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Occurrence of anatoxin-a(s) during a bloom of *Anabaena crassa* in a water-supply reservoir in southern Brazil

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Abstract Cyanobacterial blooms and the accompanying production of cyanotoxins are a serious global problem. Toxic blooms of *Anabaena* species are common in lagoons and reservoirs of southern Brazil. Worldwide, species of the genus *Anabaena* produce the majority of the known hepatotoxins (microcystins) and neurotoxins [anatoxin-a, anatoxin-a(s), and saxitoxins]. This report links a bloom of *Anabaena crassa* in the Faxinal Reservoir, the main water supply for the city of Caxias do Sul (400,000 inhabitants) in southern Brazil, to the occurrence of anatoxin-a(s) in the water. During the bloom period, the reservoir was strongly stratified, with higher temperatures and a deep anoxic hypolimnion. Two methods for sample concentration (direct and complete extraction) were tested, and direct extraction of samples proved to be more efficient. Water samples collected during the bloom showed 9% acetylcholinesterase inhibition at 50 mg mL^{-1}, corresponding to 0.61 μg of anatoxin-a(s) per gram of lyophilized powder. At these concentrations, symptoms of neurotoxicity and mortality were not observed in tests with Swiss albino mice. Although the concentrations of anatoxin-a(s) in the Faxinal Reservoir were low, these results are important because this is the first record of the toxin for *A. crassa*. Furthermore, this cyanotoxin is not yet included in Brazilian legislation for drinking-water monitoring, because of the lack of information about toxicity levels and risk calculation for oral doses. The data presented here contribute to the basis for the future inclusion of this toxin in Brazilian legislation for drinking-water quality control, and for the development of analytical methods for this toxin.

Keywords Acetylcholinesterase · Cyanobacteria · Cyanotoxin · Subtropical

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Introduction

Cyanobacterial blooms cause social, economic, and environmental problems. In Brazil, such blooms have been increasing in intensity and frequency, with cyanobacteria dominating throughout the year in many reservoirs (e.g., Beyruth 2000; Huszar et al. 2000; Calijuri et al. 2002; Bouvy et al. 2003; Vieira et al. 2005). Blooms may also cause aesthetic problems, deoxygenation, production of odor and taste in water, and, frequently, the formation of toxins (Whitton 1992). Cyanobacteria are capable of producing hepatotoxic peptides, neurotoxic alkaloids, and a wide spectrum of other selectively bioactive biotoxins (Carmichael 1992; Rapala 1998).

Cyanotoxins have received attention from researchers of water suppliers and human-health sectors because of: (a) the increase in numbers of toxic blooms in reservoirs; (b) the discovery of new toxins and their associated risks; and (c) the increase in cases of acute and chronic poisoning in

domestic animals and in human beings (Molica et al. 2005). In Brazil, cyanotoxins have been reported in several studies: microcystins (Azevedo et al. 1994; Matthiensen et al. 2000; Minillo et al. 2000; Vieira et al. 2005; Sotero-Santos et al. 2008; Ferrão-Filho et al. 2009), cylindrospermopsin (Carmichael et al. 2001), saxitoxins (Lagos et al. 1999; Yunes et al. 2003; Molica et al. 2005; Ferrão-Filho et al. 2009), and anatoxin-a(s) (Monserrat et al. 2001; Yunes et al. 2003; Molica et al. 2005). However, anatoxin-a has never been found at detectable levels (Molica et al. 2005; Colvara 2005). The genus *Anabaena* includes common filamentous bloom-forming cyanobacteria of world-wide distribution, and some species are able to produce toxins, such as hepatotoxins (microcystins) and neurotoxins [anatoxin-a, anatoxin-a(s), and saxitoxins] (Codd et al. 1997, Sotero-Santos et al. 2008).

Anatoxin-a(s) is a natural organophosphate that acts as a non-competitive inhibitor of acetylcholinesterase (AChE) activity, preventing it from hydrolyzing the neurotransmitter acetylcholine. The letter "s" corresponds to the characteristic symptom of salivation caused in vertebrates, mainly in doses close to LD_{50} levels (20–40 μg kg^{-1}; Matsunaga et al. 1989). As a consequence, acetylcholine remains attached to the membrane receptors, resulting in continuous muscle stimulation. When respiratory muscles are also affected, death may occur by convolution, suffocation, and lack of oxygen in the brain (Matsunaga et al. 1989; Carmichael and Falconer 1993).

The method to estimate anatoxin-a(s) levels uses the AChE inhibition assay, which was originally designed to estimate organophosphate herbicides, but is also used with cyanobacterial bloom extracts. Monserrat et al. (2001) and Barros et al. (2004) used a complete sample extraction prior to assessing AChE inhibition; whereas Henriksen et al. (1997) and Molica et al. (2005) used a simplified extraction. Therefore, it is necessary to evaluate methods and to determine the best conditions for this enzyme assay.

Toxic blooms of *Anabaena* species are common in Brazilian aquatic environments, mainly in water-supply reservoirs and lagoons (e.g., Sant'Anna and Azevedo 2000; Monserrat et al. 2001; Yunes et al. 2003; Barros et al. 2004). The occurrence of anatoxin-a(s) has been reported from the United States, Canada, and Denmark (USEPA 2001). This toxin is mainly related to the deaths of dogs, birds, ducks, and other domestic animals caused by blooms of *Anabaena lemmermannii* (Henriksen et al. 1997) and *A. flos-aquae* (Mahmood et al. 1988; Cook et al. 1989). Toxic blooms of *A. crassa* (Lemmermann) Komárková-Legnerová are common in reservoirs of the city of Caxias do Sul (Frizzo et al. 2004). Cyclic blooms (spring and summer) of this species were observed between 2002–2004 in the Faxinal Reservoir, the main reservoir of the city (Yunes et al. 2005; Becker et al. 2008), which services approximately 63.3% of the population. In Brazil, there exists no record of neurotoxicity from anatoxin-a(s). However, there are possible indications of its presence in cases reported from the states of Rio Grande do Sul (Monserrat et al. 2001; Yunes et al. 2003) and Pernambuco (Molica et al. 2005), associated with blooms of *A. spiroides* Klebahn.

Here, we report the occurrence of the neurotoxin anatoxin-a(s)-like anticholinesterase (AChE) during a bloom of *A. crassa* in the water-supply Faxinal Reservoir, southern Brazil. We also compared and evaluated methods to extract the samples in order to assess the occurrence of anatoxin-a(s) by means of the AChE enzyme inhibition assay.

Materials and methods

Study site

Faxinal Reservoir (29°05′00″ S; 51°03′30″ W) is the main water-supply ecosystem for the city of Caxias do Sul (400,000 inhabitants), in subtropical southern Brazil (Fig. 1). The reservoir was constructed in 1992, lies at an elevation of 700 m a.s.l, and has a surface area of 3.1 km^2. It is deep (z_{max}=30 m), warm monomictic, and mesotrophic (TP 1.07 μM and chlorophyll *a* 14 μg L^{-1}—epilimnion annual mean; Becker et al. 2008). The regional climate is temperate, without a dry season (type Cfa; Köppen 1936). The annual historical mean temperature, provide the full seasonal range, is 16°C, and total annual rainfall ranges between 1,800 and 2,200 mm.

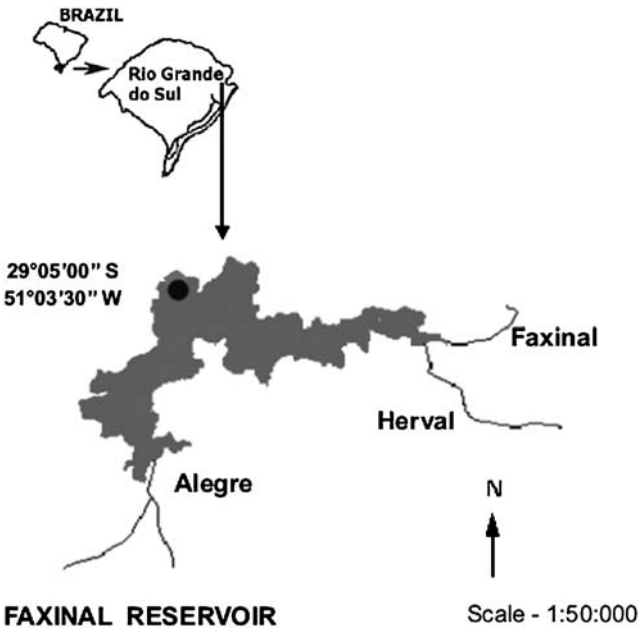

Fig. 1 Faxinal Reservoir showing the sampling station (*filled circle*) and the tributaries: Alegre, Herval, and Faxinal rivers

Sampling

Water samples were taken during a bloom of *A. crassa* in January and February/2004. Sampling followed the protocols of the water company (SAMAE—Serviço Autônomo Muncipal de Água e Esgoto) algal monitoring program, at the surface, at the water-intake sampling station (Fig. 1). Vertical profiles of temperature, dissolved oxygen, pH, and conductivity were measured with a Horiba model U-10 multiparameter probe, at 1-m intervals from the surface to the bottom (30 m).

Samples for phytoplankton analyses were collected biweekly at the surface, with a Van Dorn bottle (2 L), at a fixed time (09:00 AM). Phytoplankton samples were fixed with neutral Lugol's solution. Samples for cyanotoxin analyses were collected with a plankton net (25 μm mesh size) to concentrate the material, and were immediately frozen at −30°C.

Sample analysis

Phytoplankton was counted with the aid of a Leica-DMIL inverted microscope (Utermöhl 1958) at ×400 magnification. The units (cells, colonies, and filaments) were enumerated in random fields (Uhelinger 1964), and at least 100 specimens of the most frequent species were counted ($p<0.05$, Lund et al. 1958). The species of *Anabaena* was identified by descriptions from Kamárková-Legnerová and Cronberg (1992) and Komárek and Anagnostidis (1989).

Anatoxin-a(s) analysis

Anatoxin-a(s) was detected through either a direct extraction prior enzyme assay (Henriksen et al. 1997; Molica et al. 2005) as optimized by Barros et al. (2004), or through a complete sample extraction (Monserrat et al. 2001). In both cases, the determination of the AChE enzymatic activity used the methods recommended by Ellman et al. (1961) and their inhibition of AChE (acetylcholinesterase) as used by Barros et al. (2004). Lyophilization was done using the whole sample (water and cells) as collected in the reservoir and transported frozen to the laboratory. Lyophilization was run in MicroModulyo equipment (Edwards, UK) until the samples were completely dry.

The direct extraction was done by subjecting the lyophilized material to a single-extraction procedure to release the cell toxins. Different from the technique used by Henriksen et al. (1997) and Molica et al. (2005), we replaced the ethanol extraction of the cells by a simple process of sonification as developed by Barros et al. (2004). The procedure for the direct extraction is described below:

Direct extraction 50 mg of lyophilized cyanobacterial cells was dissolved in 1 mL of distilled water of pH 3.3 and sonified, on ice, for 1 min at 20 kHz (Ultrasonic Processor Model GE 50). This suspension was then centrifuged for 5 min at 10,000 rpm (Eppendorf 5415C, USA), and the supernatant was used in a concentration of 50 mg mL^{-1} in the enzyme (AChE) assays.

The complete sample extraction was done following the method of Barros et al. (2004). These authors divided the so-called indirect extraction into three procedures or phases: Extraction, semi-purification, and concentration, as described below:

Extraction 100 mg lyophilized material was dissolved in 40 mL of ethanol (Merck) to make a 0.25% solution (*w*/*v*). After 10 min in an ice bath, the suspension was sonified for 1 min at 20 kHz (Ultrasonic Processor Model GE 50), resulting in an ethanolic extract of the original sample.

Semi-purification The ethanolic extract was filtered four times through acetate filters (Whatman, 0.45 μm) and the filtrate was evaporated in a rota-evaporator at 40°C. The sample was then resuspended in 10 mL chloroform and placed in a separation funnel where 20 mL (2 mL each time) of deionized water at pH 3.3 was used to wash the sample. During each wash, the sample was shaken manually for 90 s, and the aqueous fraction was separated and saved. The aqueous fraction thus obtained, totaling

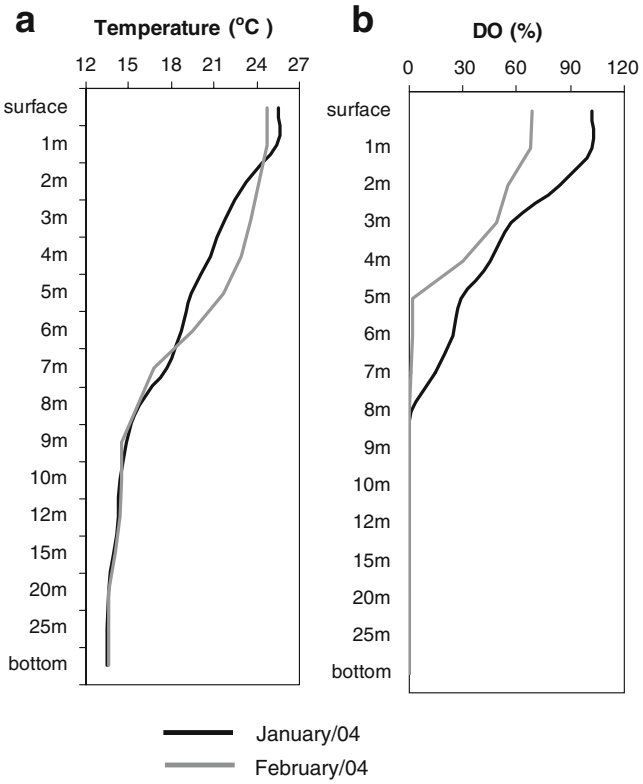

Fig. 2 Profile of water temperature (**a**) and dissolved oxygen (**b**) on January 11 and February 09, 2004, in Faxinal Reservoir

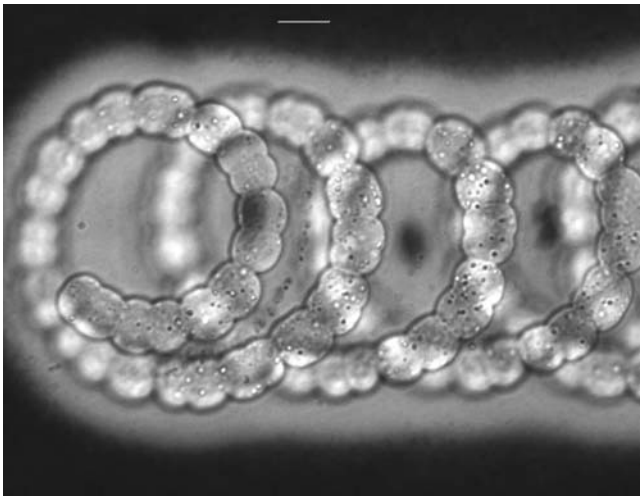

Fig. 3 Light micrographs of *Anabaena crassa* natural population showing regular coiled trichome and the mucilage. *Scale bar* 10 μm

20 mL, was then passed through a tC18 cartridge (Sep-Pak, Waters Milford, USA) previously activated by 10 mL absolute methanol and followed by 10 mL deionized water at pH 3.3, reaching a flow of 1 mL min^{-1} controlled by a Peristaltic Pump.

Concentration The aqueous eluate was concentrated in a rota-evaporator at 40°C to a final volume of 2 mL. This procedure was used in the enzyme assays, referring to the concentration of 50 mg mL^{-1}.

Mouse bioassays were done using male Swiss albino mice (19–20 g body weight), through intraperitoneal injection (1 mL), corresponding to a dose of 1,000 mg kg^{-1} of lyophilized cyanobacteria powder extract/mouse body weight. Control animals were injected with 1 mL 0.9% $NaCl_2$, pH 7.0 and observed for at least 24 h.

Results

During the study period (January and February 2004), the Faxinal Reservoir was strongly stratified, with higher temperatures (up to 24°C) in the epilimnion (Fig. 2a) and a deep anoxic hypolimnion (Fig. 2b). Mean densities of *A. crassa* (Fig. 3) during the bloom period were 71,750 cells mL^{-1} in January and 23,900 cells mL^{-1} in February. The decline of the bloom could be tracked through the weekly measurements of species density (Fig. 4).

Table 1 Sample names, collection site and species identification

Origin of samples	Collection site	*Anabaena*´s species
Caxias do Sul, RS, Brazil—2004	Faxinal Reservoir	*Anabaena crassa* (Lemmermann) Komárková-Legnerová
Rio Grande, RS, Brazil—1995	Ornamental FURG Lake	*Anabaena spiroides* Klebahn

Samples collected in the reservoir containing *A. crassa* (Table 1) in the second week of January 2004 contained cell densities between 55,000 and 65,000 cells mL^{-1}. Samples of *A. spiroides* from Rio Grande, state of Rio Grande do Sul (RS) collected in 1995 had a cell density higher than 1 million cells $mL^{-1.}$

Anticholinesterase activity was analyzed in both samples. The phytoplankton net sample of *A. crassa* from Caxias do Sul, RS showed 9% AChE inhibition at an assay concentration of 50 mg mL^{-1} (Table 2), corresponding to 0.61 μg of anatoxin-a(s) per gram of lyophilized powder. At these concentrations, symptoms of neurotoxicity or mortality were not observed in mouse bioassays.

In order to compare these assays, we also included and analyzed the sample of *A. spiroides* collected in 1995 in Rio Grande (Table 1), using different lyophilized *Anabaena* cell concentrations as a positive control (Table 3). Both *Anabaena* samples showed positive AChE inhibition; however, at the same concentration (50 mg mL^{-1}), the *A. spiroides* samples from Rio Grande achieved 100% inhibition. In fact, this sample contains a very powerful AChE inhibitor factor, which at concentrations from below 6.2 mg L^{-1} of lyophilized bloom powder reached a level of

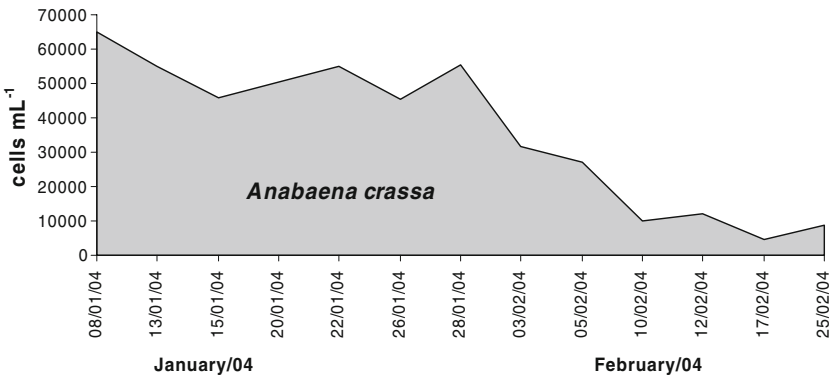

Fig. 4 *Anabaena crassa* density during January and February of 2004, in Faxinal Reservoir

Table 2 AChE inhibition of the Caxias do Sul, RS (2004) sample at a single concentration of 50 mg mL^{-1}

Method	% Inhibition	SD control	SD sample
Indirect extraction	1.6	4	3
Direct extraction	9	3	7

SD standard deviation

nearly 100% AChE inhibition. The so-called indirect or complete extraction procedure was used by Monserrat et al. (2001) and Barros et al. (2004) previously, and in the present study, we compared both extraction procedures. The values obtained in each extraction were described for the samples from Caxias do Sul, 2004 (Table 2) and Rio Grande, 1995 (Table 3). Higher AChE inhibition was observed in both samples obtained by the direct extraction method (Tables 2 and 3).

In the sample from Rio Grande, a dose–response correlation between sample concentration and AChE enzyme inhibition was apparent. Enzyme assays at decreasing concentrations revealed that a level as low as 12.5 mg mL^{-1} was still sufficient to produce almost 100% AChE inhibition. Also, for all concentrations except 0.19 mg mL^{-1}, higher AChE inhibitions were attained by applying the direct-extraction technique.

Discussion

The dominance of *A. crassa* in the summer of 2004 in Faxinal Reservoir constituted a steady state (equilibrium phase) of the population, as reported by Becker et al. (2008). The dominance of this species was strongly related to the water-column stratification, higher temperatures, and soluble reactive phosphorus (SRP) in the epilimnion. However, by means of the weekly sampling program, it was possible to track the decline of this bloom of *A. crassa* in February 2004.

The anatoxin-a(s) concentrations found in the samples of *A. crassa* were considered low, because the LD_{50} of the toxin is 20 μg kg^{-1} for mice, and no mortality occurred in the bioassays. The anatoxin-a inhibition factor present in the cells of *A. crassa* from Faxinal Reservoir is among those considered as very low by Barros et al. (2004). Therefore, the blooms in 2004 posed no risk of contamination by anatoxin-a. Water from Faxinal Reservoir can still be considered not harmful, because of the low anatoxin-a(s) concentrations detected. However, this result is important, because it is the first record of anatoxin-a(s) from the species *A. crassa*. Continued monitoring of Faxinal remains essential, in view of the potential genetic and physiological variability of the species, and of the increase in the occurrence of *A. crassa* and other spiral forms of the genus in water-supply reservoirs, in southern Brazil. Subsequent to this study in 2004, *A. crassa* has not recurred in Faxinal Reservoir (SAMAE, personal communication) up to the time of publication of the present report.

Production of anatoxin-a(s)-like AChE inhibitors has been observed only in species of *Anabaena*, including *Anabaena flos-aquae* Brébisson *ex* Bornet et Flahault (Mahmood and Carmichael 1986) and *A. lemmermannii* Richter (Henriksen et al. 1997; Onodera et al. 1997). In Brazil, the few records of the occurrence of anatoxin-a(s) are restricted to single or mixed blooms of *A. spiroides* Klebahn (Monserrat et al. 2001; Molica et al. 2005; Yunes et al. 2003; Barros et al. 2004) and *Anabaena planctonica* Brunnthaler and *Anabaena circinalis* Rabenhorst *ex* Bonet and Flahault (Yunes et al. 2003; Barros et al. 2004).

Brazilian legislation for potable water (Brazil 2000, 2004) established the maximum concentration for microcystins (1 μg L^{-1}) and recommended it for saxitoxins (3 μg L^{-1}) and cylindrospermopsins (15 μg L^{-1}). However, anatoxin-a(s) was not included. The stated reasons were that this toxin had not been reported from Brazilian drinking-water supplies and because of the lack of information about its toxicity levels, calculation of the risk from oral ingestion (LD_{50}), and possible difficulties in extraction of the toxin, as also revealed in the present report.

In order to confirm the effectiveness of this low AChE enzyme inhibition shown by the sample from Caxias do Sul taken in 2004, a positive control was applied using the Rio Grande 1995 sample previously studied by Monserrat et al. (2001) and Barros et al. (2004). Increasing concentrations of this sample were applied in order to produce a precise dose–response curve. An important step has been achieved in the process of recognizing the limitation of anatoxin-a(s) extraction. While several semipolar and nonpolar solvents were applied during sequential steps by the complete extraction procedure, it appeared that direct extraction

Table 3 AChE inhibition for the Rio Grande, RS, Brazil (1995) sample using sample concentrations from 100 to 0.19 mg mL^{-1}

Sample concentration (mg mL^{-1})	100	50	25	12.5	6.2	3.12	1.56	0.78	0.39	0.19	AChE
Indirect extraction	30.2	12.4	10.5	1.9	0.5	0	0	0	0	0	Inhibition (%)
Direct extraction	100	100	100	99	91	69	43	13	0.8	0	

using a single water sonification was more effective. The amount of the anti-AChE factor extracted was significantly higher under this condition. Although no effective tests of cyanobacterial extraction under different solvents are available, the reason for the increased efficiency appears to be that the direct method substantially reduces the losses of the anti-AChE factor because the sample is less manipulated.

After the passage of legislation in 2004, the occurrence of anatoxin-a(s) of *A. spiroides* Klebahn was recorded in Tapacurá Reservoir in northeastern Brazil (Molica et al. 2005), and in blooms of *A. crassa* in Faxinal Reservoir, as reported here. These records and the improvements in methodology will constitute a basis for future inclusion of this toxin in the requirements for analysis of this cyanotoxin in legislation for drinking-water quality control in Brazil and perhaps other countries.

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