxins quantified using HPLC. Eight species were identified belonging to the Phormidiaceae, Pseudanabaenaceae and Nostocaceae families. This is the first study in Brazil that shows that strains from the *Geitlerinema* genus correspond to at least three phylogenetic lineages, which possibly correspond to three distinct species to be subsequently reclassified. The strains that showed one of the genes involved in the cyanotoxins production were analyzed by HPLC and *Geitlerinema amphibium*, *Geitlerinema lemmermannii*, *Cylindrospermum stagnale* and *Phormidium uncinatum* were identified as producing one or more saxitoxins variants. Thus, this is the first report of saxitoxins production for those first three species and the first report in Brazil for *P. uncinatum*.

1. Introduction

Among approximately 150 described genera of cyanobacteria, 40 are known to produce toxic compounds that can impact terrestrial and water-based organism (Van Apeldoorn et al., 2007). These toxins can cause dermatitis, gastroenteritis, others have antimicrobial and cytotoxic activities and some inhibit microalgae and macrophytes growth (Codd et al., 2005). A subset of these toxins, known as cyanotoxins, has attracted significant scientific and management attention due to their toxicity to humans and animals. Cyanotoxins include: neurotoxins (saxitoxins, anatoxin-a, and anatoxin-a(s)) and hepatotoxins (microcystins, cylindrospermopsin and nodularins). The biological function and ecological role of these toxins for cyanobacteria are still unclear (Chorus and Bartrum, 1999); however, some recent studies on microcystins suggest protection against reactive oxygen species (Zilliges et al., 2011).

Globally planktonic cyanobacterial blooms, which occur generally in lentic or semilotic water bodies, have been considered a risk to the health of humans and animals for many decades (Chorus and Bartrum, 1999). In Brazil, this perception was intensified after the fatal poisoning caused by microcystins in at least 65 renal patients in a hemodialysis clinic in Caruaru, Pernambuco, Brazil (Carmichael et al., 2001). As a result of this

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tragedy, monitoring of planktonic species of cyanobacteria and cyanotoxins in drinking water supply reservoirs is mandatory in Brazil (BRASIL, 2011). Ordinance 2.914/11 requires that when cyanobacterial cells concentrations exceed 20,000 cells per milliliter, analysis of microcystins and saxitoxins must be undertaken in tap water and it also recommends analysis of anatoxin-a(s) and cylindrospermopsin.

However, little is known about the extent and frequency of toxin production by benthic cyanobacteria in Brazil despite records of deaths and poisonings of various animals around the world caused by benthic species (Edwards et al., 1992; Mez et al., 1997; Hamill, 2001; Gugger et al., 2005; Izaguirre et al., 2007; Quiblier et al., 2013). Benthic cyanobacteria are now known to produce most of the cyanotoxins produced by planktonic species, including: anatoxin-a and homoanatoxin-a (Edwards et al., 1992; Hamill, 2001; Gugger et al., 2005; Wood et al., 2007), saxitoxins (Onodera et al., 1997; Teneva et al., 2005; Smith et al., 2011) cylindrospermopsins (Seifert et al., 2007), microcystins (Mez et al., 1997; Mohamed et al., 2006; Izaguirre et al., 2007; Mohamed and Al Shehri, 2010) and nodularins (Wood et al., 2012).

Baker et al. (2001) reported a case in which pieces of benthic *Phormidium* mats detached from the sediment and entered in the public water supply. Bioassays conducted on mice found that the samples with *Phormidium* were producing toxins, which could not be identified. Wood and Young (2011) recently used an *in situ* technique known as solid phase adsorption toxin tracking technology (SPATT) and showed that cyanotoxins are released into the water from benthic cyanobacterial mats, highlighting the risk they may pose when present in drinking water supplies.

Correct identification of benthic cyanobacteria would help to identify potential toxin producers species, however, the identification is challenging when based solely on morphological features, particularly the Oscillatoriales order. Many benthic species are from this order and recent researches have demonstrated the benefits of using a polyphasic approach for their identifications (Komárek, 2003; Willame et al., 2006; Heath et al., 2010).

In Brazil few studies have been undertaken on benthic species. The only studies published so far were those of Fiore et al. (2009), who reported the production of microcystin-LR by a strain of *Fischerella* sp. isolated from a small reservoir supply in Piracicaba, São Paulo State, and Genuário et al. (2010), who reported the production of microcystin-YR by a strain of *Nostoc* sp. also isolated from a reservoir in Piracicaba. Thus, the aim of the present study was to isolate benthic cyanobacteria from the Mundaú river basin, Pernambuco State, and the Environmental Protection Area of Araripe, Ceará State to verify the presence of microcystins and saxitoxins, the main toxins found in Brazil, in the isolates and use a polyphasic approach for their identification.

2. Materials and methods

2.1. Sites and sample collection

Samples were collected on May 2011 from seven sampling sites distributed in rivers and drinking water reservoirs in the Mundaú River basin in the Pernambuco semi-arid region and three sites in the Environmental Protection Area (EPA) of Araripe, Ceará, Brazil (Table 1). At each site, sediment samples or benthic scrapings from rocks, macrophytes or the walls of the dams were collected using sterile spatulas. Samples were placed in sterile plastic bags (NASCO, 100 mL) and transported to laboratory in coolers at ambient temperature. In the laboratory, aliquots of samples were transferred to 15 mL borosilicate glass tubes containing 5 mL of ASM-1 medium for subsequent isolation, also, aliquot of each sample was fixed in acetic Lugol (5%) for later species identification.

2.2. Culture and strains isolation

Tubes containing the field samples were vortexed for 1 min to separate the aggregated trichomes. Aliquots were placed on glass slides and observed under an optical microscope (Zeiss AxioScope.A1) at 200× magnification. Free floating trichomes were transferred successively using a Pasteur pipette to drops of ASM-1 medium (Gorham et al., 1964) until only one trichome was left in the drop. Each single trichome was transferred to a 5 mL borosilicate glass tubes containing 5 mL of ASM-1 medium and cultured in chambers (Marconi MA402) at $25 \pm 0.1\ ^{\circ}C$, 80 μmol photons $m^{-2}\ s^{-1}$ – 12 h light:dark.

Additionally, aliquots of the previous isolated samples were spread on Petri plates containing ASM-1 solid medium (1% agar) aiming to remove any planktonic contamination (*e.g.* planktonic algae). The plates were observed under an inverted microscope (Zeiss Axiovert 40 CFL) and solely trichomes were removed from the plates using a sterile platinum loop and transferred to glass tubes containing 5 mL of ASM-1 medium and incubated using the conditions described above. The isolation process of the 45 strains took six months.

Successfully isolated strains were cultured for approximately ten days in 2 liter flasks containing 1.6 L of ASM-1 medium at $26 \pm 2\ ^{\circ}C$, 80 μmol photons $m^{-2}\ s^{-1}$, 12 h dark:light and aeration to obtain sufficient biomass for toxins analysis and DNA extraction. At the end of the exponential growth phase or early stationary phase, an aliquot of the material was removed for DNA extraction and the remainder centrifuged (18,000 × *g*, 15 min; Thermo Scientific Evolution RC) and the samples frozen (−18 °C) for subsequent lyophilization (Terroni, LD1500) and toxins extraction.

2.3. Taxonomic identification

Morphological features including trichome and filament sizes, cells width and length, species motility and, when present, heterocytes and akinetes were used for species identification according to the specific literature. At least 50 cells and 30 trichomes dimensions were measured for each strain. Whenever possible, natural population samples were also used to confirm species identification. Photos and measurements were taken using Axion Vision 40 software, V. 4.8.2.0.

2.4. Extraction, amplification and DNA sequencing

DNA was extracted from the 45 isolated strains using the Cetyl Trimethyl Ammonium Bromide (CTAB) method described in Rogers and Bendich (1985). PCR amplifications were performed in Eppendorf Mastercycler® Pro thermocycler, using Invitrogen Supermix® following manufacturer's recommendations. The 16S rDNA was amplified using the primers 27F1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 809R (5′-GCTTCGGCACGGCTCGGGTCGATA-3′) (Jungblut et al., 2005). All primers were purchased from Integrated DNA Technologies, Inc. PCR amplicons used for sequencing were purified using PureLink commercial kit (Invitrogen, Carlsbad, CA, USA).

2.5. Sequences analysis and phylogenetic

The 16S rDNA region of the strains was sequenced bidirectionally (Macrogen Inc., Seoul, Korea). Sequences were aligned using ClustalW and manually inspected in the BioEdit program (Hall, 1999) and their similarity compared using the BLAST (Basic Local Alignment Search Tool) with the other sequences in GenBank. Phylogenetic trees were constructed using Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods, with the PAUP version 4.02b (Swofford, 1998), and Bayesian Inference (BI), using MrBayes

Table 1
Cyanobacteria sampling sites from Mundaú River basin, Pernambuco, and Environmental Protection Area (EPA) of Araripe dams, Ceará, Brazil.

Site codes	Sample location	Habitat	Taxa	GPS
P-01	Mundaú, Pernambuco	Benthos in a stream	*Phormidium uncinatum*	08°53′16.0″ S 036°31′10.2″ W
P-02	Mundaú, Pernambuco	Benthos in a stream	*Microcoleus* sp.	08°55′02.8″ S 036°30′11.0″ W
P-03	Mundaú, Pernambuco	Benthos in a stream	*Phormidium uncinatum* *Pseudanabaena catenata*	08°55′59.3″ S 036°29′19.1″ W
P-04	Mundaú, Pernambuco	Benthos in a reservoir	*Geitlerinema amphibium*	08°56′42.9″ S 036°29′25.8″ W
P-05	Mundaú, Pernambuco	Benthos in a river	*Phormidium uncinatum*	09°07′54.5″ S 036°18′44.3″ W
P-06	Mundaú, Pernambuco	Benthos in a spring	*Cylindrospermum stagnale*	08°53′33.4″ S 036°29′24.0″ W
P-07	Mundaú, Pernambuco	Benthos in a spring	–	08°53′34.4″ S 036°29′26.2″ W
P-08	EPA of Araripe, Ceará	Benthos in a pond	*Phormidium breve*	07°39′17.5″ S 039°17′05.9″ W
P-09	EPA of Araripe, Ceará	Benthos in a pond	*Geitlerinema amphibium*	07°39′24.8″ S 039°15′44.9″ W
P-10	EPA of Araripe, Ceará	Benthos in a pond	*Geitlerinema amphibium* *Geitlerinema splendidum*	07°39′10.2″ S 039°16′20.8″ W

3.1.2 software (Huelsenbeck and Ronquist, 2001). Bootstrap analysis of 1000 replicates was performed for NJ and MP trees and the parameters for BI were fixed in two runs with 10,000,000 generations, four Markov chains and tree sampling each 1000 generations. The best evolution model for the dataset was determined by ModelTest 3.06 software (Posada and Crandall, 1998) according to Akaike's information criterion and resulted in the selection of GTR+I+G model.

2.6. Detection of the sxt*A* and mcy*E* genes

To identify whether the strains had the potential to produce microcystins and saxitoxins, genes involved in their synthesis, *mcy*E and *sxt*A, were amplified using the methods described in Jungblut and Neilan (2006) and Ballot et al. (2010), respectively. The strains ITEP-A1 (*Cylindrospermopsis raciborskii*) (data not shown) and NPLJ-4 (*Microcystis aeruginosa*) (Ferreira et al., 2010), known as saxitoxins and microcystin producers, respectively, were included as positive controls.

2.7. Analysis of toxins by high performance liquid chromatography (HPLC)

The high performance liquid chromatograph (HPLC) used for analysis of microcystins and saxitoxins consisted of three pumps (LC-20AT, LC-20AD, LC-20AD), degasser (20A5-DGU), system for post-column derivatization, oven (CTO-20A), fluorescence detector (RF-10A_{XL}), UV–vis detector with diode array (SPD-M20A), autosampler (SIL-20A_{HT}) controller system for connecting with computer modules (CBM-20A) and LC Solution software for data acquisition and processing (Shimadzu Proeminance).

2.7.1. Microcystins

Subsamples (approximately 100 mg) of each lyophilized samples was added to 75% methanol (10 mL). The solutions were stirred (1 h), and then centrifuged (12,000 × *g*, 10 min; Thermic Scientific, ST16R). The supernatant was transferred to a beaker and the procedure repeated twice, reducing the extraction time to 30 min (Fastner et al., 1998). The two extractions were combined and dried under warm air and resuspended in 0.5 mL of 20% methanol and frozen (−18 °C) until HPLC analysis. For analysis, a C18 column (250 mm × 4 mm; 5 μm), oven at 40 °C and a gradient of water:acetonitrile containing 0.05% each of trifluoroacetic acid were used (Lawton et al., 1994). The monitoring of chromatograms was at 238 nm and the absorption spectrum of the peaks monitored between 190 and 300 nm. The microcystin identification was taken by its absorption spectrum and comparison with a microcystin-LR standard (Abraxis).

2.7.2. Saxitoxins

The methods described by Oshima (1995) were used for the extraction and chromatographic analysis of saxitoxins with slight modifications. The extraction was carried out with lyophilized material (approximately 200 mg) which was added to acetic acid (5 mL, 0.05 M). The solution was stirred (1 h) and then centrifuged (12,000 × *g*, 20 min, Hettich MIKRO 200R) and the supernatant separated into a beaker. This procedure was repeated twice and, the supernatant combined and used for the chromatographic analysis. The clean-up step with C18 cartridge was not performed since previous analysis showed it was not necessary. The chromatographic analysis consisted of an on-line system of post-column derivatization with TFE teflon tube (10 m length and 0.5 mm diameter) kept under 75 °C and monitoring with fluorescence detector (330 nm emission, 390 nm excitation). For each group of saxitoxins analogs (GTXs and neosaxitoxin/saxitoxin/dc-saxitoxin) were used specific mobile phases. The mobile phases consisted of 2 mM 1-heptanesulfonic acid in 10 mM ammonium phosphate buffer (pH 7.1) for gonyautoxin (GTXs) group and 2 mM 1-heptanesulfonic acid in 30 mM ammonium phosphate buffer (pH 7.1) containing 2.5% of acetonitrile for saxitoxin (STXs) group, both were pumped at a flow rate of 0.8 mL min^{-1}. In all cases the eluate from the column (250 mm × 4.6 mm i.d., Shim-pack CLC-C8(M), 5 μm) was continuously oxidized with 7.0 mM periodic acid in 10.0 mM potassium phosphate buffer (pH 9.0) during passing through Teflon tubing and the reaction was stopped using acetic acid (0.5 M), both pumped at 0.4 mL min^{-1}. The saxitoxins analogs identification occurred by comparison with the retention times of the standards and, always necessary, samples were spiked with standards. Also, in order to avoid false identification chromatographic analysis was repeated, but the oxidizing solution and acetic acid were replaced by ultrapure water and the behavior and retention time of the analogs peaks were also compared to the standards (Lagos et al., 1999). The standards used in the saxitoxins analyses were neosaxitoxin, saxitoxin, dc-saxitoxin and GTX1/4, dc-GTX2/3, GTX2/3 (NRC Halifax, Canada). The C-toxins were not identified in this study.

3. Results

3.1. Occurrence and morphological characterization

Benthic cyanobacteria were present at nine of the ten sampling sites. In the Mundaú River basin, six of the seven sites had one or more species of cyanobacteria, and in the EPA of Araripe all sites had one or more cyanobacterial species (Table 1).

In total, 45 strains were isolated, 24 from the Mundaú River basin and 21 from EPA of Araripe. Eight species were identified, seven of which belong to Oscillatoriales order. Three pertain to the family Phormidiaceae (*Phormidium breve* (Kützing ex Gomont) Anagnostidis et Komárek 1988, *Phormidium uncinatum* Gomont ex Gomont 1892, and *Microcoleus* sp. Desmaziéres ex Gomont, 1892) and four Pseudanabaenaceae (*Pseudanabaena catenata* Lauterborn

1915, *Geitlerinema amphibium* (Agarh ex Gomont) Anagnostidis 1989, *Geitlerinema splendidum* (Greville ex Gomont) Anagnostidis 1989, and *Geitlerinema lemmermannii* (Woloszynska) Anagnostidis 1989). Strains of *G. amphibium* showed intense oscillating movement, whereas the strains of *G. splendidum*, *P. breve* and *P. uncinatum* showed sliding movement. Only one species of the order Nostocales, family Nostocaceae (*Cylindrospermum stagnale* (Kützing) ex Bornet et Flahault 1888) was identified (Fig. 1). The main morphological features of the isolated species are described in Table 2.

3.2. Molecular analysis

A region of the 16S rDNA (550 bp) was amplified for 38 strains. The sequences produced were compared with sequences deposited in GenBank NCBI by BlastN tool, and the closest sequences were retrieved to the phylogenetic analyses.

In the 16S rDNA tree (Fig. 2), *Geitlerinema* strains formed distinct lineages, termed lineages I and III, as described by Perkerson et al. (2010), and lineage *Geitlerinema* (L.G.), following Kirkwood et al. (2008). The lineage I (L.I) grouped the strains *Geitlerinema splendidum* BC 125, 126 and 127 with *G. splendidum* 0ES34S4 and *Geitlerinema* sp. 0SO37S2 with 56%, 56% and 0.99 support values for NJ, ML and BI, respectively. The similarity of DNA sequences among the members of L.I varied from 91.3 to 100%.

The lineage III grouped the strains of *Geitlerinema amphibium* with five reference strains of *Limnothrix* (CHAB709, CHAB759, PMC272.06, CENA111 and CENA110) and a clade of *Geitlerinema* (0ES36S4 and 1ES37S1) with 100%, 99% and 0.92 support values. The similarity among the strains of the clade was from 97.2 to 100%.

Strains morphologically identified as *Geitlerinema lermmermannii* (BC 131, 132, 133, 134, 135, 136 and 137) grouped with the species *Geitlerinema carotinosum* GSP167-2 and *Geitlerinema amphibium* GSP1373 with 86%, 69% and 0.93 support (NJ, ML and BI) forming the clade L.G. In this group, the similarity among the sequences varied from 89.3 to 100%.

The strains BC 122, 123 and 124, morphologically identified as *Pseudanabaena catenata*, clustered with *Planktolyngbya limnetica* PMC271.06 (100% for NJ, 98% for ML and 1.0 for BI). Strains of *Phormidium* (BC 105, 106, 107, 108 and 110) formed a very well supported clade with *Phormidium autumnale* (CYN120 and CYN122) and *Phormidium cf. uncinatum* (CYN105 and CYN111). Strains BC 102, 104, 138, 139 and 140, identified as *Microcoleus* sp., formed a supported separate group.

The order Nostocales formed a separate clade. Strains BC 144, 145 and 146, identified according to their morphology as *Cylindrospermum stagnale*, clustered with high support values (98% for NJ, 99% for ML and 1.0 for BI) to reference strains of *Cylindrospermum* sp. (PMC185.03, 186.03 and 238.04).

An approximately 628 bp region of the *sxtA* gene, was amplified in ten strains (BC 105, BC 115, BC 131, BC 133, BC 135, BC 136, BC 137, BC 144, BC 145 and BC 146) (Table 3). The amplicons from strains BC 144, BC 145, BC 146 and ITEP-A1 were sequenced and showed high identity (99%, 95%, 98% and 95%, respectively) with the *sxtA* from *Cylindrospermopsis raciborskii* T3 (EU629178 .1). In the case of the *mycE* gene, an approximately 472 bp region was amplified in four strains (BC 124, BC 144, BC 145 and BC 146). The amplicons from these strains were sequenced and showed high identity (99%, 99% and 98%, respectively) with the *mycE* gene from *Microcystis aeruginosa* FCY-28 (JQ290095.1).

3.3. Analysis of toxins by high performance liquid chromatography (HPLC)

Analyses of high performance liquid chromatography (HPLC) identified the presence of saxitoxins in five strains (Table 3). The variants neosaxitoxin (neoSTX), decarbamoylsaxitoxin (dc-STX) and saxitoxin (STX) were identified only in the strain BC 144 (*Cylindrospermum stagnale*) (Fig. 3A and Table 3), with concentrations of 0.49, 0.46, and 0.46 ng mg^{-1} dry weight, respectively. Whereas the gonyautoxin 1 (GTX1) was identified in four strains, BC 144 (1.71 ng mg^{-1} dry weight; Fig. 3B) and BC 145 (0.59 ng mg^{-1} dry weight; *C. stagnale*), BC 105 (6.72 ng mg^{-1} dry weight; *Phormidium uncinatum*) and BC 131 (2.68 ng mg^{-1} dry weight; *Geitlerinema lemmermannii*) (Fig. 4B and C). The gonyautoxin 4 (GTX4) was observed only in the strain BC 115 (*Geitlerinema amphibium*; Fig. 4D), 0.62 ng mg^{-1} dry weight. The retention time of some peaks have coincided with dc-GTX3 standard. However, in the analysis performed with ultrapure water instead oxidant and acetic acid solutions, the behavior of the peaks were different from that the standard. No microcystins were detected by HPLC in tested strains.

Fig. 1. Pictures of the isolated strains from rivers and reservoir at Mundaú, Pernambuco and EPA of Araripe, Ceará. (A) BC 124 (*Pseudanabaena catenata*); (B) BC 102 (*Microcoleus* sp.); (C) BC 106 (*Phormidium uncinatum*); (D) BC 108 (*P. breve*); (E) BC 126 (*Geitlerinema splendidum*); (F) BC 134 (*G. lemmermannii*); (G) BC 141 (*G. amphibium*); (H) BC 144 (*Cylindrospermum stagnale*). Scale bar = 10 μm.

Table 2
Main characteristics of the isolated strains from rivers and reservoir at Mundaú, Pernambuco and EPA of Araripe, Ceará. [a]Only vegetative cells otherwise indicated. H = heterocytus; () = average.

Isolates	Cell organization	Trichomes/cell morphology	Cell size (μm)[a]		
			Length	Width	Length/width
G. amphibium					
BC 111; BC 112; BC 113; BC 114; BC 115; BC 116; BC 117; BC 118; BC 119; BC 120; BC 121; BC 128; BC 129; BC 130; BC 141; BC 142; BC 143	Cells forming trichomes arranged in mats with intense oscillating movement	Straight or slightly waved trichomes, not constricted, not attenuated; apical cell cylindrical with rounded poles; cylindrical cells; without aerotopes	2.70–7.50 (5.01)	1.30–2.40 (1.85)	1.40–4.72 (2.73)
G. splendidum					
BC 125; BC 126; BC 127	Cells forming trichomes arranged in mats with sliding movement	Straight or slightly waved trichomes, generally curved at the apex, attenuated, not constricted; attenuated apical cells, with rounded poles; without aerotopes	3.98–6.98 (5.6)	2.56–3.34 (2.92)	1.37–2.44 (1.94)
G. lemmermannii					
BC 131; BC 132; BC 133; BC 134; BC 135; BC 136; BC 137	Cells forming trichomes arranged in mats with oscillating movement	Straight or slightly waved trichomes, generally curved at the apex, attenuated, not constricted; attenuated apical cells, with acuminated poles; without aerotopes	3.10–6.51 (4.86)	1.90–2.61 (2.22)	1.56–2.90 (2.30)
P. catenata					
BC 122; BC 123; BC 124	Cells forming matted trichomes. Attached to the glass (biofilm)	Slightly waved trichomes, constricted, not attenuated; cylindrical apical cell, with rounded poles; without aerotopes	1.57–3.37 (2.66)	1.56–2.02 (1.79)	0.87– 2.10 (1.48)
Phormidium uncinatum					
BC 105; BC 106; BC 107	Cells forming matted trichomes, attached to the glass (biofilm). Membranous aspect. Sliding movement	Straight or curved trichomes, usually curved at the apex, not attenuated, not constricted; attenuated apical cell, calyptra ± rounded, without aerotopes	1.54–4.02 (2.65)	5.18–6.83 (6.43)	0.26–0.60 (0.40)
Phormidium breve					
BC 108; BC 109; BC 110	Cells forming matted trichomes, attached to the glass (biofilm). Membranous aspect. Sliding movement	Generally straight trichomes, slightly attenuated, not constricted; rounded conical apical cell with granules at the cross walls, without calyptras and aerotopes	2.37–3.91 (3.00)	3.97–5.63 (4.89)	0.43–0.98 (0.62)
Microcoleus sp.					
BC 102; BC 103; BC 104; BC 138; BC 139; BC 140	Cells forming matted trichomes	Cylindrical and straight trichomes. Isodiametric cells, usually conical apical cells	5.34–8.84 (6.97)	3.05–4.04 (3.48)	1.51–2.51 (2.00)
Cylindrospermum stagnale					
BC 144; BC 145: BC 146	Cells forming densely matted trichomes, attached to the glass (biofilm) with mucilaginous aspect	Straight trichomes, constricted, not attenuated; subcylindrical cells; elongated or oval heterocytes, oblong or subcylindrical akinetes	3.8–5.47 (4.81) H: 6.3–8.2 (7.3)	3.06–4.36 (3.76) H: 4.6–5.4 (5.05)	0.96–1.78 (1.27)

4. Discussion

4.1. Morphology and phylogeny

Isolates of *Geitlerinema amphibium*, *Geitlerinema splendidum* and *Geitlerinema lemmermannii*, whose identifications were supported by morphological characters, showed cell dimensions, apical cell and motility consistent with Komárek and Anagnostidis (2005). However, phylogenetic analyses based on 16S rDNA sequences showed that these species actually form distinct lineages, suggesting that the genus behave as paraphyletic and polyphyletic, as already observed in previous studies (Willame et al., 2006; Bittencourt-Oliveira et al., 2009; Perkerson et al., 2010; Hašler et al., 2012).

Analyzing the phylogeny of the genera *Geitlerinema* and *Limnothrix*, Perkerson et al. (2010) divided them into four distinct lineages: lineage I, II, III and IV. The lineage I was characterized as lineage the *Geitlerinema sensu* Anagnostidis (1989) and lineage II was represented only by species of *Limnothrix*. The lineage III presented the characteristics of both genus, *Geitlerinema* and *Limnothrix*, however there are no morphological, ecological or biochemistry autapomorphies to correspond to the highly supported cluster recovered *via* 16S rDNA sequence data. Finally, the lineage IV clustered only the species of *Geitlerinema* from

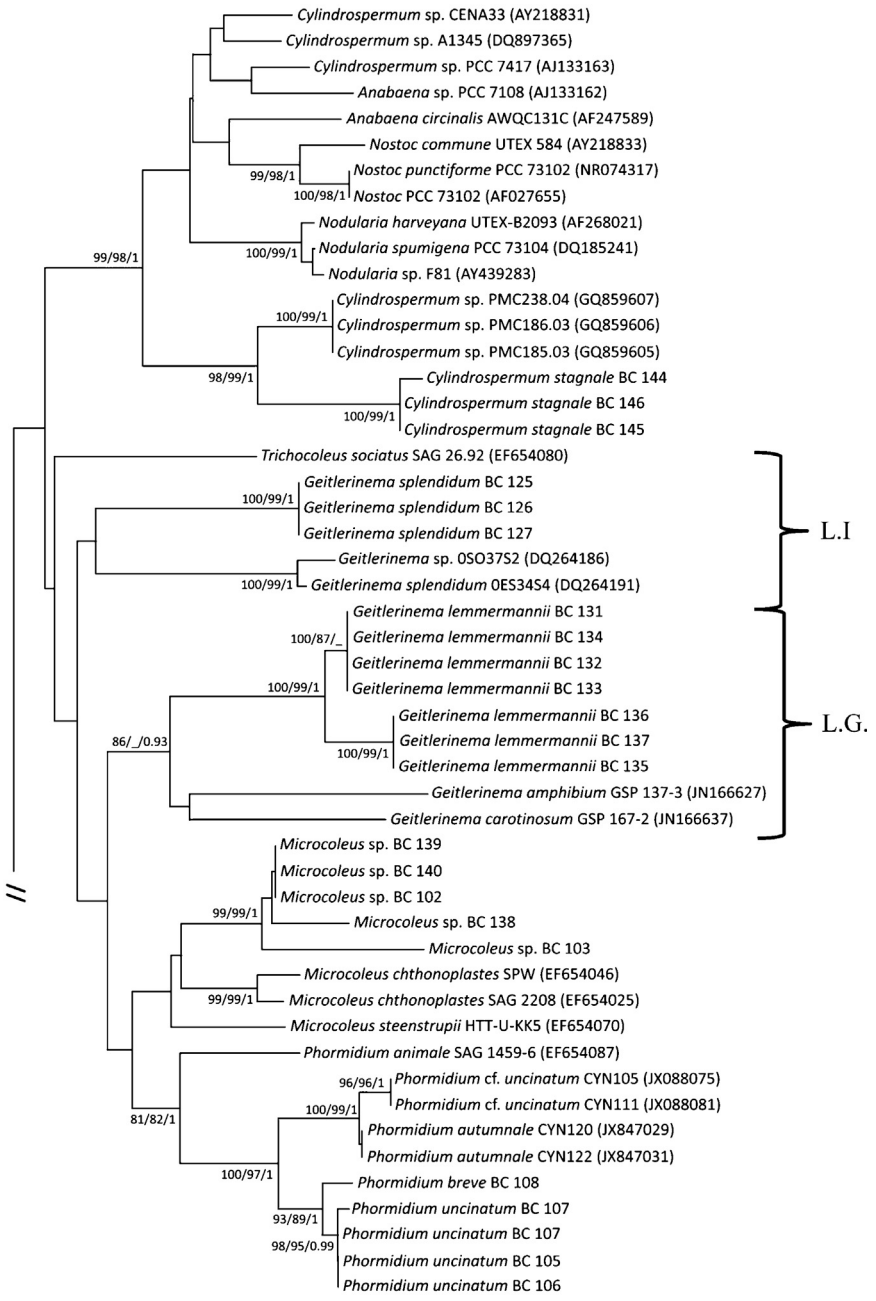

Fig. 2. Phylogenetic tree based on partial sequences of the 16S rDNA (550 bp). The tree was constructed using the methods of Neighbor-Joining (NJ), Maximum Likelihood (ML) and Bayesian Inference (BI). Bootstrap values >50% are indicated on the nodes. L. = lineage; *G.* = *Geitlerinema*.

marine habitats. The lineages I and III were also found in this work, along with a third lineage that was not observed in the work of Perkerson et al. (2010), but recognized by Kirkwood et al. (2008), who termed it as *Geitlerinema* lineage. The results of the phylogenetic analysis and the similarity of the sequences suggest the existence of more than one generic entity, reinforcing the concept about the paraphyly and polyphyly of *Geitlerinema* and its doubtful relationship with *Limnothrix*. Nevertheless, it is necessary to increase the number of sequences and improve the support level of the groups to present a more robust conclusion.

The strains BC 122, 123 and 124 were identified as *Pseudanabaena catenata* and showed morphological features similar to the populations described by Sant'Anna et al. (2007), although populations of Mundaú River have lower values for the cell length/width ratio. Such changes can be associated with the culture conditions, due to variations in the availability of nutrients or light (Cermeno et al., 2006). These strains have formed their own clade in the 16S rDNA tree and grouped with *Planktolyngbya limnetica* PMC271.06, which, according to Thomazeau et al. (2010), is sister of *Pseudanabaena*.

The strains BC 108, 109 and 110 were identified as *Phormidium breve*, whereas BC 105, 106 and 107 showed morphological features of *Phormidium uncinatum*, except for the apex curvature which is less extended and gradual, what might be associated with culture conditions. The highly supported clade formed by different *Phormidium* species with *Phormidium autumnale* strains (Fig. 2) was recently confirmed by morphological and ultrastructure analyses (Comte et al., 2007; Palinska and Marquardt, 2008; Wood et al., 2012).

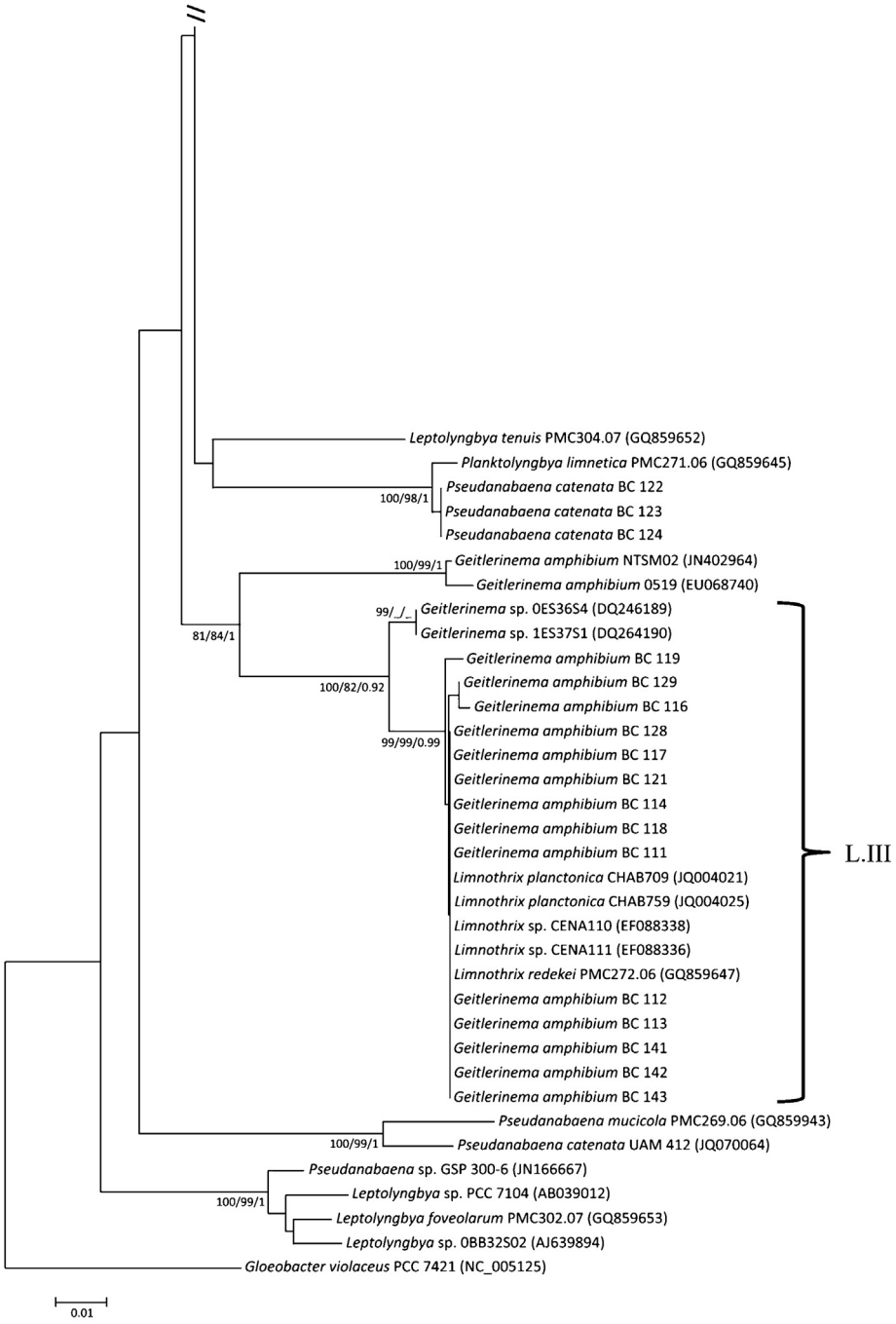

Fig. 2. (*Continued*).

Strunecký et al. (2013) consider that *P. autumnale* strains that have a specific 11 nucleotide insert actually pertain to *Microcoleus vaginatus* (Vaucher) Gomont. The relationship of the other strains in the cluster with genus *Microcoleus* has to be further evaluated.

Hašler et al. (2012), in a phylogeny study with species of *Geitlerinema*, *Phormidium* and *Microcoleus*, observed that such genera behave polyphyletic, and the results of this work corroborate these findings, and also suggest the taxonomic revision of these groups.

Despite having found only two akinetes in natural population, the strains BC 144, 145 and 146 were identified as *Cylindrospermum stagnale*. This genus is considered an indicator of unpolluted environments (Komárek, 2003), confirming the preservation of the sampling site from which they were isolated, source of the Mundaú River. Three strains formed an isolated clade near the species of the order Nostocales in phylogenetic analysis, grouping with strains of *Cylindrospermum* sp. (PMC185.03, 186.03 and 238.04) with 98%, 99% and 1.0 of support (NJ, Ml and BI) (Fig. 2). Sant'anna et al. (2006) draw attention to the fact that the genus is not well known.

4.2. Cyanotoxins

The *mcyE* gene, one of those involved in the synthesis of microcystins, amplified in four strains (Table 3), but HPLC analysis did not identify the presence of microcystin in any of the strains analyzed. Several studies report the same situation, in which part

Table 3
Detection of genes involved in the microcystins (*mcyE*) and saxitoxins (*sxtA*) production, and HPLC analysis of the isolated strains in the present study. – = negative, + = positive, NT = not tested. *Ph.* = *Phormidium*; *P.* = *Pseudanabaena*.

Strains code	Location	Taxa	Toxins			
			Genes		HPLC	
			mcyE	*sxtA*	Microcystins	Saxitoxins
BC 102	Mundaú	*Microcoleus* sp.	–	–	–	NT
BC 103	Mundaú	*Microcoleus* sp.	–	–	–	–
BC 104	Mundaú	*Microcoleus* sp.	–	–	–	NT
BC 105	Mundaú	*Ph. uncinatum*	–	+	NT	GTX1
BC 106	Mundaú	*Ph. uncinatum*	–	–	–	NT
BC 107	Mundaú	*Ph. uncinatum*	–	–	NT	NT
BC 108	EPA Araripe	*Ph. breve*	–	–	–	–
BC 109	EPA Araripe	*Ph. breve*	–	–	NT	–
BC 110	EPA Araripe	*Ph. breve*	–	–	NT	NT
BC 111	Mundaú	*G. amphibium*	–	–	NT	–
BC 112	Mundaú	*G. amphibium*	–	–	NT	NT
BC 113	Mundaú	*G. amphibium*	–	–	–	NT
BC 114	EPA Araripe	*G. amphibium*	–	–	–	–
BC 115	EPA Araripe	*G. amphibium*	–	+	NT	GTX4
BC 116	EPA Araripe	*G. amphibium*	–	–	NT	NT
BC 117	EPA Araripe	*G. amphibium*	–	–	–	NT
BC 118	EPA Araripe	*G. amphibium*	–	–	NT	–
BC 119	EPA Araripe	*G. amphibium*	–	–	NT	NT
BC 120	EPA Araripe	*G. amphibium*	–	–	NT	NT
BC 121	EPA Araripe	*G. amphibium*	–	–	NT	NT
BC 122	Mundaú	*P. catenata*	–	–	–	–
BC 123	Mundaú	*P. catenata*	–	–	–	NT
BC 124	Mundaú	*P. catenata*	+	–	–	NT
BC 125	EPA Araripe	*G. splendidum*	–	–	NT	–
BC 126	EPA Araripe	*G. splendidum*	–	–	NT	NT
BC 127	EPA Araripe	*G. splendidum*	–	–	NT	NT
BC 128	Mundaú	*G. amphibium*	–	–	NT	NT
BC 129	Mundaú	*G. amphibium*	–	–	NT	–
BC 130	Mundaú	*G. amphibium*	–	–	–	NT
BC 131	EPA Araripe	*G. lemmermannii*	–	+	NT	GTX1
BC 132	EPA Araripe	*G. lemmermannii*	–	–	NT	–
BC 133	EPA Araripe	*G. lemmermannii*	–	+	NT	–
BC 134	EPA Araripe	*G. lemmermannii*	–	–	–	NT
BC 135	EPA Araripe	*G. lemmermannii*	–	+	NT	–
BC 136	EPA Araripe	*G. lemmermannii*	–	+	NT	–
BC 137	EPA Araripe	*G. lemmermannii*	–	+	NT	–
BC 138	Mundaú	*Microcoleus* sp.	–	–	NT	NT
BC 139	Mundaú	*Microcoleus* sp.	–	–	NT	NT
BC 140	Mundaú	*Microcoleus* sp.	–	–	–	NT
BC 141	Mundaú	*G. amphibium*	–	–	–	NT
BC 142	Mundaú	*G. amphibium*	–	–	–	–
BC 143	Mundaú	*G. amphibium*	–	–	–	NT
BC 144	Mundaú	*C. stagnale*	+	+	–	neoSTX/dc-STX/STX/GTX1
BC 145	Mundaú	*C. stagnale*	+	+	–	GTX1
BC 146	Mundaú	*C. stagnale*	+	+	–	–

of the *mcy* cluster is found, but the presence of the toxin is not confirmed by analytical methods (Mankiewicz-Boczek et al., 2006; Saker et al., 2007; Bittencourt-oliveira et al., 2010). This fact could also be associated with a microcystin concentration below the detection limit (Bittencourt-oliveira et al., 2010), but the extractions were made from lyophilized material of a high biomass. Bittencourt-oliveira and Molica (2003), claim that the genotypes responsible for producing toxins are not always expressed, which might have occurred to the strains BC 124, BC 144, BC 145 and BC 146.

The results of this study presented the first report of saxitoxins production by *Geitlerinema* and *Cylindrospermum* species, beyond the first description in Brazil for the production of this metabolite by *Phormidium* (Figs. 3 and 4 and Table 3). So far, saxitoxins have only been identified in Brazil in strains of *Cylindrospermopsis raciborskii* (Lagos et al., 1999; Molica et al., 2002). There are several reports in the literature of the production of saxitoxins by species of cyanobacteria (Humpage et al., 1994, 2010; Yin et al., 1997; Lagos et al., 1999; Pomati et al., 2000; Molica et al., 2005; Rapala et al., 2005; Ballot et al., 2010). However, most reports are associated with planktonic cyanobacteria, and it is rare studies of benthic species, although some of these species have already been identified as toxins producers (Onodera et al., 1997; Teneva et al., 2005; Smith et al., 2011; Quiblier et al., 2013; Harland et al., 2014).

The gene cluster involved in the cyanobacteria saxitoxins production was identified by Kellmann et al. (2008). The *sxtA* gene amplification occurred in 10 of the 45 isolated strains (Table 3). In contrast, the presence of saxitoxins variants was confirmed by HPLC in only five strains. Ballot et al. (2010) also reported that some strains were positive for the presence of the *sxtA* gene, but they did not find saxitoxins variants by HPLC/mass spectrometry. Moustafa et al. (2009) hypothesized that several strains of *Anabaena circinalis* saxitoxins producers lose the ability to produce the toxin over time, leading to coexist in the environment of producing and non-producing strains of saxitoxins. This mechanism implies that the positive *sxtA* strains may have lost part of the cluster involved in the production of saxitoxins (Ballot et al., 2010).

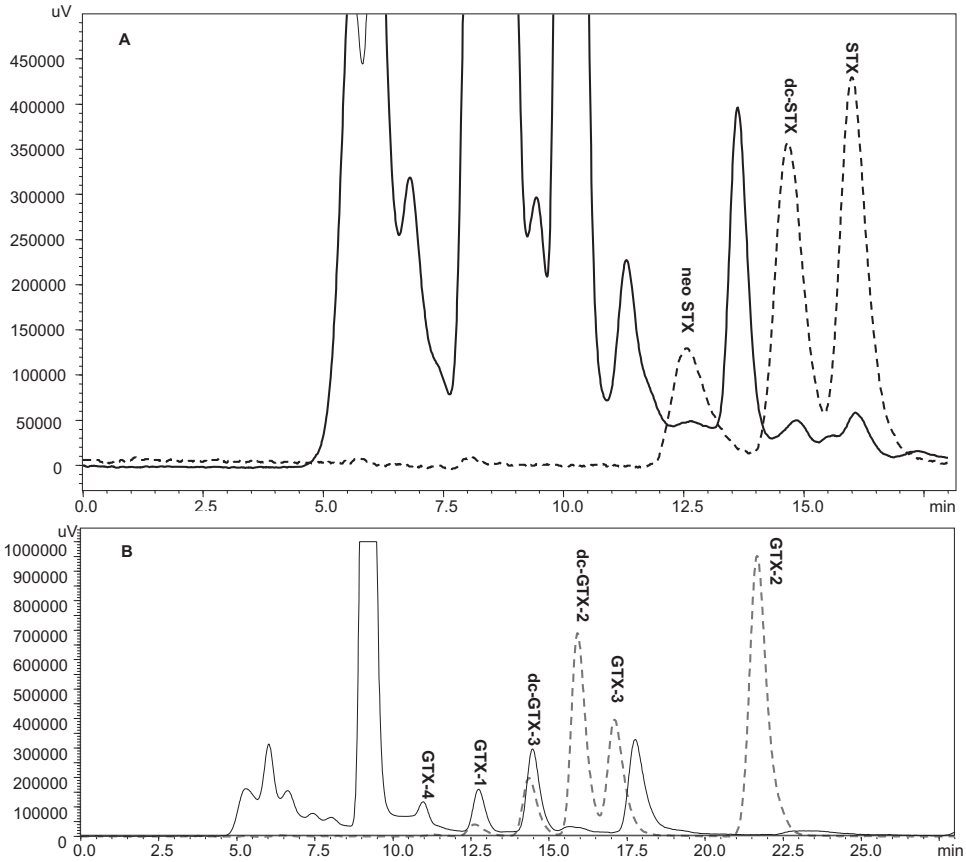

Fig. 3. Saxitoxins and gonyautoxins chromatogram analysis by high performance liquid chromatography (HPLC). (A) The presence of neosaxitoxin (neoSTX), decarbamoylsaxitoxin (dc-STX) and saxitoxin (STX); (B) gonyautoxin 1 (GTX1) in the strain BC 144 (*Cylindrospermum stagnale*). Dashed line = saxitoxins standard; black line = sample.

The strains BC 105 (*Phormidium uncinatum*), BC 115 (*Geitlerinema amphibium*), BC 131 (*Geitlerinema lemmermanni*) and BC 145 (*Cylindrospermum stagnale*) presented only one saxitoxin variant, while BC 144 (*C. stagnale*) presented four (Table 3). Previous studies have identified more than one saxitoxins variants in all strains analyzed (Humpage et al., 1994; Negri and Jones, 1995; Carmichael et al., 1997; Onodera et al., 1997; Pomati et al., 2000; Molica et al., 2002; Pomati et al., 2004; Ballot et al., 2010), except for the work of Smith et al. (2011), which identified only one saxitoxin (STX) in the species *Scytonema cf. crispum*. In contrast, it has been identified over 20 saxitoxins analogs in cyanobacteria and dinoflagellates (Oshima, 1995; Chorus and Bartrum, 1999) and we only used six standards in the chromatographic analysis in the present study. Therefore, strains that were positive for the *sxtA* gene and negative for HPLC, may be producing other analogs, different from the standards used, which could not be identified or do not have part of the cluster involved in the production of these toxins.

Prior to this study, saxitoxin production by species of *Phormidum* had not been confirmed. Teneva et al. (2005) studied five species of *Phormidum* and analyzed the presence of saxitoxins using an ELISA assay, however, the amounts found responded very close to the detection limit. Previous studies involving *Phormidium* have identified the presence of several analogs of the neurotoxin anatoxin-a (anatoxin-a, homoanatoxin-a, dihydro-anatoxin-a and dihydro-homoanatoxin-a) and such toxins are related to many animals deaths throughout the world (Gugger et al., 2005; Wood et al., 2007; Heath et al., 2010; Puschner et al., 2010; Wood and Young, 2011, 2012; Faassen et al., 2012), and microcystins (Gugger et al., 2005; Wood et al., 2006; Jungblut et al., 2006; Mohamed et al., 2006). Quiblier et al. (2013) state that the fact that microcystins and homo/anatoxin-a are the most commonly found toxins among the benthic cyanobacteria may be related to sampling biases in toxin analysis.

Sant'Anna et al. (2008) found the toxicity of *Geitlerinema amphibium* in mice bioassays, but the toxin was not identified. Other studies have also reported the toxicity of *Geitlerinema* species with mice bioassays, however, the symptoms of the animals were not similar to those caused by known toxins (Dogo et al., 2011; Bernard et al., 2011; Rangel et al., 2012). Nevertheless, in the present study, the production of GTX4 and GTX1 was confirmed by HPLC in BC 115 (*G. amphibium*) and BC 131 (*Geitlerinema lermmermannii*), respectively (Fig. 4), and both also have the gene involved in the saxitoxins production (Table 3), and this is the first report of saxitoxins production for these species.

The presence of *mcyE* and *stxA* genes was confirmed in BC 144 and BC 145 strains (*Cylindrospermum stagnale*); however, only saxitoxins analogs were identified by HPLC. The strain BC 144 showed the highest amount of saxitoxins variants, a total of four (STX, neoSTX, dcSTX, and GTX1) (Table 3), while in BC 145 only GTX1 was identified. The production of toxins by *Cylindrospermum* was only reported by Sivonen et al. (1989), who identified the presence of anatoxin-a. Thus, this work recorded for the first time saxitoxins production by *Cylindrospermum*.

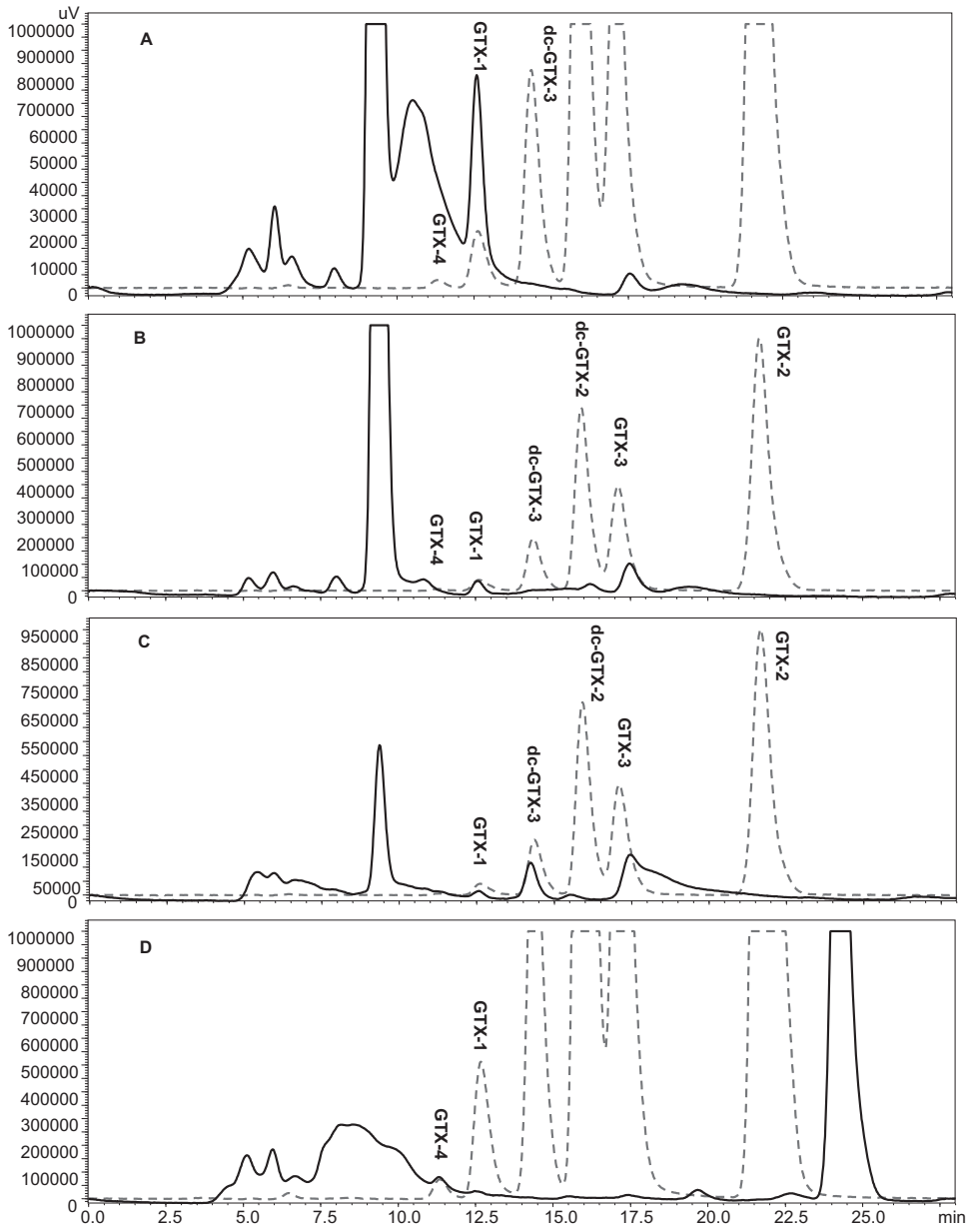

Fig. 4. Gonyautoxins chromatogram analysis by high performance liquid chromatography (HPLC). (A–C) The presence of GTX1 in the strains BC 105 (*Phormidium uncinatum*), BC 131 (*Geitlerinema lemmermannii*), BC 145 (*C. stagnale*), respectively; (D) the presence of GTX4 in the strain BC 115 (*G. amphibium*). Dashed line = saxitoxins standard; black line = sample.

5. Conclusions

Recent works (Komárek & Anagnostidis, 2005; Comte et al., 2007; Palinska and Marquardt, 2008; Heath et al., 2010; Wood et al., 2012; Hašler et al., 2012; Strunecký et al., 2013) have shown the need for taxonomy revision of some Oscillatorialean species. The present study corroborates with this proposal, since the *Geitlerinema*, *Pseudanabaena* and *Microcoleus* species were found to be polyphyletic. As an example of such necessity, this research showed that *Geitlerinema* strains correspond to at least three lineages, and that possibly correspond to three distinct species to be subsequently reclassified.

Moreover, it is necessary to define type strains based on phylogenetic studies for species of cyanobacteria, and making available sequences of these strains in GenBank, NCBI, so you can move forward in taxonomic knowledge of these organisms. It is suggested that while the type strains of cyanobacteria species are not defined, due to nomenclature complications and scientific names priority, it has to be established phylogenetic lineages and the sequences of these isolates deposited in GenBank, such as some recent publications.

In blooms events of planktonic cyanobacteria species, the cyanotoxins pose a risk to the environment and to human and animal health. And, in general, little attention has being given to benthic cyanobacteria species in relation to their ability to produce the same toxins. The results of this study, as well as other authors (Heath et al., 2010; Wood and Young, 2011), demonstrate the need to intensify the studies on benthic cyanobacteria in order to assess what are the real risks of toxin contribution by benthic species in rivers and reservoirs.

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