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A rapid bioassay for detecting saxitoxins using a *Daphnia* acute toxicity test

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A new Daphnia bioassay, as an alternative to the mouse bioassay, is able to detect effects of fast-acting, potent neurotoxins in raw water.

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ABSTRACT

Bioassays using *Daphnia pulex* and *Moina micrura* were designed to detect cyanobacterial neurotoxins in raw water samples. Phytoplankton and cyanotoxins from seston were analyzed during 15 months in a eutrophic reservoir. Effective time to immobilize 50% of the exposed individuals (ET_{50}) was adopted as the endpoint. Paralysis of swimming movements was observed between ~0.5–3 h of exposure to lake water containing toxic cyanobacteria, followed by an almost complete recovery of the swimming activity within 24 h after being placed in control water. The same effects were observed in bioassays with a saxitoxin-producer strain of *Cylindrospermopsis raciborskii* isolated from the reservoir. Regression analysis showed significant relationships between ET_{50} *vs.* cell density, biomass and saxitoxins content, suggesting that the paralysis of *Daphnia* in lake water samples was caused by saxitoxins found in *C. raciborskii*. *Daphnia* bioassay was found to be a sensitive method for detecting fast-acting neurotoxins in natural samples, with important advantages over mouse bioassays.

1. Introduction

Cyanobacterial blooms are increasing worldwide as a consequence of eutrophication and global warming (Paerl and Huisman, 2008). Toxic cyanobacteria can seriously impair water quality, threatening human health as well as environmental resources worldwide (Chorus and Bartram, 1999; Chorus, 2005; Burch, 2008). For safety reasons, the World Health Organization (WHO, 2003) established guides for human exposure to cyanotoxins, including the major routes of exposure such as oral and dermal routes through drinking water and recreational water use (Falconer, 1999).

The invasive cyanobacterium *Cylindrospermopsis raciborskii* has been spreading quickly in North America and Europe in the last 10–15 years (Chapman and Schelske, 1997; St Amand, 2002; Gugger et al., 2005; Conroy et al., 2007). This species is well adapted to high temperatures and high nutrient loading, conditions common in tropical regions, including both shallow, well-mixed lakes and deeper, thermally stratified reservoirs (Huszar et al., 2000; Yunes et al., 2003). Unlike the North American (Burns et al., 2000), Australian (Griffiths and Saker, 2003) and Thai (Li et al., 2001) strains, which produce cylindrospermopsin, Brazilian strains of *C. raciborskii* isolated to date produce saxitoxins (Lagos et al., 1999; Molica et al., 2002, 2005). These toxins can be highly toxic and in high enough doses, lethal to animals and humans (Landsberg, 2002). Although there have not been any documented human deaths related to *C. raciborskii*, there have been many serious hospitalizations related to exposure to cylindrospermopsin (Griffiths and Saker, 2003). Cases of intoxication of humans by saxitoxins have been related to consumption of marine shellfish contaminated with saxitoxins from dinoflagellates (Landsberg, 2002) but no case of human intoxication involving freshwater saxitoxin producers have been documented to date.

Saxitoxins are potent paralytic agents, blocking the influx of sodium ions (Na^+) through excitable membranes, effectively interrupting the formation of the action potential (Levin, 1991; Kao, 1993; Cestèle and Catterall, 2000). This mechanism of action has been studied for mammals such as mice (Carmichael, 1992; Kao, 1993), and for invertebrates such as squid and crayfish (Adelman et al., 1982). The ultimate effect is, therefore, the rapid paralysis of muscles, leading to respiration arrest and death in mammals (Carmichael, 1992; Kao, 1993) and to motor incoordination in fish

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(White, 1977; Lefebvre et al., 2005). Microcystins, the most widespread and best studied of the cyanobacterial toxins, disrupt cell function and lead to acute liver failure in vertebrates (Carmichael, 1992). In contrast to saxitoxins, microcystins do not cause rapid paralysis, although they can cause rapid mortality when injected into mice (mouse bioassay). Thus, these differences in the mechanism of action can be used in bioassays as a cue to separate the effects of these two types of toxins.

As a consequence of the increasing concern with toxicity of cyanobacteria, several techniques have been developed to detect cyanotoxins in the water, including the simple and inexpensive mouse bioassays and more sophisticated analytical methods that directly quantify toxins, such as High Performance Liquid Chromatography (HPLC), mass spectroscopy (MS), phosphatase assay (PPase) and Enzyme Linked Immunosorbent Assay (ELISA) (Chorus and Bartram, 1999). However, all these methods have, in general, sensitivity/selectivity issues relative to each other, and there is no consensus about the best method for detection of cyanotoxins as a routine basis. Mouse bioassays, for example, are nonspecific and have low sensitivity (μg detection level) whereas ELISA is very sensitive (pg level) but is also very unselective. Other methods are very sensitive and selective, such as HPLC and LC/MS but require very expensive equipment and expertise (Harada et al., 1999).

Other screening methods include the toxicity bioassays with aquatic organisms, but these methods have been neglected and have received little attention from the scientific community. In fact, there have been few studies suggesting the use of aquatic organisms for detection of cyanotoxins, and most of them have focused on microcystins (Törökné et al., 2000; Tarczinska et al., 2001; Sabour et al., 2002; Drobniewska et al., 2004). Recently, two studies pointed out the potential use of cladoceran species for the detection of saxitoxins in the water, giving its high sensitivity (Ferrão-Filho et al., 2008, 2009).

Although almost three decades of research on zooplankton–cyanobacteria interaction has passed (Lampert, 1981, 1987), the hypothesis of the chemical defense role of cyanobacteria against zooplankton grazing was not properly addressed. Nevertheless, a great deal of studies report a variety of effects of cyanobacteria on zooplankton, in particular on the cladocerans group, mostly on its survivorship, growth and fecundity (DeMott et al., 1991; Rohrlack et al., 1999a,b; Ferrão-Filho et al., 2000; Lürling, 2003; Wilson and Hay, 2007), but also on its feeding process and mobility (DeMott and Moxter, 1991; Haney et al., 1995; Rohrlack et al., 2005; Ferrão-Filho et al., 2008). Despite that, potential effects of cyanotoxins in these aquatic invertebrates, that feed directly on phytoplankton cells and are naturally exposed to toxic cyanobacteria, is still poorly understood. This is a quite important question if we want to use these organisms for biomonitoring of cyanotoxins in the aquatic environment.

The aim of this study was to develop and validate a protocol for detection of saxitoxins in raw water samples using the planktonic cladocerans *Daphnia pulex* Leydig and *Moina micrura* Kurs. For this purpose, we carried out a 15 month water sampling in the eutrophic Funil Reservoir (RJ, Brazil) and performed rapid (2–3 h) acute tests with these samples for detecting paralysis and used ET_{50} (Effective Time to immobilize 50% of the individuals) as an endpoint. In order to validate the method, we performed regression analysis between ET_{50} and cyanobacterial cell density, biomass and saxitoxins content.

2. Material and methods

2.1. Sampling site and phytoplankton analysis

Built in the 1960s for electricity generation and recreational use, Funil Reservoir is a eutrophic reservoir situated on the Paraíba do Sul river valley, near the city of Resende (RJ, Brazil). The reservoir also contributes to the water supply of the Rio de Janeiro City and surroundings. For this reason, the reservoir has been monitored since 2002 for blooms of cyanobacteria (Ferrão-Filho et al., 2009). We conducted a 15 month sampling program at one station near the dam including two periods: from April 2005 to March 2006 and from October 2006 to March 2007. On each sampling date, 5–20 L of raw water was transferred to the lab on ice for measuring sestonic microcystins and saxitoxins, and for toxicity bioassays with cladocerans. Water samples were also fixed with Lugol's solution for phytoplankton analysis (Utermöhl, 1958). Phytoplankton biovolume ($mm^3\ L^{-1}$) was estimated by multiplying the density of each species by the average volume of its cells, according to Hillebrand et al. (1999), and specific biomass was expressed in mg (wet weight, WW) L^{-1}, assuming a specific density of phytoplankton cells of 1.0 g cm^{-3} (Edler, 1979).

2.2. Culture of C. raciborskii

A strain of *C. raciborskii* (CYRF-01) was isolated from the reservoir and was maintained in ASM-1 medium (Gorham et al., 1964), in batch cultures with aeration, pH = 8.0, 23±1 °C, light intensity of 40–50 μE $m^{-2}\ s^{-1}$ and a 12/12 h light:dark cycle. This strain has been used in other studies and has been reported to produce saxitoxins (Ferrão-Filho et al., 2007). The cultures were kept in the exponential growth phase through the replacement of medium once a week. The cell counts were performed on a Fuchs-Rosenthal hemacytometer. For establishing the average cell size and filament size, measurements of at least 50 filaments were made and the number of cells in each filament was counted. Filaments length for CYRF-01 strain varied between 77 and 310 μm (Mean ± SD = 172.75 ± 55.71 μm) and cell length varied between 7.2 and 10.9 μm (Mean ± SD = 9.32 ± 1.14 μm). Cell biomass was estimated by the same method as for phytoplankton.

2.3. Toxin analyses

Samples for microcystins and saxitoxins were taken by filtering a variable volume of water (4–19 L, depending on the season and algal density) onto glass fiber filters (Sartorius®, Goettingen, Germany). The analysis of saxitoxins of strain CYRF-01 was carried out with lyophilized material from the culture. Microcystins and saxitoxins were analyzed by HPLC according to methods described in Ferrão-Filho et al. (2009). Microcystins, if present, were expressed as concentration of MC-LR equivalents (Chorus and Bartram, 1999), with a detection limit of 0.5 μg L^{-1}. Saxitoxins, if present, were expressed as concentration of STX equivalents (Oshima, 1995). Only STX, NEO and GTX(1-4) variants were analyzed. The detection limits for saxitoxins variants are: STX = 0.89 ng L^{-1}, NeoSTX = 2.33 ng L^{-1}, GTX-1 = 1.03 ng L^{-1}, GTX-2 = 0.72 ng L^{-1}, GTX-3 = 0.21 ng L^{-1}, GTX-4 = 0.24 ng L^{-1}.

2.4. Cladoceran cultures

Two cladocerans were used in the experiments: a clone of *D. pulex* Leydig that was obtained from Carolina Biological Supply (NC, USA) and a clone of *M. micrura* Kurs that was isolated from an oligotrophic reservoir in Rio de Janeiro, Brazil, which has no cyanobacterial blooms and exhibited high water quality (Soares et al., 2008; Ferrão-Filho et al., 2009). Both species were cultivated as clonal cultures for several generations prior to the experiments, using mineral water as the culture medium and *Ankistrodesmus falcatus* (Chlorophyceae) as food (0.5 mg C L^{-1}), under dim light, 12/12 h light/dark cycle and ~23 °C. Only *M. micrura* cultures received 20–30% of filtered lake water as previous studies showed that this species did no grow well only in mineral water (Ferrão-Filho et al., 2009).

2.5. Acute toxicity bioassays

These bioassays were designed to detect immobilization (i.e. paralysis) of the cladocerans exposed to reservoir water containing seston and to intact cells of the strain CYRF-01. Bioassays consisted of two phases: the *exposure phase* – in which 10 newborns (>24 h) were placed into 30 ml test tubes and exposed for 2–3 h to the experimental concentrations (reservoir raw water or cells of the strain CYRF-01 diluted in mineral water, with 3 replicates for each treatment) and checked for the number of active swimming individuals after 0.5, 1, 2 and 3 h; and the *recovery phase* – in which all individuals (including the paralyzed ones) from both raw water and CYRF-01 treatments were transferred to "clean" water (mineral water + food) and checked after 15–24 h. To avoid false positives, due to the presence of any other neurotoxic contaminant dissolved in the lake water, controls (3 replicates) with only filtered reservoir water were run in parallel. At the end of the recovery phase, the number of active swimming, immobilized and dead individuals was counted. Food (*A. falcatus*), at a concentration of 0.5 mg C L^{-1}, was added to all treatments.

2.6. Statistical analyses

Effective time to immobilize 50% of the individuals (ET_{50}) was calculated only for the undiluted reservoir water (100% treatment) by use of Probit Analysis (SPSS Statistical Package, v. 8.0). The effective time (or lethal time, LT_{50}) is an endpoint that have been used in some studies about the effects of toxic cyanobacteria on

cladocerans (Ferrão-Filho et al., 2000; Guo and Xie, 2006) and effects of toxic dinoflagellates on rotifers (Wang et al., 2005). This endpoint is convenient when it is not easy to standardize a time for the calculation of the effective concentration (EC_{50}) (Ferrão-Filho et al., 2000). Also, many studies have pointed out that EC_{50} varies with time and therefore is not a reliable endpoint for regulatory purposes (Kooijman and Bedaux, 1996; Jager et al., 2006). For the strain CYRF-01, the EC_{50} was calculated for 2 h exposure time using Probit analysis. The ET_{50} was also calculated for each concentration of strain CYRF-01.

Regression analyses were performed between ET_{50} and cyanobacterial cell density, biomass and saxitoxins content. For seston, logarithmic regression was performed for ET_{50} *vs.* cyanobacterial cell density and for ET_{50} *vs.* phytoplankton biomass, and first order polynomial regression was performed for ET_{50} *vs.* saxitoxin content. For strain CYRF, logarithmic regression was performed for ET_{50} *vs.* cyanobacterial cell density, biomass and saxitoxins content. The regression analysis was performed with Sigma Plot 2000 (v.6.1) program.

3. Results

3.1. Toxin contents of seston and strain CYRF-01 and phytoplankton composition

Saxitoxins content in reservoir's seston varied between 0.0003 and 3.48 μg Eq.STX L^{-1}. The saxitoxin variants found in seston were STX (in all months), neoSTX, GTX1, GTX2 and GTX4 (less often), and GTX3 (in 1 month). In two samples analyzed, the strain CYRF-01 contained 32.0–52.0 μg STX g^{-1} and 47.7–55.0 μg GTX1 g^{-1}. Microcystins were not detected in seston samples.

The cyanobacterial assemblage was composed of five species, with *Anabaena circinalis* Rabenhorst ex Bornet et Flahault (0–88%), *C. raciborskii* (Woloszyńska) Seenayya et Subba Raju (4–99%) and *Microcystis aeruginosa* (Kützing) Kützing (0.5–85%) comprising most of the biomass.

3.2. Effects of seston samples on the mobility of cladocerans

The immobilization effect was observed in every month during the exposure to reservoir water, with the animals being paralyzed within a few hours, or even in less than 1 h, depending on the season (Figs. 1 and 2). There was a clear dose–response effect, with animals being paralyzed faster in the higher concentrations of reservoir water. After the animals were transferred to control water, in most cases recovery took place in a few hours and was almost complete after 24 h. Mortality rates were very low at the end of the experiment, varying from zero to <10% in most periods. Higher mortality rates were found in November/05 (17% for *D. pulex*) and in March/06 (23% for *M. micrura*), only in the highest concentration.

3.3. Effects of the strain CYRF-01 on the mobility of cladocerans

The same effect was observed with the exposure to the strain CYRF-01 (Fig. 3). The exposure to only 0.05 mg L^{-1} (~464 cells mL^{-1}) of the strain CYRF-01 caused immobilization of about 50% of *D. pulex* in 3 h, while *M. micrura* started to be immobilized only with 0.22 mg L^{-1} (~2000 cells mL^{-1}). The EC_{50} was 0.23 mg L^{-1} (2133 cells mL^{-1}) for *D. pulex* and 0.96 mg L^{-1} (8905 cells mL^{-1}) for *M. micrura* in 2 h exposure. Recovery was evident, with most animals recovering swimming after 15–24 h. Mortality rates were negligible in all concentrations (<10%).

3.4. Results of the statistical analyses

Table 1 shows the equations and statistical results of the regression analyses. For *D. pulex*, ET_{50} showed a significant relationship with cell density of *C. raciborskii*, both for field samples and the strain CYRF-01 (Fig. 4a). There was also a significant relationship between ET_{50} and *C. raciborskii* biomass (Fig. 4b). The regression between ET_{50} and the saxitoxins content of seston showed a significant relationship when a first order polynomial function was applied, showing a sharp decrease in ET_{50} in the range of 0.0003–0.003 μg L^{-1}, and stabilizing after 0.01 μg L^{-1} (Fig. 4c). In the case of strain CYRF-01, there is a significant relationship between ET_{50} and the saxitoxins content of *C. raciborskii*, with a logarithmic function as the best fit.

For *M. micrura*, there was not a good relationship between ET_{50} for seston and any of the independent variables (Fig. 4d–f). However, ET_{50} related significantly with cell density, biomass and saxitoxins content of the strain CYRF-01, even though only the higher concentrations could be used in the analyses due to the impossibility to calculate ET_{50} for the lower concentrations.

As expected, no significant relationship was found between ET_{50} and cell density or biomass of other cyanobacteria present in the reservoir's seston.

4. Discussion

Toxic effects of saxitoxin-producer phytoplankton on crustacean zooplankton have been reported in many studies in the marine environment (Ives, 1985, 1987; Huntley et al., 1986; Sykes and Huntley, 1987; Teegarden and Cembella, 1996; Dutz, 1998; Colin and Dam, 2004) but few studies have reported effects on freshwater zooplankton (Haney et al., 1995; Nogueira et al., 2004; Ferrão-Filho et al., 2007, 2008, 2009; Soares et al., 2009). Ives (1985) has reported a 'loss of motor coordination' in marine copepods exposed to toxic dinoflagellates but it was not clear if this effect was related to the swimming behavior or to the feeding process of these animals.

Nogueira et al. (2004) showed that *Daphnia magna* exposed to filaments of *Aphanizomenon issatschenkoi* (1.2×10^6 cells mL^{-1}) accumulated up to 0.38 pmol PST $animal^{-1}$ and decreased survival relative to control with food (*A. falcatus*) but not to starved animals, concluding that *A. issatschenkoi* affects the fitness and growth of *D. magna* due to toxic effects or unsuitable food quality but that these effects could not be attributed to starvation. On the other hand, Soares et al. (2009) showed that *D. magna* had slightly longer survivorship in suspensions with the strain CYRF-01 (*C. raciborskii*) as the sole feed than in medium without food, showing that starvation was not the cause of death. However, these authors showed that the fitness (i.e. body growth and population growth rates) and clearance rates of this daphniid were depressed only in high proportions (75–100%) of this cyanobacterium, and concluded that energy limitation, not toxicity, might be the dominant factor affecting growth of large-bodied cladocerans. In both these studies with saxitoxin-producer strains any effect on mobility was reported.

The paralysis effect observed in our bioassays with reservoir water and with the strain CYRF-01 was clearly compatible with the mechanism of action of saxitoxins and incompatible with the reported responses of zooplankton to microcystins. This specific, reversible effect was described for freshwater cladocerans in a previous study by Ferrão-Filho et al. (2008) and was regarded as a toxic effect of saxitoxins, paralyzing the muscles of the second antennae, which is responsible by the swimming movements of the animals. In another study, Haney et al. (1995) reported a reduction in the thoracic appendage beating rate and an increase in rejection rate of particles by the postabdomen of *Daphnia carinata* when exposed to a filtrate of a saxitoxin-producing strain of *Aphanizomenon flos-aquae* and to purified saxitoxin. Similarly to our results, they found that animals recovered after the medium containing saxitoxins was replaced by control medium. These authors concluded that the chemicals in *A. flos-aquae* act as a chemosensory cue, causing behavioral change in feeding activities rather than by a direct inhibition of motor activity. However, in accordance with

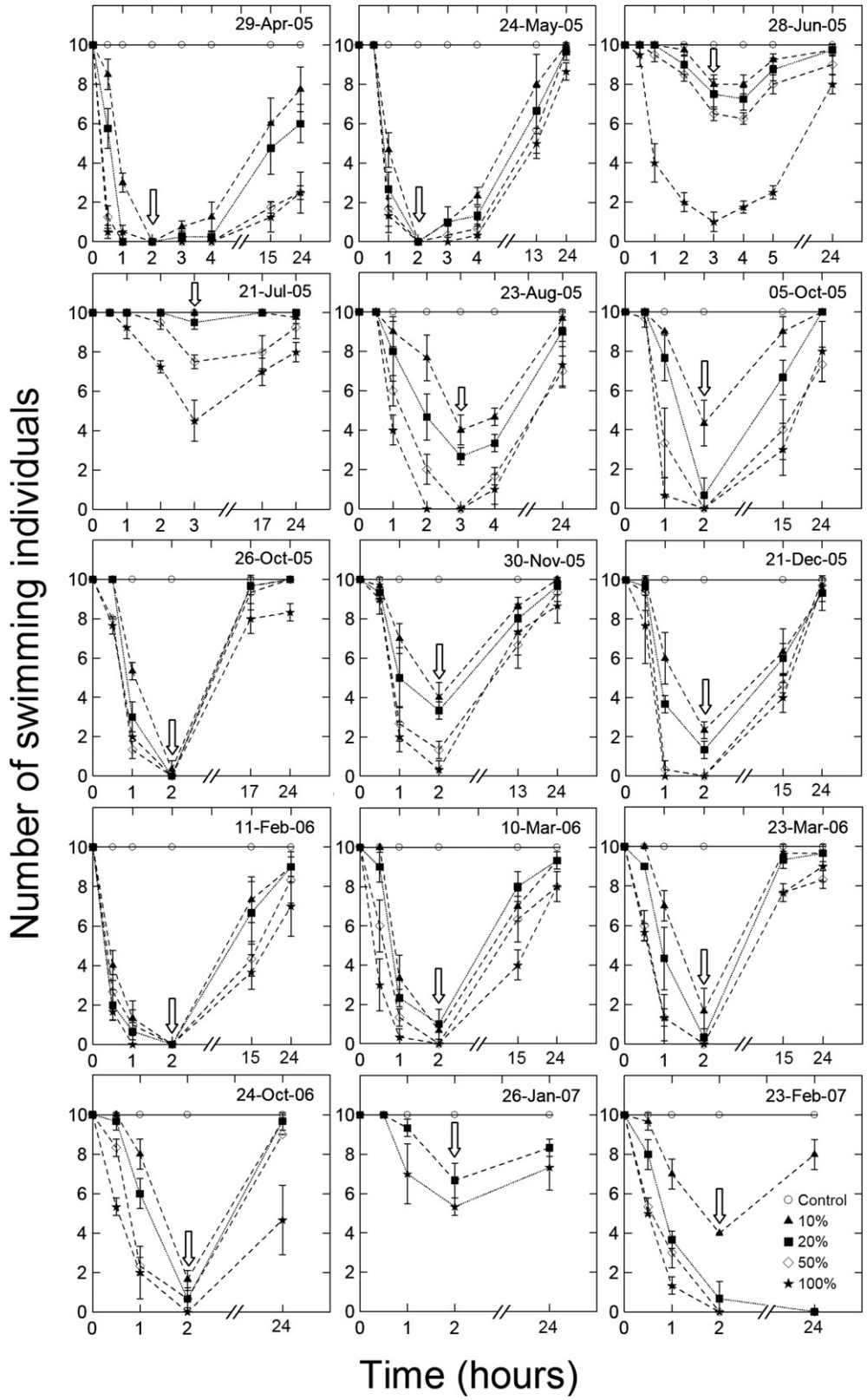

Fig. 1. Results of the acute bioassays with *D. pulex* carried out during the 15 month sampling period in Funil Reservoir. Bioassays consisted of two phases: the *exposure phase*, when the animals were exposed for 2–3 h to raw water (10–100%); and the *recovery phase*, when the animals were transferred to 'clean water' (mineral water + food) and followed until 24 h. Controls consisted of animals exposed to filtered reservoir water. White arrows indicate the transition from the exposure to the recovery phase.

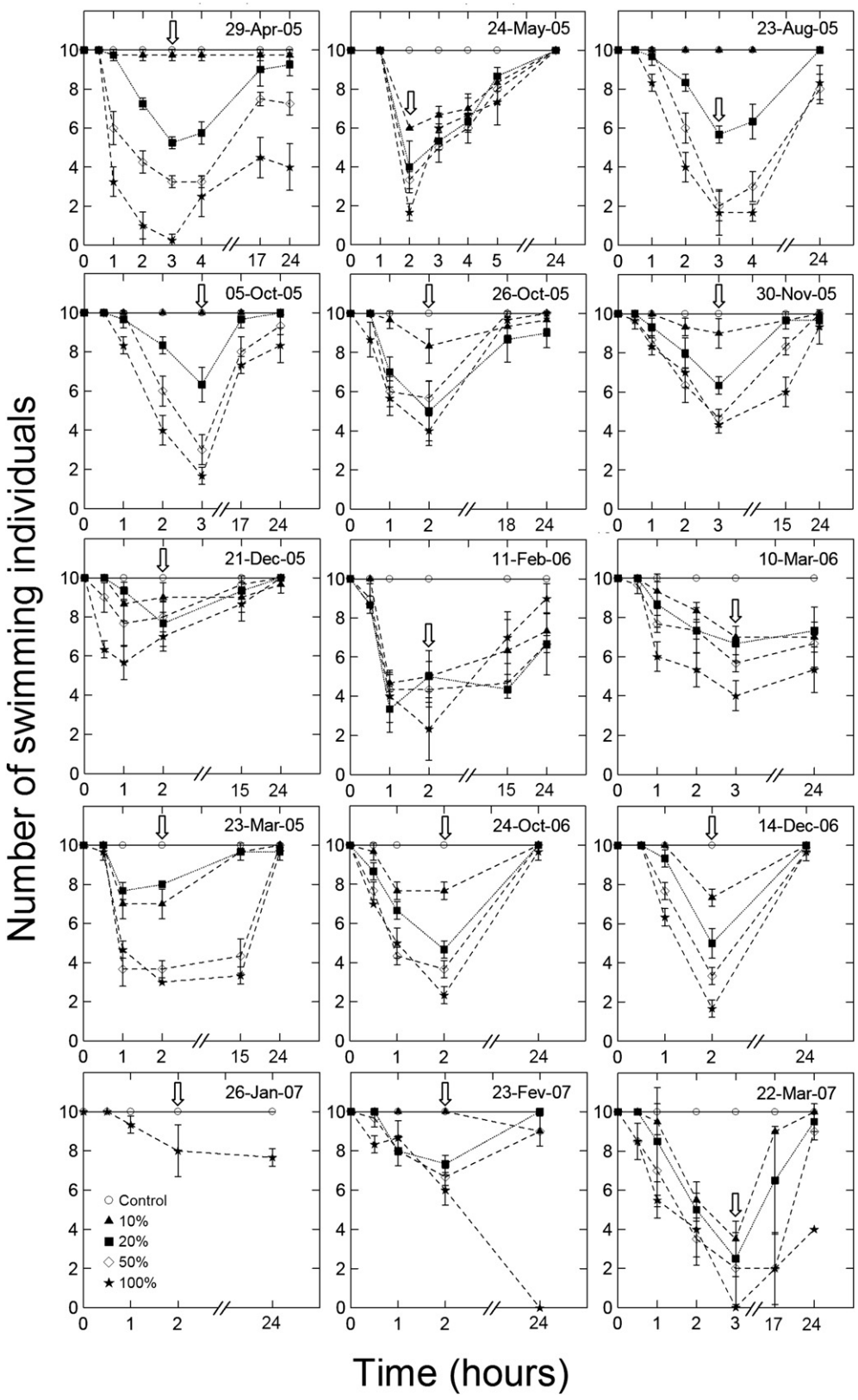

Fig. 2. Results of the acute bioassays with *M. micrura* carried out during the 15 month sampling period in Funil Reservoir. The same design was adopted as for *D. pulex* bioassays.

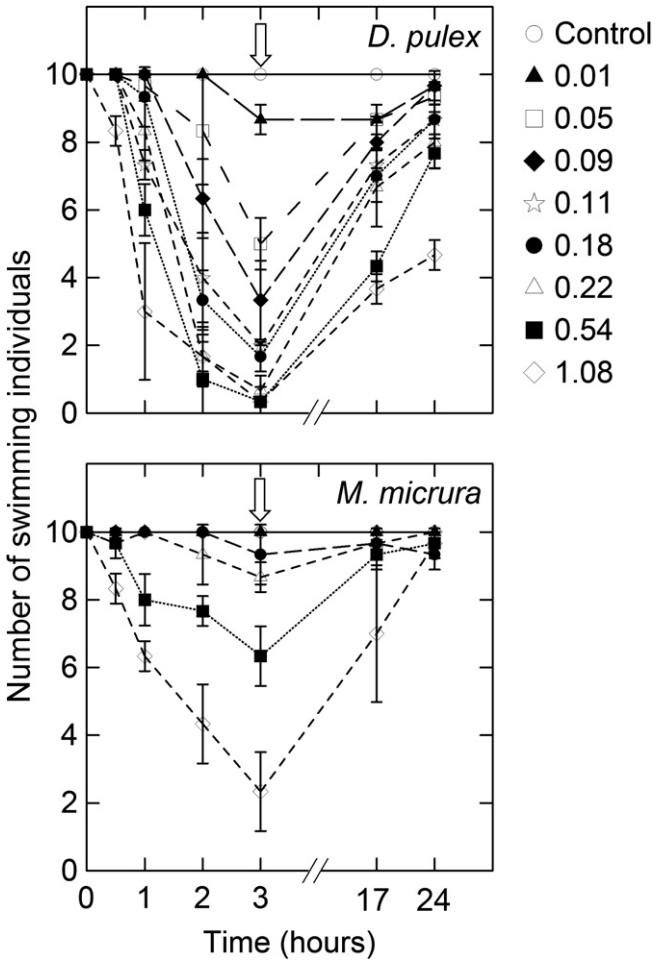

Fig. 3. Results of the acute bioassays with *D. pulex* and *M. micrura* exposed to the strain CYRF-01. The animals were exposed for 3 h to different concentrations (mg L^{-1}) of cells and after that transferred to 'clean water' (mineral water + food) and followed until 24 h. Controls consisted of animals exposed to mineral water + food. The white arrow indicates the transition from the exposure to the recovery phase.

Ferrão-Filho et al. (2008), our results suggest that chemicals produced by *C. raciborskii* really act as a direct inhibitor of motor activity, as shown by the inhibition effect on mobility of cladocerans. This effect is probably caused by saxitoxins produced by this cyanobacterium, since a non saxitoxin-producing strain of *C. raciborskii* (NPLP-1) caused no effect on cladocerans' mobility (Ferrão-Filho et al., 2008).

In spite of the paralysis effects observed are suggestive of saxitoxins action, there is always the possibility that other, non-identified compounds, both in seston and in strain CYRF, may be acting on cladocerans. Certainly, a test with purified saxitoxins would help to improve our knowledge on the mechanism of action of these toxins in cladocerans. However, the absorption of toxins directly from the water seems to be low, leading to underestimation of effects and use of high, not ecologically relevant toxin concentrations (DeMott et al., 1991; Reinikainen et al., 2002; Oswald et al., 2008; Berry et al., 2009). Also, since cyanotoxins are endotoxins and its uptake is primarily in the digestive tract, trough the ingestion and digestion of cells (Rohrlack et al., 2005), the ecological meaning of such tests with pure toxins is questionable.

In all tests, controls with filtered reservoir water caused no effect on cladorerans, showing that the toxic agent was in the particulate fraction. Thus, effects of dissolved compounds in the water, such as organophosphate pesticides, which can induce paralysis, are less likely. This emphasizes that cyanobacterial biomass, which comprised most of seston biomass, was the main cause of paralysis. Also, *A. circinalis*, another potential saxitoxin producer, was present in Funil reservoir, but was mainly in the form of large (>100 μm) and spiraled tricomes, which makes the ingestion of their cells by cladocerans unlikely. On the other hand, filamentous cyanobacteria such as *C. raciborskii* and *Planktothrix* spp. can be easily broken by *Daphnia* into small, edible size during the filtration process (Bouvy et al., 2001; Oberhaus et al., 2007; personal observation).

Since microcystins mechanism of action is quite different from saxitoxins, leading to death of the animals in a scale of days (Ferrão-Filho et al., 2000; Reinikainen et al., 2002), it is unlikely that microcystins are causing the paralysis effect. Also, microcystins were not detected in seston, leading to the conclusion that saxitoxins produced by *C. raciborskii* were the main paralyzing agent in seston. Thus, the bioassay does not detected microcystins, and this was the reason why ET_{50} was not correlated with other abundant cyanobacteria (especially *Microcystis*).

The regression analyses showed that, at least in the case of *D. pulex*, ET_{50} can be considered a good predictor of cell density, biomass and saxitoxins content of seston and strain CYRF-01 (i.e. *C. raciborskii*). Also, the relationship between ET_{50} and cell density/biomass approached a linear response which means that the

Table 1
Statistical results and equations for the regression analyses between ET_{50} *vs.* cell density and biomass of *C. raciborskii*, and ET_{50} *vs.* saxitoxins content of seston and the strain CYRF-01.

Variables	Type of regression	R-squared	*P*-value	Equation
D. pulex – Seston				
ET_{50} *vs.* cell density	Logarithmic	0.688	0.0001	$ET_{50} = -0.367$ Ln(cell dens.) + 4.424
ET_{50} *vs.* biomass	Logarithmic	0.687	<0.0001	$ET_{50} = -0.367$ Ln(biomass) + 1.073
ET_{50} *vs.* saxitoxins	Non-linear; 1st order polynomial	0.870	<0.0001	$ET_{50} = 0.565 + 0.000514(STX)^{-1}$
D. pulex – CYRF-01				
ET_{50} *vs.* cell density	Logarithmic	0.874	0.0020	$ET_{50} = -0.568$ Ln(cell dens.) + 6.197
ET_{50} *vs.* biomass	Logarithmic	0.885	0.0020	$ET_{50} = -0.562$ Ln(biomass) + 1.015
ET_{50} *vs.* saxitoxins	Logarithmic	0.917	0.0007	$ET_{50} = -0.531$ Ln(STX) − 1.391
M. micrura – Seston				
ET_{50} *vs.* cell density	Logarithmic	0.060	0.4956	–
ET_{50} *vs.* biomass	Logarithmic	0.077	0.3173	–
ET_{50} *vs.* saxitoxins	Logarithmic	0.022	0.5956	–
M.micrura – CYRF-01				
ET_{50} *vs.* cell density	Logarithmic	0.999	0.0112	$ET_{50} = -2.352$ Ln(cell dens.) + 23.240
ET_{50} *vs.* biomass	Logarithmic	0.999	0.0148	$ET_{50} = -2.380$ Ln(biomass) + 1.7544
ET_{50} *vs.* saxitoxins	Logarithmic	0.991	0.0591	$ET_{50} = -2.702$ Ln(STX) − 10.536

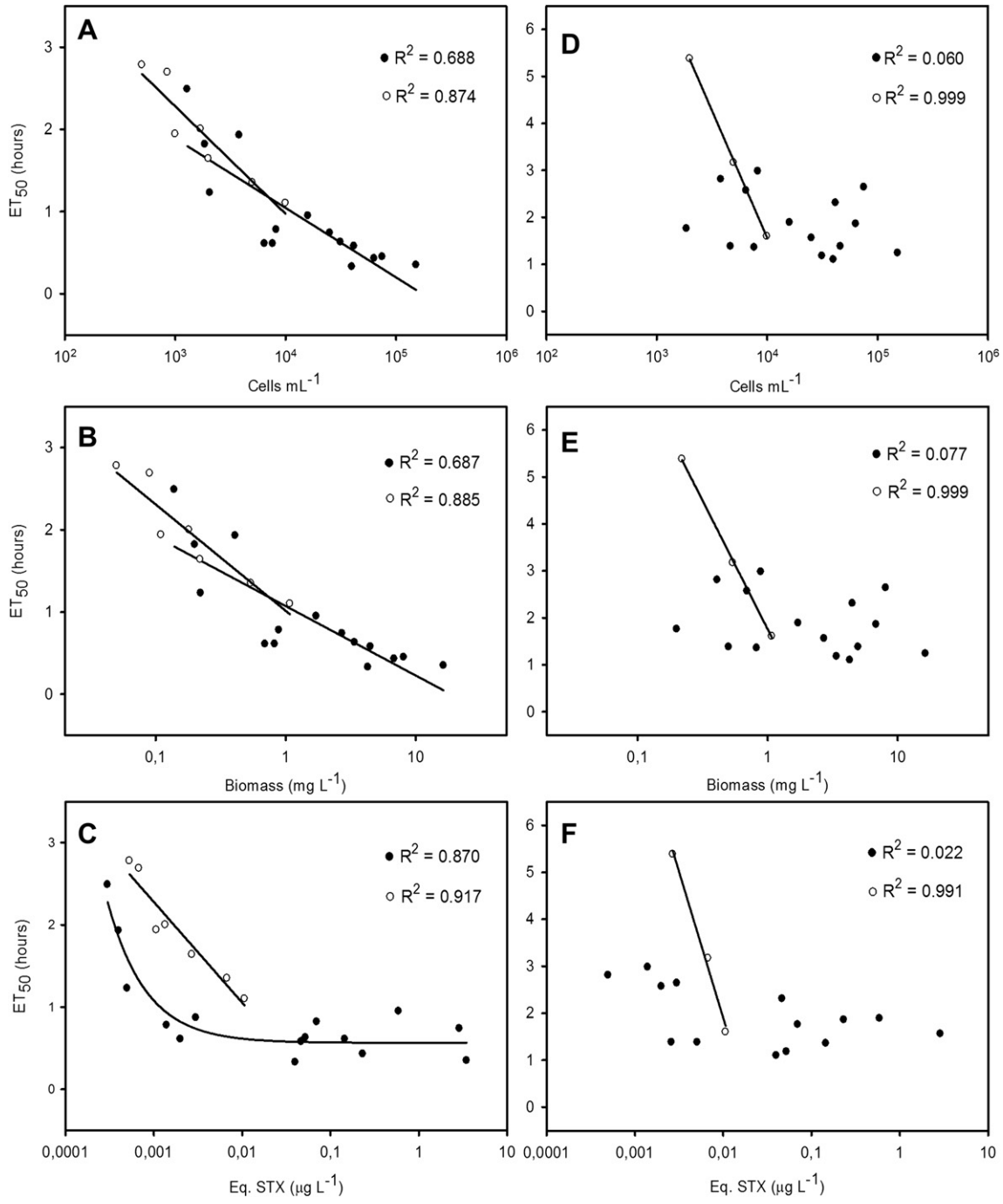

Fig. 4. Plots of ET_{50} *vs.* cell density and bioamass of *C. raciborskii* and ET_{50} *vs.* saxitoxins content of the reservoir's seston and the strain CYRF. Best regression fits are shown. Black circles refer to seston samples and open circles to strain CYRF-01. A–C, *D. pulex*; D–F, *M. micrura*. See Table 1 for statistical results and equations.

bioassay can be validated for cell densities between 10^2–10^5 cells mL^{-1} and biomasses between 0.05 and 16 mg L^{-1} (fresh weight). Also, the relationship between ET_{50} and saxitoxins content of seston showed a linear response between 0.3 and 3.0 ng Eq.STX L^{-1}. After that, there was a stabilization of the response, with no further decrease in ET_{50}. This reflects a saturation response (i.e. threshold) in the absorption of toxins, due to bioaccumulation, and after a certain toxin concentration the effect takes about the same time to occur. For the strain CYRF-01, there was a similar response in the range of cell density and biomass tested, with a good agreement with the lower concentrations of seston. Also, the relationship between ET_{50} and saxitoxins approached a linear response which means that a dose–response relationship is expected for toxin concentrations in the range of ~0.3–10 ng Eq.STX L^{-1}.

The response to strain CYRF-01 was a little lower than predicted for seston, which may represent differences in toxin composition between cells. Although strain CYRF-01 was isolated from the same reservoir, it presented only two saxitoxin variants (STX and GTX1), while seston presented a more varied toxin composition along the sampling period. The most toxic variants are STX and NEO while GTX variants have lower levels of toxicity (Shimizu, 1987). Thus, it is likely that other, more toxic strains were present in the reservoir

water. Furthermore, we cannot rule out the possibility that other, unidentified compounds may be present in the seston samples, which can have additive toxic effects.

Among the detection methods for paralytic shellfish toxins (PST), the AOAC mouse bioassay (AOAC, 1997) is the only internationally, legally accepted method for PST detection. In this bioassay, 100 g of a macerated shellfish meat extract is injected intraperitoneally into a ~20 g test mouse. The toxicity of the solution is established by measuring the time from injection to the mouse's last gasping breath, using a table of dose/death time relationships and correcting for the precise weight of the test animal. Substantial errors can result in estimating dose at long or short death times, and sample extracts therefore need to be diluted by trial and error to achieve death times in the range of 5–7 min. Test results can be expressed as mouse units (MU) or can be calibrated against pure saxitoxin and expressed as mg saxitoxin equivalents per 100 g of shellfish meat. The method is relatively easy to perform and requires no special equipment or expertise. The major disadvantages are: 1) test entails severe suffering of test animals; 2) death of animals is the method endpoint, which conflict with Animal Welfare Directive in several countries; 3) large variation in results between laboratories; 4) sex, weight and lineage of mice influence the test results; 5) poor precision (±20%) and insensitivity (detection limit is 40 μg saxitoxin/100 g); 6) the test cannot be validated (Lehane, 2000).

Another detection method for PST is the bioassay using a cultured neuroblastoma cell line (Okumura et al., 2005), modified from the bioassay system previously established by Manger et al. (1993). Although these bioassays specifically and sensitively detect the bioactivity of the cells affected by Na^+ channel blocking agents such as PST, they require longer cell incubation times (i.e. 6–8 h), expensive reagents, great expertise and more sophisticated equipment.

Although the previous bioassays have been used successfully for detection of PST, both organisms do not represent the natural and ecologically relevant exposure route of these toxins. On the other hand, zooplankton is exposed to these toxins in the field through its diet (i.e. phytoplankton), making them surrogate to test with natural seston samples. The main advantages of this *Daphnia* bioassay are: 1) the low quantity of sample needed (just a few milliliters of raw water); 2) inexpensive and require few material, no sophistication and expertise; 3) faster result compared to the standard 48-h *Daphnia* bioassay; 4) no conflict with Animal Welfare Directive; 5) no age and sex influences since the standard test only uses newborns and parthenogenetic females; 6) high sensitivity.

Another argument in favor of this rapid *Daphnia* bioassay is its specificity for detecting the reversible mode of action of saxitoxins. While in the mouse bioassay the ultimate effect is death, due to paralysis of the diaphragm and respiration arrest, in the *Daphnia* bioassay animals stay alive, immobilized on the bottom of the test tube, and can recover after being placed in clean medium. Other neurotoxins may have different effects on zooplankton. In previous tests with an anatoxin-a(s) producer strain of *Anabaena spiroids* (ITEP-024), both intact cells and extracts caused no paralysis effect on these same cladocerans, despite of the fact that this strain was very toxic in mouse bioassay (Molica et al., 2005). In fact, we have not tested any anatoxin-a-producer strain yet, and results with this toxin are also needed to know its mode of action in cladocerans. Therefore, the proper knowledge of these specific modes of action in *Daphnia* will provide a cue for differentiating toxins in raw water samples.

The sensitivity level of the bioassay with *D. pulex* was in the range of 0.3–10.0 ng Eq.STX L^{-1}, which is in a similar range of that found in the study of Ferrão-Filho et al. (2008) with another STX-producer strain of *C. raciborskii* (T3). As in the previous study, *D. pulex* was much more sensitive than *M. micrura*, although *M. micrura* was also very sensitive in concentrations ranging from 2.7 to 10.7 ng Eq.STX L^{-1} of the strain CYRF-01. Thus, the results found in this study show that the high sensitivity of *D. pulex* make it a good choice for biomonitoring of saxitoxins in water supply reservoirs.

Recently, Brazilian legislation has established a requirement for drinking water analyses (Portaria 518/04/MS from the Ministry of Health) regarding cyanobacterial toxins, setting guideline values for microcystins, saxitoxins and cylindrospermopsin, along with biomass monitoring programs. Guideline value of 1 μg L^{-1} for microcystins was adopted as mandatory, while values of 3 and 15 μg L^{-1} for equivalents of saxitoxins and for cylindrospermopsin, respectively, was included as recommendations. Also, toxicity testing or toxin analysis is required when cell counts exceed 20,000 cells mL^{-1} or 2 mm^3 L^{-1} biovolume (Burch, 2008).

Reports of PST in raw water samples from several reservoirs in Brazil suggest an increasing trend of these toxins in drinking water supplies (Yunes et al., 2003; Molica et al., 2002, 2005; Ferrão-Filho et al., 2009), and indicate the urgent need for a development of detection methods that can be easily applied by environmental agencies. In Brazil, the National Council of Environment (CONAMA 357/05) has established guidelines for water quality standards and has recommend the use of standard toxicity tests or another scientifically recognized method for accessing water quality of water bodies (CONAMA, 2005). However, standard toxicity tests utilized in Brazil does not take into account the specificities of the cyanotoxins and their mechanisms of action. Thus, we suggest that this fast, simple and cheap bioassay is applied not only by our environmental agencies, but worldwide, for detection of cyanotoxins in raw water.

5. Conclusions

This rapid bioassay with *Daphnia* proved to be an effective method for detecting saxitoxins in raw water, even in very low biomass and toxin levels. This relatively simple, fast and inexpensive method is a promising alternative to other screening methods (e.g. mouse bioassay) for detection of saxitoxins in raw water samples. Nevertheless, some specificities such as the time of observations (i.e. less than 24 h intervals), the use of filtered lake water controls and the observation of recovery must be taken into account to distinguish the mode of action of the toxins present.

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