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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