PRESENCE OF DSP-TOXINS IN *Prorocentrum lima* (EHRENBERG) DODGE IN CUBA

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ABSTRACT

The presence of Dinophysistoxin-1 (DTX-1) is determined for the first time, like a possible toxin associated to the ciguatera in waters of the North coast of Havana, was measured by High Performance Liquid Chromatography (HPLC-FLD). *Procentrum lima* (Dinophycea, Prorocentrales) in association with *Padina* sp, is the producing species of this toxin. Counts of cells were made of obtained in environmental natural (1675 cell.g^{-1} algae wet weight) and cultivated in medium K (65 000 Cell/ Ml). The results of toxin extracts from cultivated cells and natural, showed concentrations of 7.15 pg DTX1/cell and 4.2 pg DTX1/cell respectively. The potential distribution of DTX-1 all over the NW Cuban coast, considering that the environmental conditions are very similar, is highly probable.

Key words: algas nocivas y/o tóxicas; ciguatera; toxins; *Prorocentrum lima*; ASW, Cuba.

RESUMEN

Se determina por primera vez la presencia de Dinophysistoxin-1 (DTX-1) como una possible toxina asociada a la ciguatera en aguas de la costa norte de La Habana, fue medida por cromatografía líquida de alta resolución (HPLC-FLD). *Prorocentrum lima* (Dinophycea, *Prorocentrales*), en asociación con *Padina*. sp es la especie productora de esta toxina. Fueron obtenidos conteos de las células del medio natural 1675 cell.g^{-1} peso húmedo de macroalgas) y de las cultivadas en medio K (65 000 Cell/ mL). Los resultados de los extractos de las toxinas de las células cultivadas y las del medio natural mostraron concentraciones de 7.15 pg DTX-1 /cél y 4.2 pg DTX-1/cél respectivamente. Es altamente probable la presencia y distribución potencial de DTX-1 NW de Cuba considerando que las condiciones ambientales son muy similares.

Palabras claves: harmful algae; ciguatera; toxinas; *Prorocentrum lima*; ASW, Cuba.

Prorocentrum. lima (Ehrenberg) Dodge is a neritic, estuarine species with world-wide distribution (Faust, 1991; Heredia *et al.* 2002, Steidinger and Tangen, 1996, Heredia *et al.* 2002, Nascimento, *et al.* 2005). Cells can be found in temperate (Lebourd, 1925; Schiller, 1933; Carter, 1938) as well as tropical oceans (Fukuyo, 1981; Steidinger, 1983; Carlson, 1984; Faust, 1990). This species occurs in sand (Dodge, 1985), attached to surface of red and brown algae and benthic debris (Fukuyo, 1981; Steidinger, 1983 and Carlson, 1984), associated with coral reefs (Yasumoto *et al.* (*et al.* 1980); Bomber *et al.* 1985 and Carlson and Tindall, 1985), or can be found attached to floating detritus in mangroves (Faust, 1991).

P. lima is a toxic dinoflagellate species known to produce a number of toxic substances as fast-action toxin (FAT) (Tindall *et al.* 1989); prorocentrolide (Torigoe et al. 1988); diarrheic shellfish poison (DSP) toxins (Yasumoto et al. 1987); okadaic acid (OA) (Murakami et al. 1982; Lee *et al.* 1989; Marr *et al.* 1992); Dinophysistoxin-1 (DTX1) (Marr *et al.* 1992); Dinophysistoxin-2 (DTX2) (Hu *et al.*, 1993), and Dinophysistoxin-4 (DTX4) (Hu *et al.*, 1995).

In Cuba, ciguatera is one of the main causes of food intoxication mostly during summer, being the NW region the most affected. Despite this situation, the first researches on dinoflagellates associated with ciguatera, started in the 1990's Valdés *et al.* 1992 and later by Delgado, *et al.* 2000, 2002 and Popowski *et al.* 2001, nevertheless no studies have been done to corroborate the toxins associated to dinoflagellates and this type of poisoning.

The objective in this work is to evaluate the toxicity of *P. lima*, found in a zone of the NW coast of Havana City, Cuba, on extracts of cultivated

(controlled environment) and *in situ* (natural) cells over *Padina* sp substrate.

MATERIALS AND METHODS.

Samples collection

Padina sp was collected, in June 2003, by snorkeling according the procedure proposed by Quod *et al.* (1995) in littoral NW of La Havana city (Fig. 1). The macroalgae was collected in plastic bags, and was vigorously shaken, to remove the epiphytic dinoflagellates. The suspension was passed through three successive 250, 140 and 20 µm mesh sieves. The fraction last retained (20 µm) was utilized to isolation of *P. lima*. Cells of *P. lima* were identified by a Zeiss inverted microscope.

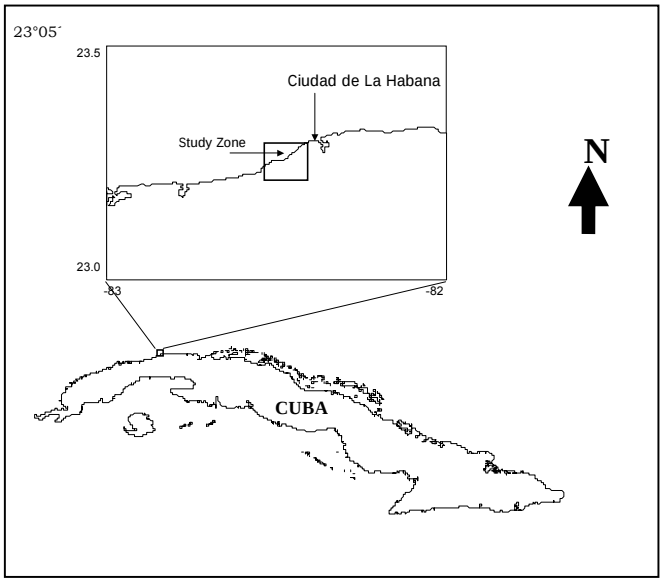

Fig. 1. Localization of sampling site, north of Havana City

The isolation of *P. lima* was realized by micropipettes under an inverted microscope Olympus at 120X magnification. Each isolated cell was placed in a microplate well followed by successive washings with sterilized seawater. They were transferred to bottles of 5 mL and 75 mL successively filled with medium K (Keller and Guillard, 1985). All this process was made on a 12:12 light:dark cycle (fluorescent lamp of 40 W), and constant temperature of 22 ± 1ºC. Cells counting were made at the beginning and at the end of the culture. After 27 days of incubation, the culture reached that was harvested for centrifugation for the toxin analysis.

Chemicals

Standard toxins for dinophysistoxin-1 (DTX-1) and, deoxycholic acid (DOCA) were purchased from SIGMA (Sigma Chemical Co, St, Louis, Mo, USA). 9-antryldiazomethane (ADAM) was purchased from Funakoshi Pharmacy (Tokyo, Japan). HPLC grade solvents (acetonitrile, acetone, methanol, chloroform, HCl, acetic acid) were purchased from Fisher Scientific (New Jersey, U.S.A.). The SEP-PAK® cartridges for solid phase extraction of silica and C-18 were purchased from Waters Corporation (Division of MILLIPORE, Milford, Ma. USA). Water of high purity grade, was obtained by elution through an ion exchange cartridge and then by boiling for 2 hours with nitrogen bubbling.

Derivatization of DSP phycotoxins with ADAM

The ADAM derivatives of standards and sample toxins, to be used in the HPLC measurements, were carried out according to previous described method (García et al., 2004). Briefly, the microalgae extract residues or standards were treated with a freshly prepared solution of 0.1% ADAM (in 100 µL of acetone and 400 µL of methanol) (Lee *et al.* 1989). After one hour at 25ºC in the dark, the sample was evaporated to dryness and the residue was diluted in 200 µL CH_2Cl_2/hexane, 1:1 (v/v) and then transferred into a 500 mg Silica gel SEP PAK® cartridge. The system was washed successively with 5 mL of CH_2Cl_2/hexane, 1:1 (v/v) and 5 mL CH_2Cl_2. Finally, was eluted with 5 mL of CH_2Cl_2/methanol, 1:1 (v/v). The last fraction was evaporated to dryness, dissolved in 1 mL methanol, and then 10 µL was injected and analyzed by HPLC with fluorescent on line detection (HPLC-FLD).

Chromatographic conditions for HPLC analysis of DSP toxins.

The HPLC chemical analysis was performed on a Shimadzu Liquid Chromatograph System equipped with a pump (Shimadzu LC-6A), a rheodyne injector (7725i Rheodyne. Cotati, Ca. USA), and a fluorescence detector (Shimadzu RF-535). Ten microliters of toxin derivatives were injected on a reversed phase column Supelcosil LC-18 (5 µm; 25 cm x 4 mm) (Supelco, Bellefonte, PA. USA). An isocratically mobile phase of $CH_3CN/CH_3OH/H_2O$ 8:1:1 (v/v) with a flow rate of 1 mL/min were run at room temperature. The excitation and emission wavelengths were set at 365 and 415 nm respectively. Peaks in the resulting chromatograms were identified by comparison with the retention

times of DSP phycotoxin analytical standards. This method corresponds to a High Performance Liquid Chromatography with fluorescent on line detection (HPLC-FLD) with pre-column derivatization.

Sample preparation

Cell pellets (*P. lima*) of natural environment and those of the culture were treated twice by freeze-thawing procedures. They were suspended in 1 mL of aqueous methanol 80% and sonicated for 1 min at room temperature. After centrifugation the supernatant was decanted and the pellet was re-extracted twice with 1 mL aqueous methanol 80%. The supernatants were combined into a 5 ml glass vial and evaporated in Speed Vac SC 210A with refrigerated vapor trap RVT400 (SAVANT). The residue was dissolved in 2 mL of 80% methanol and extracted with 2 mL of hexane. The methanolic layer was dried and preserved frozen until preparation of PDAM derivatives.

RESULTS

Characterization of *P. lima* under culture.

The microscope analysis showed a growth of 65 000 cells.mL^{-1} of *P. lima* cultured in K medium. It was also confirmed the taxonomic characteristics of the species by the depression in V shape in the anterior part, as well as the pores distributed on the valvar and marginal surface of the cells, except in the central part.

Analysis of DSP of *P. lima* in culture and in the natural environment.

The quantitative analysis for HPLC of the toxins of *P. lima*, confirmed the presence of diarrheic toxins (DTX-1) for the first time in the waters of the Cuban platform.

The concentration of DTX1 in the cultured cells and those of the natural environment are shown in the Fig. 2 A-B. The DTX1 Chromatogram standard is in Fig. 3. The toxin content for cultivated cells was 7.15 pg/cell, and for natural cells 4.2 pg/cell (g^{-1}algae wet weight).

DISCUSSION

The morphologic characteristics of *P. lima* observed in the cultured cells are similar to those reported by other authors in organisms isolated in other regions (Fukuyo, 1981; Faust, 1991; Heredia *et al.* 2002).

The toxin concentration falls within the range reported for this species in other regions of the world. Lee *et al.* (1989) reported variations in the concentration of DTX1 between 6 and 14.3 pg/cell obtained in five clones of *P. lima* isolated from the coasts of Galicia, Spain. Bravo *et al.* (2001) in studies also made on the cultures of *P. lima,* report average values of 1.01 pg/cell in the extract (PL6V) and 12.45 pg/cell. (PL9V). In the coasts of the Mexican Pacific Heredia *et al.* (2002) reports values higher than ours (19.5 pg/cell).

The presence of this toxin in *P. lima* in the zone of study, demonstrates the risk by which the events of ciguatera in the area take place. If it is considered, as stated by Morton and Norris (1990), and Jackson *et al.* (1993), that the environmental conditions of high temperatures, salinity and light intensity can determine an increase of the toxicity in this species, since these environmental conditions prevail most of the year in waters of the Cuban platform, there is a continuous high risk of DSP events related to *P. lima*.

Further work will be directed to growth rates and toxin variability in *P. lima* from Cuban platform.

ACKNOWLEDGEMENTS.

We want to leave to certainty of our gratefulness to Lic. Frank Abel Alfonso and Celia Ma. Rosquete, by field work and sample processing. To all the technical staff of the Biochemical Laboratory of Membrane, Physiology and Biophysics Department, Medicine Faculty, University of Chile, with whom was possible this work. This study was supported by FONDECYT 1020090, DID, Universidad de Chile, CSMAR 02/5-2, by the Fisheries Research Center of Cuba and by the National Council of Science and Technology (CONACYT grant number 122843) by a grant awarded to G. Delgado.

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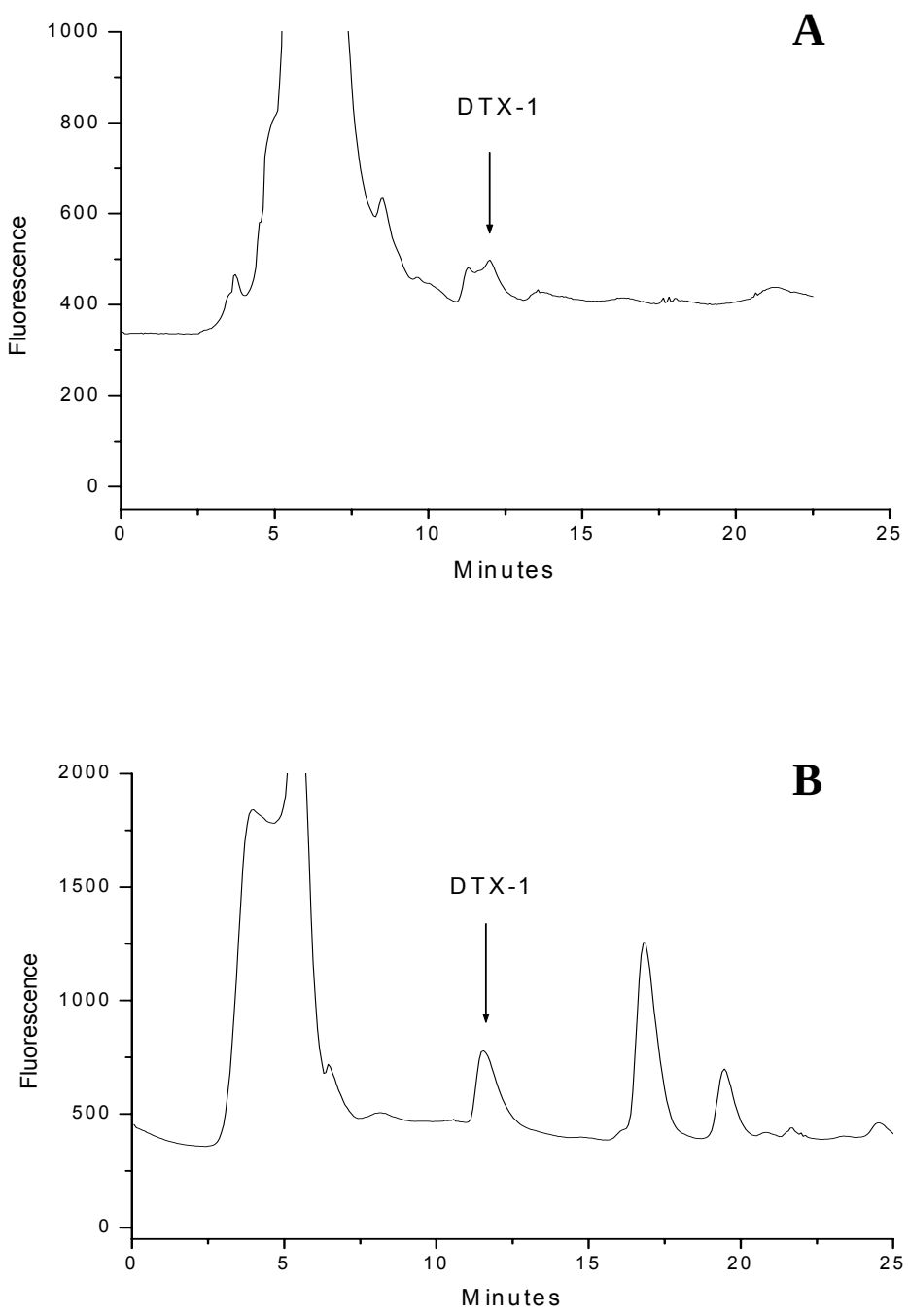

Fig. 2. HPLC of DTX-1 toxins from the *Prorocentrum lima* in culture (A) and in natural (B) samples

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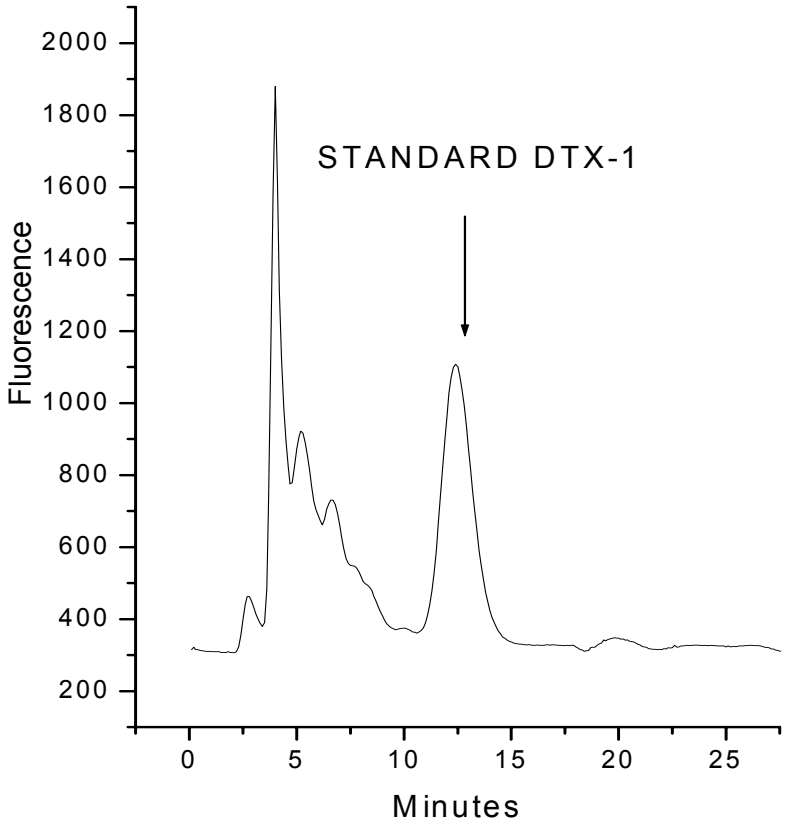

Fig. 3. . Chromatogram of DTX-1 toxin standard

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Aceptado: 18 de octubre del 2005