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First Report of Microcystins and Anatoxin-a Co-occurrence in San Roque Reservoir (Córdoba, Argentina)

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Abstract The aim of this study was to evaluate the presence of microcystin-LR, microcystin-RR, microcystin-YR, and the neurotoxin anatoxin-a in water samples collected monthly during 1 year in San Roque reservoir (Córdoba, Argentina) to identify the environmental factors that could promote the presence of these cyanotoxins. The HPLC-UV and MS/MS analysis showed the presence of microcystin in most of the sampling times, even when Cyanobacteria were subdominant. Microcystin concentrations varied from not detectable levels to 119.0 μg L^{-1}. Thus, they frequently surpassed the guidelines suggested by WHO for drinking water (1 μg L^{-1}) and recreational exposure (20 μg L^{-1}). To the extent of our knowledge, this is the first report of anatoxin-a in freshwaters in South America. Anatoxin-a concentrations varied from not detectable levels to 6.6 ng L^{-1}, a thousand times below the provisional guideline adopted by New Zealand for drinking water. Microcystin showed significant correlation with *Microcystis* and *Pseudoanabaena* while anatoxin-a correlated with *Oscillatoria* and *Anabaena* counts. Linear discriminant analysis showed that higher pH levels and more variable chlorophyll-a concentrations were measured in San Roque reservoir when cyanotoxins were present. Lower inorganic nitrogen concentrations were observed in autumn, when the prevalence of *Anabaena* became significant in Cyanobacteria composition and highest anatoxin-a levels were measured. The observed dynamic of phytoplankton going together with the cyanotoxins occurrence could be explained by the hypothesis of cyanotoxins acting as allelopathic compounds. The microcystin levels measured plus the presence of anatoxin-a show the need of stronger management efforts to preserve human and wildlife health.

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1 Introduction

Cyanobacteria are the dominant phytoplankton group in eutrophic freshwater bodies worldwide. However, no single factor has been directly related to bloom occurrence. A stable water column, warm water, high epilimnetic nutrient concentration (phosphorous, nitrogen, and organic compounds) low N/P ratio, low CO_2 availability, and low grazing rates by large zooplankton are advantageous conditions for the development of these blooms (Amé et al. 2003; Zurawell et al. 2005; Wilhelm et al. 2011).

Cyanobacterial blooms have adverse effects on freshwater aesthetics and recreation, and they are also associated with a number of other water-related problems, including foul odors, drinking water treatment, and fish kills due to oxygen depletion and ammonia release as cyanobacteria decay (Chorus et al. 2000).

A potentially hazardous consequence of cyanobac terial blooms is the production of potent toxins. Their toxic mechanisms to vertebrates are used to separate them into hepatotoxins (microcystins (MC) and nodularins), neurotoxins (anatoxin-a (ANTX), -a(s), homoanatoxin, saxitoxins), cytotoxins (cylindros permopsin), dermatotoxins (lyngbyatoxin), and irritant toxins (lipopolysaccharides) (Wiegand and Pflugmacher 2005). The frequency of toxicity in cyanobacterial blooms found in field studies have varied from 25 to 95 % in all situations investigated (Chorus 2001). The most ubiquitous cyanotoxins are the MC, a group of more than 85 cyclic hepapeptides that share the general structure cyclo-(D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-), in which X and Z represent variable L-amino acids, and Adda refers to the β-amino acid residue of 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. For example, when the variable amino acids are leucine and arginine, the MC is indicated as MC-LR (Sivonen and Jones 1999; Svrcek and Smith 2004; Del Campo and Ouahid 2010).

ANTX is a potent neurotoxin produced by several cyanobacterial genera, namely *Anabaena*, *Aphanizom enon*, *Microcystis*, *Planktothrix*, *Raphidiopsis*, *Arthrospira*, *Cylindrospermum*, *Phormidium*, *Nostoc*, and *Oscillatoria* (Osswald et al. 2007 and other authors therein referenced). Although ANTX is not the more frequent cyanotoxin worldwide, it has to be regarded as a health risk that can be fatal to terrestrial and aquatic organisms due to its high toxicity.

The occurrence of cyanobacterial toxic bloom is well-known in several countries. However, a lack of information exists with respect to South America, with a small number of official reports and/or published data for the majority of countries (Dörr et al. 2010). The presence of MC was reported in Argentina, Brazil, Chile, and Uruguay but according to Dörr et al. (2010), the low amount of existing data suggests deficient monitoring programs or unreported data in the countries where such programs are carried out. Moreover, reports about the presence of neurotoxins in South America waterbodies are limited to saxitoxins in Brazil (Lagos et al. 1999; Yunes et al. 2003; Molica et al. 2005; dos Anjos et al. 2006; Ferrão-Filho et al. 2009) and anatoxin-a(s) (Monserrat et al. 2001; Yunes et al. 2003; Molica et al. 2005; Becker et al. 2010). ANTX at detectable levels has never been reported in the scientific literature for South America.

San Roque reservoir (Córdoba, Argentina) is the main supplier of drinking water for Córdoba city and is a means of producing electric power. It is also an important recreational area, thus promoting an increase in the urbanization of the lake surroundings. Drainage from agricultural and urban areas has contributed to increased eutrophication of the water. Cyanobacterial blooms have occurred for about 30 years, as either mixed or single blooms of *Microcystis aeruginosa*, *Aphanizomenon flos-aquae*, *Anabaena circinalis*, and *Anabaena flos-aquae* at variable time intervals and with different intensities (Scarafia et al. 1995; Pizzolón et al. 1997).

From October 1998 to June 2002, 35 samples were collected during bloom events. The presence of MC-LR and MC-RR was confirmed in 97 % of studied cases, with concentrations ranging from 5.8 to 2,400 μg g^{-1} of freeze-dried material. Though the occurrence was very similar for both toxins, the highest concentrations correspond to MC-RR (Amé et al. 2003). Similar results were reported by Ruibal Conti et al. (2005) in samples collected from 1998 to 2000 in the same reservoir. MC concentration varied from very low (<0.050) to 450 μg L^{-1} in the center of the reservoir to 923 μg L^{-1} on the East shore.

The occurrence of MC and ANTX, either alone or together, should always be considered as a potential health hazard to the waterbody users. The study of

phytoplankton composition, environmental factors and cyanotoxins occurrence will provide a more comprehensive view on the bloom proliferation, function as well as production of these toxins that could help to develop effective management of the reservoir. Thus, the main goal of this study was to evaluate the presence of three common microcystins (MC-LR, MC-RR, and MC-YR) and anatoxin-a in water samples in San Roque reservoir and identify the environmental factors that could promote the presence of these cyanotoxins in this water body. A specific composition of the phytoplankton, average pH levels over 7.9, and lower inorganic nitrogen concentration could be the keys to cyanotoxins occurrence.

2 Material and Methods

2.1 Site Description and Sampling

San Roque reservoir (31°21′S, 64°30′W) is an artificial lake with a storage capacity of 350 hm^3. The surface area of the lake is 2,478 ha with a maximum depth of 35.5 m. It has two main tributaries: the San Antonio River (estimated annual mean flow= 3 $m^3\ s^{-1}$) and the Cosquín River (estimated annual mean flow=4.5 m $m^3\ s^{-1}$). It also has two minor tributaries: Los Chorrillos Stream (estimated annual mean flow=1.2 $m^3\ s^{-1}$) and Las Mojarras Stream (estimated annual mean flow=0.5 $m^3\ s^{-1}$; Wunderlin et al. 2001). Two monitoring sites were selected for this study: station 1 located in the mouth of San Antonio River (a heavily visited recreational area) and station 2 nearby the main water intake for Córdoba city (1.3 million of inhabitants). Water samples from both sites were collected monthly since February 2006 to March 2007 with the exception of April 2006 due to bad weather conditions.

2.2 Water Quality Analysis

The following water quality parameters were determined to establish the environmental conditions of sampling area. Analytical methods were standard; APHA (1998) method numbers are cited in parentheses. Measured parameters include: chlorophyll-a (10200-H-spectrophotometric), dissolved oxygen (DO, 4500-O G), pH (4500-H + B), total inorganic nitrogen (TIN, Ammonia (4500-NH_3 F); nitrates: 4500-NO_3^-E; Nitrites: 4500-NO_2^-B), total inorganic phosphorus (TIP, 4500-P-D), temperature (2550-B), and transparency (Secchi disk; Chapman 1992). Dissolved oxygen, pH, and temperature were monitored in the field with a Horiba U-23 Multiparameter Meter (Kyoto, Japan).

For the analysis of phytoplankton composition, samples were preserved in 3‰ Lugol's solution immediately after collection and enumerated with a standard microscope equipped with a Whipple grid following method 10200 C F proposed by APHA (2005).

2.3 Microcystins Extraction

For MCs analysis, two water samples (1 L) were taken at each sampling station from the surface, stored in dark glass bottles, ice refrigerated, and transported to the laboratory within 4 h. Then, water samples were filtered through membrane filters (0.45-μm pore size; Millipore, USA) using a vacuum pump. The filters were air-dried, packed in aluminum foil and stored at −20 °C. Filtered water samples were maintained in dark glass bottles at −20 °C until analysis (Ballot et al. 2005; Cazenave et al. 2005). Determination of MCs in water was carried out according to Amé et al. (2010). The applied method was modified from Harada et al. (1988). Briefly, 0.45-μm air-dried filters were placed in glass tubes, covered with 1.5 mL of 5 % acetic acid and sonicated for 5 min in an ultrasonic bath. The suspension was centrifuged at 9300×*g* for 3 min, supernatant was retained and the filters re-extracted as before. Combined supernatants were centrifuged at 9,300×*g* for 10 min. Centrifuged supernatants were applied to a C-18 solid-phase extraction cartridge (LiChrolut RP-18, 500 mg, Merck), which was previously conditioned with methanol (10 mL) and 5 % acetic acid (10 mL). The cartridge was washed with 10 mL of 10, 20, and 30 % aqueous methanol and toxins were eluted with 3 mL of pure methanol. The eluate was evaporated to dryness under reduced pressure (40 °C, 0.3 Torr) and resuspended in 200 μL of methanol prior to high-performance liquid chromatography (HPLC) analysis (Amé et al. 2003, 2010).

For the determination of free dissolved MCs, water samples (1 L) were conditioned with 5 % acetic acid and applied to C-18 solid-phase extraction cartridge (LiChrolut RP-18, 500 mg, Merck) previously washed

with methanol and further conditioned with 5 % acetic acid. Toxins were eluted using methanol (3 mL, HPLC grade). The eluate was evaporated to dryness at 40 °C under reduced pressure, suspended in 200 μL methanol (HPLC grade), and analyzed by HPLC (Amé et al. 2003; 2010).

Total MCs content in water was obtained by the addition of cellular and dissolved MC amount in the water sample.

2.4 Anatoxin-a Extraction

For ANTX extraction from water samples, the methods described by Araoz et al. (2005) and Dagnino and Schripsema (2005) were combined and applied with some modifications. In a typical run, 25 mg of freeze-dried cells were placed in 1.7-mL plastic tubes, extracted with 1.5 mL of 50 mM acetic acid, and sonicated for 5 min in an ultrasonic bath (Ney 300 Ultrasonik, USA). The suspension obtained was stirred with a Vortex (Boeco, Germany) for 1 min and centrifuged at 12,000×*g* for 15 min, retaining the supernatant and reextracting the pellet as before. The pH of the combined supernatants was adjusted to 9 with 6 M NH_4OH and then 3 mL of chloroform were added. After intensive shaking, the extract was maintained for 15 min at −20 °C and centrifuged at 10,000×*g* for 15 min. The chloroform phase was then separated, evaporated under N_2, and resuspended in 500 μL of methanol prior to HPLC analysis. This extraction is based in the fact that most alkaloids, as ANTX, are present in plants in the form of salts of organic acids. In consequence, they can be easily extracted by weak acidic solutions, as 50 mM acetic acid. If a base is added to the extract (i.e., NH_4OH), the alkaloids are converted to basic forms that are now soluble in organic solvents (like chloroform), which are further evaporated prior to analysis.

2.5 Microcystins and Anatoxin-a Analysis by HPLC-UV and MS/MS

The quantification of MC was performed by HPLC with UV detection, in according to our previous experience (Amé et al. 2003). HPLC was run on a Hewlett-Packard system equipped with a KONIC UV–VIS spectrophotometer, using a 4.6×250 mm Microsorb-MV 100 C18 (VARIAN, USA) column, with acetonitrile, 0.05 % trifluoracetic acid (50:50) as mobile phase, flow rate at 0.8 ml min^{-1}, column temperature at 20 °C, and UV detection at 238 nm. The identity of different variants of MC was confirmed by HPLC coupled to ESI-MS/MS according to Amé et al. (2010). Quantification was performed using external standard method, with pure MC dissolved in methanol (Sigma-Aldrich, USA), showing good linearity (R^2>0.995) for all the MC variants. The limit of detection (LOD) was taken at a signal to noise ratio of 3 (*S*/*N*=3), while the limit of quantification (LOQ) was taken as *S*/*N*=10. This calibration procedure affords a LOD=0.2 μg mL^{-1} and a LOQ=0.6 μg mL^{-1} in the injected solution, which are in good agreement with values reported in the literature (Moollan et al. 1996). Considering 1 L of water sample, we obtain a calculated LOD=0.04 μg L^{-1} and a LOQ=0.12 μg L^{-1}. Both samples and standard solutions were analyzed by triplicate.

Recovery percentages were evaluated from spiked samples. Thus, water samples were spiked with 1 and 10 mg L^{-1} pure MC, followed by SPE extraction and further HPLC–MS/MS. Recovery percentage in water samples was always over 85 % which are in good agreement with values reported in literature (Barco et al. 2005).

For the quantification of ANTX, a Varian 1200 triple quadrupole equipped with an ESI ion source was operated in positive mode using nitrogen as both drying (21 psi, 300 °C) and nebulizing gas (50 psi); voltages: needle 5,000 V and shield 600 V.

ANTX were recorded using MRM mode by selecting characteristic ANTX ions at the first quadrupole (Q1): 166 (ANTX, $[M+H]^+$). This ion was fragmented in Q2 using Ar (1.8 mTorr) and −10 V as collision energy to produce the ion at *m*/*z* 149, probably arising from the elimination of NH_3 from parent ion. Both remaining parent ion and *m*/*z* 149 fragment were separated at Q3 and detected at 1,800 V.

ANTX was quantified by HPLC coupled to ESI-MS/MS using a column Varian Polaris 5-μm C18-A (50×2.0 mm). Solvent delivery was performed at 0.25 mL min^{-1} (pumps: Varian Prostar 210 Dynamax), using 0.1 % formic acid in ultrapure water (A) and HPLC-grade methanol supplemented with 0,1 % formic acid (B). HPLC started with 20 % B, changing to 80 % B within 12 min, held by 5 min, returning to 20 % B in 1 min and keeping this condition for seven additional minutes to achieve column stabilization before next run (total run time 25 min). Samples and standard solutions were introduced in HPLC using a

Varian ProStar 410 autosampler equipped with a 20-μL loop. Quantification was performed using external standard method, with pure ANTX dissolved in ultrapure water (1 to 100 μg L^{-1}), showing good linearity (R^2>0.9986). Considering intra-day variation of noise from the ESI source, we decided to establish the limit of detection (LOD) at S/N=10, while the limit of quantification (LOQ) was taken at a S/N=80. This calibration procedure affords a LOD=1.3 μg L^{-1} (26 pg ANTX on column) and a LOQ=3.9 μg L^{-1} (78 pg ANTX on column). Considering that we started ANTX extraction from 25-mg freeze-dried cells, experimental LOD was 50 ng g^{-1} with a LOQ of 160-ng g^{-1} freeze-dried cells. Recovery percentage from freeze-dried cells was also evaluated, spiking with 50 pg of ANTX. Recoveries were always above 85 %, measured by triplicate. Samples and standard solutions were also analyzed by triplicate. The content of lyophilized material in water samples was also determined, then ANTX concentrations are expressed in microgram per liter. Calculated LOD and LOQ in water samples were 0.20 and 0.50 ng L^{-1}, respectively.

2.6 Statistics

All values are expressed as mean±standard deviation. Normal distribution for data was analyzed by Shapiro Willks test, while Levene test was used to check the homogeneity of variance. One-way ANOVA followed by Tukey test were carried out for comparing different treatments. Pearson correlation test was used to establish association between different variables. We applied linear discriminant analysis (LDA) according to our previous experience (Wunderlin et al. 2001; Amé et al. 2003; Monferrán et al. 2011). LDA can be used if it is known in advance of which particular groups objects are members. The LDA technique built up a linear discriminant function for each group. This function had the form shown in Eq. (1) (Johnson and Wichern 1992; Vandeginste et al. 1998).

$$f(Gi) = k_i + \sum_{j=1}^{n} w_{ij} p_{ij} \quad (1)$$

where i is the number of groups (G, two in this case); k_i is the constant inherent in each group; n is the number of parameters used to classify a set of data (water sample) into a given group (in this case n represents the number of analytical parameters used to assign a water sample into a given group); $w_{i,j}$ is the weight coefficient assigned by LDA to a given selected parameter ($p_{i,j}$); and $p_{i,j}$ is the analytical value of the selected parameter. LDA was applied to the experimental data, which had previously been standardized in order to improve the performance of the allocation rule.

The InfoStat/P software (2001) was employed in all cases. Significance was accepted for p<0.05.

3 Results

3.1 Water Quality

3.1.1 Physical, Chemical, and Biological Conditions

The descriptive statistics for the physical, chemical and biological variables measured in water are summarized in Table 1.

Chlorophyll-a levels varied between sampling stations, although significant differences were observed only during spring. At station 1, the highest concentrations were registered in summer and autumn while at station 2, observed values were similar in summer, autumn, and spring with lower levels during winter. As expected, the phytoplankton counts showed a similar trend with higher values during summer at station 1 and summer and spring at station 2.

Water was fully saturated with oxygen during summer and spring probably due to increased photosynthesis by the phytoplankton (Amé et al. 2010). Non-significant differences were observed between sampling sites and seasons when dissolved oxygen values were compared. Temperature ranged between 13.6 and 26.3 °C at station 1 and between 12.8 and 24.9 °C at station 2. Significant differences were found for this parameter at station 2 between seasons (Table 1). Similar TIN and TIP levels as well as pH values have been observed in other mesotrophic or eutrophic waterbodies (Ryding and Rast 1992; Amé et al. 2010). TIN levels showed significant differences between seasons at station 1. The higher values were measured during winter. As well during winter and spring, TIN were significantly different between stations (Table 1). Even when TIP showed superior levels in summer than during other seasons, at station, 1 it was significantly different to

Table 1 Water quality parameters in water from San Roque Reservoir evaluated during the studied period

Parameter	Season	Station 1	Station 2
Chlorophyll-a (μg L^{-1})	Annual	79.27±95.35	27.30±16.73
	Summer	151.17±133.61	29.65±14.47
	Autumn	113.93±93.15	25.09±21.34
	Winter	18.21±0.00	6.62±0.00
	Spring	3.26±0.12*	31.81±19.23
DO (mg L^{-1})	Annual	9.20±3.05	8.59±3.80
	Summer	11.86±5.44	11.75±6.89
	Autumn	7.93±1.41	6.54±2.98
	Winter	6.60±0.00	5.95±0.00
	Spring	9.12±0.29	8.72±2.35
pH	Annual	8.19±0.97	7.63±0.41
	Summer	8.47±0.04* a,b	7.70±0.26
	Autumn	8.92±1.18 b	7.64±0.36
	Winter	8.19±0.27 a,b	8.14±0.37
	Spring	6.84±0.37 a	7.33±0.39
Phytoplankton (cell mL^{-1})	Annual	1.3E+7±2.3E+7	7.6E+6±1.1E+7
	Summer	4.3E+7±4.5E+7	1.3E+7±1.1E+7
	Autumn	1.0E+7±9.4E+6	1.4E+6±9.7E+5
	Winter	3.9E+6±2.0E+6	2.4E+6±9.2E+5
	Spring	6.5E+5±1.6E+5	1.0E+7±1.6E+7
Temperature (°C)	Annual	19.9±5.0	20.9±4.8
	Summer	25.0±1.9	24.5±0.4 b
	Autumn	21.3±5.4	20.1±5.2 b
	Winter	14.0±0.4	13.2±0.6 a
	Spring	21.3±0.9	23.3±1.2 a,b
Total Inorganic Nitrogen (mg L^{-1})	Annual	0.97±1.12	0.40±0.33
	Summer	0.42±0. 29 a	0.47±0.63
	Autumn	0.15±0.07 a	0.17±0.08
	Winter	3.29±0.00* b	0.43±0.09
	Spring	1.18±0.26* a	0. 50±0.24
Total Inorganic Phosphates (mg L^{-1})	Annual	0.11±0.49	0.09±0.05
	Summer	0.17±0.03 b	0.15±0.0 7
	Autumn	0.08±0.01 a	0.07±0.04
	Winter	0.13±0.00 b	0.05±0.03
	Spring	0.06±0.01 a	0.09±0.02
Transparency (m)	Annual	0.8±0.2	1.40±0.31
	Summer	0.7±0.3*	1.5 ±0.2 b
	Autumn	0.9±0.5	1.7 ±0.2 b
	Winter	0.8±0.1*	1.4±0.1 a,b
	Spring	0.6±0.1*	1.0±0.1 a

Different letters indicate mean significant differences between seasons

*$p<0.05$ significant differences between monitoring stations

autumn and spring but not for winter. At station 2, TIP levels were not significantly different between seasons. In the same way, significant differences were observed for pH among seasons only at station 1. The highest

values were measured in autumn while the lowest were observed in spring. Significant differences between sites were only detected in summer (Table 1). Transparency was always lower at station 1. However, significant differences between seasons were only found for this parameter at station 2 with minor levels during spring (Table 1).

3.1.2 Phytoplankton Community Dynamics

Figure 1a, b show the phytoplankton community dynamics at stations 1 and 2, respectively. Six algae classes have been identified in San Roque reservoir showing different prevalence at each monitoring station. At station 1, the prevalence of Cyanobacteria during monthly monitoring was observed from December to March (summer–autumn), of Dinophyta in May (autumn) and of Crysophyceae from June to November (winter–spring). At station 2, the prevalence of Cyanobacteria was observed during the same period (summer–autumn) but also in June. Crysophyceae was the dominant algae class in May (autumn) and Criptophyta from August to November (winter–spring).

Chlorophyta as well as Dinophyta were present almost during the whole year but never at high abundances (Fig. 1a, b). Euglenophyta were only found in a smaller proportion during January at station 1.

Among Cyanobacteria, four genera were identified in San Roque reservoir during the studied period (Fig. 1c, d). *Microcystis* sp. was the most abundant genus from December to March (summer–autumn) at both stations. This dominance was coincident with Cyanobacteria prevalence in the reservoir. The genus *Pseudoanabaena* sp. contributed the largest proportion of the Cyanobacteria in October, while *Anabaena* sp. became the dominant species from May to August at station 1 (autumn–winter) and from June to August and during November (autumn–winter–spring) at station 2. The cyanobacteria *Oscillatoria* was dominant during November but only at station 1. Cyanobacteria were not found at San Roque Dam in September sampling.

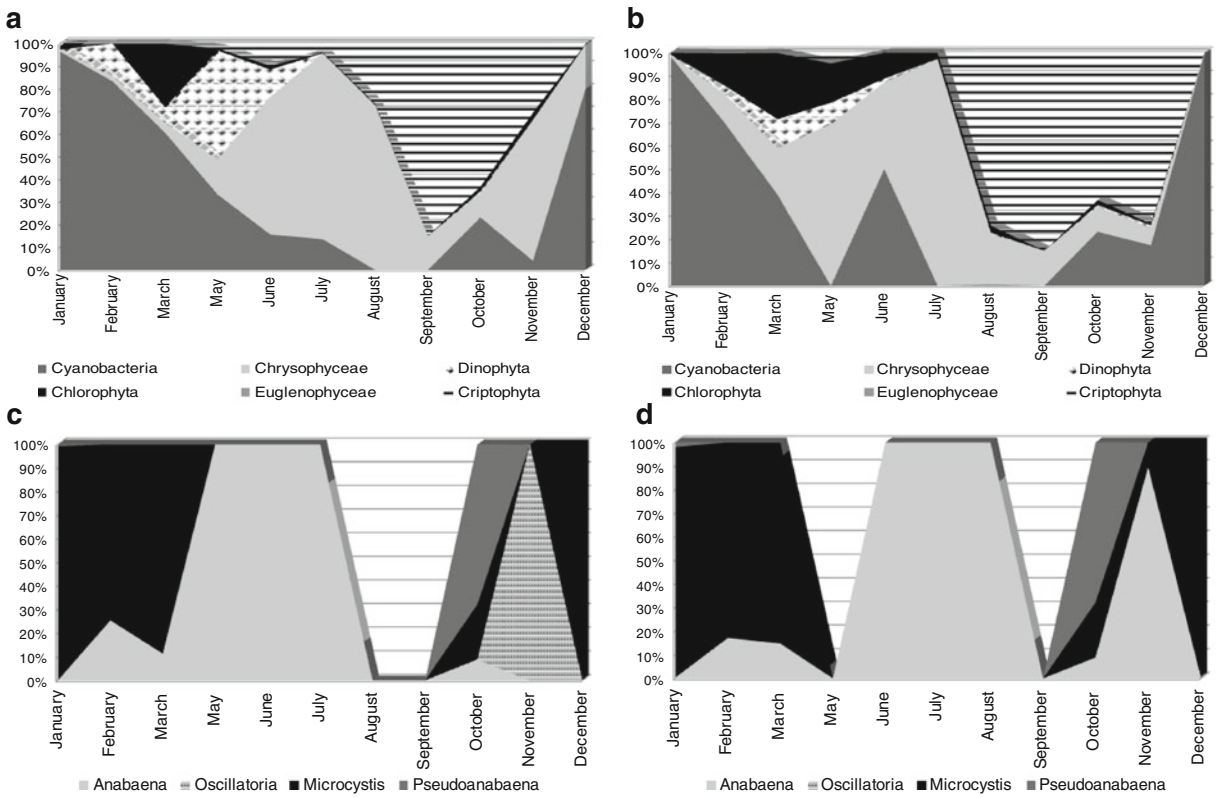

Fig. 1 Phytoplankton and Cyanobacteria dynamics in San Roque reservoir. **a** Phytoplankton composition at station 1; **b** Phytoplankton composition at station 2; **c** Cyanobacteria composition at station 1; **d** Cyanobacteria composition at station 2

3.2 Cyanotoxins in San Roque Reservoir

Total MC, MC-LR, MC-RR, MC-YR, and ANTX concentrations in water from San Roque reservoir during the studied period are shown in Table 2.

The HPLC-UV and MS/MS analysis showed the presence of microcystin in most of the sampling times, even when Cyanobacteria were subdominant. Total MC concentrations varied from not detectable levels to 119,00 μg L^{-1}. The average total MC amount was higher at station 2. However, this parameter showed clear seasonality by monitoring station reaching higher values during summer at station 1 and during spring at station 2. Due to high variability, none of these differences were statistical significant ($p<0.05$). For instance, at station 1 the lowest levels of total MC were observed in January (6.23±2.01 μg L^{-1}) while the highest concentrations were measured in February (12.18±3.94 μg L^{-1}). At station 2, lower concentrations were observed in September (0.20±0.28 μg L^{-1}) while the higher values were detected in December (119.00±0.21 μg L^{-1}).

Three variants, MC-LR, MC-RR, and MC-YR, were identified in water samples of San Roque reservoir. MC-RR was the main toxin found in cellular and dissolved fractions (5.54±24.29 and 0.15±0.41 μg L^{-1} cellular and dissolved respectively). MC-LR showed higher

Table 2 Total MC, MC-LR, -RR, –YR and anatoxin-a concentrations in water from San Roque Reservoir, evaluated during the studied period

Parameter	Season	Station 1	Station 2
Total MC (μg L^{-1})	Annual	2.17±3.98	8.84±31.71
	Summer	9.21±4.21	0.67±0.74
	Autumn	0.49±0.22	<LOD
	Winter	0.51±0.67	0.19±0.16
	Spring	0.15±0.21	20.23±48.39
MC-LR (μg L^{-1})	Annual	0.51±1.25	0.11±0.18
	Summer	2.02±2.86	0.27±0.25
	Autumn	0.25±0.22	<LOD
	Winter	<LOQ	0.15±0.21
	Spring	<LOD	<LOQ
MC-RR (μg L^{-1})	Annual	1.59±3.79	8.62±31.77
	Summer	7.19±7.06	0.40±0.50
	Autumn	<LOD	<LOD
	Winter	0.42±0.73	<LOD
	Spring	0.15±0.21	19.92±48.54
MC-YR (μg L^{-1})	Annual	0.07±0.23	0.11±0.30
	Summer	<LOD	<LOD
	Autumn	0.25±0.43	<LOD
	Winter	<LOD	<LOQ
	Spring	<LOD	0.25±0.45
Anatoxin-a (ng L^{-1})	Annual	0.68±1.30	<LOQ
	Summer	<LOD	<LOQ
	Autumn	2.70±1.60	<LOQ
	Winter	<LOQ	<LOQ
	Spring	<LOD	<LOQ

LODs: MCs 0.04 μg L^{-1}, ANTX 0.20 ng L^{-1}; LOQs: MCs 0.12 μg L^{-1}, ANTX 0.50 ng L^{-1}

Different letters indicate mean significant differences between seasons

<*LOD* below detection limit, <*LOQ* below quantification limit

levels of cellular toxin (0.19±0.52 and 0.08± 0.32 μg L^{-1} cellular and dissolved respectively) while MC-YR was only detected in the cellular fraction (0.10 ±0.27 μg L^{-1}). MC-LR, MC-RR, and MC-YR did not show significant differences between dissolved and cellular content ($p<0.05$).

MC variants identified in San Roque reservoir showed different dominance at each monitoring station. At station 1, the prevalence of MC-RR was observed during winter, spring, and summer, while autumn was differentiated by the co-dominance of MC-LR and MC-YR (Table 2). At station 2, the co-occurrence of MC-LR and MC-YR was observed in winter, while MC-RR dominance was observed during spring and summer. MC were not detected at station 2 during autumn (Table 2). Once again, the high variability observed did not allow to detect statistical significant differences ($p<0.05$).

Spatial and temporal variations were also observed for ANTX concentrations. This cyanotoxin was always present at station 2 but below quantification limits (between the limit of detection=0.20 and 0.50 ng L^{-1}). In contrast, at station 2, maximum levels of ANTX were observed during autumn, it was detected but below LOQ in winter and it was not detected at all during spring and summer (Table 2).

The co-occurrence of both MC and ANTX was always dependent on the monitoring station. At station 1, the presence of both cianotoxins was observed during March, May, and July (autumn and winter), while at station 2 was reported in February, July, and October (summer, winter, and spring).

3.3 Associations Between Cyanotoxins Presence and Water Quality

Pearson correlation analysis was used to establish associations between cyanotoxins (total MC and ANTX) in water and the number and composition of the phytoplankton community. Total MC content showed a significant correlation with the phytoplankton quantity ($r=0.46$, $p<0.05$) and the proportion of Cyanobacteria in the phytoplankton community ($r=0.51$, $p<0.05$). In contrast, ANTX concentration showed a significant correlation with Dynophyta and Chlorophyta composition of the phytoplankton community ($r=0.53$ and $r=0.48$ respectively, $p<0.05$).

A similar statistical analysis was run in order to determine associations between cyanotoxins (total MC and ANTX) in water and the Cyanobacteria composition. Total MC content showed a significant correlation with *Pseudoanabaena* sp. ($r=0.55$, $p<0.05$) and *Microcystis* sp. ($r=0.51$, $p<0.05$). In contrast, ANTX levels were positive associated with *Oscillatoria* sp. and *Anabaena* sp. presence ($r=0.48$ and $r=0.40$ respectively, $p<0.05$).

Afterward, in order to point out which parameters of water quality could be associated with the presence or absence of cyanotoxins in San Roque reservoir, we used stepwise linear discriminant analysis (LDA; Wunderlin et al. 2001; Amé et al. 2003; Monferrán et al. 2011).

Thus, the first LDA was performed by considering the presence or absence of the cyanotoxins measured as grouping variable and 19 independent variables including chemical parameters and phytoplankton composition of water samples. This analysis allows 88 % discrimination among water samples collected in presence or absence of cyanotoxins (data not shown), affording three descriptors: pH, chlorophyll-a, and *Anabaena* sp. cells for distinguishing between both situations. Box and whisker plots and canonical coefficients for the selected parameters pointed out by LDA are shown in Fig. 2 and Table 3, respectively. As can be seen from Fig. 2, pH and chlorophyll-a show higher mean values in presence of cyanotoxins while *Anabaena* sp. presents a wider dispersion of cells counts when the toxins were not detected.

Subsequently, two additional LDA were performed to determine associations between measured parameters of water samples and the presence of either Total MC or ANTX.

Results evidenced that the water of San Roque reservoir has different characteristics when MC occurs (75 % different, data not shown). Parameters that mainly point out those differences include both chemical parameters (pH) and phytoplankton composition (Chlorophyta, Crysophyceae, and Dynophyta percentage). Canonical coefficients for these four selected parameters are also shown in Table 3. The presence of MC in water samples was associated with an increase on pH values and a decrease in Chlorophyta and Crysophyceae composition of the phytoplankton community. No changes were observed for mean Dynophyta percentages (Fig. 3).

The following LDA was carried out considering the occurrence of ANTX as grouping variable. The classification matrix for this analysis afforded a 92 % right

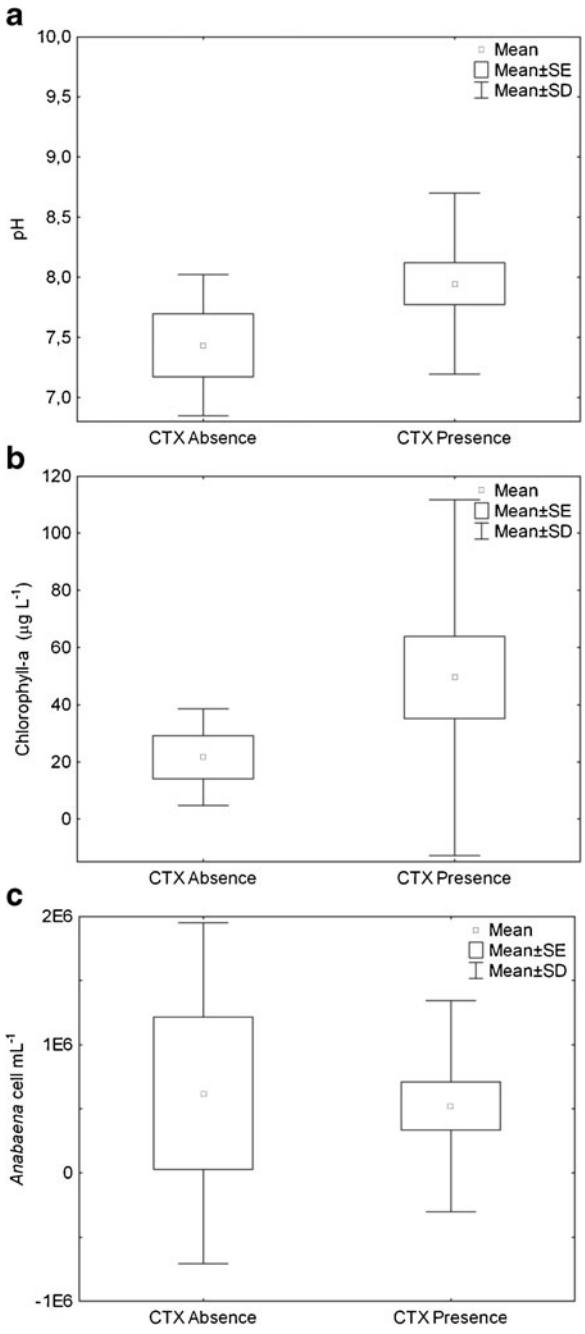

Fig. 2 *Box* and *whisker* plots showing mean, SE and SD of parameters indicated by LDA as significant to discriminate between water samples with or without cyanotoxins (MC and ANTX, either together or alone). **a** pH; **b** chlorophyll-a; **c** *Anabaena* cells

discrimination between water samples collected in absence and presence of ANTX. The variables that mainly indicate those differences are again chemical parameters (pH and total Inorganic nitrogen) and phytoplankton community composition (Crysophyceae and Criptophyta percentage as well as *Microcystis* sp. and *Pseudoanabaena* sp. cells). Canonical coefficients for these parameters are shown in Table 3. Box and whisker plots (Fig. 4) show that ANTX presence was also associated with higher pH levels (7.66±0.57 during absence; 8.33±0.83 during presence). On the contrary, lower TIN levels were associated with ANTX presence (0.91±1.00 mg L^{-1}during absence; 0.59±1.10 mg L^{-1}during presence). ANTX occurrence was also associated with changes in phytoplankton composition showing higher proportion of Crysophyceae and lower amounts of Criptophyta cells. Among Cyanobacteria, a decrease in *Microcystis* sp. and no significant changes in mean *Pseudoanabaena* sp. cells counts were associated with the presence of ANTX.

Finally, a LDA was carried out considering the co-ocurrence of both MC and ANTX as grouping variable. The application of this statistical analysis allows 88 % discrimination among water samples in presence or absence of the cyanotoxins (data not shown), affording seven descriptors: pH, DO, chlorophyll-a, Cyanobacteria, and Dynophyta percentage as well as *Microcystis* sp. and *Pseudoanabaena* sp. cells for distinguishing between water samples collected during both scenarios. Once again, pH points out the differences between the presence and absence of cyanotoxins with higher values associated with the occurrence of them. A similar pattern was observed for DO, chlorophyll-a, Dynophyta percentage, and *Pseudoanabaena* sp. cells. The mean composition on Cyanobacteria of the phytoplankton remains without important changes while the mean value for *Microcystis* cells diminishes during the presence of MC and ANTX (Fig. 5).

4 Discussion

Many studies indicate that an increase in average temperature could lead to a higher frequency of toxic cyanobacterial blooms in the future (Paerl and Huisman 2008; O'Neil et al. 2012).

Blooms of cyanobacteria are often comprised of toxic and non-toxic strains of the same or different species. Population dynamics and the factors influencing their relative contributions to cyanobacteria and phytoplankton communities are still poorly understood (El-Shehawy et al. 2012). The role played by environmental factors on

Table 3 Classification functions (Eq. (1)) calculated by linear discriminant analysis

Parameter	Cyanotoxins		MC		ANTX		MC and ANTX	
	Absence[a]	Presence[a]	Absence[a]	Presence[a]	Absence[a]	Presence[a]	Absence[a]	Presence[a]
pH	−1.08666	0.283382	−1.00511	0.416676	−0.66152	1.32389	−1.16438	3.49669
Cyanobacteria %							−0.40375	1.19909
Chlorophyta %			0.85679	−0.353287				
Crysophyceae %			0.59353	−0.243038	−1.16823	2.34757		
Dynophyta %			0.55005	−0.230597			0.32967	−1.00285
Criptophyta %					0.50255	−0.99060		
Microcystis					0.55158	−1.08730	0.66927	−1.99266
Anabaena	1.22227	−0.317784						
Pseudoanabaena					−0.65984	1.30452	−0.27866	0.81706
Total inorganic nitrogen					1.10781	−2.22378		
DO							−0.27300	0.82780
Chlorophyll-a	−0.66952	0.175917					0.42915	−1.28317
Constant (Eq. (1))	−2.0505	−0.266504	−1.73952	−0.431182	−0.79688	−2.65827	−0.47705	−3.08089

[a] Discriminant function coefficients for presence and absence of cyanotoxin correspond to $w_{i,j}$ as defined in Eq. (1)

the presences of potentially toxic species and toxin production is not clear as results are contradictory and the specific effects of single or combined factor vary in different environments (Rinta-Kanto et al. 2009).

Through the present study, it was intended to contribute to the comprehension of this complex phenomenon by identifying abiotic environmental conditions and phytoplankton composition that could be associated with the presence or absence of MC and ANTX either isolated or together.

San Roque reservoir has been classified as a eutrophic waterbody with elevated concentrations of nutrients and an associated high biomass production (Amé et al. 2003; Cazenave et al. 2005; Ruibal Conti et al. 2005). As expected, during warmer seasons (summer, autumn, and spring) higher chlorophyll-a and phytoplankton counts were measured. The dynamics of the phytoplankton community showed the dominance of Cyanobacteria during months with higher temperatures and of Chrysophyceae and Criptophyta during the colder ones, pointing out that period with the probable higher risk associated with the presence of cyanotoxins. However, as a consequence of the frequent cyanobacterial occurrence in San Roque reservoir, almost all samples contained MC, even when Cyanobacteria were subdominant. Although the frequency of MC-positive samples is one of the highest among similar surveys worldwide (Chorus 2001), MC concentrations were usually below 10 μg L^{-1} and only rarely exceeded 100 μg L^{-1}. This study's survey showed that, as previously stated, the threat associated with MC in water rises in spring and summer, when significantly higher MC were measured (Amé et al. 2003).

Recreational exposure to cyanotoxins can pose substantial hazards to public health. However, confusing factors such as the age of users, many potential exposure pathways, and varying durations of exposure (Chorus et al. 2000; WHO 2003) mean that guidelines for acceptable recreational exposure range from the more conservative 20 μg L^{-1} MC suggested by WHO (2003) to as high as 100 μg L^{-1} MC (Fromme et al. 2000). In the present study, MC concentrations frequently surpassed the level suggested by WHO for drinking water (1 μg L^{-1}), exceeded the suggested recreational exposure guideline and are in good agreement with other MC levels observed in South America (Dörr et al. 2010).

When analyzing the presence of different MC variants, the pattern observed was dissimilar between sampling stations. Even when MC-RR was the dominant variant, MC-YR and MC-LR dominated during different seasons (autumn at station 1, winter at station 2). This is likely a consequence of different cyanobacterial species and strain compositions during different seasons (Znachor et al. 2006). Further studies on the genetic diversity of cyanobacteria population in this freshwater

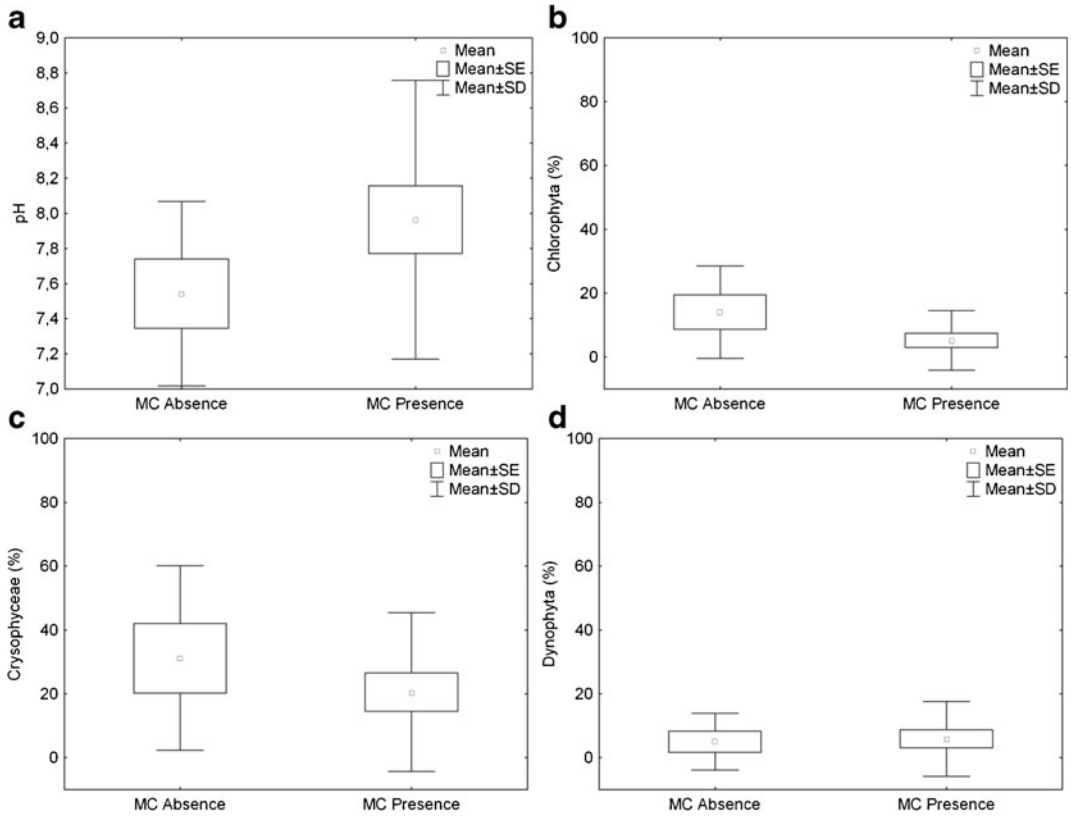

Fig. 3 *Box* and *whisker* plots showing mean, SE and SD of parameters indicated by LDA as significant to discriminate between water samples with or without MC. **a** pH; **b** Chlorophyta %; **c** Crysophyseae %; **d** Dynophyta %

body could reveal the interactive influences between dynamics in cyanobacteria cells and cyanotoxins and environmental factors (Xu et al. 2011).

Despite early characterization studies on ANTX (Devlin et al. 1977), the detection of ANTX in the field is rare, and impeded by the rapid degradation of these toxins to form non-toxic compounds and by the availability and complexity of the methods of detection (Gugger et al. 2005). Nevertheless, this toxin has been reported in North America, Europe, Africa, Asia, and New Zealand. Osswald et al. (2007) reviewed the occurrence of ANTX published until 2005. Concentrations found in different water bodies varied from 0.01 to 444 μg L^{-1} or from 0.003 to 8,000 μg g^{-1} DW (Osswald et al. 2007 and authors therein referenced). The levels of ANTX in San Roque reservoir should be considered among the lowest detected.

New Zealand adopted a provisional national allowed value for drinking water of 6 μg L^{-1} (MEMH 2009). Maximum values found in the present study are a thousand times below these limits. Nevertheless, it was present along the complete studied period at station 2 and during autumn and winter at station 1. ANTX toxicity should never be disregarded. To the extent of our knowledge, this is the first report of detection of ANTX in freshwater sources in South America and responsible authorities also have to be aware of this toxin considering it as a potential cause of animal and human fatalities. Risk of intoxication with ANTX is, as well as MC, of significant concern to wildlife and aquatic species.

Most environmental samples are composed of several codominant species and additional subdominant species (Znachor et al. 2006). Thus, it is difficult to attribute cyanotoxin production to one particular species. In the present study, MC concentration showed a strong positive correlation with phytoplankton quantity and Cyanobacteria as dominant species in this community. Moreover, total MC content showed also significant correlation with *Microcystis* sp. and *Pseudoanabaena* sp. counts. Both cyanobacteria have been described as cyanotoxin producers (Chorus and

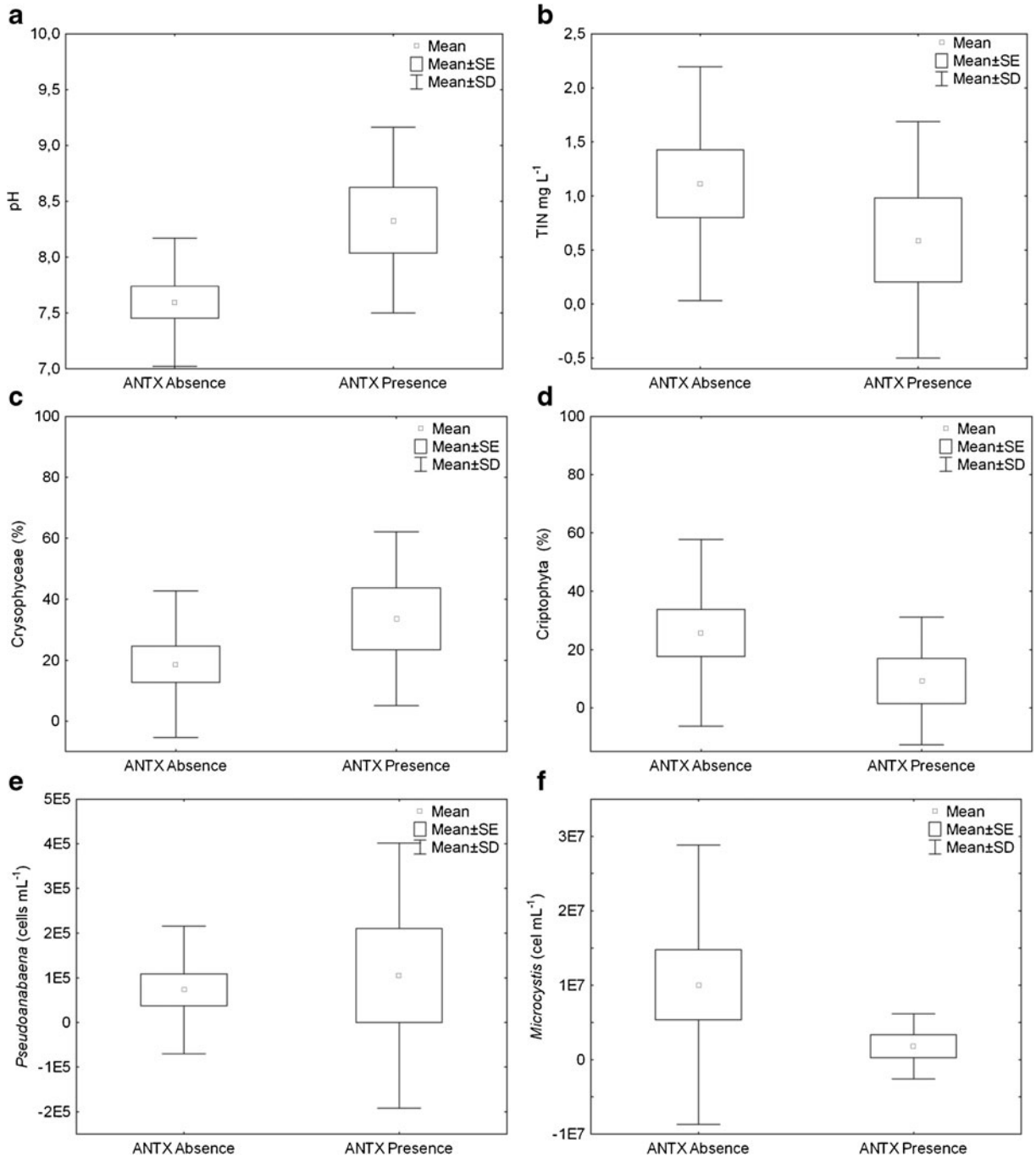

Fig. 4 *Box* and *whisker* plots showing mean, SE and SD of parameters indicated by LDA as significant to discriminate between water samples with or without ANTX. **a** pH; **b** total inorganic nitrogen (TIN); **c** Crysophyseae %; **d** Criptophyta %; **e** *Pseudoanabaena* cells; **f** *Microcystis* cells

Bartran 1999; Oudra et al. 2001; Marsalek et al. 2003). Moreover, similar positive correlations in *Microcystis*-dominated samples were found in other studies (Znachor et al. 2006; Davis et al. 2009).

Different results were obtained for ANTX since higher concentrations of this toxin were significantly correlated with an increase of Dynophyta and Chlorophyta composition on the phytoplankton community instead of

Cyanobacteria. Nonetheless, a strong positive correlation between ANTX concentration and *Oscillatoria* sp. and *Anabaena* sp. suggests that they were the major ANTX-producing cyanobacteria in San Roque reservoir.

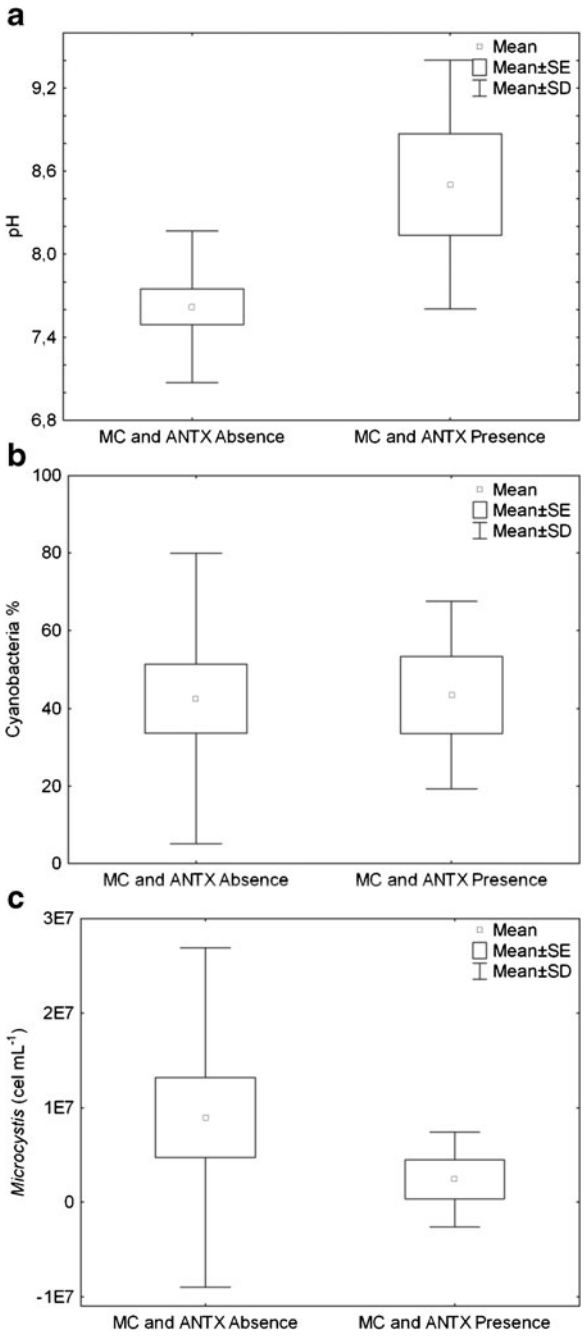

Fig. 5 *Box* and *whisker* plots showing mean, SE and SD of selected parameters indicated by LDA as significant to discriminate between water samples with or without MC and ANTX. **a** pH; **b** Cyanobacteria %; **c** *Microcystis* cells

In a natural habitat, many parameters interact to produce a specific consequence. We decided to use LDA, a multivariate statistical technique, to evaluate the difference between water samples collected in presence or absence of cyanotoxins (MC and ANTX, either alone or together). LDA has advantages over other multivariate techniques because it allows the quantification of the differences between groups and also provides an indication of those parameters strongly associated with these differences.

In all the analysis performed, the two groups of water samples could be differentiated with high certainty (75 to 92 %) and up to 7 of the 19 measured parameters expressed the main differences between these two groups (Table 3).

From Figs. 3 to 5, it can be seen that higher pH levels were measured when cyanotoxins (MC, ANTX, or both) were present. Average pH levels were around 7.9 when MC were present, around 8.2 when ANTX were present, and 8.5 when both toxins were present. This result is in good agreement with other reports (Wicks and Thiel 1990). The pH values are usually higher during a bloom event due to the photosynthesis of algae and cyanobacteria. In return, a high pH condition is advantageous to the growth of cyanobacteria with their carbon-concentrating mechanisms (Kardinaal and Visser 2005). This eventually increases the toxin production as the cells grow under favorable condition (Orr and Jones 1998; Kaebernick and Neilan 2001).

Moreover, higher and more variable chlorophyll-a concentrations were observed in those water samples with cyanotoxin presence (Fig. 2). The correlation between cyanotoxin and chlorophyll-a concentrations in water have been largely discussed. Previous studies in San Roque reservoir showed that an abundance of cyanobacteria was not necessarily connected with the presence of high amounts of MC (Amé et al. 2003). Davies et al. (2009) found that only two out of five studied lakes displayed mildly significant correlations between chlorophyll-a concentrations and concentrations of MC, while only three showed significant correlations between total cyanobacterial cell counts and MC concentrations. Considering that all phytoplankton contain chlorophyll-a and nearly every major species of cyanobacteria has both toxic and non-toxic strains (Chorus and Bartran 1999), this result is quite expected.

Total inorganic nitrogen in water samples was significant to discriminate between samples with or without ANTX. Inorganic nitrogen depletion in the water

was shown to support the development of filamentous N_2-fixing cyanotacteria as *Anabaena* rather than *Microcystis* (Znachor et al. 2006). Lower TIN levels were observed in autumn when the prevalence of *Anabaena* became significant in Cyanobacteria composition. This change was accompanied with the higher ANTX levels.

The composition of the phytoplankton seems to be another crucial environmental factor to determine the presence of the cyanotoxins. The Pearson correlation analysis showed a strong correlation between Cyanobacteria dominance in the phytoplankton community with MC content in water samples. However, LDA indicate also that a decrease in Chlorophyta and Crysophyceae composition of the phytoplankton was significant to differentiate water samples with or without MC. In contrast, ANTX presence in water was observed when an increase in Crysophyceae composition of the phytoplankton occurred; while a lower amount of Criptophyta cells were present. The co-occurrence of both MC and ANTX occurred with an augment in Dynophyta composition. Therefore, a dynamic of cyantoxins presence could be taking place in San Roque reservoir going together with changes in the phytoplankton. During summer months, the prevalence was of Cyanobacteria and MC. In autumn and the beginning of winter, the composition of the phytoplankton turn to a more diverse community (increasing mainly Chrysophy .ceae and Dynophyta) accompanied by the emergence of ANTX. Late winter is dominated by Dynophyta with less risk associated to cyanotoxin presence, returning in spring slowly to summer scenario.

Finally, LDA indicate *Pseudoanabaena* sp. and *Microcystis* sp. cell counts to discriminate between samples containing or not ANTX, as well as having or not MC plus ANTX. For both situations, an increase in average and variability of *Pseudoanabaena* sp. cells as well as a decrease in average and variability of *Microcystis* sp. cells were observed when ANTX or MC plus ANTX were present.

The biological function of cyanotoxins is still not well understood, although some theories have been put forward. Among them, there are good hints for allelopathic mode of action of cyanobacterial secondary metabolites within a lake phytoplankton community (Wiegand and Pflugmacher 2005; El-Shehawy et al. 2012). The observed dynamic of phytoplankton going together with MC and ANTX occurrence in San Roque reservoir could be explained by the hypothesis of cyanotoxins acting as growth regulators for non-toxic strains of the same species as wells as other phytoplankton giving toxin-producing cyanobacteria a better opportunity for successful adaptation (Sedmak and Kosi 1998). This possible function of cyanotoxins may help cyanobacteria to adapt to environmental changes and various challenges of the climatic fluctuations.

As stated by El-Shehawy et al. (2012), integrated approaches of genetic, ecological, and physiological studies are still necessary to understand bloom proliferation, function, production, and accumulation of cyanotoxins as well as the impacts of global warming cyanotoxicity.

5 Conclusions

This study has shown the dynamic of phytoplankton and cyanotoxins in San Roque reservoir along a complete year study. In this waterbody *Microcystis* sp. and *Pseudoanabaena* sp. are presumably the major producer of MC as well as *Oscillatoria* sp. and *Anabaena* sp. of ANTX. Moreover, changes in the phytoplankton composition as well as some abiotic factors were also associated to cyantoxins occurrence indicating the probable allelopathic function of these toxins.

The threat associated with MC in San Roque reservoir rises in spring and summer, when higher MC concentrations were measured. This is the first report of ANTX presence in South America waters, and even when low concentrations of this toxin were detected, its presence should always be considered as a potential health hazard to humans, aquatic animals, livestock and wildlife. The results here presented could be helpful not only for the local authorities, but from worldwide.

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