JPR Advance Access published September 8, 2016

Journal of
Plankton Research

plankt.oxfordjournals.org

J. Plankton Res. (2016) 00(00): 1–15. doi:10.1093/plankt/fbw066

Cyanobacteria diversity in alkaline saline lakes in the Brazilian Pantanal wetland: a polyphasic approach

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Received August 25, 2015; accepted August 11, 2016

Corresponding editor: Pia Moisander

Shallow alkaline saline lakes are often found in the Brazilian Pantanal and although their extreme conditions are adverse to many phytoplankton organisms, they may support growth of dense cyanobacterial blooms. Here, we tested the hypothesis of reduced cyanobacterial diversity in these stressful environments, investigating changes between rainy and dry periods and analyzed which methodology would be best suited to assess this community. The cyanobacteria were studied in three lakes at three time periods, using morphological (microscopy) and molecular approaches (DGGE and ARDRA analyses of 16S rRNA gene segments). All methods showed that cyanobacteria dominance was higher during dry periods, probably as a consequence of intensified selective pressure by the increased solute concentration. *Anabaenopsis elenikinii* was the major bloom-forming cyanobacterium, followed by *Arthrospira platensis*. Interestingly, these two species never coexisted, but differentiated stages of one were observed during dominance of the other, suggesting that they may be excluding competitors. These life-stage differences, only detected through microscopic examination, show the importance of this approach. However, the detection of small-unseen picocyanobacteria and mainly the possibility to access population genotype diversity reinforce the relevance of molecular techniques. Therefore, this polyphasic methodology was particularly suitable to assess cyanobacteria communities in these extreme environments.

KEYWORDS: Pantanal wetland; alkaline saline lakes; cyanobacteria; ARDRA; DGGE; *Arthrospira*; *Anabaenopsis*; Nhecolândia

INTRODUCTION

Cyanobacteria are photosynthetic prokaryotes with a broad physiological plasticity and a long evolutionary history that have allowed them to disperse and colonize a range of different environments on Earth, including extreme habitats where other forms of life are rarely

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found (Ballot *et al.*, 2004; Taton *et al.*, 2006; Bahl *et al.*, 2011; Coman *et al.*, 2011).

The Pantanal wetland is located in the central portion of South America, mostly in Brazil, and is recognized as one of the largest continental wetlands in the world, with an area of approximately 160 000 km² (Por, 1995; Keddy and Fraser, 2005). Nhecolândia is the second largest region of the Pantanal wetland (24 000 km²) characterized by a large environmental heterogeneity due to the abundance of shallow lakes with different characteristics ranging from freshwater to saline. The extreme conditions of the alkaline saline lakes are known to hinder the growth of many organisms (Grant and Sorokin, 2011; Bell, 2012), nevertheless, some cyanobacteria species can develop and form blooms in these environments (Ballot *et al.*, 2004; Krienitz and Kotut, 2010; Santos and Sant'Anna, 2010).

The alkaline saline lakes from the Pantanal wetland (known as "salinas") are shallow (maximum depth is 2–3 m) and small (generally 500–1000 m in diameter), located in round depressions surrounded by 2–3 m high sand hills, which prevent their superficial connection to other lakes during flood periods (Furquim *et al.*, 2010). Several authors (Barbiéro *et al.*, 2002; Furquim *et al.*, 2010; Furian *et al.*, 2013) have suggested a recent origin of the saline lakes in the Pantanal, contradicting previous studies based on paleoecological explanations that linked the high salt concentration to ancient phenomena, particularly the alternation of wet and arid phases during the Pleistocene (Ab'Saber, 1988). Barbiéro *et al.* (2002) showed that all waters from the Nhecolândia belong to the same chemical family and their geochemical variability results from present-day evaporative concentration of freshwater. According to Barbiéro *et al.* (2002), the alkaline lakes are surrounded by sub-surface rises of green horizons with low permeability. These horizons have a high sodium equivalent and are cemented by amorphous silica that decreases the water infiltration rate and favors solute accumulation in the lake. Recently, Furian *et al.* (2013) confirmed that the coexistence of both saline and freshwater lakes in the region occurs as a result of the distribution of the soil cover that induces differential hydrological regimes. They showed that the average quantity of water flowing annually towards the lakes depends on the relative height of the greenish material with respect to the regional freshwater table, which decreases with increasing height of the green horizon that acts as a hydrological threshold.

The resulting elevated alkalinity, conductivity and pH of these lakes explain the absence of aquatic plants, fishes and also the low diversity in zooplankton communities (Mourão *et al.*, 1988; Medina-Júnior and Rietzler, 2005). Since cyanobacteria have developed mechanisms to tolerate conditions that are adverse to the majority of other species (Klähn and Hagemann, 2010), their study in this extreme environment has an ecological and evolutionary relevance. Many authors agree that alkaline saline lakes are driven by dense populations of cyanobacteria (Grant and Sorokin, 2011), which turn these lakes among the most productive systems in the world (Bell, 2012).

There are few studies on the diversity of cyanobacteria in alkaline saline lakes from the Nhecolândia Pantanal (Malone *et al.*, 2007; Santos and Sant'Anna, 2010) to our knowledge just one that utilized a polyphasic approach (Andreote *et al.*, 2014). In the present investigation, we provide additional genetic information to these previous works, include quantitative data about the phytoplankton community and evaluate its changes in different seasons focusing on ecological aspects.

Microscopy techniques, like morphological characterization and cell counting, are traditional methods that allow the study of the phytoplankton community (Spiegelman *et al.*, 2005); however; morphology alone cannot always properly reflect the ecological entities found in a system (Nübel *et al.*, 1999). Morphological variations as a result of differential gene expression (Castenholz and Norris, 2005) have already been reported, as well as the existence of genetic differences that remained undetected through the observation of morphological variations (Yoshida *et al.*, 2008).

The use of the 16S subunit of the ribosomal RNA gene has opened new possibilities in the study of microbial community diversity via molecular approaches (Spiegelman *et al.*, 2005), allowing a more complete understanding of the changes observed in their composition. Thus, the need of a polyphasic approach has been emphasized to improve the knowledge of these communities (Willame *et al.*, 2006).

In this study we tested the hypothesis that alkaline saline lakes from the Pantanal wetland have a reduced phytoplankton diversity, which further decreases during the dry season, when salinity levels increase as a consequence of evaporative concentration and fewer species are able to persist. In order to test this hypothesis, we assessed the diversity of the cyanobacterial community of three saline lakes from the Nhecolândia region (Pantanal, Brazil) using traditional identification by microscopy, Denaturing Gradient Gel Electrophoresis (DGGE) of amplified 16S rRNA segments and Amplified Ribosomal DNA Restriction Analysis (ARDRA), in samples obtained at different periods. Both molecular methods were followed by sequencing. The combination of morphological and molecular approaches improved the description of the community, providing new information about the genetic diversity and ecological dynamics of a distinct group of organisms in one of the world's largest, but still poorly studied, wetlands (Por, 1995; Keddy and Fraser, 2005).

METHOD

Sampling

The sampling was carried out in the Nhecolândia region of the Brazilian Pantanal in three alkaline saline lakes located at the Nhumirim farm: "Salina da Reserva" (18°57′37"S; 56°37′25"W), "Salina do Meio" (18°58′ 27"S; 56°38′51"W) and "Salina da Invernada" (18°58′ 59"S; 56°39′42"W). These are shallow, non-stratified and permanent alkaline saline lakes. They are situated at a short distance from each other: 3 km from Salina da Reserva to Salina do Meio, 1.7 km from Salina do Meio to Salina da Invernada. Salina do Meio is placed between the other two (Fig. 1).

Samples were taken in three different periods: in October 2011, at the end of the flooding, after a late rainy season; in April 2012, during the dry season; and in March 2013, during an atypical dry season. The periods of rainfall are quite different from year to year in the Pantanal and as a consequence the seasonal flooding and drought patterns may shift over the years.

Temperature, pH, conductivity, dissolved oxygen and salinity were measured *in situ* with a multi-parameter probe (YSI 556) sub-surface (around 10 cm depth). Water samples were collected at the same depth in the middle of each lake, stored on ice in polyethylene bottles and transported to the laboratory in less than 2 h. In the laboratory the samples were immediately filtered through a 47 mm diameter, 0.7 μm pore-size glass fiber filter (GF1, Macherey and Nagel, Germany). The filters were stored at −20°C until further analyses. Unfiltered water samples were also frozen for subsequent analyses. A fraction of 100 mL of each sample was stored in a glass flask and immediately preserved with Lugol's iodine solution, for phytoplankton counting.

Chemical analyses

Total alkalinity was obtained by titration according to the method of Gran (1952). Free CO_2 was accessed as described in Kempe (1982) and modified by Hamilton *et al.* (1995). Turbidity was determined according to APHA (1998). Dissolved nutrients were analyzed using standard colorimetric techniques (APHA, 1998). The content of cations and anions was measured with an ion chromatograph DIONEX ICS-1100 (Thermo Scientific, Waltham, USA). Filters were used to obtain total, organic and inorganic suspended solids by gravimetric analysis (APHA, 1998).

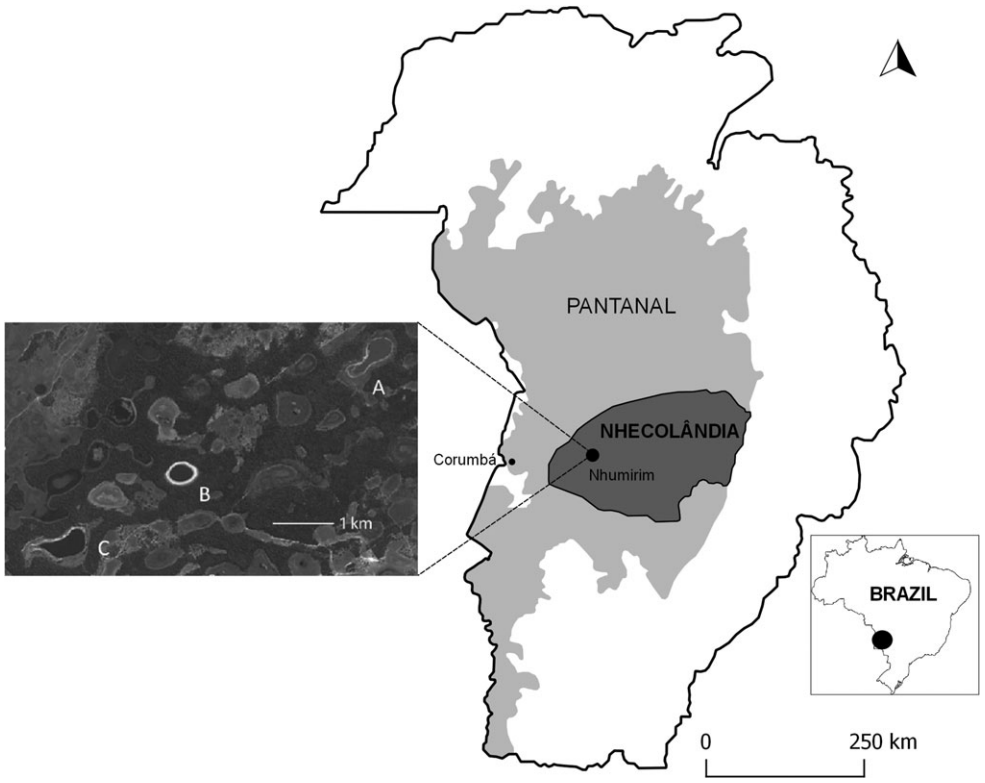

Fig. 1. Location of the Nhecolândia Pantanal in Brazil, and satellite image of the three lakes of study: A – Salina da Reserva; B – Salina do Meio; C – Salina da Invernada. White sandy beaches that are especially visible in Salina do Meio surround the alkaline saline lakes.

Cell counting and biovolume

Phytoplankton quantification was performed under an inverted microscope (Zeiss Axio Observer D1) according to the Utermöhl technique (Utermöhl, 1958). The total biovolume of each species was calculated by multiplying the cell number by the mean cellular volume of each species, obtained using geometrical models (Rott, 1981).

DNA extraction

The DNA was extracted from frozen filters following the method described by Kurmayer *et al.* (2003), with few modifications. One quarter of each filter was subjected to an osmotic shock treatment by immersion in a sucrose buffer (25% w/v sucrose, 50 Mm Tris-HCl, 100 Mm EDTA, pH 8) on ice for 2 h and cell lysis was promoted by the addition of lysozyme (5 mg/mL, 1 h, 37°C). In order to degrade cellular proteins, proteinase K (100 µg/mL) in sodium dodecyl sulfate (2% v/v) was added and filters were incubated overnight at 55°C. A phenol:chloroform:isoamyl alcohol solution (25:24:1, v/v) was subsequently used to precipitate the proteins and isolate the DNA. The DNA was precipitated in 100% ethanol and the pellet obtained was rinsed with 70% ethanol. The DNA was finally resuspended in TE (10 mM Tris-HCl, pH 8, and 1 mM EDTA). The DNA extract was quantified by a spectrophotometer, at 260 nm and 280 nm, and its quality checked in 1% (w/v) agarose gel, stained with ethidium bromide.

Denaturing gradient gel electrophoresis

The PCR reaction for DGGE analysis was performed with (GC-) CYA359F, CYA781R (a) and CYA781R (b) primers (Nübel *et al.*, 1997). Primer sequences are given in Table I. For the PCR, about 20 ng of the extracted genomic DNA were added to 50 µL of the amplification mixture, with a final concentration of 250 nM of the forward primer and 125 nM of each reverse primer, 1X DreamTaq Buffer (Thermo Scientific, Waltham, USA), 0.625 mM $MgCl_2$, 4 µg/µL of non-acetylated bovine serum albumin, 0.25 mM of deoxynucleotides and 0.025 U/µL of DreamTaq DNA polymerase (Thermo Scientific, Waltham, USA). Thermal cycling conditions were 95°C for 3 min followed by 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, repeated for 30 cycles with a final extension of 72°C for 10 min. The reactions were run in a Bio-Rad MyCycler (Foster City, California, USA). PCR products were visualized on a 1% agarose gel (w/v) and quantified using Low DNA Mass Ladder (Invitrogen, Carlsbad, USA). After the quantification using the program Image Lab 5.0 (Bio-Rad Laboratories, Hercules, USA), approximately 400 ng of PCR product were loaded in each DGGE well. The DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA), with a 6% acrylamide gel (acrylamide/bis 37.5/1) (w/v) and a denaturing gradient from 40% to 60%. The 100% denaturing solution was composed of 7 M urea and 40% formamide (v/v). The samples were prepared by adding 4 µL of loading dye solution (0.09% bromophenol blue, 0.09% xylencyanol, 60% glycerol, 60 mM EDTA) to 36 µL of the PCR product. The electrophoresis was performed at 60°C, with a constant voltage of 80 V during 959 min. The gel was stained with SYBR Gold 1X (Invitrogen, Carlsbad, USA), for 30 min.

The bands were visualized under UV-light using DigiDoc-It Imaging System (UVP Company, Upland, USA). Each band was excised using a razor blade, previously sterilized. After an overnight elution in 50 µL of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA), the DNA from the excised bands was re-amplified using the CYA359F, CYA781R (a) and CYA781R (b) primers. The resulting amplification products were purified with 100% ethanol/125 mM EDTA (Ethanol/EDTA Precipitation Clean-up Protocol as per the Applied Biosystems Sequencing Guide) and submitted to sequencing (Macrogen Inc., Korea).

Table I: Primers used in this study

Primer[a]	Sequence (5′ to 3′)	Reference
CYA356F	GTGGGGAATTTTCCGCAA	de la Torre *et al.* (2003)
1391 R	GACGGGCGGTG(G/T)GTRCA	Turner *et al.* (1999)
(GC-) CYA359F[b]	GGGGAAT(C/T)TTCCGCAATGGG	Nübel *et al.* (1997)
CYA781R(a)	GACTACTGGGGTATCTAATCCCATT	Nübel *et al.* (1997)
CYA781R(b)	GACTACAGGGGTATCTAATCCCTTT	Nübel *et al.* (1997)

[a]F (forward) and R (reverse) refer to the primer orientation in relation to rRNA genes.
[b]A 40-nucleotide G-C clamp 5′-CGC CCG CCG CGC CCC GCG CCG GTC CCG CCG CCC CCG CCC G-3′ was added to the 5′ end of the forward primers indicated with (GC-).

Cloning and amplified ribosomal DNA restriction analysis

The PCR reaction for cloning was performed using the CYA356F and 1391R primers, whose sequences are given in Table I. The PCR mix was the same as previously described for DGGE and about 20 ng of the extracted genomic DNA were added to 50 µL of the amplification mixture. Thermal cycling conditions were 95°C for 3 min followed by 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, repeated for 30 cycles. PCR

products were visualized on a 1% agarose gel (w/v) and the bands excised were purified with phenol:chlorophorm:isoamyl alcohol (25:24:1, v/v). The purified product was inserted in the vector pJET 1.2/blunt (Thermo Scientific, Waltham, USA) and then transformed into TOP 10 chemically competent *Escherichia coli* strains through the heat shock method. The positive colonies were selected from a LB agar plate (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar), containing ampicillin (50 μg/mL), and submitted to a colony PCR using the CYA356F and 1391R primers, the same reagent mix concentration and PCR cycles described above. Each PCR product was treated with the restriction enzyme BsuRI (HaeIII) (Thermo Scientific, Waltham, USA) in a mixture containing a final concentration of 0.04 U/μL BsuRI and 1X BufferR (10 mM Tris-HCl, 10 mM MgCl2, 100 mM KCl, 0.10 mg/mL bovine serum albumin). After 4 h at 37°C each mixture was loaded in a 2% agarose gel (w/v) under standardized electrophoresis conditions (10 V/cm, 25 min), stained in ethidium bromide. Plasmids from clones with different Restriction Fragment Length Polymorphisms (RFLP) were extracted through the Minipreparation method (Birnboim and Doly, 1979), submitted to a new PCR and the 16S rRNA fragments were sequenced (Macrogen Inc., Korea), after a purification. More than one plasmid with the same banding pattern was sequenced to test the sensitivity of the digestion method.

In order to verify the sufficiency of clone sampling, the nonparametric estimators of asymptotic richness Chao1 and Chao2 (Chao, 2005) were calculated and rarefaction curves were constructed (data not shown) using the EstimateS 9.0 Software (Colwell, 2013).

Sequence processing and phylogenetic analysis

Purified amplicons obtained from DGGE and ARDRA were sequenced in a 23 ABI 3730 XLs, by dye-terminator sequencing with the corresponding amplification primers, to allow sequencing of both complementary DNA strands (Macrogen, Korea). The 16S rRNA gene sequenced fragments were assembled into contigs using the Genome Assembling Program (Gap4) and the Pregap4 (Bonfield *et al.*, 1995). The sequences were trimmed by considering only bases with a quality score above 30. The resulting consensus sequences were submitted to a BLAST on the NCBI GenBank and the best-matched sequences were retrieved. A summary of the BLAST results is shown in Tables SI and SII (see supplementary material). All sequences in this study (from DGGE bands and ARDRA) and homologous sequences obtained by BLAST were aligned by ClustalW, manually edited obtaining 396 aligned positions for DGGE and 976 positions for ARDRA. To verify the robustness of this analysis, phylogenetic trees were constructed with Neighbor-joining and Maximum-parsimony algorithms implemented by Mega 6.0 Software (Tamura *et al.*, 2013), applying the Maximum Composite Likelihood model and the Subtree-Pruning-Regrafting (SPR) method, respectively, and using the complete deletion of gaps and missing data. Bootstrap resampling was performed using 1000 replications. The 16S rRNA gene sequences are deposited in the NCBI GenBank under accession numbers KP219020 to KP219044 for DGGE isolated bands and KP219045 to KP219084 for clones.

Statistical methods

A principal component analysis (PCA) was performed in order to extract the main trends among the variables, from a data pool that comprised environmental parameters and total biovolume of the cyanobacteria. All parameters were log transformed prior to statistical analyses. The biotic data were also transformed, and the zero values in the species matrix were considered as $1e^{-6}$, prior to log transformation. An Analysis of Similarity (ANOSIM) was also performed on these data, in order to detect potential differences between the sampling periods. Generalized Linear Models (GLM) were constructed to verify significant relationships between cyanobacteria richness and the sampling period or the methodological approach used. The richness data exhibited a Poisson distribution and, for this reason, the minimum model was contrasted to the null model by ANOVA, using a chi-square test. All statistical analyses were performed using R 3.1.0 for Windows (R Core Team, 2014).

RESULTS

Climatic and physico-chemical characteristics

Table II summarizes the most important environmental parameters of each saline lake in the different sampling periods.

In October/2011, "Salina da Invernada" showed the lowest values for alkalinity, conductivity, turbidity, chloride, sodium and potassium concentration. In March/2013 "Salina da Reserva" presented the highest values for conductivity, turbidity, sodium and potassium concentration. In the same period, the highest value of

Table II: Abiotic variables of the saline lakes from Nhecolândia Pantanal wetland in the period of study

	Salina da Reserva			Salina do Meio			Salina da Invernada		
	Oct/11	Apr/12	Mar/13	Oct/11	Apr/12	Mar/13	Oct/11	Apr/12	Mar/13
Alkalinity (HCO_3 μeq/L)	17 003.3	28 528.0	78 713.1	22 781.1	33 919.9	78 713.1	14 061.3	24 278.3	38 911.4
Conductivity (μS/cm)	2089.0	3562.0	9630.0	4596.0	8100.0	8260.0	1617.0	3251.0	3700.0
Turbidity (NTU)	177.6	173.6	3622.5	190.0	143.5	441.0	54.3	139.2	3150.0
Chloride (mM)	3.6	4.2	10.3	26.3	24.6	27.3	1.2	2.1	2.2
Sodium (mM)	14.7	20.3	40.1	36.6	37.8	32.8	11.6	15.9	19.3
Potassium (mM)	6.4	8.5	20.0	6.9	10.6	9.0	4.5	6.3	11.0
pH	9.0	9.7	9.5	10.2	9.6	8.9	9.1	9.7	9.0
Water temper. (°C)	25.8	29.0	33.7	27.2	39.0	36	28.0	37.0	36.4

chloride concentration was registered in "Salina do Meio".

The pH did not show a large variation among sites or periods. Mean pH for all the samples was 9.4, with the lowest registered in Salina do Meio in March/2013 (8.9) and the highest in this same lake in October/2011 (10.2). Mean water temperature, considering the three lakes, was highest in April/2012 (35°C) and in March/2013 (35.4°C) and the lowest average temperature was in October/2011 (27°C).

Fig. 2 presents the results obtained by the multivariate analysis PCA. Alkalinity, conductivity, total nitrogen, total phosphorus and temperature were the main variables associated with the first axis. Organic Suspended Material, total biovolume of cyanobacteria and sulfate were the variables that better explained the variability of the second axis. Together these two axes extracted 76.12% of the total variability, and 51.96% was explained just by the first axis.

The three different sampling periods (October/2011, April/2012 and March/2013) are clearly separated in the PCA two-dimensional plot (Fig. 2), but the ANOSIM test did not show a significant difference among the three groups ($R = 0.218$; $P = 0.102$), because of the existing variability within them.

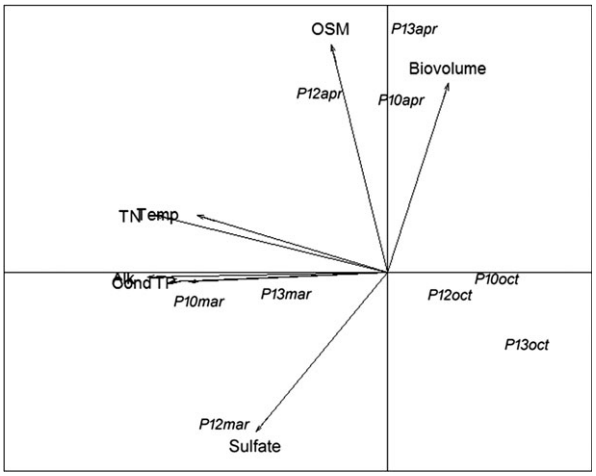

Fig. 2. PCA ordination of environmental variables and cyanobacteria biovolume of the three alkaline saline lakes, during three sampling periods. (Alk = alkalinity, Biovolume = total biovolume of Cyanobacteria, Cond = Conductivity, OSM = Organic Suspended Material, Temp = water temperature, TN = total nitrogen, TP = total phosphorus; P10: Salina da Reserva; P12: Salina do Meio; P13: Salina da Invernada.)

Microscopic analysis of the phytoplankton community

The relative abundance of the most representative phytoplankton groups, measured as relative percentage of the total phytoplankton biovolume, is shown in Fig. 3. Table III presents a summary of the data obtained by microscopy for each lake and sampling period: relative abundance, total biovolume and richness of cyanobacteria, and the dominant phytoplankton species. In Table SIV (supplementary material) the biovolume of all observed cyanobacteria and their respective differentiated stages (hormogonia and akinetes) can be found.

Cyanobacteria, mainly represented by the species *Anabaenopsis elenkinii* or *Arthrospira platensis*, was the dominant phylum in six of the nine samples analyzed (Fig. 3, Table III). The highest cyanobacteria biovolumes were registered in April/2012, in Salina do Meio and Salina da Invernada. Cyanobacterial blooms were observed in all three sampling periods at Salina da Reserva, and in March/2013, it was the largest. In April/2012, the dominant species was *A. platensis*, while in October/2011 and March/2013 was *A. elenkinii*.

Probable hormogonia of *A. platensis* were detected in samples of Salina da Reserva (October/2011) and Salina do Meio (April/2012) during blooms of *A. elenkinii*. Hormogonia are short filaments that detach from the parental trichome and whose differentiation is related to environmental changes and perturbations. Akinetes (resting forms) of *A. elenkinii* were usually detected, either

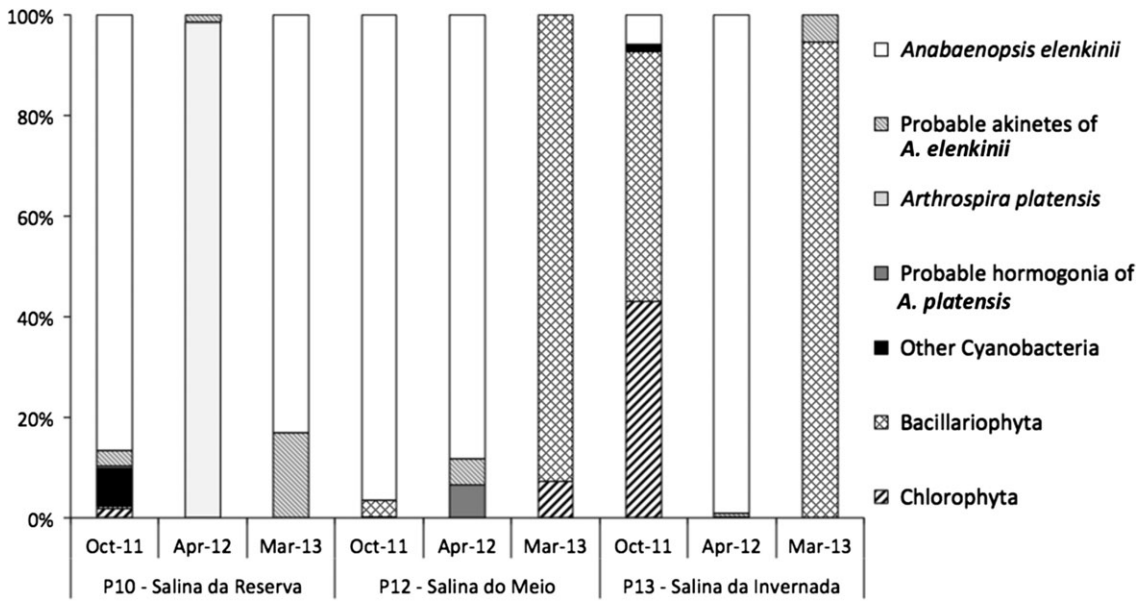

Fig. 3. Relative abundance of the main phytoplankton components based in biovolume quantification, in the three alkaline saline lakes during three sampling periods. (Other Cyanobacteria: *Aphanocapsa* sp., *Coelosphaerium punctiferum*, *Dolichospermum* sp., *Merismopedia tenuissima*, Nostocales sp1, Oscillatoriales sp1, *Pseudanabaena galeata*, *Romeria caruaru*, *Microcystis* sp.; Bacillariophyta; *Nitzschia* sp.)

Table III: Summary of data based on microscopy identification and quantification of the phytoplankton species, and molecular richness measured as numbers of BLAST identified cyanobacterial sequences, isolated from DGGE bands and ARDRA profiles

	Salina da Reserva			Salina do Meio			Salina da Invernada		
	Oct/11	Apr/12	Mar/13	Oct/11	Apr/12	Mar/13	Oct/11	Apr/12	Mar/13
Cyanobacteria (%)	97	100	100	96	100	0.00	7	100	5
Total cyanobacteria (µm³)	9E+07	3E+08	2E+09	2E+08	6E+08	0.00	1E+05	7E+08	2E+05
Microscopy cyanobacterial richness	7	2	1	1	3	0	7	3	2
DGGE bands	4	2	2	3	1	1	3	1	2
ARDRA profiles	7	2	2	5	1	2	2	2	1
Dominant species	*A. elenkinii*	*A. platensis*	*A. elenkinii*	*A. elenkinii*	*A. elenkinii*	*Nitzschia* sp.	*Nitzschia* sp.	*A. elenkinii*	*Nitzschia* sp.

attached to the filaments or as isolated forms (Fig. 3, Table SIV). Interestingly, numerous akinetes of *A. elenkinii* were identified during a bloom of *A. platensis* in Salina da Reserva (April/2012).

Molecular analysis based on the cyanobacteria 16S rRNA gene

A total of 24 sequences were obtained from DGGE bands and 14 corresponded to *A. elenkinii* (6 from Salina da Reserva, 6 from Salina do Meio and 2 from Salina da Invernada), 4 to *Synechococcus* sp. (2 from Salina da Reserva and 2 from Salina da Invernada), 3 to *Microcystis* sp. (all from Salina da Invernada), 1 to *A. platensis* (from Salina da Reserva) and 2 to plastids of Bacillariophyta (both from Salina do Meio). After BLAST analyses, some sequences from DGGE bands occupying different positions revealed similarity to a single strain deposited in the NCBI GenBank. For example, seven bands in different positions (A1.2, B2, B3, B4, B5, B7.2 and B8) corresponded to *A. elenkinii* AB2006/20 (AM773307.1); two (A2 and A3) to *A. elenkinii* NIVA-CYA 501 (AM773309.1: three (C1, C2 and C4) to *Microcystis aeruginosa* KLL-C004 (KP726249.1) and two (C5 and C6) to *Synechococcus* sp. Suigetsu-CG2 (AB610891.1) (see supplementary material, Tables SI and SIII). This has similarly already been observed for environmental PCR products separated by DGGE (Nikolausz *et al.*, 2005). BLAST analyses indicated intraspecific diversity in *A. elenkinii*, through the detection of three sequences corresponding to three different strains (AB2006/20 isolated from lake Texcoco, in Mexico;

NIVA-CYA 501 from Kazinga Channel, in Uganda; and AB2002/37 from Lake Sonachi, in Kenya) and also in three sequences of *Synechococcus* spp. strains (Suigetsu-CG2 from a saline lake in Japan; BS2 from Lake Taihu in China and CENA 140 isolated from a Brazilian mangrove) and two belonging to *Microcystis* spp. (KLL-C004 from Israel and CENA120 from the Brazilian Salto Grande reservoir).

From ARDRA analysis, we sequenced 40 clones, corresponding to 11 different restriction patterns. The consensus 16S rRNA sequences were assembled using, whenever possible, two representatives of each ARDRA pattern (ribotype). In few cases, different cyanobacterial RFLP patterns were identified after sequencing as belonging to the same taxa, as for example in *A. elenkinii* that showed seven different patterns, representing intraspecific variation of the restriction fragments. For each alkaline lake, the ribotype accumulation curves as a function of the number of clones displayed a hyperbolic tendency towards a saturation curve, indicating that new ribotypes would not be retrieved by the addition of more clones (curves not shown). The highest amount of RFLP patterns corresponded to *A. elenkinii* sequences, consistent with those observed in the analysis of the DGGE bands.

The number of different ARDRA profiles and DGGE bands found for each identified species revealed the low diversity of the cyanobacteria community in these alkaline saline lakes. Only in samples from October/2011, during the rainy period, were more than two different genotypes of cyanobacteria detected by ARDRA and DGGE (Table III).

All the genotypes detected by DGGE were also confirmed by ARDRA, but the last method was more sensitive to detect some genotypes, such as the ones that exhibited close relationship to 16S rRNA gene sequences belonging to *Merismopedia* and *Synechocystis*. A larger diversity of genotypes could be accessed through ARDRA than with DGGE, as it is shown in the more robust phylogenetic tree based on the ribotype sequences, because of the higher number of aligned positions by the ARDRA (see Methods). However, according to the GLM analysis there was no significant difference between the richness found by DGGE, ARDRA or the Utermöhl method ($P = 0.38$).

The phylogenies were inferred through the Maximum Parsimony Tree and Neighbor-joining methods on the 16S rRNA gene sequences obtained by DGGE or ARDRA. Qualitatively similar tree topologies were observed when using the two algorithms; however, bootstrap values were better for the Maximum Parsimony Tree that was used to generate the trees represented in Figs 4 (DGGE) and 5 (ARDRA). Closely related sequences from the GenBank were also included in the dataset. A larger diversity of genotypes could be assessed through ARDRA than DGGE, as it is shown in the phylogenetic tree based on the ribotype sequences.

Anabaenopsis elenkinii was the most abundant genotype in both trees [cluster (1), Figs 4 and 5] and the different alignments and clustering patterns within these sequences confirmed the existence of intraspecific genetic diversity of the dominant morphotype observed in most samples (Fig. 3). Several genotypes of *Synechococcus* [cluster (2) Figs 4 and (2a) 5], *Merismopedia* [cluster (2b), Fig. 5] and *Synechocystis* [cluster (5), Figs 4 and 5] were also detected, indicating the importance of the picocyanoplankton in this environment.

Furthermore, the primers used in both molecular techniques also amplified the plastid gene from the bacillariophycean *Nitzschia* [cluster (6), Figs 4 and 5], which was also detected by microscopy and was dominant in certain samples (Fig. 3).

DISCUSSION

Alkaline saline lakes from the Pantanal wetland exhibit elevated alkalinity, pH and salt content, which prevent many organisms from colonization (Mourão *et al.*, 1988; Medina-Júnior and Rietzler, 2005; Santos and Sant'Anna, 2010). The harsh conditions of alkaline saline lakes can even increase during dry periods, as observed in our study (Table II) and can be very stressful for most phytoplankton species (Estrada *et al.*, 2004; Jungblut *et al.*, 2005). In general, organisms able to tolerate elevated pH and excess of ions present in the water adjust their cellular content to be in osmotic equilibrium with the outside medium (Oren, 2000, 2007) and develop strategies to maintain the internal pH lower than the external environment, as particular cell walls, membranes and enzymes (Bell, 2012).

Interestingly in our study, the most extreme cyanobacterial blooms were found during the driest period, when the highest values of alkalinity, salinity and temperature were registered (Table II, Fig. 3). Nutrient concentration (TN and TP) followed the same pattern of increase at high temperatures (Fig. 2), as a possible result of the desiccation process, and the consequent size reduction of the water body and evaporative concentration of solutes. Furian *et al.* (2013) described the changes in alkaline saline lakes of the Pantanal during the dry season. Because of the low-permeable green horizons that surround these lakes (see Introduction), under influence of high evaporation rates and with very little or no inflows of freshwater, even of sub-surface water, the content of salts and nutrients tended to increase during dry periods.

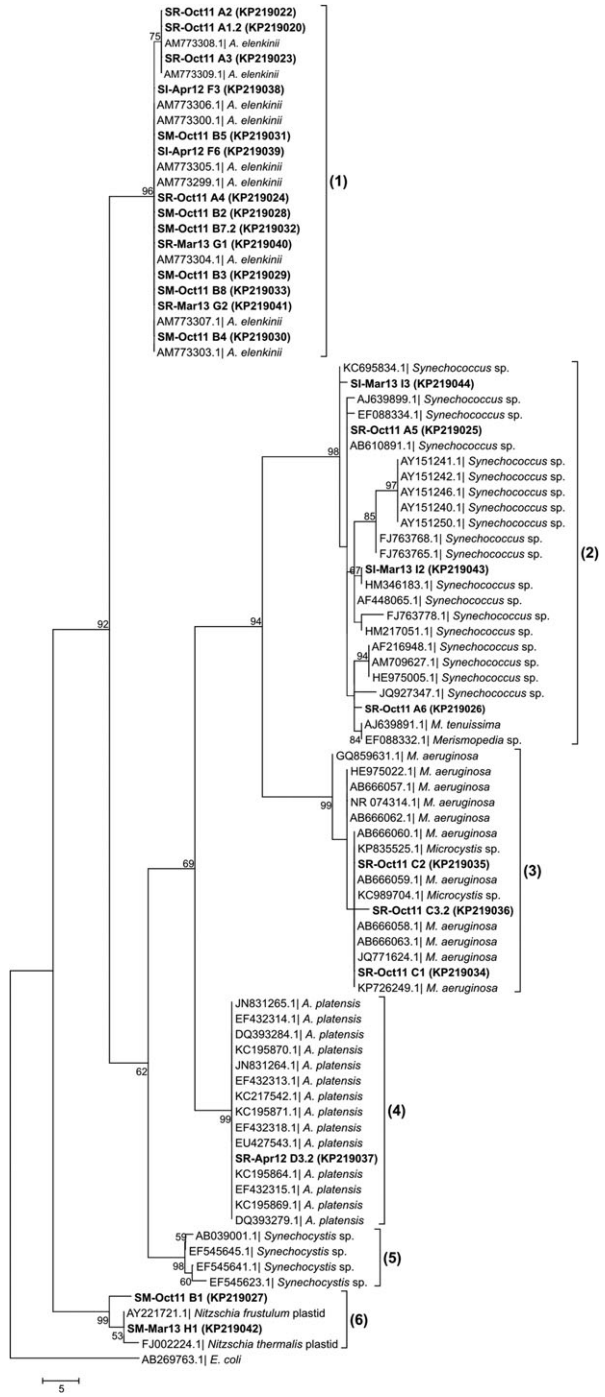

Fig. 4. Maximum parsimony tree based on 16S rRNA sequences from isolated DGGE bands. Representative 16S rRNA sequences were obtained from the GenBank. Those obtained in this work are given in bold letter (GenBank accession numbers in parenthesis). (Samples are identified as following: SI = Salina da Invernada, SM = Salina do Meio, SR = Salina da Reserva.) Qualitatively similar tree topologies were observed when using a Neighbor-joining algorithm.

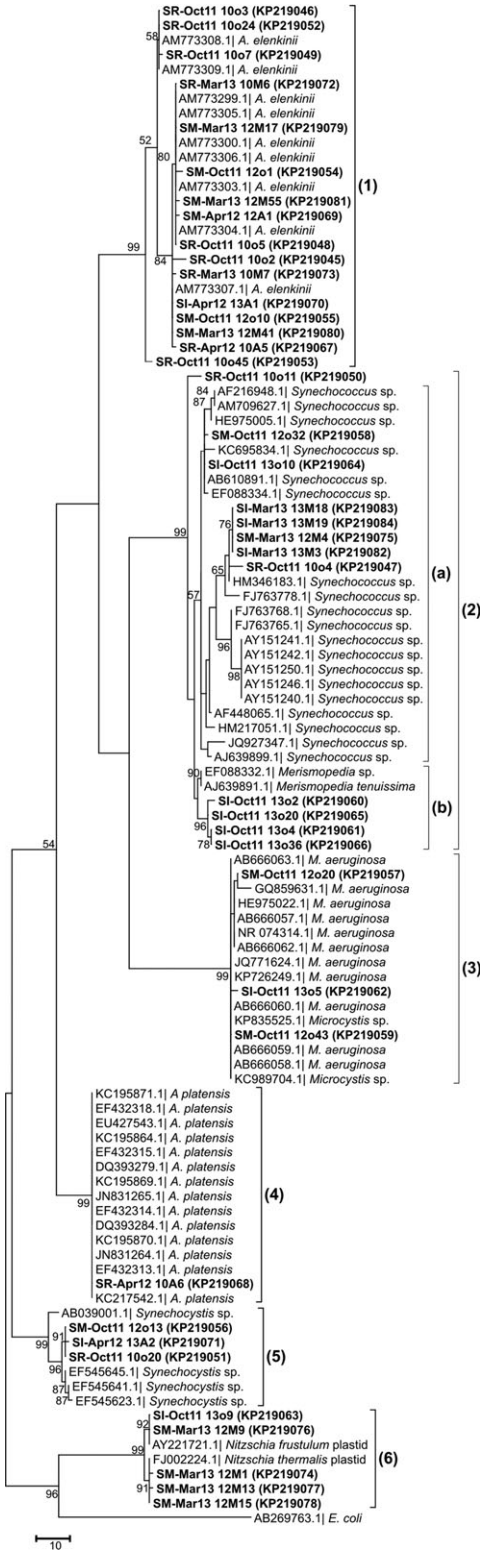

Fig. 5. Maximum parsimony tree based on 16S rRNA sequences from isolated ARDRA genotypes. Representative 16S rRNA sequences were obtained from the GenBank. Those obtained in this work are given in bold letter (GenBank accession numbers in parenthesis). (Samples are identified as in Fig. 3). Qualitatively similar tree topologies were observed when using a Neighbor-joining algorithm.

It is well known that cyanobacteria are favored under high nutrient conditions, especially when nitrogen and phosphorus are elevated (Giani *et al.*, 2005; Paerl and Paul, 2012). In alkaline saline lakes as well, thanks to their physiological adaptations, alkalophile cyanobacteria are the main primary producers (Grant and Sorokin, 2011). However, in these systems their dominance may also be explained by the reduced interactions with other species (interspecific competitors and predators) that are rare or absent as the result of the strong selective pressure of the environment. As Horváth *et al.* (2014) highlighted, salinity can be a major structuring factor in aquatic habitats, which constrains species composition, resulting in communities of low complexity where few tolerant species are able to grow and ensure high biomass production in the absence of antagonistic interactions.

Santos and Sant'Anna (2010), comparing three lakes in the Pantanal with different salt levels, observed that cyanobacteria richness was higher in the alkaline lake (Salina do Meio) than in the two freshwater systems. They collected samples in the dry and rainy periods, during 2004–2007, and detected the presence of *A. elenkinii* in all the samples from Salina do Meio, species that promoted blooms especially during the dry season. Vegetative cells and probable hormogonia of *A. platensis* were also registered in samples from Salina do Meio and in "Salitrada Campo Dora", a lake with intermediate water conductivity between the three studied (mean value of 874 µS/cm). Küfner (2014), when investigating the phytoplankton community of 14 freshwater lakes in the same region and same time of our study, observed that the relative cyanobacterial contribution to the total phytoplankton was not very high in the freshwater lakes, but increased during dry periods. The author found that cyanobacteria reached a maximum of 9% of the phytoplankton richness, while chlorophytes, for example, represented up to 34% of the community. In contrast, in our saline lakes, a low number of cyanobacteria species represented up to 100% of the total community (Table III), thus proving the strong selection pressure exerted by the alkaline saline environment on the community and supporting the idea that the absence of antagonistic interactions may favor dominance of few and more tolerant species (Horváth *et al.*, 2014).

In our study, the dry periods were represented by less diverse communities than those observed at the end of the inundation period, as confirmed by the GLM analysis. The significant decrease ($P < 0.001$) in cyanobacteria richness supports our initial hypothesis that even fewer species would be able to tolerate the intensified extreme conditions during drier periods. A similar pattern was observed in saline lakes across the world, such as the solar salterns in Spain (Estrada *et al.*, 2004) and the meltwater ponds in Antarctica (Jungblut *et al.*, 2005).

The two dominant species observed in this study during cyanobacterial blooms are already known for their ability to tolerate high conditions of salinity, conductivity and alkalinity. *Anabaenopsis elenkinii* has been previously suggested as an indicator of alkaline saline lakes in the Nhecolândia Pantanal, where it is extremely abundant, while it is rarely found in freshwater lakes of the same region (Santos and Sant'Anna, 2010). *Anabaenopsis elenkinii* has also been reported in other tropical ecosystems around the world, being preferentially found in alkaline and saline lakes, such as Lake Sonachi in Kenya, Lake Texcoco in Mexico (Ballot *et al.*, 2008), or the rift valley lakes in Kenya (Krienitz and Kotut, 2010), but also in some freshwater habitats, such as the Kazinga Channel in Uganda (Ballot *et al.*, 2008). The strains that showed the best BLAST similarity with the sequences isolated from Pantanal are those found by Ballot *et al.* (2008) in Kenya, Uganda, and Mexico (Fig. 4 and Fig. 5). Santos *et al.* (2011), studying the effects of different pH values on growth and morphology of a strain of *A. elenkinii* isolated from the Nhecolândia Pantanal, reported higher growth rates at pH values of 10.5, indicating that high pH may be a key variable promoting the presence of *A. elenkinii* blooms in these environments. Additionally, as all other Nostocales species, *A. elenkinii* can fix nitrogen through heterocysts, but the predominance of this species in the alkaline lakes of the Pantanal seems not to be related with nitrogen availability, since during the period of study the total nitrogen concentration in these three lakes was always very high (average 2.7 mM, min 0.3 and max 13.4 mM), thus nitrogen was probably never limiting the growth of other potential competing species. However, even if for this reason our present data did not show any statistical correlation between nitrogen and the heterocystous *A. elenkinii*, the changes observed in the dominance of N-fixing and non-fixing species suggest that this could be an interesting topic for further research in these environments.

The salinity tolerance of *A. platensis*, the other dominant cyanobacterium in our lakes, has been extensively studied, particularly because of the economic importance of this species (Vonshak and Tomaselli, 2000). The cells of *A. platensis* are a significant source of protein and vitamins and have been used, for example, to produce food supplements (Ayachi *et al.*, 2007). The genus *Arthrospira* is ubiquitous and naturally grows in water bodies with different salinity in tropical and subtropical regions. Typical populations of *A. platensis* have been described in alkaline lakes in Africa and populations of *Ar. maxima* in Lake Texcoco in Mexico (Ciferri, 1983).

Arthrospira platensis has been reported as a model organism for investigating the adaptation of photosynthetic organisms to extreme environments. Pogoryelov *et al.* (2003) found that this cyanobacterium exhibits a pH-dependent sodium requirement for the photosynthetic electron transport and reported that its cell functions in this cyanobacterium are optimized at high pH (9.5–11.5) and at salinity levels even higher than those described in our study (150–250 mM Na^+).

When cyanobacterial blooms were not observed, the Bacillariophyceae *Nitzschia* was detected as the most abundant photosynthetic organism. There are not many studies on Bacillariophyta in saline lakes, but Santos and Sant'Anna (2010) also reported the association of a Bacillariophyceae species with *A. elenkinii* blooms. Hecky and Kilham (1973) observed *Nitzschia frustulum* being favored in high alkalinity conditions in African saline lakes as a result of its wide salinity tolerance and of the reduced competition from other diatom species. Servant-Vildary and Roux (1990), studying salt lakes in the southern Bolivian Altiplano, and Fritz *et al.* (1993), studying lakes ranging from freshwater to hypersaline in the northern Great Plains of North America, reported a high correlation of diatom assemblages with ionic elements and salinity, respectively. Krienitz and Kotut (2010) also observed the appearance of *Nitzschia* in alkaline saline lakes in Kenya, following crashes of the dominant cyanobacteria species. Although the number of samples in our study is too small to characterize our observation as a trend, the occurrence of *Nitzschia* in alkaline saline lakes in the Pantanal finds support in the results described in these previous studies, which have already confirmed halotolerance in some *Nitzschia* species.

As we reported in the results, the primers used in our molecular analyses, specific for the 16S rRNA gene of cyanobacteria, were able to detect *Nitzschia* plastids in the samples where this species was very abundant. This is not unexpected since chloroplasts of eukaryotic organisms evolved from cyanobacteria cells (Falkowski *et al.*, 2004), and Presting (2006) already reported the existence of 16S rRNA gene sequences shared among algal plastids and cyanobacteria. The result observed, however, is important since it confirmed the presence of diatoms by a different methodology.

Akinetes of *A. elenkinii* were reported several times during this study (Fig. 3 and Table SIV). Akinetes are dormancy forms resulting from a physiological state of hibernation, which is broken through germination when suitable environmental conditions are restored (Komárek, 2005). During blooms, we detected some akinetes of *A. elenkinii* still attached to the filaments. The same akinetes, however as isolated cells, were also observed when no bloom and no filaments of this species were present (Salina da Reserva, in April/12), that is, when the environmental conditions were no longer appropriate to its growth. In this same sample however, molecular analyses detected the presence of *A. elenkinii* DNA (Fig. 5), even though only resting forms of this species were found by microscopy (Fig. 3 and Table SIV). This amplified DNA may be associated to the akinetes that were observed by microscopy. Ramm *et al.* (2012) have successfully isolated DNA from akinetes of different cyanobacterial species using lysozyme and proteinase K. They were able to amplify the 16S rRNA sequences and identify the akinetes. We used a similar protocol and it is probable that the sequences of *A. elenkinii* obtained originated from the observed akinetes.

Interestingly, during an extreme bloom of *A. platensis* in Salina da Reserva, when this species represented 88.7% of the total phytoplankton biovolume (Fig. 3), only abundant dormant akinete forms of *A. elenkinii* were observed and no vegetative cells. This may be evidence that these two species are excluding competitors, exploiting similar niches. For example, in two other samples (Salina da Reserva in October/11 and Salina do Meio in April/12) a strong bloom of *A. elenkinii* was recorded. At the same time, probable *A. platensis* hormogonia, whose induction to differentiation can be related to stressful environmental conditions (Meeks and Elhai, 2002), were detected. This could suggest that *A. elenkinii* and *A. platensis* are excluding competitors, unable to coexist once they occupy very similar niches (Hardin, 1960). Competitive exclusion of cyanobacterial species has already been reported in saline environments such as the Great Salt Lakes (Roney *et al.*, 2009), where the halotolerant *Aphanothece halophytica* seemed to be suppressed by the presence of *Nodularia spumigena*. Krienitz and Kotut (2010) also observed frequent shifts in dominant populations of cyanobacteria in alkaline saline lakes in Kenya. The dominant cyanobacterium *Arthospira* was periodically replaced by *Anabenopsis* and the authors discussed the importance of these shifts for the increasing episodes of Lesser Flamingo die-offs, because of the different nutritional quality of these two species. Laboratory experiments would be important to provide clues and to test the hypothesis of competitive exclusion between these two cyanobacteria found in the Pantanal lakes.

The distinction of the vegetative cells and resting stages, akinetes or hormogonia was possible only by microscopy. These different forms are distinct physiological states of the same species, resulting from differential gene expression when exposed to favorable or stressful growth conditions. Therefore, molecular approaches based solely on the detection of the 16S rRNA gene could not discern

between vegetative and other life-stages given that both belong to the same genotype.

No significant differences were found when comparing the numerical richness obtained by the three methods employed ($P = 0.32$), but it is clear that the molecular analyses allowed access to a distinct group of cyanobacteria not detected through microscopy, the picocyanobacteria. These organisms, whose size ranges from 0.2 to 2 μm, can represent an important fraction of the ecological community but are often unnoticed under light microscopy, leading to their underestimation in the phytoplankton community, unless specific microscopic techniques are used for their detection (Havskum *et al.*, 2004). In this work, the intense blooms promoted by few species and the consequent need for sample dilution for phytoplankton quantitative analyses may have reduced the recognition of the more inconspicuous and less abundant cyanobacteria, which were, however, detected through molecular methods. The detection of 16S rRNA gene sequences closely related to *Synechococcus* [cluster (2), Figs 4 and 5], *Merismopedia* [cluster (2b), Fig. 5] and *Synechocystis* [cluster (5), Figs 4 and 5] are evidence of the importance of the picocyanobacteria in these alkaline saline alkaline lakes. In a study based on morphological and molecular identification of isolated strains, Andreote *et al.* (2014) also described picocyanobacteria in five alkaline saline lakes from the Nhecolândia Pantanal.

One of the greatest advantages of applying molecular approaches for ecological studies is the enhanced ability to detect and identify microorganisms in nature (Amann *et al.*, 1995). Usually a larger diversity is found by molecular techniques when compared to the traditional morphological methods (Taton *et al.*, 2003; Estrada *et al.*, 2004; Willame *et al.*, 2006). Molecular methods not only allow the detection of very small organisms, overlooked through microscopy, but also permit the recognition of cryptic species and of different genotypes that display similar morphology within a population (Ernst *et al.*, 2003). In our study, for example, different DGGE bands and ARDRA profiles with high similarity to *A. elenkinii* strains showed different BLAST results (see supplementary material), probably representing distinct genotypes. The presence of distinct genotypes in the same population has been reported for several cyanobacterial taxa (Moore *et al.*, 1998; Melendrez *et al.*, 2011; Piccini *et al.*, 2011), and they may be very important to improve the survival chance of the species in a changing environment. Different genotypes in a population, also called ecotypes, are known to have divergent ecological preferences resulting in physiological adaptations that can lead to the dominance or prevalence of one or more ecotypes according to the growth conditions available in specific environments (Carlson *et al.*, 2009). We observed that, during the bloom, one genotype of *A. elenkinii* was always the most abundant, but the existence of the less abundant genotypes, representing different ecotypes, may characterize an important adaptive strategy of this species.

As a fingerprinting technique, DGGE provided a rapid and good profile of the environmental community composition for all samples. Due to its practicality, it is an advisable method for large temporal or spatial variability studies but, since DGGE usually does not show a high amplification efficiency of the isolated bands, it is more suitable for comparative rather than descriptive approaches. In comparison to DGGE, ARDRA is a time-demanding method, but it produces longer sequences with higher quality, therefore it is worthy especially for the description of less diverse communities. The genotypes identified through ARDRA confirmed those isolated in the DGGE bands; however, they allowed the construction of a more robust phylogenetic tree, detecting a larger genotypic diversity that included strains closely related to *Synechocystis* and *Merismopedia* and different 16S rRNA gene sequences from of *A. elenkinii*, *Synechococcus* and *Microcystis*. This difference probably relies on the distinct amplification efficiency of the methods, as well as on the fact that ARDRA sequences included more alignment positions (see Methods).

CONCLUSIONS

In summary, this study shows a prevailing dominance of cyanobacteria in alkaline saline lakes in the Nhecolândia Pantanal and remarkably dense blooms during dry periods. This evidence reinforces the versatility and high adaptability of some cyanobacteria species, particularly abundant in alkaline and saline environments, which are able to cope with extreme conditions. This is the first study analyzing the phytoplankton community in saline lakes of the Pantanal through morphological and molecular methods, and it contributes with new and reliable information to this barely known, yet extremely important wetland. The three different techniques used for the study of the cyanobacterial biodiversity produced the same trends, but the importance of a polyphasic study is emphasized by the fine distinction that each approach provided. For example, different physiological states, such as the appearance of resting stages, were easily detected by microscopic analyses, but not through the molecular methods based on 16S rRNA gene amplification. On the other hand, molecular approaches allowed a better detection of inconspicuous species, like picocyanobacteria, and were also essential for the

detection of different genotypes in the environmental populations. This study shows that the use of polyphasic techniques produced a more complete and reliable picture of the community.

DATA ARCHIVING

The 16S rRNA gene sequences are deposited in the NCBI GenBank under accession numbers KP219020 to KP219044 for DGGE isolated bands and KP219045 to KP219084 for clones.

ACKNOWLEDGEMENTS

We are especially grateful for D.C.L. Kufner, M.D. Oliveira and EMBRAPA staff for assistance on sampling and for chemical analyses of water samples. We also thank G. Patrício, R. Cortês and C. Leal from the Department of Sanitary and Environmental Engineering (DESA-UFMG) for help with DGGE analyses.

FUNDING

Scholarship to N.B.C. from Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG). We acknowledge the financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), with a grant to A.G. and the cooperation of Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) Pantanal.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org.

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