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Toxins in the freshwater cyanobacterium *Cylindrospermopsis raciborskii* (Cyanophyceae) isolated from Tabocas reservoir in Caruaru, Brazil, including demonstration of a new saxitoxin analogue

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Cyanobacteria can produce biotoxins that are significant hazards to humans. After the intoxication incident in 1996 at the city of Caruaru, Brazil, a phytoplankton-monitoring programme was established at its main water supply, the Tabocas reservoir. Data obtained during 1997 and 1998 revealed the dominant species at Tabocas to be *Cylindrospermopsis raciborskii,* which was responsible for a massive bloom observed in July–October 1998. Laboratory cultures of isolate ITEP-018 demonstrated highly toxic properties, mice inoculated with this strain exhibited the same symptoms as those of paralytic shellfish poisoning, with an acute lethal effect of 9.3 mouse units mg^{-1} of dry cells. Several saxitoxin analogues were identified in these cultures, specifically saxitoxin (3.3 mol% total toxin content), gonyautoxin 6 (6.4 mol%), decarbamoylsaxitoxin (8.5 mol%), neosaxitoxin (17.1 mol%), and a new saxitoxin analogue, which proved to be the major product of the Tabocas strain, accounting for 64.6 mol% of the toxin present in the sample analysed. Additionally, decarbamoylneosaxitoxin was detected by liquid chromatography–mass spectrometry. *Cylindrospermopsis raciborskii* strain ITEP-018 thus produces at least five saxitoxin analogues, including the most toxic ones as assessed by mouse bioassay.

INTRODUCTION

Cyanobacteria commonly bloom in eutrophic reservoirs and some produce powerful biotoxins, including hepatotoxins (e.g. microcystins) and neurotoxins (e.g. paralytic shellfish toxins and anatoxins). Consequently, their presence in water supplies is potentially dangerous to animals (Beasley *et al.* 1989) and humans (Kuiper-Goodman *et al.* 1999).

In February 1996, 131 patients experienced visual disturbances, nausea and vomiting after routine haemodialysis treatment at the haemodialysis centre in Caruaru, in the state of Pernambuco, Brazil. Subsequently, 100 patients developed acute liver failure and 76 of these died. By December 1996, a common cause was established for 52 of these deaths, and the complex of symptoms was called 'Caruaru Syndrome' (Jochimsen *et al.* 1998; Carmichael *et al.* 2001). The working hypothesis that toxins produced by cyanobacterium were responsible for these deaths was strongly supported by the discovery of microcystins in liver samples from 39 patients (Carmichael *et al.* 2001).

After the Caruaru incident, Pernambuco State established water-quality monitoring programmes, resulting in an apparently significant rise in the number of reported bacterial blooms in drinking water reservoirs. Such a monitoring was established for Tabocas reservoir, one of the water sources for the city of Caruaru, and included programme data on phytoplankton, the toxicity of cyanobacteria and limnological variables. During the period November 1997 to October 1998, *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenayya & Subba Raju was identified as the dominant phytoplankton species in the Tabocas reservoir, and a massive bloom of this species was observed from July 1998 to October 1998 (Bressan 2001). Cyanobacterial levels were also monitored in other reservoirs throughout the state; these data showed that *Cylindrospermopsis* Seenayya & Subba Raju spp. were the main bloom-forming cyanobacteria during the period 1997–1999 (Bouvy *et al.* 1999, 2000). Huszar *et al.* (2000) also reported *Cylindrospermopsis* sp. dominance in some reservoirs of Pernambuco State in 1995 and 1996.

This paper reports analyses of toxins produced by a strain of *C. raciborskii* (ITEP-018) isolated from the Tabocas reservoir. Toxins were identified by high-performance liquid chromatography with postcolumn fluorescence derivatization (HPLC–FLD) and also by a liquid chromatography–mass spectrometry technique (LC–MS). Production of cylindrospermopsin by ITEP-018 was also tested using an HPLC–Diode Array Detector.

MATERIAL AND METHODS

Sampling site, organism isolation and culture

Tabocas reservoir is located at latitude 08°06′15″S, longitude 36°13′23″W in the semiarid region of the State of Pernambuco

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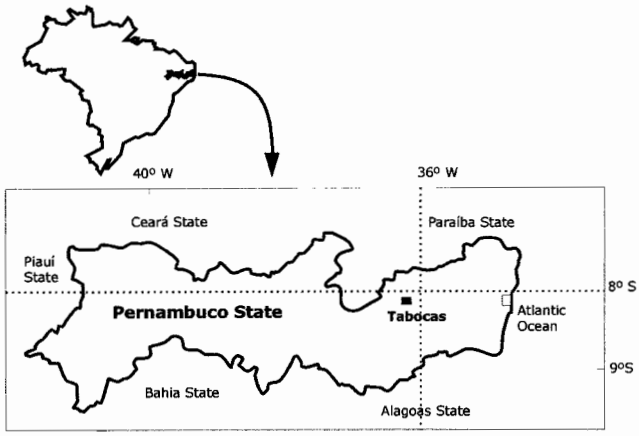

Fig. 1. Map and location of Tabocas reservoir in the State of Pernambuco, Brazil, South America.

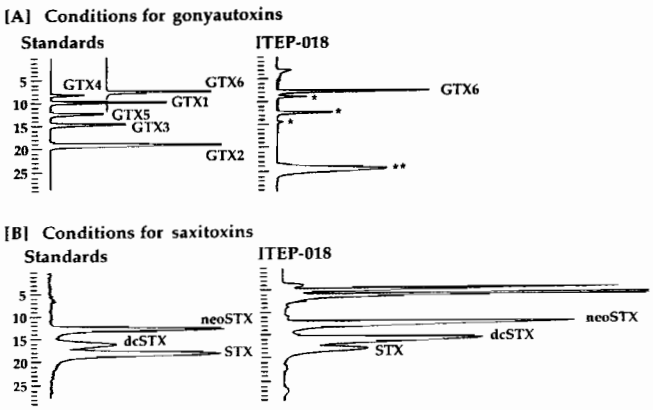

Fig. 2. HPLC–FLD chromatograms of GTXs and STX standard saxitoxin mixtures (left) and *C. raciborskii* strain ITEP-018 (right), analysed under conditions appropriate for gonyautoxins (A) and for saxitoxins (B). The retention times of the peaks marked with asterisks did not match any of the known saxitoxin analogues. The major peak marked with two asterisks (X in Fig. 3) corresponds to a 64.6 mol% of the total toxin content (A, right).

(Fig. 1). This reservoir was completed in 1973 and has a storage capacity when full of 13.6×10^6 m^3. *Cylindrospermopsis raciborskii* strain ITEP-018 was isolated from a water sample collected there in March 1998. A single trichome was transferred by Pasteur pipette to a culture tube (100 × 12 mm) containing 5 ml sterile ASM-1 medium (pH 8.0) (Gorham *et al.* 1964). The culture was maintained at 26 ± 1°C, with a photon flux density of 40 μmol m^{-2} s^{-1} (QSL-100; Biospherical Instruments, San Diego, California) from cool-white fluorescent tubes on a 12 : 12 h light–dark cycle. After *c.* 30 d, all the trichomes were transferred into fresh medium. The culture was nonaxenic.

To obtain sufficient biomass for toxin analysis, the strain was cultivated in 9 litre Carboy culture flasks containing 8 litres of ASM-1 medium, under 80 μmol photons m^{-2} s^{-1} with a 12 : 12 h light–dark cycle. Cultures were harvested in late-exponential phase by centrifugation and the cells were freeze dried, weighed and then stored at −18°C.

The taxonomic characteristics of the strain ITEP-018 agreed well with those described for *C. raciborskii* and reported in the literature (Branco & Senna 1991). The length of the trichomes varies with the stage of growth and culture conditions (data not shown).

Cylindrospermopsin analysis

The protocol described by Hawkins *et al.* (1997) was adopted for cylindrospermopsin extraction and analysis. Briefly, 45 mg of freeze-dried cells were extracted with 5% acetic acid and centrifuged for 10 min at 4000 × *g* followed by chromatographic analysis using an Allsphere ODS-2, 5 μm, 250 × 4.6 mm internal diameter column (Alltech) and detection at 260 nm. The retention times and UV spectrum (200–300 nm) of the major peaks were compared with a cylindrospermopsin standard kindly provided by Dr Assaf Sukenik (Kinneret Institute, Haifa, Israel).

Paralytic shellfish poisoning toxin extraction

Freeze-dried cells (170 mg) were extracted twice by sonication in 6 ml of 0.5 M acetic acid and subsequent centrifugation at 5000 × *g* for 5 min. Twelve millilitres of chloroform–methanol (1 : 1, v/v) were then added and the mixture was shaken vigorously and centrifuged. The aqueous layer was then removed and concentrated in a Speed Vack (SC210A, SAVANT). The residues were resuspended in 50 mM acetic acid and stored cold until analysis.

Mouse bioassay

The toxicity of each sample was determined by the Association of Official Analytical Chemists (AOAC) method for paralytic shellfish poisoning (PSP) (Cunniff 1995). Male mice (CF1 and ddY strains, with a 20 g body weight) were injected intraperitoneally (i.p.) with a 1 ml extract of the cultured material and symptoms of poisoning and survival times were recorded. Toxicity was expressed in mouse units (MU), where 1 MU is defined as the amount of toxin required to kill a mouse of 20 g body weight within 15 min after i.p. injection.

HPLC–FLD analysis of PSP toxins

HPLC–FLD analysis was performed based on the work of Oshima (1995) and Lagos (1998) on a silica-base reversed phase column (150 × 4.6 mm internal diameter, 5 μm; Prodigy C8, Phenomenex). Mobile phases of 1 mM tetrabutylammonium phosphate (pH 5.8) (for the C toxin group), 2 mM 1-heptanesulfonic acid in 10 mM ammonium phosphate buffer (pH 7.1) [for the gonyautoxin (GTX) group], or 2 mM 1-heptanesulfonic acid in 30 mM ammonium phosphate buffer (pH 7.1) containing 3% of acetonitrile (v/v) [for the saxitoxin (STX) group], were pumped at a flow rate of 0.8 ml min^{-1}. In all cases, the eluate from the column was continuously oxidized with 7 mM periodic acid in 10 mM potassium phosphate buffer (pH 9.0; 0.4 ml min^{-1}) while passing through Teflon tubing, and then acidified with 0.5 M acetic acid (0.4 ml min^{-1}) before entering a fluorescence detector (excitation 330 and emission 390 nm). To investigate the possibility of false identification of toxins, further analyses were carried out according to Onodera *et al.* (1996).

HPLC–electron spray ionization mass spectrometry analysis of PSP toxins

Chromatographic conditions for HPLC–ESIMS (electron spray ionization mass spectrometry) analysis were the same as those reported by Lagos *et al.* (1999). A reversed-phase Develosil ODS-UG-5 column (5 μm, 150 × 2.0 mm internal diameter, Nomura Chemicals, Seto, Japan) was used, with mobile phases of aqueous 10 mM heptafluorobutyric acid and acetonitrile at ratios of 8:2 for STXs and 95:5 for GTXs (v/v), eluted isocratically at a flow rate of 0.20 ml min^{-1}. Full-scan spectra were acquired in the positive ion peak centroid mode over the mass ranges of *m/z* 200–450 for STXs and *m/z* 200–650 for GTXs.

RESULTS

Mouse bioassay

The extract of ITEP-018 produced an acute lethal effect when tested by mouse bioassay; the neurological symptoms of PSP were observed, including nervousness, ataxia, convulsions and paralysis. Mice died of respiratory failure in a few minutes. The total toxicity measured by the AOAC mouse bioassay for PSP was 9.30 (N = 5) MU mg^{-1} of dry cells.

Cylindrospermopsin analysis

No cylindrospermopsin was detected using HPLC; the retention times and UV spectra (200–300 nm) of the peaks were compared with the cylindrospermopsin standard. HPLC–Diode Array Detector analysis also failed to detect any cylindrospermopsin (data not shown).

HPLC–FLD analysis of PSP toxins

HPLC–FLD chromatograms of strain ITEP-018, together with those of standards, are shown in Fig. 2. Five major toxins were found in the extract of ITEP-018, showing a total content of 3.63 μg toxin mg^{-1} dry cyanobacteria. Saxitoxin (STX) was a minor PSP toxin, representing 3.3 mol% of the sample, followed by gonyautoxin 6 (GTX6) (6.4 mol%), decarbamoylsaxitoxin (dcSTX) (8.5 mol%), and neosaxitoxin (neoSTX) (17.1 mol%). The principal PSP toxin was a new STX analogue with a retention time (Rt) of 25 min (indicated by ** in Fig. 2A, right panel), which did not match any of the known saxitoxin analogues. The new analogue accounted for 64.6 mol% of the total toxin profile of the ITEP-018 sample. The toxin profile of the sample is shown in Fig. 3.

No peak was observed corresponding to the *N*-sulfocarbamoyl-11-hydroxysulphate toxin group (C toxins), showing that strain ITEP-018 does not contain this type of toxin.

The presence of the STX analogues was confirmed by spiking the sample with standards and also by reanalysis under different oxidizing conditions (Onodera *et al.* 1996). The fluorescence intensities of the four peaks changed in the same manner as the respective standard toxins, neoSTX and GTX6 increasing and dcSTX and STX essentially disappearing. Under mild oxidative conditions, the peak fluorescent intensity of the major new STX analogue increased, as with neoSTX, which indicates a compound having a skeleton common to STX analogues (Onodera *et al.* 1996; Lagos *et al.* 1999).

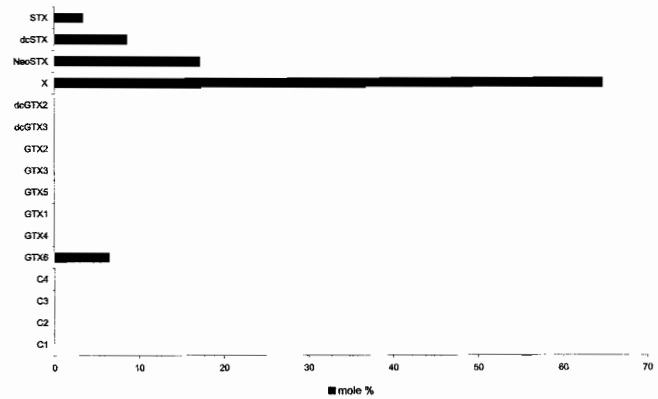

Fig. 3. PSP toxin profile (mol%) of strain ITEP-018 from Tabocas reservoir. X corresponds to the major new STX analogue marked with two asterisks in the HPLC–FLD chromatogram shown in Fig. 2A, right. Note the absence of C toxins.

The presence of significant amounts of new STX analogues is a distinctive feature of the toxin profile of strain ITEP-018 and makes this *C. raciborskii* toxin profile unique (Fig. 3). In addition this strain showed the highest total toxicity (4.74 MU mg^{-1} dry cells) reported until now for a PSP-producing *C. raciborskii* strain (as calculated by HPLC–FLD) because the total toxicity calculated by HPLC–FLD of the T1 (*C. raciborskii*) strain isolated from Amparo City, Sao Paulo, Brazil, showed a toxicity of only 0.81 MU mg^{-1} dry cells (Lagos *et al.* 1999). The HPLC–FLD toxicity reported here does not include the toxicity of the new STX analogue. Hence, the total toxicity of the sample measured by mouse bioassay (9.30 MU mg^{-1} of dry cells) is underestimated by the HPLC–FLD method, because the specific toxicity of the new STX analogue has not been reported and so cannot be included in the calculation.

HPLC–ESIMS analysis

Figure 4 shows LC–mass chromatograms of the ITEP-018 extract analysed under conditions appropriate for the saxitoxin group. According to this analysis, the saxitoxin analogues were detected as protonated forms ($[M + H]^+$ ions) of *m/z* 257, 273, 300, 316 and 426, corresponding to dcSTX (Rt = 9.28 min), dcneoSTX (Rt = 9.43 min), STX (Rt = 9.38 min), neoSTX (Rt = 9.43 min), and the new saxitoxin analogue, respectively. All showed identical retention times when compared with the toxin standards. The toxin contents, calculated from peak areas, were nearly equivalent to those obtained from HPLC–FLD analysis. The toxin dcneoSTX was not detected by HPLC–FLD, because this toxin cannot be resolved satisfactorily under our chromatographic conditions (Lagos 1998). But this toxin was detected and quantified by LC–MS analysis; the ratio of neoSTX–dcneoSTX was *c.* 2:1 in the sample.

The four STX analogues' molecular ions appear in the mass spectrum analysis with retention times of 9.2–9.5 min (Fig. 5). Here, the most prominent signal is the one corresponding to neoSTX, with a molecular ion of *m/z* 316.1, followed by STX (*m/z* 300.0), dcneoSTX (*m/z* 273.0) and dcSTX (*m/z* 257.1). Moreover, lower protonated molecular ions with *m/z* 239.1 and 255.1 also appear; these correspond to the dehydrated ions ($[M–H_2O^-H]^+$) for dcSTX and dcneoSTX, respectively.

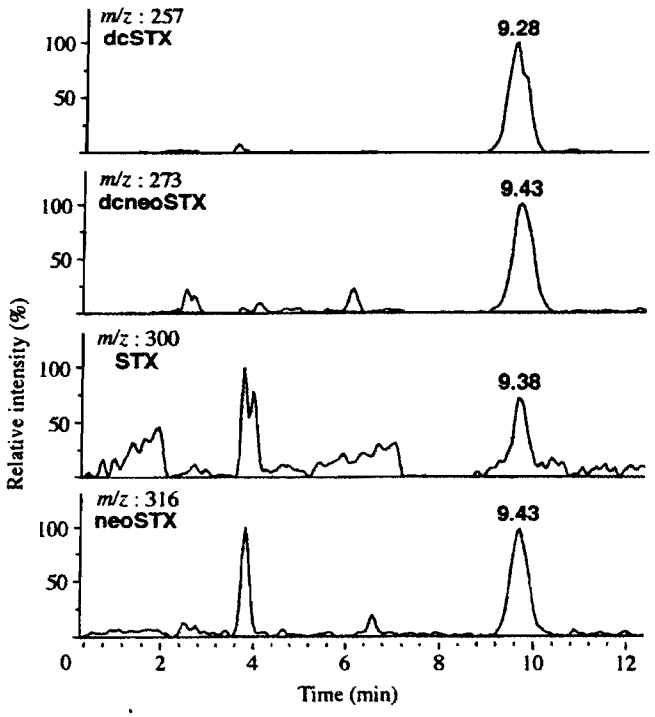

Fig. 4. Mass chromatograms of $[M + H]^+$ ions of the ITEP-018 extract analysed in selected ion monitoring (SIM) mode. The protonated forms detected were *m/z* 257, 273, 300, 316 (and 426), corresponding to dcSTX (Rt = 9.28 min), dcneoSTX (Rt = 9.43 min), STX (Rt = 9.38 min), and neoSTX (Rt = 9.43 min), respectively.

Figure 6A exhibits the data from LC–MS analysis of the same sample under the conditions for the gonyautoxin group. This figure shows the LC–mass chromatograms of the ITEP-018 extract traced at *m/z* 396, $[M + H]^+$ for GTX6. Two peaks (5.27 min) were observed with exactly the same retention time as the GTX6 standard (chromatogram not shown). The mass spectra obtained at 5.27 min by subtraction of background are

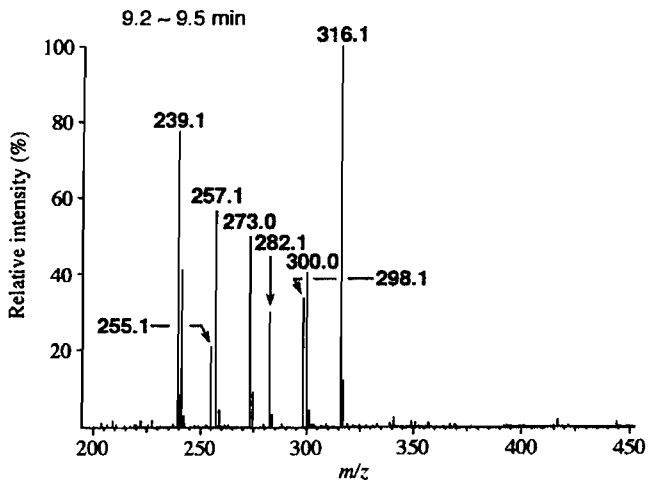

Fig. 5. Mass spectra obtained by background subtraction of full-scan mass chromatograms. At retention times in the range of 9.2–9.5 min, the molecular ions detected were neoSTX (*m/z* 316.1), STX (*m/z* 300.0), dcneoSTX (*m/z* 273.0) and dcSTX (*m/z* 257.1). Lower protonated molecular ions with *m/z* 239.1 and 255.1 correspond to the dehydrated ions ($[M–H_2O–H]^+$) for dcSTX and dcneoSTX, respectively. The *y*-axis gives relative intensity in percent.

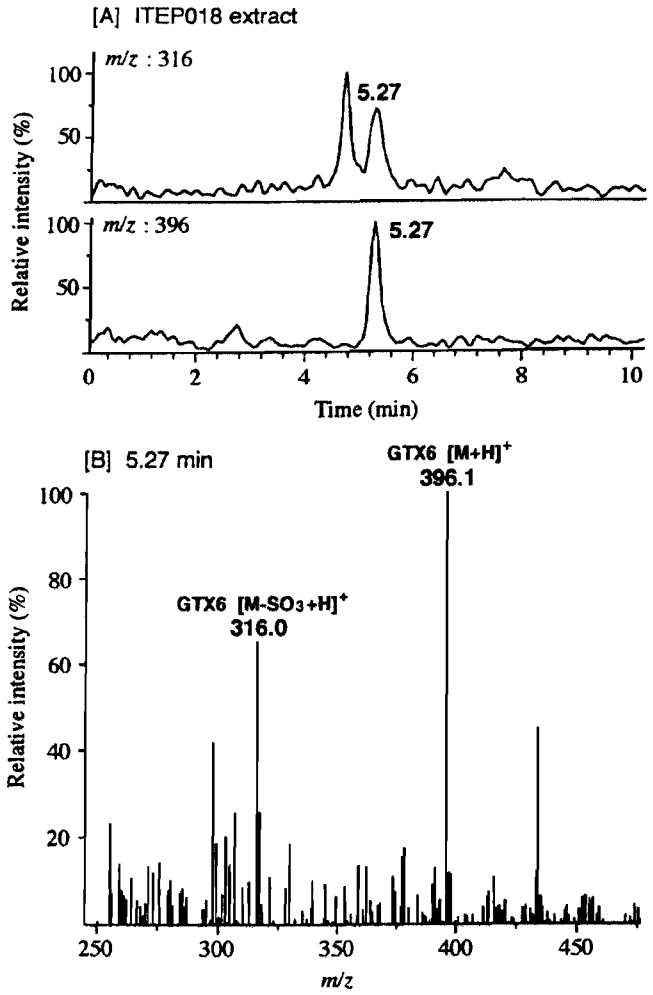

Fig. 6. LC–MS analysis of ITEP-018 extract under gonyautoxin group conditions. (A) Mass chromatograms at *m/z* 316 and *m/z* 396. Peaks at 5.27 min show the same retention times as standard GTX6. (B) Mass spectrum obtained by background subtraction at 5.27 min. *m/z* 396.1 and *m/z* 316.0 correspond to the molecular ions of GTX6 and GTX6-SO_3, respectively. The *y*-axis gives relative intensity in percent.

shown in Fig. 6B (below). In the mass spectrum analysis of GTX6, in addition to the protonated ion at *m/z* 396.1, a second predominant signal was observed at *m/z* 316.0 (80 mass units lower than the parent protonated ion). The molecular ion at *m/z* 316 can be easily accounted for as a structure that has lost a SO_3 moiety from the side chain R_4 at the Carbon 14 of GTX6. This signal is characteristic of the STX analogues that possess a *N*-sulfocarbamoil moiety (Oshima 1995; Lagos 1998).

DISCUSSION

Strain ITEP-018 from Tabocas reservoir is highly toxic when tested by mouse bioassay (9.30 MU mg^{-1} dry cells). To our knowledge this toxicity is the highest reported among cyanobacteria known to produce PSP toxins, which include *Aphanizomenon flos-aquae* Ralfs *ex* Bornet & Flahault (Mahmood & Carmichael 1986), *Lyngbya wollei* Farlow *ex* Gomont from North America (Onodera *et al.* 1997; Yin *et al.* 1997),

Anabaena circinalis Rabenhorst *ex* Bornet & Flahault from Australia (Onodera *et al.* 1996), *C. raciborskii* from Brazil (Lagos *et al.* 1999), *A. flos-aquae* from Portugal (Pereira *et al.* 2000), and *Planktothrix* Anagnostidis & Komárek sp. from Italy (Pomati *et al.* 2000).

The presence of PSP toxins in cyanobacteria has thus been established in five genera. Comparing the ITEP-018 toxin profile with those of other cyanobacterial PSP-producers reported in the literature, ITEP-018 is unique. First because a new STX analogue is reported, second because according to the HPLC–FLD analysis this new toxin represents the 64.5 mol% of the total PSP toxins, and last because this strain shows a complex PSP toxin profile including four STX analogues detectable by HPLC–FLD (STX, dcSTX, neoSTX and GTX6) and a fifth only detectable by LC–MS (dcneoSTX).

No cylindrospermopsin was detectable by HPLC analysis in ITEP-018. So, the total toxicity of the sample can be attributed only to STX-analogue toxins. Because the toxicity calculated by HPLC–FLD analysis corresponded to only 51% of the toxicity evaluated by mouse bioassay, the remainder should be associated to the new STX-analogue specific toxicity, which corresponds to 64.6 mol% of the sample.

Because Carmichael *et al.* (2001) have reported the presence of cylindrospermopsin in the carbon, sand and resins of the filters of the haemodyalisis clinic, it is plausible to propose that there is a cylindrospermopsin producer among Tabocas reservoir phytoplankton. Among the cyanobacterial species (11 in total) described by Bressan (2001) for this reservoir from November 1997 to October 1998 during limnological studies, *C. raciborskii* is the only one known (from elsewhere in the world) to produce cylindrospermopsin; however, no Brazilian strains of *C. raciborskii* have as yet been shown to be cylindrospermopsin producers.

Finally, we advise strongly that attention is paid not only to hepatotoxins but also to neurotoxins in cyanobacterial blooms, because the toxicity found in this *C. raciborskii* strain is the highest reported for a cyanobacterium, indicating potential animal and human intoxication. Recently, Kaas & Henriksen (2000) pointed out that PSP toxins may be more widespread in freshwater environments than has previously been assumed.

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