Occurrence and toxicity of *Microcystis aeruginosa* (Cyanobacteria) in the Paraná River, downstream of the Yacyretá dam (Argentina)

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Abstract: Cyanobacteria constitute the main toxin producers in inland water ecosystems and have extensive global distribution. The presence of hepatotoxins in aquatic environments is hazardous to human and animal health; even though the presence and identification of hepatotoxic microcystins in rivers and reservoirs of the world have been confirmed by several studies in the last few years. Herein, we studied the abundance and toxicity of *Microcystis aeruginosa* in the Argentine section of the Paraná River at the beginning of the Middle Paraná (Corrientes Hydrometer), near Corrientes city (27º28´ S - 58º51´ W) and approximately 220 km downstream of the Yacyretá dam (High Paraná). The Paraná River basin, with a drainage area of 3.1 x 10^6 km^2 and 3 965 km in length, is the second largest catchment of South America, after that of the Amazon. The Paraná River is the main source of drinking water supply for the Northeastern Argentine region. Phytoplankton samples were collected and environmental variables were measured in a monthly basis (exceptionally fortnightly), from March 2004 to June 2008. Fifty-eight samples were analyzed for phytoplankton density and biomass. Five samples were used for toxicity testing; the latter were obtained during the cyanobacteria blooms from 2005 to 2008. Phytoplankton counts were performed with an inverted microscope, and biomass was expressed as biovolume. Bioassays with mice and high-performance liquid chromatography (HPLC) analysis were performed to evaluate the presence of cyanotoxins. Phytoplankton mainly consisted of Cryptophyta, Chlorophyta and Bacillariophyta. *Microcystis aeruginosa* was identified during the warmer months each year (November to March). Density varied between 189 and 25 027 cells/mL (1-10 colonies/mL) and biomass from 0.34 to 44 mm^3/L. Taking into account the number of cells, the highest abundance occurred in April 2004 (25 027 cells/mL), coinciding with the largest biovolume (44 mm^3/L). All mice subjected to intraperitoneal injections with samples obtained during bloom episodes showed positive results for the presence of hepatotoxins. Three microcystins variants: LR, RR and [D-Leu^1] Mcyst-LR were detected by analysis with semi-preparative high-performance liquid chromatography with diode array detector system (HPLC-PDA). This constitutes the first report of microcystins recorded during *M. aeruginosa* blooms in the Argentine stretch of the Paraná River at the beginning of the Middle Paraná (Corrientes Hydrometer), approximately 220 km downstream of the Yacyretá dam (High Paraná). Rev. Biol. Trop. 64 (1): 203-211. Epub 2016 March 01.

Key words: *Microcystis aeruginosa*, hepatotoxins, microcystins, Yacyretá dam.

Cyanobacteria, having wide global distribution, constitute the main toxins producers in inland water ecosystems (Chorus & Bartram, 1999). The massive developments of cyanobacterial populations have also become important because of the increasing eutrophication in freshwater of all continents (Komárek, 2006). This fact, together with climate change,

is evident in the frequent production of water blooms (Paerl & Huisman, 2008).

Livestock, wildlife, and pet deaths due to ingestion of water containing toxic cyanobacterial cells or toxins released by these have been extensively documented. Recently, human poisonings have also been reported (Azevedo et al., 2002).

The presence and identification of microcystins in the Río de la Plata river of Argentina was confirmed in different studies along the last ten years (De León & Yunes, 2001; Andrinolo et al., 2007; Younes et al., 2011).

The Paraná River basin, shared by Brazil, Bolivia, Paraguay and Argentina, with a drainage area of 3.1 x 10^6 km^2 and 3 965 km in length, is the second largest of South America, after that of the Amazon. Near Corrientes City (Corrientes Hydrometer), the Paraná River had an average annual discharge of 16 941 m^3/s between 1904 and 1994 (Orfeo & Stevaux, 2002). Several reservoirs (approximately 23), situated in cascade (Bonetto, Wais, & Castello, 1989) occupy its main channel, mainly in Brazil (Upper Paraná). The construction of the Yacyretá dam on the High Paraná, one of the largest impoundments built between Argentina and Paraguay, was finished in April 1990, although the first turbine did not start operating until September 1st, 1994. This caused a significant impact on aquatic communities of the reservoir and those downstream (Garrido, 1999; Meichtry de Zaburlín, 1999; Peso & Bechara, 1999; Neiff, Poi de Neiff, Patiño, & Basterra de Chiozzi, 2000; Domitrovic, Bechara, Jacobo, Flores Quintana, & Roux, 1994; Zalocar de Domitrovic, Devercelli, & García de Emiliani, 2007a).

The first blooms of *Microcystis aeruginosa* (Kütz.) Kütz were recorded during the summer of 2004, after ten years of operation of the Yacyretá dam. These were carried about 300 km downstream the dam, on the left bank of the river and expanded in backwater areas or on the riverbanks, during the prolonged period of low waters of the Paraná River at that time (water level about 2.5 m). Blooms continued to be observed on the left bank of the river near Corrientes city during 2005, 2006 and 2007 (Zalocar de Domitrovic & Forastier, 2007, 2008).

Given that the Paraná River is the main source of drinking water supply for the Northeastern Argentine region, and that these cyanobacteria are potentially toxic, the aim of this study was to conduct a phytoplankton monitoring in order to detect cyanobacteria blooms, ascertain their toxicity and identify the cyanotoxins they produce.

MATERIALS AND METHODS

Sampling design and limnological characteristics: The study was carried out on the left bank of the Argentine section of the Paraná River (Corrientes Hydrometer) near Corrientes city (27º28´ S - 58º51´ W), approximately 220 km downstream of the Yacyretá dam. Monthly (exceptionally fortnightly) samples were collected at one site (about 300 meters from the coast of Corrientes city) from March 2004 to June 2008.

The methods used to collect and quantify the phytoplankton samples followed established standards (Vollenweider, 1974; Sournia, 1978). Samples for phytoplankton analysis were collected at subsurface level in 500 mL bottles, fixed with Lugol's acidified solution and quantified in an inverted microscope (Utermöhl, 1958; Lund, Kipling, & Le Cren, 1958). Biomass was expressed as biovolume (mm^3/L) by calculating the volume of a geometric form resembling that of the cells of the colony (Hillebrand, Dürselen, Kirschtel, Pollingher, & Zohary, 1999).

Environmental variables such as temperature, water transparency (with 25 cm diameter Secchi disk), pH (pH meter Metrohm AG Herisau), conductivity (conductometer YSI 33 SCT) and dissolved oxygen concentration (Oxygen meter YSI 54 A) were also measured *in situ* during all the samplings.

For cyanotoxin extraction, *Microcystis* colonies were collected with a 50 µm phytoplankton net during blooms of the warmer months (February 2005, January and December

2006, November 2007 and January 2008), obtaining a total of five samples. To allow cell lysis and the release of toxins, the samples were subjected to three freezing/thawing cycles and then centrifuged at 5 000 r.p.m in a Rolco G20 centrifuge, discarding the precipitate (Chorus & Bartram, 1999).

Toxicity bioassays with mice: The laboratory mice (*Mus musculus* CF-1 lineage Romanelli), used in the assays, were obtained from the Facultad de Ciencias Veterinarias of the Universidad Nacional del Nordeste (Argentina). Eighteen male mice weighing 20-23 g (analytical balance Sartorius, max. cap. 100 g, Resol. 0.001 g) were separated into six groups, and one was utilized as Control. Each group was treated with each sample extract. Toxicity was tested by intraperitoneal injection of 0.1 and 1.0 mL of lysed cyanobacteria. Lysed cyanobacteria were passed through 0.45 µm Osmonics filters before being injected. The injected animals were observed by recording their symptoms on an ongoing basis during the first three hours, and then every 60 minutes for eight hours. After 24 hours, they were sacrificed and their livers removed and examined for signs of hepatotoxicity (Andrinolo et al., 2007). Studies were conducted in accordance with international protocols for laboratory animal care (National Research Council, 1985). Experimental designs were also approved by the institutional committee and followed standard protocols for animal welfare.

External appearance, size and color were also recorded at the same time. The livers were immediately fixed in Bouin's solution for 24 hours and then placed in alcohol 70 % for their preservation and subsequent histopathological analysis. To this end, samples were embedded in paraffin, cut into 5 µm thick sections with a microtome, stained with hematoxylin-eosin, mounted with Canada balsam (Culling, 1975) and analyzed with a Olympus BX41 optical microscope with 100X, and 400X magnification.

Analysis by high-performance liquid chromatography (HPLC): The aqueous extracts obtained from the 2005 and 2008 blooms were initially filtered using 0.45 µm filters to prevent the entry of particles into the HPLC equipment and then through Sep Pack C 18 filters previously activated with MEOH 100 %, microcystins were eluted with MEOH 80 % (Andrinolo et al., 2007).

Quantitative Chromatographic analysis of microcystins was performed by HPLC (Shimadzu) with a photodiode array detector and C18 column (Hyperprep HS, 5 µm pore, 250 × 10 mm). The column was equilibrated with a mixture composed of 65 % of solution A [water with 0.0 5 % (v/v) trifluoroacetic acid (TFA)] and 35 % of solution B [acetonitrile with 0.05 % (v/v) TFA]. The mobile phase consisted of a discontinuous gradient of solutions A and B. The flow rate was 1.0 mL/min. Microcystins were identified on the basis of their UV spectra and retention time. The presence of the RR and LR variants was confirmed by comparison of retention times of the peaks produced by the standards of SIGMA Chemicals (St. Louis, MO, USA). [D-Leu^1] Mcyst-LR were purified from *Microcystis aeruginosa* strain CAAT 2003.

RESULTS

Cyanobacterial abundance: Between 2004 and 2008, the phytoplankton was dominated by Cryptophyta, Chlorophyta and Bacillariophyta. Cyanobacteria were recorded during the warmer months each year (November to March), with high density of *Microcystis aeruginosa* (Fig. 1).

The density of *M. aeruginosa* colonies ranged from 1 to 10 col/mL. The highest abundance taking into account the number of cells (25 027 cells/mL) occurred in April 2004, coinciding with the largest biovolume (44 mm^3/L).

The environmental temperature ranged between 13 ºC and 41 ºC (25.9 ± 5.4 ºC), while water surface temperature ranged between 15

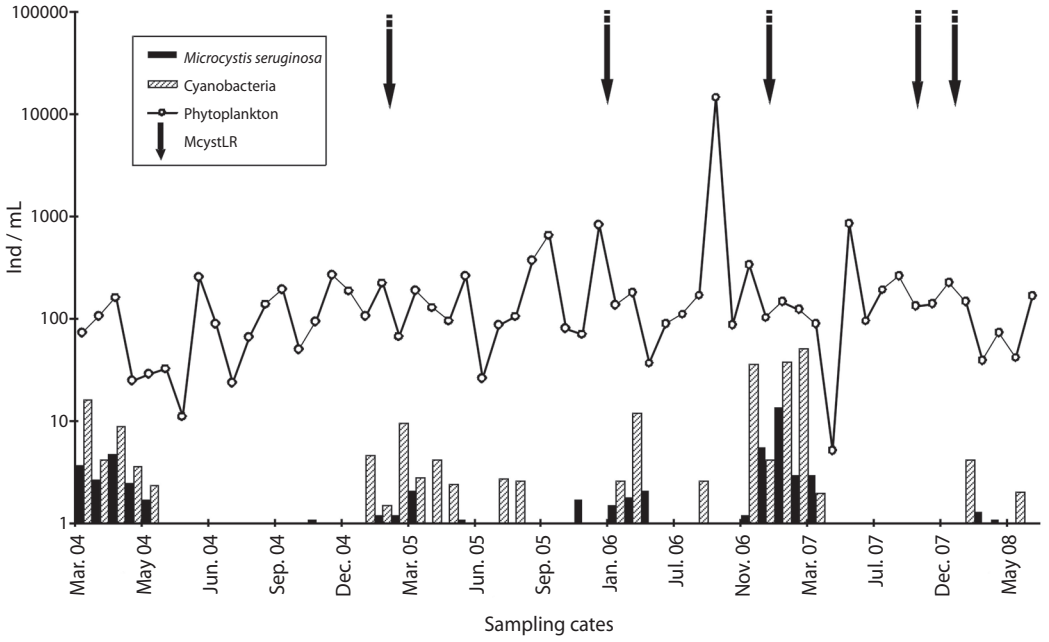

Fig. 1. Occurrence of microcystin LR and variation in density (ind./mL) of total phytoplankton, cyanobacteria and colonies of *Microcystis aeruginosa* in the Paraná River.

and 31 ºC (24 ± 5 ºC, n= 75). The conductivity ranged from 35 to 105 µS/cm (59 ±22 µS/cm; n= 75), the pH ranged from 6.03 to 10.4 u (7.4 ± 0.5 u), and the transparency (Secchi disk) ranged between 1.05 and 2.10 m (1.16 ± 0.47 m; n= 75). The water level of the Paraná River at the Corrientes Hydrometer fluctuated between 2.2 (11 330 m^3/s) and 6.5 m (31 086 m^3/s). The environmental variables during the samplings in which cyanobacteria blooms were present are shown in Table 1.

Toxicity bioassays with mice: All mice injected with samples obtained during bloom episodes from 2005 to 2008 exhibited symptoms corresponding to hepatotoxins. These included piloerection, abdominal contraction and deep breathing, among others. Only mice injected with lysed cyanobacteria from January 2006 died. Death occurred between 60 and 70 minutes after injection. In the post-mortem examination, the extracted liver was found to be larger than that of Controls, with a dark and hemorrhagic aspect. Liver weight was between 10 and 12 % higher than its initial counterpart.

Histopathologically, injuries consistent with hepatotoxins were identified. The liver showed alteration in its architecture and

TABLE 1
Environmental variables of the Paraná River measured during the collection of *Microcystis aeruginosa* samples

Date	Temperature (ºC)	Secchi disk (m)	pH (u)	Conductivity (µS/cm)	Dissolved Oxygen (mg/L)	Hydrometric level (m)
08-02-05	28.5	1.20	7.6	75	7.0	3.9
04-01-06	28.0	1.63	10.4	50	7.3	4.0
28-12-06	29.1	1.35	7.7	55	8.4	3.6
13-11-07	22.0	1.10	7.6	55	8.0	3.0
23-01-08	29.5	2.10	8.1	55	7.8	3.1

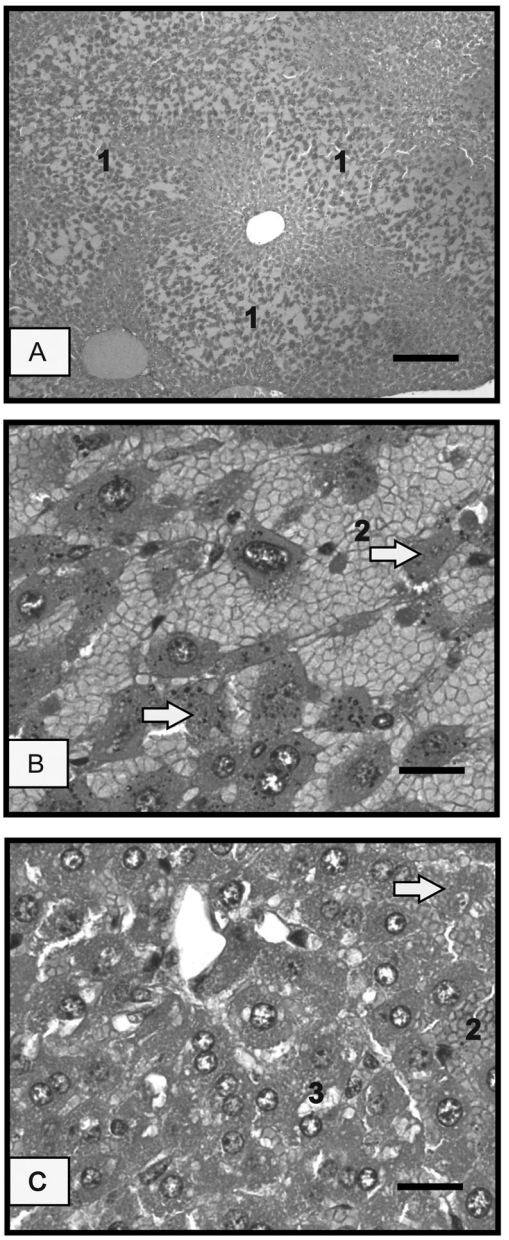

Fig. 2. Histological sections of liver from a mouse injected with a lysed extract of *Microcystis aeruginosa* (**A-C**). Congestion and hemorrhage (1-2). Hepatocyte necrosis (white arrow) and cytoplasma vacuolization of hepatocytes (3). Scales: 21 µm.

hepatocytes necrosis (Fig. 2A, Fig. 2B and Fig. 2C); in some cases, congestion and zonal hemorrhage at the level of the lobules (Fig. 2A) was also observed. Hepatocytes exhibited irregular shape, cytoplasmic swelling and vacuolization and necrosis in some cells (Fig. 2 B, Fig. 2C).

Microcystins detection by HPLC: All samples analyzed showed presence of microcystins, with three variants: LR, RR and [D-Leu^1] Mcyst-LR. Most of them were LR and [D-Leu^1] Mcyst-LR, being the former present in all blooms. The concentration of LR ranged from 0.09 µg/L on February 8th, 2005 to 1.9 µg/L in the summer of 2008, while [D-Leu^1] Mcyst-LR ranged from 37.7 µg/L on January 4th, 2006 to 6.9 µg/L on December 28th, 2006. Mcyst-RR was the variant with the lowest concentration and sporadic presence (Table 2). The chromatograms obtained from material from the *M. aeruginosa* blooms and the peaks of the microcystins variants recorded are shown in Figure 3.

DISCUSSION

The density and composition of the phytoplankton of the Paraná River presented fluctuations between 1976 and 2008. Since 1976 (when studies on the Argentine section of the Paraná River were initiated) three taxonomic groups characterized the phytoplankton: Bacillariophyta, Chlorophyta and Cyanobacteria (Zalocar de Domitrovic & Vallejos, 1982; Bonetto, Zalocar de Domitrovic, & Vallejos, 1982; Zalocar de Domitrovic & Maidana, 1997). Cyanobacteria presented maximum density only in spring and summer, when hydrometric levels were lower than 3.50, and they were represented by *Cylindrospermopsis raciborskii*, *Planktolyngbya subtilis* and *Dolichospermum spiroides*, without obvious formation of blooms (Zalocar de Domitrovic, Poi de Neiff, & Casco, 2007b). *Microcystis aeruginosa* was below the detection limits of the counting method, and it appeared sporadically in concentrated samples collected with plankton meshes (Zalocar de Domitrovic & Forastier, 2007, 2008).

A comparative study of the phytoplankton before and after the operation period of the Yacyretá dam (Zalocar de Domitrovic et al,

TABLE 2
HPLC-PDA analysis of microcystins in water samples collected in the Paraná River during *Microcystis aeruginosa* blooms

	Mcyst-LR (µg/mL)	Mcyst-RR (µg/mL)	[D-Leu^1] Mcyst-LR (µg/mL)
08-02-05	0.09	0.04	*
04-01-06	0.2	1.23	37.7
28-12-06	0.4	*	6.9
13-11-07	0.1	*	9.6
23-01-08	1.9	*	17

(*) Undetectable.

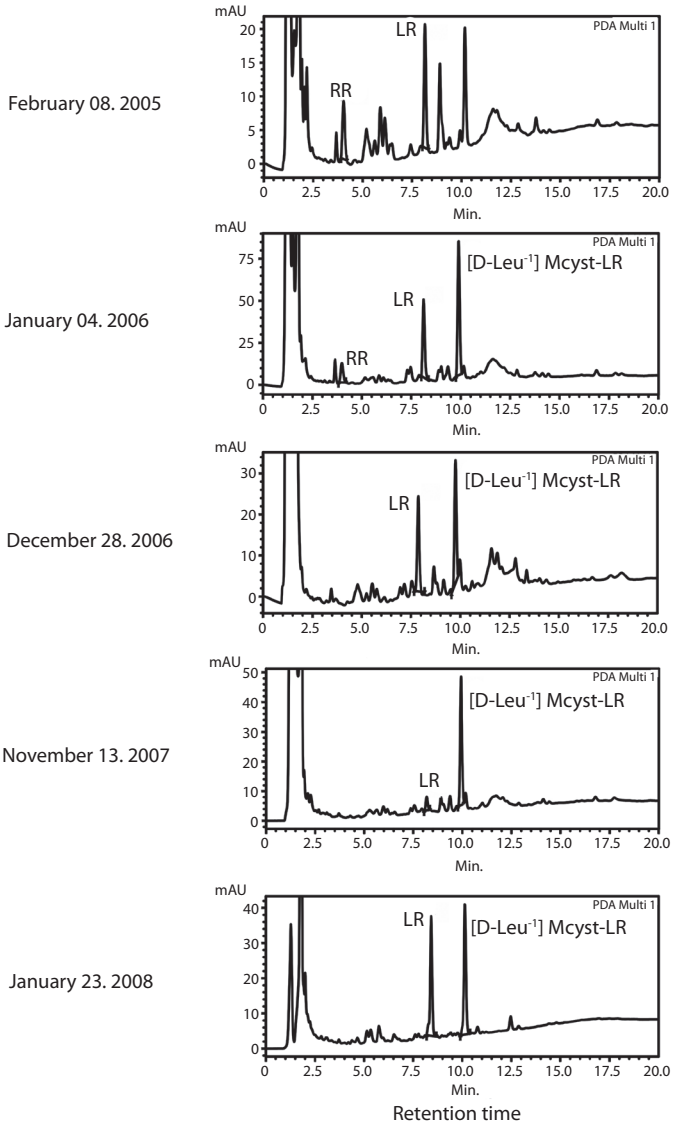

Fig. 3. HPLC-PDA chromatograms (λ- 238) of cyanobacterial bloom samples dominated by *Microcystis aeruginosa* obtained from the Paraná River.

2007b) pointed out changes in the structure and density of the community, 220 km downstream of the dam. One year after the filling stage of the reservoir, between 1995 and 1996, the presence of Cyanobacteria and Bacillariophyta decreased, while that of Cryptophyta and Chlorophyta increased (Zalocar de Domitrovic et al., 2007b). Similar results were observed in the Yacyretá dam during the filling stage, mainly due to the changes caused by the embankment, such as decreased flow and water turbulence (Meichtry de Zaburlín, 1999, 2002).

The first blooms of *M. aeruginosa* were observed after ten years of operation of the Yacyretá dam, between February and April 2004, coinciding with a prolonged period of low water of the river (Zalocar de Domitrovic & Forastier, 2007; Meichtry de Zaburlín, Llano, & Martens, 2011). Following this period, other blooms were observed between 2005 and 2008 (Forastier, 2012).

The microcystins detected in the Paraná River match those previously recorded during blooms with dominance of the genus *Microcystis* (Park et al., 1998). *M. aeruginosa* blooms also occurred in the Río de la Plata, where microcystin production was detected (Andrinolo et al., 2007; De León & Yunes, 2001). The microcystin variants LR, RR and [D-Leu^1] Mcyst-LR, identified in the Paraná River during this study, are similar to those found in the Río de la Plata river, on the coast of the city of Ensenada (Andrinolo et al., 2007).

Studies by Pizzolón, Tracanna, Prósperi and Guerrero (1999) have shown the presence of cyanotoxins in different rivers and reservoirs of Argentina, whereas this is the first record of microcystins with three variants (LR, RR, and [D-Leu^1] Mcyst-LR) in the Paraná River. LR and RR variants, which are typical of blooms dominated by the genus *Microcystis* (Park et al., 1998; Welker, Sigrid & Steinberg, 1999), were recorded during all the years of the present study, mainly during the warmer months. LR is the most toxic variant for the human population, whereas RR is characterized by extensive power of biomagnification in some organisms, such as fish (Cazenave, Bistoni, Pesce, & Wunderlin, 2005). The formation of surface scums and the accumulation on the shorelines may multiply the concentration by several orders of magnitude to the hot spot locations (Welker et al., 1999), mainly if the water bodies are used as recreation sites.

Considering the alert levels suggested by the WHO (World Health Organization) and other countries (Andrinolo & Ruiz, 2011; Calijuri, Alves, & Alves Dos Santos, 2006; Chorus & Bartram, 1999), the concentration levels of microcystins in this study did not exceed said alert levels for water bodies used as a source of drinking water or recreation.

Among the taxonomic groups comprising the phytoplankton of the Paraná River, Cyanobacteria are present in low density throughout the year. *M. aeruginosa* blooms occur in warm periods, mainly in summer and prolonged low water of the river, as observed in 2004. These blooms, developed on the Yacyretá reservoir (10 years after the dam initiated operation), are transported down the river over a distance of about 300 km and disappear about 50 km after the confluence with the Paraguay River. This is the first record of hepatotoxins, microcystins LR, RR, and [D-Leu^1] Mcyst-LR for the Paraná River on the left bank near Corrientes city, Argentina.

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RESUMEN

Presencia y toxicidad de *Microcystis aeruginosa* **(Cianobacteria) en el río Paraná, aguas abajo de la represa Yacyretá (Argentina).** Las Cyanobacterias constituyen el principal productor de toxinas en ecosistemas acuáticos y tienen una amplia distribución mundial. La presencia e identificación de microcistinas hepatotóxicas en ríos y embalses de todo el mundo fue confirmada por diferentes estudios durante los últimos años. La presencia de hepatotoxinas en cuerpos de agua son riesgosas para

la salud humana y animal. Se estudió la abundancia y toxicidad de *Microcystis aeruginosa* (Kütz.) Kütz. en el río Paraná (Argentina), cerca de la ciudad de Corrientes (27°28' S - 58°51' W), aproximadamente a 220 km aguas abajo de la represa Yacyretá. La cuenca del río Paraná, con un área de drenaje de 3.1 x 10^6 km^2 y 3 965 km de longitud, es la segunda mayor cuenca de Sudamérica, después del Amazonas. El río Paraná es la principal fuente de abastecimiento de agua potable para el Nordeste de la República Argentina. Los muestreos se realizaron mensualmente (excepcionalmente fueron quincenales) con medición de variables ambientales, entre Marzo 2004 y Junio 2008. Se tomaron un total de 58 muestras para analizar la densidad y biomasa del fitoplancton; mientras que cinco muestras fueron utilizadas en ensayos de toxicidad, estas últimas fueron obtenidas durante floraciones de cianobacterias entre 2005 y 2008. Los recuentos de fitoplancton fueron realizados con un microscopio invertido y la biomasa fue expresada como biovolumen. Para determinar la presencia de cianotoxinas se utilizaron bioensayos con ratones y análisis con Cromatografía líquida de alta resolución (HPLC). El fitoplancton estuvo representado principalmente por Cryptophyta, Chlorophyta y Bacillariophyta. Cyanobacteria fue dominante durante los meses cálidos de cada año (Noviembre a Marzo), con alta densidad de *Microcystis aeruginosa*. La densidad de *M. aeruginosa* varió entre 189 y 25 027 cells/mL (1-10 colonies/mL) y la biomasa entre 0.34 y 44 mm^3/L. Teniendo en cuenta el número de células, la mayor abundancia ocurrió en abril 2004 (25 027 cells/mL), coincidiendo con el gran biovolumen (44 mm^3/L). Todos los ratones inyectados intraperitonealmente presentaron síntomas correspondientes a hepatotoxicidad. Tres variantes de microcystinas: LR, RR y [D-Leu^1] Mcyst-LR, fueron detectadas por análisis de cromatografía líquida de alta resolución con detector de diodos (HPLC-PDA). Este es el primer trabajo de microcistinas registradas durante las floraciones de *M. aeruginosa* en el tramo argentino del río Paraná en los inicios del Paraná Medio (Hidrómetro Corrientes), aproximadamente a 220 km aguas abajo de la represa de Yacyretá (Alto Paraná).

Palabras clave: *Microcystis aeruginosa*, hepatotoxinas, microcistinas, represa de Yacyretá.

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