Cyanobacterial Neurotoxins from Southern Brazilian freshwaters

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Blooms of Cylindrospermopsis raciborskii and Anabaena spiroides are studied in relation to their toxins composition, geographical locations and other characteristics of the waters in the Southern region of Brazil. All forms of Cylindrospermopsis were PSTs producers, with a similar profile of the toxins. Anabaena blooms, are studied in relation to the production of anatoxin-a(S). In all samples containing Anabaena spiroides, a positive result was found when the AChE inhibition technique was used. Methods applied for both studies are very convenient for the monitoring of this large region and gives a reasonable view of the present situation of water reservoirs in Southern Brazil.

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## SAXITOXINS FROM *CYLINDROSPERMOPSIS* FORMS

Introduction

Harmful Algal Blooms (HABs) are very common, but alarming nuisance events which happens throughout the world (1). Brazilian water supplies as a result of a recent legislation, the Portaria 1469, Brazilian Health Ministery (2), have been hidrobiologically surveyed. Several toxic cyanobacterial from the genera *Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya* e *Planktothrix* (*Oscillatoria*) were identified and were able to produce neurotoxic compounds known as PSTs (Paralytic Shellfish Toxins). The PSTs are basically constituted of a tetrahydropurine, with more than 26 structures already described. They can be classified in three groups, according to the net charge, at neutral pH: (a) N-sulfocarbamoyl-11-hydroxysulfate toxins (C-toxins), with a net charge of 0, (b) gonyautoxins (GTXs), with net charge of +1, and (c) saxitoxins (STXs) group with a net charge of +2 (Figure 1). These toxins have been determined quantitatively by the high performance liquid chromatography technique, with fluorescence detection by FLD-HPLC and post-column derivatization method (3). PSTs are neurotoxins that block the sodium voltage-gated channels of excitable cells, impeding neuronal transmission (4).

First report of PSTs in southern Brazilian freshwater

The hydroelectric system of "Salto" in the Southern Brazilian town of São Francisco de Paula (18,000 inhabitants, 907m above the sea level) is at least of a high importance from the electricity generation, point of view (See all locations of cities, towns, dams and rivers in Figure 2). From the Salto dam, waters flushed down into two rivers basins, the Caí and the Sinos rivers. The Caí and Sinos rivers basins together accomplish for a $5,100km^2$ and $4,300km^2$, respectively and were used for the water supply of more than 69 towns in the region, most of them situated below 100m from the sea level.

In the summer of 1999, two river-basins Caí and Sinos were deeply affected by cells generated from blooms of *Cylindrospermopsis raciborskii* several miles upstream in the Salto Hydroelectric Power System. At the Salto dam during the bloom event, the waters released 63,444 filaments mL^{-1} of *Cylindrospermopsis raciborskii* into the Caí basin and 45,300 filaments mL^{-1} in the Sinos basin.

In the Caí river, cells of *C. raciborskii* reached the uptake of water plant, at levels as high as 103.603 filaments.mL^{-1} in the town of Canela and in the Sinos river, as high as 60.000 filaments.mL^{-1} in the town of Três Coroas (5). Material collected at the highest dam (Salto) and at several uptakes, downstream in the rivers Caí and Sinos, confirmed the neurotoxicity of the cyanobacterial bloom. A standard mouse test (1) revealed levels as high as 1,57 μg L^{-1} of saxitoxin equivalents in the bloom material. Analysis for cylindrospermopsins also was carried out, but the toxin was not detected, using a
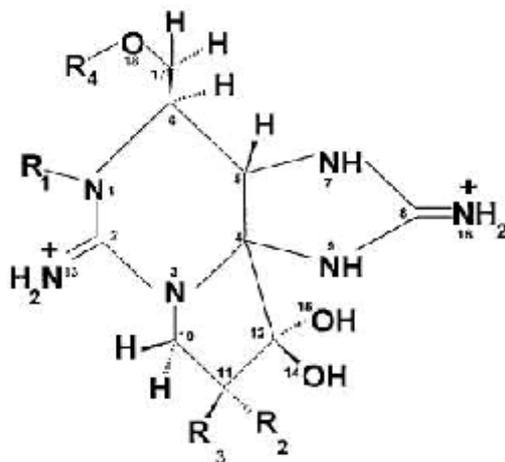
	Molecular Weight $(g.mol^{-1})$	R1	R2	R3	R4
STX	301,31	H	H	H	$CONH_2$
Neo-STX	317,31	OH	H	H	$CONH_2$
GTX 1	412,36	OH	OSO_3^-	H	$CONH_2$
GTX 4	412,36	OH	H	OSO_3^-	$CONH_2$
GTX 2	396,36	H	OSO_3^-	H	$CONH_2$
GTX 3	396,36	H	H	OSO_3^-	$CONH_2$
C1	475,41	H	OSO_3^-	H	$CONHSO_3^-$
C2	475,41	H	H	OSO_3^-	$CONHSO_3^-$
C3	491,41	OH	OSO_3^-	H	$CONHSO_3^-$
C4	491,41	OH	H	OSO_3^-	$CONHSO_3^-$

Figure 1: PSTs structures: STX= saxitoxin; Neo-STX= Neo—Saxitoxin; GTX= Gonyautoxin; dc= decarbamoil.

DAD-HPLC system and standards supplied by the NRC, Australia.

1. Town of São Francisco de Paula
2. River Sinos
3. River Caí
4. Town of Camaquã
5. City of Ponta Grossa
6. City of São Paulo
7. City of Rio Grande
8. City of Caxias do Sul
9. Itaúba Dam
10. Itapeva Lake
11. Town of Maracanaú
12. Rio Negro Dam (Uruguay)

Figure 2. Map of Brazil pointing the location of the several Cities, towns, dams and rivers mentioned in the text where cyanobacterial blooms were collected.

The Camaquã bloom case

Camaquã is a town located at the Southernmost part of Brazil. The town (60,000 inhabitants) is situated at the flat margins of the Patos Lagoon, and is crossed from the Northwest by the Duro river. The Duro river is a very important economical factor for the whole region. Waters are used for rice irrigation, the most important regional resource, as well as a population water supply. Consequently, at the years 1960, a huge concrete dam was built upstream in the Duro river. The dam produced an approximately 1,600 acres of land filled by water raised up to 59 m above the sea level. Since the year 2000, the Duro dam has been monitored for the occurrence of toxic cyanobacteria.

In may 2000, the waters at the dam presented levels of *Cylindrospermopsis raciborskii* of 490 filaments.mL^{-1} and *Pseudoanabaena limnetica* of 653 filaments.mL^{-1}. The sample was evaluated in a standard mouse toxicity test and presented typical

symptoms of neurotoxicity. That sample was analyzed by a FLD-HPLC system and presented levels of Neo-STX of $0.101 \mu g \ g^{-1}$; GTX1 of $1.8 \ \mu g \ g^{-1}$ and GTX2 of $0.6 \ \mu g \ g^{-1}$. The data confirmed a mouse lethal dose near $25 \ \mu g \ kg^{-1}$ b.w.

From February till March, 2002 the dam developed another cyanobacterial bloom. At that period the highest levels of filaments reached more than 30,000 filaments of *C. raciborskii*, predominantly. The mouse toxicity test confirmed the neurotoxicity symptoms, and a further analysis by FLD-HPLC, confirmed the existence of Neo-Saxitoxin and GTX 2-3 peaks. Although the technique used at this moment was different from the one of the year 2000. The latter analysis confirmed the toxic mode (PST-like) of the cyanobacterial bloom occurring at the Duro reservoir.

Other cyanobacteria, including *Pseudoanabaena* genera were also found in the year 2002 samples. They may account for the levels of $0.31 \ \mu g \ L^{-1}$ of microcystins equivalents.

Permanent blooms in Ponta Grossa, Paraná

The Alagados dam is a water reservoir originally meant to serve to hydroelectric generation purposes. As the demand for larger water volumes increased for the Ponta Grossa City (300,000 inhabitants), the reservoir was shared with the former state owned water company, SANEPAR. Located on the top of the "Serra do Mar" chain of mountains, 900 m above the sea level, the reservoir suffers the impact of a heavy load of organic and inorganic material released by several intensive pig and poultry farming in the surrounding areas. Due to a surface formed by a basic rocky layer, the absorption of this heavy load of organic and inorganic nutrients by the bottom sediments is insufficient and most material were flushed and carried by the rainwaters straight to the reservoir. A hydrobiological survey of the two main waterbodies, Alagados and Pitangui (an uptake point, situated several kilometres downstream) indicated the presence of a very persistent *Cylindrospermopsis raciborskii* bloom in the reservoirs (Figure 3). The analysis throughout the years revealed levels as high as $3.5 \ x \ 10^{5}$ cells.mL^{-1}.

Cyanotoxin analyses in waters from the Alagados and Pitangui uptake in the river have been done routinely since 2001. Samples of raw and finalwater were analysed. Six highly toxic structures were identified following analysis on a FLD-HPLC and post-derivatization method. STX, Neo-STX, GTX 1, GTX 2, GTX 3 and GTX4 were followed. Apparently, the present strain produces all PST variants, having Neo-STX, GTX 2, GTX3 e GTX 4 at the highest toxic levels. Mouse bioassays were also positive for neurotoxins and confirmed the toxicity of the local bloom.

The Taiaçupeba case and the Taquacetuba sample

The City of São Paulo, is the most inhabited (10 millions inhabitants) urban complex in Brazil. The City is also located at the top of the "Serra do Mar" chain of mountains at 760m above the sea level. Although the City is not near to the Central areas of the Country (Brasília – 1,015 km far), the region climate resembles more to the Southeast of
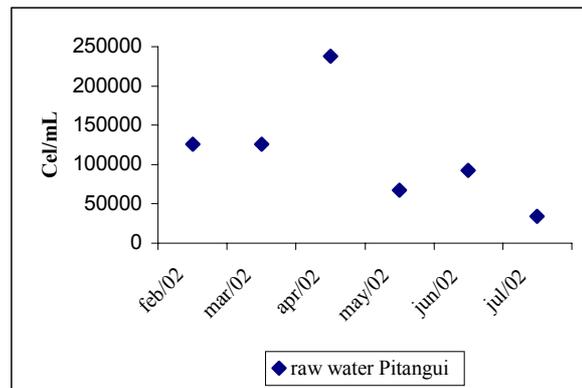
(A)
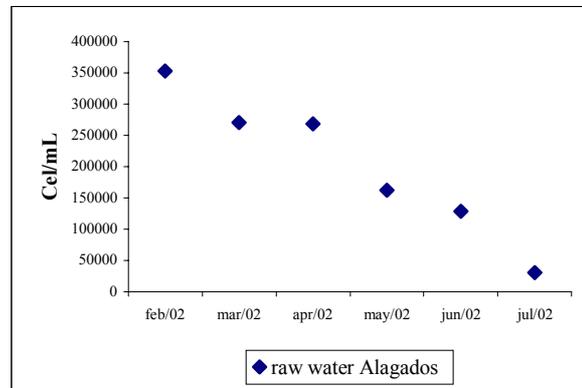
(B)

Figure 3: Average monthly counts of *Cylindrospermopsis raciborskii* for the reservoirs of (A) Alagados and (B) Pitangui. Data supplied by Claudia V. Pacheco from SANEPAR.

Brazil (subtropical) than the other regions, (temperate weather) mentioned before in the previous cases.

The Taiaçupeba reservoir is a recently built dam aimed to accumulate larger volumes of water to be treated and supplied to the East zone of the City. Prior to the end of 1998, the Taiçupeba waters were only impacted by *Microcystis* sp. blooms, with levels of hepatotoxins been kept below 1 μg L^{-1} (the WHO guideline for microcystin) in the drink water samples. After these years, following information of the Microbiology and Hydrobiology Division of SABESP, the São Paulo State Water Supplier, *Cylindrospermopsis raciborskii* blooms, became a common feature of seven of the main water reservoirs for the City (Taiaçupeba, Rio Grande, Guaraú/ Paiva-Castro, Jacareí, Jaguarí, Jundiaí and the Billings reservoir), these blooms on the contrary of others occurred, normally along the whole year.

In the Taiaçupeba reservoir, analysis of samples collected in November and December 1998 indicated levels of *C. raciborskii* varying from 6,000 to 12,820 filaments.mL^{-1}. Mouse bioassays confirmed the neurotoxicity of the samples and produced LD$_{50}$ from 38.4 mg.kg^{-1} to 130.3 mg.kg^{-1}. In this case, following the toxicity classification proposed by Lawton *et al*, 1994, one may consider these samples as from a highly toxic organism due to the relatively few number of filaments which characterised those blooms. The samples were analysed by FLD-HPLC by Geoff Eaglesham, at the NRC, in Australia and revealed levels of 17μg.g^{-1} and 24μg.g^{-1} of Saxitoxins (STX) and Gonyautoxins (GTX). The analytical system used at that moment was the pre-column derivatization method (7).

In August 2002, a further sample of raw water from the Taiaçupeba reservoir was analysed. The sample contained a large variety of harmful cyanobacterial species including *Anabaena* sp (13,000 filaments.mL^{-1}), *Microcystis* sp. (1,005 cells.mL^{-1}) and *Cylindrospermopsis* sp (5,400 filaments.mL^{-1}) and a few others. Analysis of PSTs using the post-column derivatization method[15], confirmed the presence of STX and GTX in the local blooms and discriminated precisely their concentrations as 0.01μg.L^{-1} of Saxitoxin, 0.03 μg.L^{-1} of Neo-Saxitoxin, 0.02 μg.L^{-1} of GTX 2 and 0.03μg.L^{-1} of GTX 3. The use of μg.L^{-1} as units is a requirements of the Brazilian legislation[2] for drink water quality. The sample was also tested for the presence of anatoxin-a (S). The method used was the anti-acethylcholinesterase activity technique (AChE), which will be fully described later on in this chapter. The sample showed an inhibition of 10.28% respect the controls, having near 400,000 cells.mL^{-1}. In previous works of our group[14] we pointed that over a million cells of *Anabaena spiroides* was required to obtain a 100% AChE inhibition (8).

Recently, Lagos *et al.* (9), analysed samples from *Cylindrospermopsis raciborskii* strains isolated from other water reservoirs in the City of São Paulo. The Taquacetuba arm is a part of the Billings reservoir and, as mentioned above, was also under the impact of *Cylindrospermopsis raciborskii* blooms. Although the isolate was from 1996, the toxins profile was very similar to the most recent blooms. The authors analysed the sample by a post-column derivatization system and confirmed data on an ESI- MS (Electron Spray Ionisation-mass spectrometer). Results indicated the presence of STX, GTX 2 and GTX 3 at levels of 0.28, 0.98 and 0.98 μmol g^{-1}, respectively.

Overview of the South Brazilian situation

The first aspect that must call one's attention related to the increasing occurrence of cyanobacterial blooms in the Southern regions of Brazil is related to the PST's predominance at most blooms analyzed. *Cylindrospermopsis raciborskii* is the genera most cited as a neurotoxic producer and seems to be well settled in the calm waters retained by the dams. The organism prefers very turbid waters, nutrient-rich and high pH (above 8.0). More than 10 cases of others dams in the region were reported to have *C. raciborskii* neurotoxic producing blooms while this chapter was prepared.

It seems very likely that the organism is substituting blooms of other seasonal cyanobacteria (*Microcystis, Aphanizomenon, Anabaena*) for a permanent occurrence in the dams environment. A requirement for drinkwater analysis set by the recent Brazilian Legislation (Portaria 1469) from the Ministery of Health[2], has established in the country a high demand for PSTs analysis in waters produced in the whole region. A quick screening at the profile of the several PSTs found in raw water samples in South Brazil suggests a possible trend on the PSTs spectrum. Most samples have NeoSXT, GTX 2 and GTX3 as the main component of the toxic part of the bloom. A few of them have SXT, GTX 1 and GTX 4 as the complementary part. It is possible that a mixture of these two principal toxins could be provided as standards to control contamination in the final water generated after treatment.

Anatoxins from *Anabaena* forms.

Introduction

As a result of urban development, the aquatic environment has been subjected to severe impacts from inland nutrient inputs, which in turn affects several ecological communities by the inducing of cyanobacterial blooms (10). Bloom formation is characterized by a short-term exponential growth of a generally single toxic cyanobacterium species. During the blooms, toxins are released into the water, potentially toxic to several animals, including humans. This toxicity may provoke allergic reactions and respiratory arrest. Gastroenteritis and liver and neurological malfunction are also symptoms that have been registered (11). *Anabaena* neurotoxins include analogues of the neurotransmitter acetylcholine (anatoxin-a), Ca^{2+} channel blockers (PSTs) and the acetylcholinesterase inhibitor[3] (anatoxin-a(S))(3).

Anatoxin-a(S) is a natural analogue of organophosphorus pesticides, from its chemical structure as determined by Matsunaga *et al.*, 1989 (12). Organophosphorus pesticides (OPs) are specific inhibitors of acetylcholinesterase (AChE), an enzyme known to hydrolyze the neurotransmitter acetylcholine, an important regulator of neural transmission. Because of the specificity of OPs against AChE, this enzyme is frequently employed as a biomarker for the detection of OPs in marine and freshwater environments (13, 14). Inclusive, it is the legal method adopted by the Brazilian legislation towards the analysis and control of OPs in the drink water in the Country (Portaria 1469, Brazilian Health Ministry). Toxins like anatoxin-a(S) structurally resemble an activated oxo-form of an organophosphorus molecule, allowing their detection by in vitro enzymatic systems without oxygenates, since anatoxin-a(S) is a 'ready to act' molecule, and can inhibit AChE activity without further metabolization.

It is well established that AChE inhibition by organophosphorus esters follows a pseudo-first order kinetics (15, 16). Previous assays conducted in our laboratory, showed that inhibition of brain *Odontesthes argentinensis* (silver-side fish) AChE by aqueous extracts from *A. spiroides* followed a pseudo-first order kinetics (**Figure 4**), suggesting the presence of organophosphorus-like compounds in the cyanobacteria extracts. As to date, anatoxin-a(S) is the only cyanobacteria toxin known to be structurally similar to

Ops (12), the existence of this molecule in the assayed aqueous extracts can be inferred. The estimated inhibition parameters allow us to make comparisons with other OPs molecules or anatoxin-a(S) tested in other AChE assays. Amongst different inhibition parameters, the phosphorylation constant (Kp) is considered a measure of the reactivity of organophosphorus molecules to the active site of the AchE (17). Previous work with the same lyophilized powder of *A. spiroides* showed a K_p of 0.17/min when using purified eel AchE (8). Using brain homogenates from the silver-side fish, the estimated K_p was 0.18/min, evidence for a similar reactivity of the anticholinesterase fraction in the eel and silverside AChE. It is important to note that in both studies, the extraction protocol employed was the same. The fact that some authors have found that non-critical binding proteins are not important in the inhibition of AChE from different species (18) provides further support for results obtained with a purified AChE and a non-purified one.

First toxicity test with Brazilian *Anabaena* blooms

Previously, Monserrat *et al*. (2001) (8), used a sample collected at an ornamental lake in the University Campus FURG which contained a number higher than 500,000 filaments of *Anabaena spiroides* per mL (8) . These authors reported a 100% inhibitory effect of the aqueous extract of this sample on the AChE activity of fish and crab species and a purified eel AChE, and suggested that the inhibitory factor was anatoxin-a(S). The results cited above in turn suggest that a simple *in vitro* detector system can be employed for the detection of anatoxin-a(S) in environmental water samples of a variable number of *Anabaena* filaments.

Detection of *Anabaena* toxins

Because of the high number of samples containing *Anabaena* usually received for detection of toxic compounds, the AChE activity inhibition test has become a fully useful tool for environmental analysis. Cyanobacterial bloom powders from drinking water reservoirs showed AChE inhibition ranging from 2.57 to 100% in all cases where *A. spiroides* or *Planktothrix agardhii* filaments were identified (**Table 1**). A fully positive (100% AChE inhibition) sample of *A. spiroides* described elsewhere (8) was included to give a temporary upper limit for filaments counts and %AChE inhibition. Other samples collected at the same location at different dates were included to show how variable the AChE inhibition can be concerning the differences on environmental samples. Although most blooms were composed of more than one cyanobacterium genus, filament counts presented are exclusive of *A. spiroides* These varied from 30 to 2,900 filaments. mL^{-1} with an AChE inhibition ranging from 3.73% to 28.2%   Although a linear correlation between number of filaments and AChE inhibition was not estimated, samples taken during a blooms dominated by *Anabaena circinalis* and lacking *A. spiroides* showed absence of enzyme inhibition. Environmental control of *A . spiroides* blooms still lack and also an efficient and low-cost tool to estimate how toxic blooms can be if anatoxin-a (S) is the possible toxin contained in the cells. Henriksen *et al.,* 1997 (19), overcame this situation when dealing with mixed populations of *Anabaena flos-aquae* and *A. lemmermanni* by the use of the value proposed by Matsunaga *et al,* 1989 (12) for the LD_{50} for pure anatoxina-a(S) of 20μg.kg^{-1} b.w.. Using this value the authors, considered that a LD_{50} of 2.5 mg.kg^{-1} and 150 mg.kg^{-1} b.w. for the mixed blooms would contain 8mg.g^{-1} and

0.13mg.g^{-1} of anatoxina-a(S), respectively. Following the same assumption it is possible to propose that a LD$_{50}$ of 369 mg.kg^{-1} b.w. determined with the sample collected in 1995 (**Table 1**), should contain 54.2 µg.g^{-1} of lyophilized sample. This estimate value posses a 100 % inhibitory effect on AChE activity.
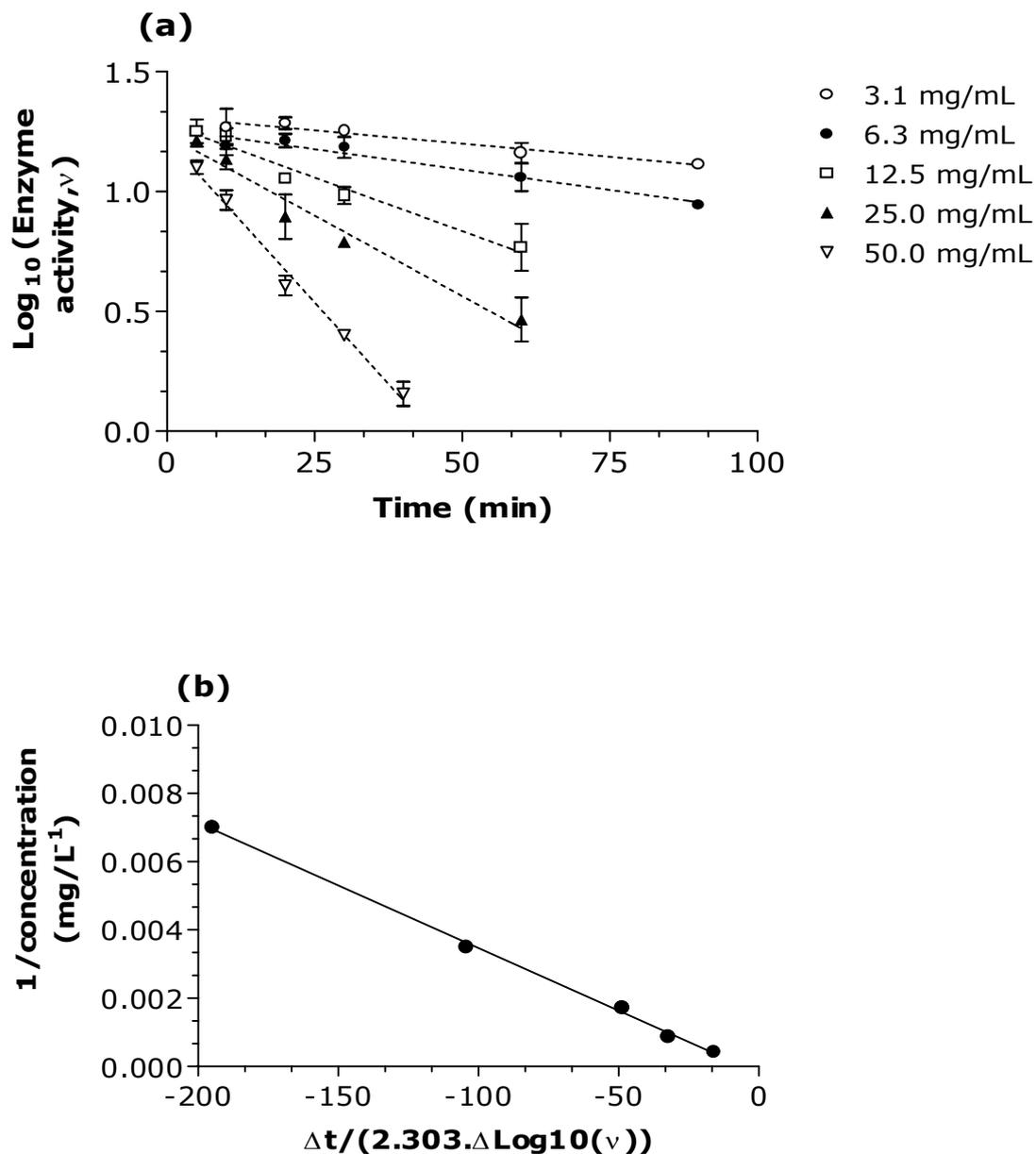

Figure 4. (a) Inhibition of acetylcholinesterase activity (v; expressed as nmoles substrate/mg protein/min) of brain from *Odontesthes argentinensis*, at different concentrations of *Anabaena spiroides* aqueous extract and incubation times. (b) Regression function employed to estimate the affinity equilibrium (Ka) and bimolecular inhibition (Ki) constants. Δt.(2.303.log10 v)$^{-1}$ represents the inverse of the slope of the natural logarithm of enzyme activity against time.

Table 1. Variation of AChE inhibition using the standard protocol in samples taken at different water reservoirs during cyanobacteria blooms.

Local of Sampling Collection date	*Filaments. mL^{-1}	Dominating cyanobacterium species or genus	AChE Inhibition (%)
Rio Grande (RS) – Brazil 05 nov 1995	500,000	*Anabaena spiroides*	100
Rio Grande (RS) - Brazil 09 jan 1997	7,556	*Anabaena spiroides*	6.41
Rio Grande (RS) – Brazil 29 dec 2001	2,900	*Anabaena spiroides*	28.2
Caxias do Sul (RS) – Brazil 20 feb 2001	30	*Anabaena spiroides, M. aeruginosa Oscillatoria sp. Lyngbya*	3.73
Caxias do Sul (RS) – Brazil 01 nov 2001	1,073	*Anabaena planctonica / Anabaena spiroides*	6.04
Caxias do Sul (RS) – Brazil 14 nov 2001	2,900	*Anabaena spiroides, Microcystis*	7.7
Ponta Grossa (PR) – Brazil 19 jun 2000	444	*Anabaena spiroides / Cylindrospermopsis sp.*	5.11
Maracanaú (CE) – Brazil 01 aug 2000	1,566	*Anabaena sp. / Planktothrix agardhii*	6.49
Itaúba (RS) – Brazil 13 jun 2000	0	*Anabaena circinalis*	0
Itapeva (RS) – Brazil 23 jun 1999	1,341	*Anabaena spiroides*	4.79
Itapeva (RS) – Brazil 03 feb 2000	300	*Anabaena spiroides / Anabaena circinalis Anabaena sp.*	8.98
Itapeva (RS) – Brazil 11 may 2000	0	*Anabaena circinalis / Anabaena sp.*	0
Rio Negro (Uruguay) 17 jan 2001	42.8	*Anabaena spiroides / Microcystis aeruginosa*	2.57
Rio Negro (Uruguay) 06 mar 2001	2,790	*Anabaena spiroides / Microcystis aeruginosa*	3.15

Genus and species (when identified) that dominated those blooms were also indicated, but counts (*) are specific for *Anabaena spiroides*.

Overview of the Southern Brazilian Situation

As shown previously, Monserrat *et al.* (2001) (8) increasing concentrations of dried *Anabaena* bloom draws a linear correlation with AChE inhibition, it is also possible to suggests reasonable values for AChE inhibition from 0 to 100% based on the levels of anatoxin-a(S) ($\mu g.mg^{-1}$) present in the environmental sample extracts from Southern Brazilian reservoirs (**Table 1**).

The methodology employed in this study is a feasible and simple method to analyze the presence of anticholinesterase compounds during cyanobacterial blooms. In all cases where *A. spiroides* was present in the samples, a level of AChE inhibition was achieved. A very high number of filaments are required to produce a sample containing a 100% inhibition of AChE. However, the levels of AChE inhibition that causes environment and water quality concern will be the ones in respects of 20,000 cells or just above - quantities considered by the WHO guidelines as reference for the environment and water quality alerts for drinking water.

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