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PRIMARY RESEARCH PAPER

Cyanobacterial community and microcystin production in a recreational reservoir with constant *Microcystis* blooms

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Abstract Cyanobacterial blooms are increasing worldwide favored by eutrophic conditions of aquatic ecosystems associated with climatic perturbations. Generally, inland lentic systems are more susceptible to the development of harmful blooms. In the Salto Grande Reservoir (Brazil), *Microcystis* is the most common bloom-forming genus along with a wide range of co-occurring and less-known cyanobacteria taxa. The cyanobacterial community and microcystin production were studied in Salto Grande Reservoir applying biological, toxicological, and molecular approaches. Thirteen cyanobacterial strains belonging to eight genera were isolated and taxonomically investigated based on morphological traits and phylogenetic analyses of their 16S rRNA gene sequence. The morphotypes identified were, in general, in agreement with their phylogeny. The presence of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) was investigated using PCR gene amplification, which were detected in 76.9 and 84.4% of the strains, respectively. Positive enzyme-linked immunosorbent assays (ELISA) reactions for microcystins were obtained only from the strain *Leptolyngbya* sp. CENA129. ELISA and high-performance liquid chromatography (HPLC) analyses of the environmental water samples showed the highest microcystin concentration during the dry and rainy seasons, respectively. This study highlights that microcystin production must be suspected in benthic forms as well as in genera that are morphologically similar but belonging to other evolutionary lineages.

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Introduction

Intensive human occupation induces deterioration of water quality due to the discharge of untreated organic/inorganic loads and sanitary sewers from domestic/industrial/agricultural effluents, resulting in nutrient-enriched waters (Sala & Mujeriego, 2001). The eutrophication of water bodies is a worldwide problem (Palus et al., 2007) and favors cyanobacterial blooms, which in turn cause hypoxia, fish kills, increase the turbidity (Waajen et al., 2014), and sometimes the toxin releasing into the waters (Paerl et al., 2001). In addition to the nutrient availability, CO_2 accessibility, high temperatures, low luminosity, high pH, low N:P ratio, buoyancy regulation, bottom-up influence, and physical changes are other factors involved in the formation of cyanobacteria blooms (Reynolds & Walsby, 1975; Smith, 1983; Niklisch & Kohl, 1989; Shapiro, 1990; Walsby et al., 1997; Caraco & Miller, 1998; Bouvy et al., 1999; Huszar et al., 2000; Briand et al., 2002; Figueredo & Giani, 2009; Soares et al., 2009). Conjunction of these factors seems more likely to induce cyanobacterial blooms than a single environmental aspect (Briand et al., 2002; Marinho & Huszar, 2002; Figueredo & Giani, 2009; Soares et al., 2009; Dantas et al., 2011). In Brazil, the eutrophic condition of Salto Grande Reservoir associated with an optimum temperature and long-term water residence time has caused cyanobacterial blooms over the years (Zanata & Espíndola, 2002). This reservoir is located in the eastern-central area of the São Paulo State, a region with high industrial and agricultural activities and elevated population density (Espíndola et al., 2004). Economically, it is considered the second and third richest region in the São Paulo State and in Brazil, respectively, with approximately 4 million people living in 44 cities (Espíndola et al., 2004).

The cyanobacteria genera *Microcystis*, *Anabaena* (including *Dolichospermum*), *Aphanizomenon*, *Cylindrospermopsis*, *Raphidiopsis*, and *Planktothrix* are documented to occur in freshwater reservoirs in Brazil (Sant'Anna & Azevedo, 2000). Among them, the genus *Microcystis* is widely reported as a bloom-forming cyanobacteria in Salto Grande Reservoir and a microcystin producer. Microcystins are cyclic heptapeptides synthesized non-ribosomally by multifunctional enzymes that include the polypeptide synthetase (PS) and polyketide synthase (PKS) modules (Nishizawa et al., 1999; Tillett et al., 2000). This cyanotoxin is associated with liver cancer leading to animal poisoning and death in humans (Falconer & Humpage, 1996; Carmichael, 1997; Kuiper-Goodman et al., 1999; Sivonen & Jones, 1999; Duy et al., 2000; Carmichael, 2001; Azevedo et al., 2002). After an incident involving the death of 76 people caused by cyanotoxin in Brazil, a specific regulation was implemented regarding the cyanobacteria and cyanotoxin content of water for public supply. The most recent regulation establishes a limit of 1 $\mu g\ l^{-1}$ microcystin in the public water supply and a weekly monitoring when the cell densities are higher than 10^4 cells l^{-1} (Brasil, 2011).

Other cyanobacteria genera such as *Aphanocapsa*, *Nostoc*, *Fischerella*, *Planktothrix*, *Cylindrospermopsis*, *Geitlerinema,* and *Lyngbya* are also known to produce microcystins (Domingos et al., 1999; Sivonen & Jones, 1999; Fiore et al., 2009; Genuário et al., 2010). Therefore, studies of the wider cyanobacterial community are of particular interest. So, the main purpose of this study was to investigate the cyanobacterial community of a tropical and eutrophic water body and its connection with microcystin production. The hypothesis is that the neglected less-known cyanobacterial genera, concerning microcystin production, can be important source of this toxin in addition to the genus *Microcystis.* For this purpose, the specific objectives were: (1) to characterize the cyanobacterial community by microscopic inspection of environmental water samples, (2) to isolate cyanobacterial strains, followed by the examination of their phylogenetic relationship (using 16S rRNA gene sequences) and by the detection of the non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) genes, (3) to detect microcystin in the environmental water samples and in the cultured cyanobacteria by high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assays (ELISA). These goals will provide a better understanding of the cyanobacterial community and will test whether more effort is needed to investigate taxa not yet linked to toxin production in a eutrophic condition.

Materials and methods

Site description and sampling

Salto Grande Reservoir is located at Americana municipality, São Paulo State, Brazil and was built during 1940s to dam water for hydroelectric power generation. Currently, its water is being used to supplement the water flow of downstream rivers during the dry season, for agricultural irrigation and recreational activities, such as boating, swimming, and angling (Espíndola et al., 2004). Sampling was performed once a month during 11 months (from April 2005 to February 2006) covering the rainy (October–March) and dry (April–September) seasons (Espíndola et al., 2004). During the sampling period, the mean precipitation in Salto Grande region was 220 and 50 mm for the rainy and dry seasons, respectively, and its flow rates were 31.0 ± 17.1 and 15.7 ± 8.0 (m^3 s^{-1}) for the rainy and dry seasons, respectively. Subsurface water samples (4 l) were collected at the edge of Praia dos Namorados (PN) (22°42′20″S, 47°16′01″W) and Iate Clube (IC) (22°43′13″S; 47°16′22″W) in Salto Grande Reservoir, following the São Paulo State Environmental Sanitation Technology Company (CETESB) technical recommendations (1987). The Secchi disk transparency, pH, total nitrogen (TN), total phosphorus (TP), and water and air temperatures were measured (American Public Health Association, APHA, 1998). One milliliter of water samples was used for cyanobacterial isolation, 100 ml was preserved in 4% formaldehyde (final concentration) for microscopic inspection, and 100 ml was fixed with Lugol's iodine solution for cell counting (Willén, 1976). The microcystin analyses were performed using 1 and 2 ml of water samples for ELISA and HPLC methods, respectively.

Chlorophyll-*a*, phytoplankton, and cyanobacterial densities

Chlorophyll-*a* was extracted using 80% ethanol (Nusch, 1980), followed by determination of absorbance at 665 and 750 nm wavelengths in spectrophotometer model Hach DR 2500 (Hach Company, Loveland, CO, EUA). Volume used for the extraction varied depending on the amount of phytoplankton biomass. Final concentration was calculated considering the total volume used. The phytoplankton and cyanobacterial cell densities were counted applying the Utermöhl counting technique (APHA, 1998) and an inverted light microscope (Leica, DM IRB, Westzlar, Germany). Colonies were disaggregated using KOH (0.1 M) at 90°C for 40 min to release the cells. Drops of Lugol's iodine solution were added to the samples to induce cell sedimentation. When necessary, transects or random fields of Utermöhl chambers were counted using Whipple disk. At least 400 cells were counted with a margin of error of 10 and 95% of confidence level.

Cyanobacterial isolation and morphological identification

One milliliter of sampled water was inoculated into a 50 ml test tube containing 9 ml of the liquid BG-11 medium (Stanier et al., 1971) that contained cycloheximide (70 mg l^{-1}) to inhibit eukaryotic cell growth (Rippka, 1988). After mixing, 10-fold serial dilutions (to 10^{-7}) were used to inoculate the test tubes containing the same medium. The tubes were incubated for 30 days at 25 ± 1°C under a 14:10 h light–dark cycle of 40 µmol photons m^{-2} s^{-1} provided by cool-white fluorescent lights. After cyanobacterial growth became visible in the liquid medium, 100 µl was spread onto the agarised BG-11 medium containing cycloheximide (70 mg l^{-1}). Repeated streaking onto fresh solid medium and microscopic observations were performed until a monospecific cyanobacterial culture was established. The morphological identification of isolated cyanobacterial strains was made using a Zeiss Axioskop 40 optical light microscope equipped with an AxioVisionLE 4.6 digital imaging system (Carl Zeiss, Jena, Germany) and the classification system devised by Komárek & Anagnostidis (1999, 2005), Komárek (2013) and Hoffmann et al. (2005).

Molecular analyses

DNA extraction, 16S rRNA amplification, and sequencing

The total genomic DNA was extracted from the cultured cyanobacterial cells using a modified cetyltrimethylammonium bromide-based (CTAB) extraction method adapted for cyanobacteria (Fiore et al., 2000). The PCR amplification and sequencing of

the 16S rRNA gene were performed as previously described (Fiore et al., 2007). The sequences obtained in this study and the related sequences retrieved from GenBank were aligned using CLUSTAL W, trimmed (16S rRNA gene matrix with a 1,445-bp length and with 888 informative sites). A total of 125 sequences were considered and used to infer the phylogeny based on the maximum likelihood (ML), neighbor joining (NJ), and Bayesian methods. Kimura two-parameter model with gamma distributed with invariant sites ($K2 + G + I$) was selected as the best fitting model to the sequence data using the model-testing function in MEGA version 5 (Tamura et al., 2011) based on a NJ tree. The $K2 + G + I$ model was used in the ML analysis while $K2$ with uniform rates was applied in the NJ since $G + I$ was not available in MEGA 5.0 for this method of reconstruction. The robustness of both of the phylogenetic trees was estimated by bootstrap analysis using 1,000 replications. General time-reversible evolutionary model with Gamma distribution and with an estimate of proportion of invariable sites (GTR + $G + I$) were selected as the fittest for the alignment by jModelTest 2.1.1 (Darriba et al., 2012). This model was applied in the Bayesian inference using MrBayes 3.2 (Ronquist & Huelsenbeck, 2003) executing six substitution types (nst = 6) combined with gamma model (invgamma). Two separate runs of four Markov chains executing 10 million generations, sampling every 100 generations were executed and 500 of sampled trees were discarded as burn-in. The tree was viewed in FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree). Given that ML, NJ, and Bayesian methods had nearly identical topologies, only ML tree is presented with the indication of bootstrap values (ML and NJ) and Bayesian (B) probabilities. The accession numbers for the new nucleotide sequences were published in the NCBI GenBank database under the accession numbers HQ380799 and KP835525–KP835536.

PCR amplification of NRPS and PKS genes

The cyanobacterial genomic DNA was used to amplify the aminoacyl-adenylation domain of NRPS and ketoacyl domain of PKS using degenerate primer sets MTF/MTR (Neilan et al., 1999) and KSF/KSR (Beyer et al., 1999), respectively. The PCR reactions and thermal cycling conditions were performed as previously described (Silva-Stenico et al., 2011).

Microcystin analyses

Enzyme-linked immunosorbent assays (ELISA)

One milliliter from cultured cyanobacteria strains and from environmental samples was used to extract microcystin by freezing (liquid nitrogen, −196°C) and thawing (warm bath, +45°C) method (Silva-Stenico et al., 2009). After toxin extraction, the samples were centrifuged for 15 min at 10,000×g. The supernatant (100 µl) was collected and analyzed by ELISA using microplate kits for microcystins (Beacon Analytical Systems, Inc., Portland, ME, USA), following the manufacturer's recommendations, with at least three replicates. The microcystin isoforms accessed by this method were LR, YR, and RR and its detection limit was 0.1 µg l^{-1}.

High-performance liquid chromatography–mass spectrometry (HPLC–MS)

The HPLC–MS analyses were performed to investigate the presence of microcystin (microcystin-LR isoform) in environmental water samples. The toxin extraction was performed as described above and purified using a pre-conditioned C_{18} SPE cartridge (Applied Separation, Allentown, PA, USA). The cartridge was washed with water (2 × 10 ml) and the toxins were eluted with 90% methanol. The aliquots were subjected to LC–MS analysis using Waters Equipment, model 2695 with UV detector model 2996 and Micromass ZQ spectrophotometer as described by Silva-Stenico et al. (2009). Pure microcystin-LR (Alexis Corporation, Lausen, Switzerland) was used to construct a standard curve to determine the microcystin concentration in environmental water sample.

Results

The abiotic and biotic variables from Salto Grande Reservoir

Physical–chemical parameters measured for both sampling sites are presented in Table 1.

Chlorophyll-*a* concentration was higher in dry than in rainy season, most likely due to the concentration of cells caused by the drought period. The mean values for dry and rainy seasons were 759.6 ± 1,369.8 and 179.4 ± 72.8 μg l^{-1} for PN and 1,034.4 ± 1,406.5 and 339.1 ± 217.0 μg l^{-1} for IC (Table 1), respectively.

The organism density indicated Cyanobacteria as the dominant group in phytoplankton community of Salto Grande Reservoir for both sampling sites almost throughout the year. At PN, Bacillariophyceae and Cryptophyceae were the main phyla in July and September 2005, respectively. At IC, Chlorophyceae dominated in May 2005, and Cryptophyceae was prevalent in June and September 2005.

The cyanobacteria cell counting revealed concentrations of 10^5 and 10^6 cells ml^{-1} for PN during the dry and rainy seasons, respectively, and concentration of 10^5 cells ml^{-1} in both seasons in IC (Table 1). The morphotypes of *Anabaena* sensu lato, *Chroococcus* spp., *Coelosphaerium* spp., *Geitlerinema* spp., *Jaaginema* spp., *Limnothrix* spp., *Merismopedia* spp., *Microcystis* spp., *Oscillatoria* spp., *Planktothrix* spp., *Pseudanabaena* spp., *Sphaerocavum* spp., *Planktolyngbya* spp., and *Radiocystis* spp. were observed in environmental samples.

Cyanobacterial isolation and morphological identification

Thirteen cyanobacterial strains were isolated from PN and IC (Table 2). Considering the morphological traits, these strains were identified as *Cyanobium* spp. (Synechococcales, Synechococcaceae), *Synechococcus* spp. (Synechococcales, Synechococcaceae), *Microcystis* spp. (Chroococcales, Microcystaceae), *Chroococcidiopsis* sp. (Chroococcales, Xenococcaceae), *Leptolyngbya* spp. (Pseudanabaenales, Pseudanabaenaceae), *Romeria victoriae* (Pseudanabaenales, Pseudanabaenaceae), *Lyngbya* sp. (Oscillatoriales, Oscillatoriaceae), and *Calothrix* sp. (Nostocales, Rivulariaceae), according to Hoffmann et al. (2005) (Fig. 1). The main morphological features of these strains are depicted in Table 3. Members of *Calothrix* spp., *Chroococcidiopsis* spp., *Cyanobium* spp., *Lyngbya* spp., *Leptolyngbya* spp., *Romeria* spp., and *Synechococcus* spp. were evidenced only by isolation method, while *Microcystis* spp. were revealed by direct microscopic inspection and cell isolation. It highlights the importance of using both techniques for better revealing the cyanobacterial community in this reservoir.

Table 1 Abiotic and biotic variables measured in Salto Grande Reservoir

Parameters	Praia dos Namorados (PN)		Iate Clube (IC)	
	Mean ± SD		Mean ± SD	
	Dry season	Rainy season	Dry season	Rainy season
Secchi transparency (m)	0.5 ± 0.2	0.6 ± 0.4	1.0 ± 0.4	0.7 ± 0.2
pH	7.6 ± 0.9	7.3 ± 0.3	7.6 ± 1.0	7.5 ± 0.5
Water temperature (°C)	23.3 ± 2.8	27.4 ± 2.7	22.9 ± 3.3	27.1 ± 1.6
Air temperature (°C)	23.8 ± 3.9	25.0 ± 2.6	22.0 ± 4.7	24.7 ± 2.5
Total phosphorus (TP) (mg L^{-1})	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
Total nitrogen (TN) (mg l^{-1})	1.1 ± 0.3	2.5 ± 2.2	2.3 ± 2.3	1.7 ± 1.0
TN:TP ratio	58.7 ± 58.8	69.0 ± 52.6	46.5 ± 23.9	64.8 ± 48.2
Cyanobacterial cells number (cells l^{-1})	804,661 ± 1,115,643.3	2,124,732 ± 4,276,887.1	515,683.6 ± 610,748.6	390,026.5 ± 436,456.4
Chlorophyll *a* (μg l^{-1})	759.6 ± 1,369.8	179.4 ± 72.8	1,034.4 ± 1,406.5	339.1 ± 217.0

The mean values and standard deviation were calculated considering the data collected from 6 and 5 months comprising the dry and rainy seasons, respectively

Mean mean values, *SD* standard deviation, *TN:TP ratio* mean values of the TN:TP ratio considering individual measurements

The 16S rRNA gene analyses and phylogeny

Thirteen novel 16S rRNA gene sequences were obtained from the isolated cyanobacterial strains. These sequences showed ≥96.1% identity against the best hit cyanobacterial sequences available in NCBI public database (Table 4). The lowest identities found were those from sequences of *Leptolyngbya* sp. CENA129 and *Lyngbya* sp. CENA128 against sequences of *Leptolyngbya* sp. CENA112 (EF088337) (96.1%) and *Nodosilinea* sp. CENA546 (KF246507) (96.7%), respectively (Table 4). *Nodosilinea* sp. CENA546 and *Leptolyngbya* sp. CENA112 sequences were generated from cyanobacterial strains isolated from Pantanal wetlands and from wastewater stabilization ponds in Brazil (Furtado et al., 2009; Andreote et al., 2014), respectively. The comparison among the 16S rRNA gene sequences of a subset of *Leptolyngbya* sensu lato and sensu stricto revealed identities lower than 95.6% when compared to the sequences of *Leptolyngbya* sp. CENA129 and *Leptolyngbya* sp. CENA131 (Table 5). The novel sequences were distributed in eight distinct clusters formed in the phylogenetic tree (Fig. 2). Members of orders Synechococcales, Chroococcales, Pseudanabaenales, and Oscillatoriales were dispersed within the tree, while representatives of the order Nostocales grouped together (99/99/1, ML, NJ, B). The clades formed in the 16S rRNA gene phylogenetic reconstruction showed congruence with the cyanobacterial identification based on the morphological traits, with the exception of *Lyngbya* sp. CENA128, *Cyanobium* sp. CENA118 and CENA122, *Synechococcus nidulans* CENA132, and *R. victoriae* CENA123.

Fig. 1 Cyanobacterial morphotypes isolated from Salto Grande Reservoir. **A** *Microcystis* sp. CENA120, **B** *Cyanobium* sp. CENA122, **C** *Calothrix* sp. CENA127, **D** *Chroococcidiopsis* sp. CENA124, **E** *Romeria victoriae* CENA123, **F** *Microcystis panniformis* CENA121, **G** *Synechococcus nidulans* CENA132, **H** *Synechococcus elongatus* CENA126, **I** *Microcystis* sp. CENA133, **J** *Lyngbya* sp. CENA128, **K** *Leptolyngbya* sp. CENA131, **L** *Leptolyngbya* sp. CENA129, and **M** *Cyanobium* sp. CENA118. *Scale bars* for all *panels* represent 10 μm, except for *picture* **F** which corresponds to 100 μm

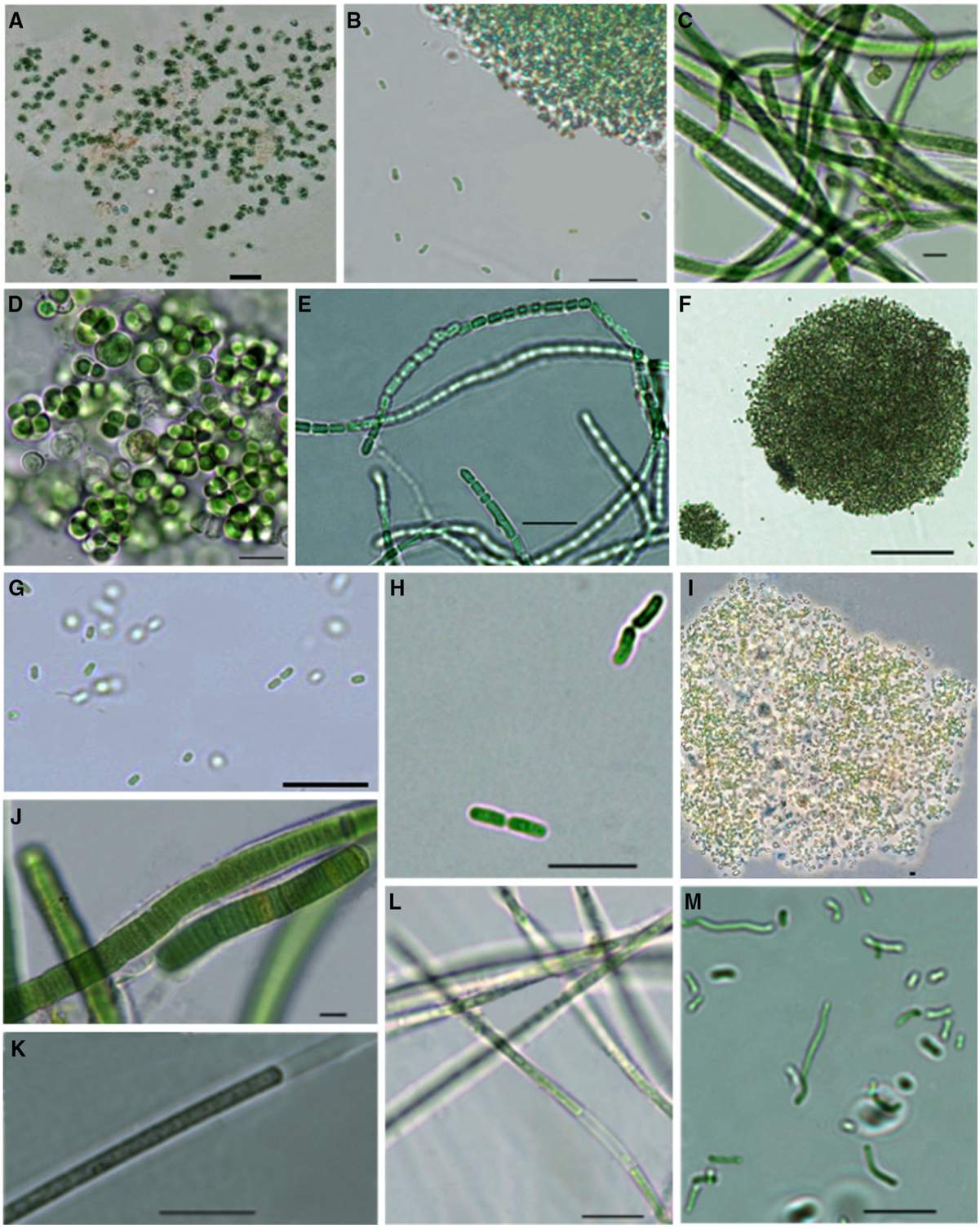
PCR amplification of NRPS and PKS gene sequences

Putative sequences coding for NRPS adenylation domain and PKS ketosynthase domain were successfully detected from 76.9 and 84.6% of the cyanobacterial DNA templates, respectively. *Microcystis* sp. CENA120, *Microcystis panniformis* CENA121, *Chroococcidiopsis* sp. CENA124, *Synechococcus elongatus* CENA126, *Calothrix* sp. CENA127, *Lyngbya* sp. CENA128, *Leptolyngbya* sp. CENA129, *Leptolyngbya* sp. CENA131, and *Microcystis* sp. CENA133 gave positive results for both NRPS and PKS genes, while the *Cyanobium* sp. CENA118

Table 2 Morphological identification of the cyanobacterial isolates from Salto Grande Reservoir

Orders[a]	Morphotypes	Strain numbers	Origin
Synechococcales	*Cyanobium* sp.	CENA118	Praia dos Namorados, April 2005
	Cyanobium sp.	CENA122	Praia dos Namorados, April 2005
	Synechococcus elongatus	CENA126	Iate Clube, April 2005
	Synechococcus nidulans	CENA132	Iate Clube, June 2004
Pseudanabaenales	*Leptolyngbya* sp.	CENA129	Iate Clube, April 2005
	Leptolyngbya sp.	CENA131	Iate Clube, June 2004
	Romeria victoriae	CENA123	Iate Clube, April 2005
Chroococcales	*Chroococcidiopsis* sp.	CENA124	Iate Clube, April 2005
	Microcystis sp.	CENA120	Praia dos Namorados, April 2005
	Microcystis panniformis	CENA121	Praia dos Namorados, April 2005
	Microcystis sp.	CENA133	Iate Clube, June 2004
Oscillatoriales	*Lyngbya* sp.	CENA128	Iate Clube, April 2005
Nostocales	*Calothrix* sp.	CENA127	Iate Clube, April 2005

[a] According to Hoffmann et al. (2005)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showed a negative PCR amplification for both gene regions. At least one of the NRPS and PKS genes were PCR detected for *Cyanobium* sp. CENA122, *R. victoriae* CENA123, and *S. nidulans* CENA132.

Microcystin analysis

The microcystin production evaluated by ELISA, positive reacted only for *Leptolyngbya* sp. CENA129 (2.31 μg l^{-1}). Given that ELISA cross reacts only against MCYST-LR, -RR, and -YR isoforms and nodularin, the remaining isolated strains, especially *Microcystis*, could be producing other isoforms than those investigated. The presence of microcystin in environmental samples assessed by the ELISA and HPLC analyses showed contradictory results. The highest microcystin concentration was detected by ELISA during dry season at both sampling sites, while by HPLC method the microcystin-LR concentration was below the detection limit during the same period. The highest concentration of microcystin-LR detected by HPLC was found at PN during the rainy season (9.2 μg l^{-1}; Table 6).

Discussion

Generally, high TP concentration (Watson et al., 1997), low TN:TP ratio (Smith, 1983), high temperature (Bouvy et al., 2000; Figueredo & Giani, 2009), and high pH (Briand et al., 2002) are reported as the most favorable conditions for the development of cyanobacterial blooms in water bodies. Among these factors, high TP concentration, high temperature, and high pH were found in the Salto Grande Reservoir (Table 1) and can be associated with cyanobacterial bloom formation. A TP concentration higher than 0.035 mg l^{-1} (Oliver et al., 1998) or higher than 0.05 mg l^{-1} (Wasson et al., 1996) is used to characterize phosphorus-enriched water bodies with a high potential for cyanobacterial growth or blooms. Based on these values and considering the concentration of 0.025 mg l^{-1} established by the Brazilian guideline (Brasil, 2005), the TP concentration in Salto Grande Reservoir exceeded the stated values, characterizing it as a phosphorus-enriched reservoir. An additional parameter used to demonstrate the trophic status of water bodies and to predict cyanobacterial bloom formation is the TN:TP ratio (Smith, 1983; Grayson et al., 1997). TN:TP ratios lower than 29 are favorable for cyanobacterial growth (Smith, 1983; ANZECC & ARMCANZ, 2000). Smith (1983) correlated cyanobacterial blooms with a low TN:TP ratio assuming that all cyanobacterial species are generally better nitrogen competitors than other phytoplankton members in an N-deprived environment. However, a TN:TP ratio higher than 29 and cyanobacterial blooms were observed in Salto Grande Reservoir, which contradicts the typical trend observed in other aquatic environments. According to some authors (Smith et al., 1987; Willén, 1992; Lathrop et al., 1998; Downing et al., 2001), cyanobacterial dominance and bloom formation can be connected more strongly to variations in P and N concentrations than to changes in TN:TP ratio. The capability of fixing atmospheric nitrogen is widespread among heterocytous-differentiating cyanobacteria such as in the genera *Anabaena* sensu lato, *Aphanizomenon*, *Cylindrospermopsis*, and *Nodularia* allowing them to grow in nitrogen-limited water bodies (Smith, 1983; Paerl et al., 2001) which in turn can cause blooms and toxin release. According to chemical analyses, Salto Grande Reservoir's water is considered a nitrogen-poor water body because TN concentration is under the limit of 3.7 mg l^{-1} established by the Brazilian guideline (Brasil, 2005). Cyanobacterial cells estimated by direct microscopic counting technique revealed 10^5–10^6 cells ml^{-1} in the Salto Grande Reservoir, confirming the prevalence of cyanobacterial blooms (>2 × 10^4 cell ml^{-1}) (Oliver & Ganf, 2000). According to Brazilian guideline, cyanobacterial cell count higher than 10^4 cell ml^{-1} requires a weekly monitoring of water quality (Brasil, 2011). The massive cyanobacterial growth in Salto Grande Reservoir is resulted from its eutrophic conditions and the advantageous photosynthetic system of cyanobacteria (Reynolds et al., 1987) enabling them to maximize their growth in a saturated condition of light, high temperature, and low water flow. Likewise, cyanobacterial blooms over the phytoplankton community are favored in high pH (7–9) (Wasson et al., 1996), such as the pH measured in the Salto Grande Reservoir's water (7.3–7.6). Moreover, the occurrence of these blooms in Salto Grande Reservoir was accompanied by a high chlorophyll-*a* concentration that surpassed the limit established for oligotrophic waters (1–10 μg l^{-1}) (Bartram et al., 1999) and the value recommended by Brazilian guideline (10 μg L^{-1}) (Brasil, 2005). Lower chlorophyll-

Table 3 Main morphological features of cyanobacterial isolates from Salto Grande Reservoir

Strains	Cell organization	Cell morphology	Colony size (μm)	Cell size (μm)		Occurrence
				Length	Width	
Calothrix sp. CENA127	Filaments entangled, sheaths hyaline, homogeneous; trichomes slightly constricted; ending in a thin hyaline hair	Cell barrel-shaped or cylindrical, heterocyte terminal, rounded or conical-rounded	–	4.0–7.5	Basal cell: 6.0–8.0 Middle cell: 4.4–7.0 Apex cell: 2.0 Heterocyte: 4.5–5.0 ϕ	C*
Chroococcidiopsis sp. CENA124	Solitary or in irregular aggregates	Spherical to irregular spheroidal enveloped by thick, firm, colorless sheaths; reproduction by baeocytes	–	2.1–5.1 ϕ		C
Cyanobium sp. CENA118	Cells arranged irregularly, densely in common, amorphous mucilage, small colonies	Straight, elongate, slightly curved or sigmoid, rod-shaped, with filamentous involution formations	58–82 × 45–54	1.0–2.0	0.6–0.8	C
Cyanobium sp. CENA122	Cells arranged irregularly, densely in common, amorphous mucilage forming spherical agglomerations	Straight or arcuate, rod-shaped	80–300 × 50–200	1.9–5.7	0.8–1.2	C
Cyanobium sp. CENA132	Cells solitary or in irregular clusters	Rod-shaped	–	1.5–2.0	1.0	C
Leptolyngbya sp. CENA129	Filaments entangled, loosely arranged; free-floating sheaths colorless (culture conditions)	Cells longer than wide, cylindrical, slightly granular content	–	1.0–3.0	1.0	P# and C
Leptolyngbya sp. CENA131	Filaments entangled, loosely arranged free-floating sheaths colorless (culture conditions)	Cells longer than wide, cylindrical, slightly granular content	–	2.0–3.5	1.6–2.0	C
Lyngbya sp. CENA128	Entangled filaments, cells forming trichomes not constricted at cross walls, sheaths colorless	Cells very short, content with small granules, apical cell with calyptra	–	2.0–2.5	10.0–12.6	C
Microcystis sp. CENA120	Cells arranged sparsely in irregular mucilaginous colonies (culture conditions)	Spherical with aerotopes	–	3.5–4.5 ϕ		P and C
Microcystis panniformis CENA121	Cells into mucilaginous spherical colonies, densely arranged	Spherical with aerotopes	78–266	3.5–4.4 ϕ		P and C

Table 3 continued

Strains	Cell organization	Cell morphology	Colony size (μm)	Cell size (μm)		Occurrence
				Length	Width	
Microcystis sp. CENA133	Colonies amorphous (culture conditions); cells densely arranged inside colonies	Spherical with aerotopes	–	3.0–3.5 ϕ		P and C
Romeria victoriae CENA123	Trichomes wavy, constricted at cross walls, solitary with 2–32 cells	Cylindrical	–	1.3–5.8	1.3–1.8	P and C
Synechococcus elongatus CENA126	Cells solitary or in irregular clusters	Straight, elongate, slightly curved or sigmoid, rod-shaped, with filamentous involution formations	–	2.9–47.0	1.2–1.3	C
Synechococcus nidulans CENA132	Cells solitary or in irregular clusters	rod-shaped	–	1.5–2.0	1.0	C

Minimum–maximum values, ϕ diameter, *C found in culture, $P^{\#}$ found in the plankton

a values during the rainy season can be explained by higher levels of precipitation and water flow during this period.

In Brazil, the occurrence of toxic cyanobacteria have been reported in an estuary (Yunes et al., 1996), in coastal lagoons (Azevedo et al., 1994; Lagos et al., 1999; Porfirio et al., 1999; Magalhães et al., 2001), and in reservoirs (Bouvy et al., 1999; Chellappa et al., 2000; Molica et al., 2002; Bittencourt-Oliveira et al., 2011, 2012; Borges et al., 2015). The majority of these studies have focused only on floristic surveys of the cyanobacterial communities without any investigation of the phylogenetic relationship among the morphotypes. The lack of genetic information hampers a more precise characterization of the morphotypes, mainly when considering the occurrence of many cryptic genera in the phylum Cyanobacteria (Perkerson et al., 2011; Strunecký et al., 2011; Zammit et al., 2012; Dvořák et al., 2015) and consequently the correlation with toxin production. According to Sant'Anna & Azevedo (2000), the most frequently found planktonic genera in Brazilian water bodies are *Microcystis*, *Anabaena* sensu lato, *Aphanizomenon*, *Cylindrospermopsis*, *Raphidiopsis*, and *Planktothrix*. Among these cyanobacterial genera, the well-known microcystin-producing genera *Microcystis*, *Anabaena* sensu lato, and *Planktothrix* were reported in Salto Grande Reservoir. In addition, the microcystin production has also been detected in the benthic genera *Fischerella* and *Nostoc* isolated from Brazilian spring water and freshwater reservoir, respectively (Fiore et al., 2009; Genuário et al., 2010).

The isolation techniques using environmental samples collected in Salto Grande Reservoir favored the cultivation of 13 benthic and planktonic cyanobacterial morphotypes. Isolation of genera not visualized in environmental samples by optical microscopy and observed taxa refractive to culture can be a consequence of the artificial culture condition applied (culture media composition, temperature, and luminosity) that can promote the development of a dormant/suppressed cells or inhibit the growth of dominant communities, respectively. In general, laboratory culture condition imposes restrictions or promotes the development of specific groups of microorganisms according to their nutritional and physical requirements (Harwani, 2013). Furthermore, picocyanobacteria, such as *Synechococcus* and *Cyanobium*, may be undetectable in optical microscopy

Table 4 Sequence identity (%) of 16S rRNA gene fragments from isolated strains compared to other cyanobacterial sequences available from GenBank

Morphotypes	16S rRNA gene fragment length (bp)	Query coverage (%)	Identity (%)	Closest match in GenBank (accession numbers)
Calothrix sp. CENA127	1,410	100	98.6	*Calothrix* sp. HA4186-MV5 (HQ847580)
		100	98.5	*Calothrix* sp. HA4395-MV3 (HQ847571)
		100	98.0	*Calothrix* sp. 2T08 (FR798918)
		100	98.0	*Calothrix parietina* 2T10 (FR798917)
Chroococcidiopsis sp. CENA124	1,416	100	100	*Chroococcidiopsis thermalis* PCC 7203 (NR_102464)
		100	99.4	*Chroococcidiopsis* sp. PCC 7431 (AB074506)
		100	98.5	*Chroococcidiopsis* sp. 9E−07 (FR798923)
		97	99.5	*Chroococcidiopsis cubana* SAG 39.79 (AJ344558)
Cyanobium sp. CENA118	1,409	100	99.7	*Aphanothece minutissima* 2LT34S03 (FM177488)
		100	99.7	*Cyanobium* sp. 1BB04S06 (AJ639896)
		100	99.7	*Synechococcus* sp. BO981502 (AF317077)
		100	99.7	*Cyanobium gracile* PCC 6307 (NR_102447)
Cyanobium sp. CENA122	1,409	100	99.7	*Aphanothece minutissima* 2LT34S03 (FM177488)
		100	99.7	*Cyanobium* sp. 1BB04S06 (AJ639896)
		99	99.7	*Synechococcus* sp. BO981502 (AF317077)
		100	99.7	*Cyanobium gracile* PCC 6307 (NR_102447)
Leptolyngbya sp. CENA129	1,415	100	96.1	*Leptolyngbya* sp. CENA112 (EF088337)
		100	95.6	Uncultured cyanobacterium 3GSCS_K18 (JX127186)
		99	95.8	Oscillatoriales cyanobacterium JSC-1 (FJ788926)
		100	95.4	*Leptolyngbya* sp. CENA103 (EF088339)
Leptolyngbya sp. CENA131	1,415	100	99.6	*Leptolyngbya* sp. CENA103 (EF088339)
		99	95.8	Oscillatoriales cyanobacterium JSC-1 (FJ788926)
		99	95.4	Uncultured cyanobacterium 3GSCS_K18 (JX127186)
		100	94.7	*Leptolyngbya* sp. 1T12c (FR798935)
Lyngbya sp. CENA128	1,412	100	96.7	*Nodosilinea* sp. CENA546 (KF246507)
		100	96.7	Uncultured cyanobacterium clone Alchichica_AL33_1_1B_03(JN825334)
		98	97.2	*Leptolyngbya* sp. BL0902(JN376076)
		100	96.6	Oscillatoriales cyanobacterium EcFYyyy400 (KC463194)
Microcystis sp. CENA120	1,420	99	99.7	*Microcystis aeruginosa* NIES-101 (FJ461750)
		99	99.7	*Microcystis aeruginosa* NIES-298 (FJ461749)
		99	99.7	*Microcystis* sp. GL260735 (AY439282)
		99	99.7	*Microcystis* sp. 205 (AY439281)
Microcystis panniformis CENA121	1,413	100	99.9	*Microcystis aeruginosa* NIES-101 (FJ461750)
			99.9	*Microcystis aeruginosa* NIES-298 (FJ461749)
			99.9	*Microcystis* sp. GL260735 (AY439282)
			99.9	*Microcystis* sp. 205 (AY439281)
Microcystis sp. CENA133	1,413	100	99.8	*Microcystis aeruginosa* NIES-101 (FJ461750)
		100	99.8	*Microcystis aeruginosa* NIES-298 (FJ461749)
		100	99.8	*Microcystis* sp. 205 (AY439281)
		99	99.9	*Microcystis flos-aquae* UWOCC N (AF139327)

Table 4 continued

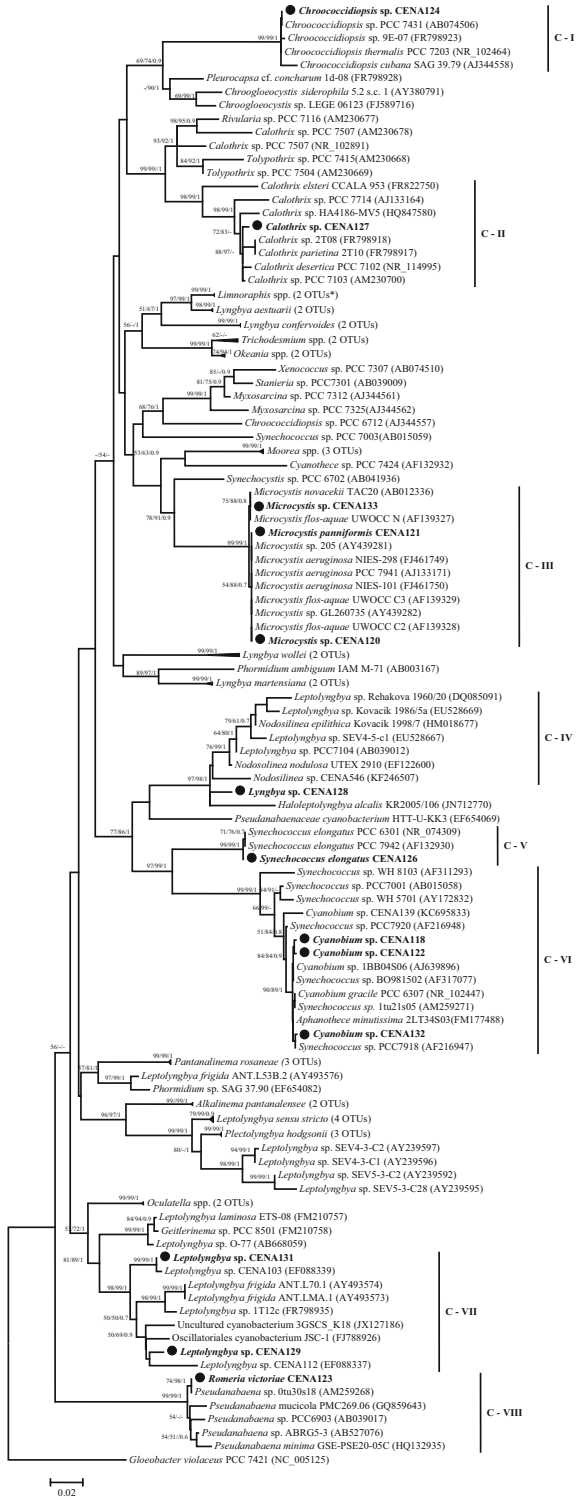
Morphotypes	16S rRNA gene fragment length (bp)	Query coverage (%)	Identity (%)	Closest match in GenBank (accession numbers)
Romeria victoriae CENA123	1,410	100	99.7	*Pseudanabaena* sp. 0tu30s18 (AM259268)
		99	97.3	*Pseudanabaena* sp. PCC 6903 (AF132778)
		100	96.7	*Pseudanabaena* sp. dqh15 (JF429939)
		98	97.3	*Pseudanabaena* sp. ABRG5-3 (AB527076)
Synechococcus elongatus CENA126	1,413	100	99.8	*Synechococcus elongatus* PCC 6301 (NR_074309)
		99	99.8	*Synechococcus elongatus* PCC 7942 (AF132930)
		99	99.7	*Synechococcus* sp. PCC 7943 (AF216949)
		100	99.4	*Anacystis nidulans* 6301 (X03538)
Synechococcus nidulans CENA132	1,409	100	99.7	*Aphanothece minutissima* 2LT34S03 (FM177488)
		99	96.6	*Synechococcus* sp. PCC7918 (AF216947)
		100	96.5	*Cyanobium gracile* PCC 6307 (NR_102447)
		100	96.5	*Synechococcus* sp. 1tu21s05 (AM259271)

Table 5 Identity matrix of 16S rRNA gene sequences among a subset of *Leptolynbya* sensu lato selected based on the phylogenetic tree

Strains	1	2	3	4	5	6	7	8	9
(1) *Leptolyngbya* sp. CENA129	–								
(2) *Leptolyngbya* sp. CENA131	95.6	–							
(3) *Leptolyngbya boryana* PCC 6306 (EF429290)	90.9	95.6	–						
(4) *Nodosolinea nodulosa* UTEX 2910 (KF307598)	90.1	90.9	88.6	–					
(5) *Plectolyngbya hodgsonii* ANT.LPR2.2 (AY493583)	90.2	89.6	95.4	88.2	–				
(6) *Leptolyngbya* sp. SEV4-3-C1 (AY239596)	90.2	89.9	96.4	88.2	96.2	–			
(7) *Oculatella subterranea* AD501/Zammit 2007/1 (HQ917688)	93.7	94.0	91.3	91.1	91.4	92.0	–		
(8) *Pantanalinema rosaneae* CENA516 (KF246483)	92.6	92.1	91.4	90.8	90.5	90.7	92.7	–	
(9) *Alkalinema pantanalense* CENA528 (KF246494)	91.4	90.2	89.2	89.0	91.1	92.3	91.0	91.8	–

inspection. The colonial and unicellular forms were the most represented group (genera *Microcystis*, *Chroococcidiopsis*, *Synechococcus*, and *Cyanobium*), followed by the filamentous non-heterocytous (genera *Leptolyngbya*, *Lyngbya,* and *Romeria*) and by the filamentous heterocytous morphotypes (genus *Calothrix*). Studies involving cell isolation, morphological, and molecular characterization of cyanobacteria are still scarce for tropical areas, especially in Brazil with regards to its different biomes and vast territory. Such studies are extremely important since they provide a better understanding of tropical cyanobacterial communities, their phylogenetic relationships, and allow an investigation of the cyanotoxin production.

The phylogenetic analyses based on the 16S rRNA gene sequences revealed that the orders Synechococcales, Chroococcales, Pseudanabaenales, and Oscillatoriales are polyphyletic whereas the order Nostocales is monophyletic (Hoffmann et al., 2005) (Fig. 2). These results are in agreement with those found in other studies for the orders Chroococcales and Oscillatoriales (Castenholz, 2001; Litvaitis, 2002; Seo & Yokota, 2003; Taton et al., 2006; Willame et al., 2006; Furtado et al., 2009). The orders Chroococcales and Oscillatoriales established by Komárek & Anagnostidis (1999, 2005)



◀**Fig. 2** Maximum likelihood phylogenetic tree based on the 16S rRNA gene sequences of cyanobacteria. The studied sequences from Salto Grande reservoir are shown in *bold* and in *black-filled circle*. A bootstrap test involving 1,000 resampling was performed. Bootstrap (greater than 50%) and probabilities values are displayed in *front* of the relevant *nodes* obtained from ML, NJ, and Bayesian methods, respectively. *Operational Taxonomic Unit

were split into four distinct orders (Synechococcales, Chroococcales, Pseudanabaenales, and Oscillatoriales) (Hoffmann et al., 2005).

Based on the 16S rRNA gene phylogeny, eight clusters containing the sequences generated in this study (C-I–VIII) (Fig. 2) were recognized. The sequences of unicellular, non-heterocytous, and heterocytous morphotypes formed four (C-I, C-III, C-V, and C-VI), three (C-IV, C-VII, and C-VIII), and one (C-II) clusters, respectively.

The cluster C-I comprised only the sequences related to *Chroococcidiopsis* morphotypes in a highly stable and supported clade (99/99/1). Although the genus *Chroococcidiopsis* has been considered a polyphyletic taxon (Cumbers & Rothschild, 2014), the sequence of *Chroococcidiopsis* sp. CENA124 was grouped with sequence of the type species *C. thermalis* according to the International Code of Nomenclature for Algae, Fungi, and Plants (ICN) (McNiell et al., 2012). Although not recognized as a type specimen by the ICN, the strain PCC 7203 adopted as reference sequence for cluster C-I for the genus *Chroococcidiopsis* taking into account Bergey's Manual of Systematic Bacteriology (Rippka et al., 2001a, b) showed 100% identity with *Chroococcidiopsis* sp. CENA124. Based on these findings, it seems legitimate to consider the Brazilian strain isolated from the Salto Grande Reservoir to be a true representative of the genus *Chroococcidiopsis*.

The cluster C-II included only those sequences corresponding to *Calothrix* morphotypes in a highly supported clade (98/99/1), including the sequence of *Calothrix* sp. CENA127. Although the genus *Calothrix* displays many genetic lineages (Sihvonen et al., 2007), the sequence of *Calothrix* sp. CENA127 was placed together with the reference sequences of *Calothrix* spp. from cluster C-I in the Bergey's Manual of Systematic Bacteriology (*Calothrix* sp. PCC 7102, PCC 7103, and PCC 7714) (Rippka et al., 2001a, b). This result confirms the morphological identification of the Brazilian strain and endorses its genetic affiliation with *Calothrix*. The sequences of the other heteropolar forms (genera *Tolypothrix* and

Table 6 Microcystin concentration (μg l^{-1}) in environmental water samples collected from Salto Grande Reservoir

Methods	Praia dos Namorados		Iate Clube	
	Mean ± SD		Mean ± SD	
	Dry season	Rainy season	Dry season	Rainy season
HPLC (LR isoform)	0.2 ± 0.4	9.2 ± 14.3	0.1 ± 0.1	2.5 ± 4.3
ELISA (LR, YR, RR isoforms)	8.5 ± 16.5	2.6 ± 2.9	6.5 ± 13.4	3.1 ± 2.6

Mean mean values, *SD* standard deviation

Rivularia) clustered together and formed the sister clade of C-II (93/92/1). Altogether, sequences of Order Nostocales (including the heteropolar, isopolar, and branched forms) formed a monophyletic cluster (99/99/1) as already reported in literature based on phylogenetic analyses of 58 concatenated core ortholog genes and on 54 whole-genome sequenced cyanobacterial strains (Larsson et al., 2011; Shih et al., 2013).

The cluster C-III grouped only sequences of *Microcystis* morphotypes in a highly stable and supported clade (99/99/1). Three of these sequences were generated from the *Microcystis* morphotypes isolated from Salto Grande Reservoir while the remaining sequences were recovered from different origins. The type species of the genus *Microcystis*, *M. aeruginosa* (Kützing, 1846) according to ICN (McNiell et al., 2012) was placed within this clade. Although not recognized as a type specimen by the ICN, the strain PCC 7941 adopted as reference sequence for the genus *Microcystis* taking into account Bergey's Manual of Systematic Bacteriology (Rippka et al., 2001a, b) grouped into this clade and showed ≥98.5% identity with *Microcystis* morphotypes isolated from Salto Grande Reservoir. This finding confirms the morphological identification of the Brazilian morphotypes. At the infrageneric level, no genetic differences were found to separate the *Microcystis* species (Fig. 2). It is known that the current morphological identification of *Microcystis* species is not supported by the phylogenetic analysis based on the 16S rRNA sequences (Neilan et al., 1997; Lyra et al., 2001; Willame et al., 2006) because this gene sequence is too highly conserved to estimate the infrageneric relationships. Despite this limitation, it is important to emphasize that some species of the genus *Microcystis* are widely known as microcystin producers. As mentioned, the *Microcystis* blooms in the Salto Grande Reservoir are constantly observed and may represent an important source of microcystin in it.

The cluster C-IV included sequences of morphotypes assigned to three different genera in a well-supported clade (97/98/1). These sequences, with the exception of the *Lynbya* sp. CENA128, belong to the genera *Haloleptolyngya* and *Nodosilinea* (Perkerson et al., 2011; Dadheech et al., 2012). These latter genera were separated from *Leptolyngbya* sensu lato a genus well known to be polyphyletic, after applying a polyphasic evaluation of selected genetic lineages (Casamatta et al., 2005; Komárek & Anagnostidis, 2005; Perkerson et al., 2011; Dadheech et al., 2012; Andreote et al., 2014; Zammit et al., 2012; Vaz et al., 2015). Despite the affiliation of *Lyngbya* sp. CENA128 sequence with the C-IV cluster, its sequence showed 16S rRNA gene identity lower than 96.8 with *Nodosilinea* sp. CENA546 isolated from Pantanal wetlands, Brazil (Andreote et al., 2014). Morphologically, the *Lyngbya* sp. CENA128 is distinguishable from *Nodosilinea* morphotypes, mainly by a comparison of their cell shapes (discoid vs. rectangular or isodiametric cells, respectively) and cell width (70 μm vs. 0.5–3.2 μm wide, respectively). The genus *Lyngbya* is also a polyphyletic group of cyanobacteria, possessing at least seven genetic lineages comprising morphotypes isolated from salt and freshwater habitats (Engene et al., 2010, 2012, 2013; Komárek et al., 2013). Considering the distinct phylogenetic position of *Lyngbya* sp. CENA128 sequence with regard to the cluster of *Lyngbya* sensu stricto (*L. confervoides*) and the freshwater and saltwater lineages (Fig. 2), this novel Brazilian sequence may represent another descendant line of the genus *Lyngbya.* This finding underscores the cyanobacterial diversity in tropical environments yet to be discovered. Although the

Lyngbya blooms are rarely mentioned in literature (Sharp et al., 2009; Komárek et al., 2013), a massive proliferation of *Lyngbya* filaments was recorded (not the *Lyngbya* sp. CENA128 strain) during this study in Salto Grande Reservoir as a consequence of its detachment from the bottom.

The cluster C-V comprised in a high supported clade (99/99/1), the sequence of morphologically identified *S. elongatus* CENA126 and sequences of the type species of the genus *Synechococcus* (*S. elongatus*) according to ICN (Nägeli, 1849). Although not recognized as a type specimen by the ICN, the strain PCC 6301 adopted as reference sequence for the genus *Synechococcus* taking into account Bergey's Manual of Systematic Bacteriology (Herdman et al., 2001) also grouped into this clade. The comparison between the 16S rRNA gene sequences of *S. elongatus* CENA126 and *S. elongatus* PCC 6301 showed a high identity (99.8%), supporting their genetic and morphological relationship. In addition to this finding, the ecology (freshwater habitat) is shared between them, corroborating the identification of the Brazilian isolate as *S. elongatus* CENA126.

The cluster C-VI comprised in a well-supported clade (99/90/1) the sequences of *Cyanobium* spp. (CENA118 and CENA122), *S. nidulans* CENA132 and other sequences from the genera *Synechococcus* and *Cyanobium* (Fig. 2). The morphologically mixed condition of this cluster stresses the difficulty in identifying these small and character-poor morphotypes by analyzing only the morphological features and lies the difficulty of reconciling the botanical and bacteriological codes. Based on the phylogenetic affiliation of the novel sequences with sequence of the type species of the genus *Cyanobium* (*C. gracile*) (Rippka & Cohen-Bazire, 1983) and the reference sequence (PCC 6307) (Fuller et al., 2003) considered at ICN and Bergey's Manual of Systematic Bacteriology, respectively, in combination with high identity among these sequences ($\geq$99.5%), the molecular evidence supports the morphological identification of these novel strain as *Cyanobium* sp. (CENA118, CENA122, and CENA132).

The cluster C-VII included the sequences from morphotypes identified as *Leptolyngbya* spp. and the sequences from two uncultured cyanobacteria in a well-supported clade (98/99/1). The comparative analysis among sequences of *Leptolyngbya* sp. CENA129 and *Leptolyngbya* sp. CENA131 against those available in GenBank showed as the best scored sequences the ones from the *Leptolyngbya* sp. CENA112 (EF088337) and *Leptolyngbya* sp. CENA103 (EF088339), respectively (Table 4). The same comparative analysis among these novel sequences with sequence of *L. boryana* PCC 6306 (EF429290) and with sequences from the morphologically related and recently described genera showed identities $\leq$93.7 (Table 5). Following the bacteriological threshold set as the cut-off point for the genus definition (95%) (Stackebrandt & Goebel, 1994; Ludwig et al., 1998), these identity values are below established limit and therefore indicates the separation of the sequences *Leptolyngbya* sp. CENA129 and *Leptolyngbya* sp. CENA131 into a new generic unit. Morphologically, the genus *Leptolyngbya* sensu lato comprises character-poor morphotypes with thin filaments and genetically is considered a polyphyletic group from which many paraphyletic lineages have originated new genera such as *Pantanalinema*, *Alkalinema*, *Oculatella*, and *Nodosilinea* (Casamatta et al., 2005; Komárek & Anagnostidis, 2005; Perkerson et al., 2011; Dadheech et al., 2012; Zammit et al., 2012; Andreote et al., 2014; Vaz et al., 2015).

The cluster C-VIII grouped in a well-supported clade (99/99/1), sequences of *R. victoriae* CENA123 and *Pseudanabaena* sp. 0tu30s18, a strain isolated from Lake Tuusulanjärvi (Rajaniemi-Wacklin et al., 2008). A subgroup of the cluster C-VIII comprised the sequences of *Pseudanabaena*, including PCC 6903, the reference sequence of the genus *Pseudanabaena* (cluster 1) (74/98/1), according to the Bergey's Manual of Systematic Bacteriology (Castenholz, 2001). The comparison against the sequence of *R. victoriae* CENA123 and those available in GenBank resulted as the highest scored hits, the sequences of *Pseudanabaena* sp. 0tu30s18 (AM259268) (99.7%) and *Pseudanabaena* sp. PCC 6903 (AB039017) (97.3%) (Table 4). Morphotypes of genera *Pseudanabaena* and *Romeria* are similar morphologically and both belong to the order Pseudanabaenales, according to the classification system proposed by Hoffmann et al. (2005). The morphological identification performed for *R. victoriae* CENA123 is in agreement with the formal description of the type specimen under the ICN (Komárek & Cronberg, 2001) especially concerning the cell number (up to 60 cells) (Table 3). Typical populations of the genus *Romeria* have been registered in freshwater reservoirs (Broa and Barra

Bonita) and in a lake (Clube da Penha) in São Paulo State, Brazil (Komárek & Komárková-Legnerová, 2007).

The NRPS and/or PKS genes were detected by PCR amplification in twelve cyanobacterial strains isolated from the Salto Grande Reservoir. The widespread occurrence of these genes indicates the genetic potential of these novel isolates for the biosynthesis of natural products, including toxins, such as microcystins (Tillett et al., 2000). The screening for microcystin production conducted using an ELISA was positive only for *Leptolyngbya* sp. CENA129. Additionally, both NRPS and PKS genes were PCR amplified in *Leptolyngbya* sp. CENA129, strongly suggesting its potential for microcystin production. The genus *Leptolyngbya* sensu lato has already been reported as a microcystin-producing group of cyanobacteria (Azevedo & Magalhães, 2002; Richardson et al., 2007; Mohamed & Al Shehri, 2010). In Brazil, the production of hepatotoxin was detected in two *Leptolyngbya* strains (NPLJ-34 and NPLJ-35) isolated from freshwater, although the specific toxin was not identified (Azevedo & Magalhães, 2002). The production of microcystin was also detected by ELISA in *Leptolyngbya* sp. CENA112, a strain isolated from the baffle tank of a waste stabilization pond system in Cajati, São Paulo State, Brazil (Furtado et al., 2009). Interestingly, in the 16S rRNA gene phylogenetic tree, the Brazilian sequences of *Leptolyngbya* spp. (CENA129 and CENA112) were grouped together and, in the 16S rRNA comparison, they share 96.1% identity (Fig. 2). The genetic potential depicted by PCR amplifications (NRPS and PKS) and the effective production of microcystin revealed by ELISA were inconsistent for the remaining strains. This result can be explained by the multifunctional capabilities of these enzymes that synthesize compounds with different biological activities (i.e., toxins, antitumoral, and anti-inflammatory compounds) or by the non-expression of the genes required for toxin production in the artificial condition imposed by the culturing techniques.

In environmental water samples, the highest microcystin concentrations were detected by HPLC and ELISA in the rainy and dry seasons at both sites (PN and IC), respectively (Table 6). The disagreement in the microcystin concentration found between these techniques may be linked to differences in the microcystin isoforms accessed by them and a possible inhibition of the immunoreactions caused by the presence of interfering soil particles in the samples, especially during the rainy season. Although most of the investigations on cyanotoxin production have focused on planktonic cyanobacteria, especially the bloom-forming genera *Microcystis*, *Planktothrix,* and *Cylindrospermopsis*, many studies have shown that benthic cyanobacteria can also produce toxins; including the genera *Phormidium*, *Schizothrix*, *Tolypothrix*, *Rivularia*, *Leptolyngbya*, *Lyngbya*, *Fischerella*, and *Nostoc* (Aboal et al., 2005; Mohamed et al., 2006; Izaguirre et al., 2007; Richardson et al., 2007; Fiore et al., 2009; Genuário et al., 2010). Despite the detection of microcystin in *Leptolyngbya* sp. CENA129 and the occurrence of sporadic blooms of *Lyngbya* spp. in Salto Grande Reservoir, the planktonic genus *Microcystis* might be the main microcystin-producing cyanobacteria in this water body because *Microcystis* blooms were observed throughout the studied period. Yet, microcystin variants were recently reported from *M. aeruginosa* LTPNA 02 isolated from this reservoir (Qi et al., 2014).

Conclusion

The eutrophic condition of Salto Grande Reservoir has favored cyanobacterial blooms formed mainly by genus *Microcystis*. In the present study, a cyanobacterial community composed of planktonic and benthic forms was cultured and studied by a polyphasic approach. These cultured strains allowed the investigation of NRPS and PKS genes which in turn can indicate their genetic potential for microcystin production. The detection of microcystin production by *Leptolyngbya* sp. CENA129 is a call for the scientific community to consider cryptic genera and the benthic forms as a potential microcystin-producing group further than those already widely reported in the literature. Microcystins were detected in environmental waters of Salto Grande Reservoir, representing a risk to other organisms present in this water body, which could culminate in fish and humans through bioaccumulation. In general, ecological and toxicological studies have been used to investigate the production of cyanotoxins in benthic and planktonic cyanobacterial groups. The combination of the constant toxic cyanobacterial blooms and the severe drought period in recent years in the Brazilian

southeast, where the Salto Grande Reservoir is located, have imposed serious restrictions on the quality and quantity of the water resources. Therefore, the application of polyphasic approach coupled with multidisciplinary studies must be conducted aiming for proper management of impacted water bodies.

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Compliance with ethical standards

Conflict of Interest The authors declare that there are no conflicts of interest.

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