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Detection of Paralytic and Diarrhetic Shellfish Toxins in Moroccan Cockles (*Acanthocardia tuberculata*)

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During the last few decades, toxic algae blooms have been a common and regular occurrence in many regions world-wide. Human health problems associated with the ingestion of shellfish containing toxic algae have been recognized for many years on a world-wide basis. The algae toxins including paralytic shellfish poisons, diarrhetic shellfish poisons and neurotoxic shellfish poisons have been the most frequent causes of human poisoning (PSP, DSP and NSP), although morbidity due to other potent neurotoxins has also been documented. Algal neurotoxins include the sodium channel blockers saxitoxin and neosaxitoxin, the phosphatase inhibitor okadaïc acid and amnesic carboxylic acid domoic acid. These toxins are commonly produced by algae which can develop under favourable conditions of water temperature, stratification, light and nutrient supply to form blooms.

In Morocco, toxic algae blooms have occurred along the Atlantic and Mediterranean coasts since 1971. On the Atlantic coast, PSP toxicity occurred in 1971, 1973, 1975, 1978, 1982, 1985 and 1986 (Tber 1983; Akalay 1995). Each time, bivalve toxicity was sufficiently high to prohibit shellfish harvesting. Acute outbreaks with human intoxication were reported along the Atlantic coast in 1971 in Casablanca and Rabat, 1975 in Casablanca, Safi and Kenitra and 1982 in Agadir. Outbreaks along the Mediterranean coast were observed in 1985 and again in 1988. Since 1988, fishing and the commercial shellfish harvesting have been prohibited on the Mediterranean coast. In November 1994, algal blooms occurred on both the Atlantic and Mediterranean coasts and caused a large number of intoxications (64) linked with shellfish consumption (Tagmouti-Talha et al. 1996). Twenty three persons were hospitalized and four died (Akalay 1995). Acute neurologic illness was associated with paresthesia of the mouth and extremities, a sensation of floating, headache, cerebellar symptoms such as ataxia and vertigo, cranial nerve dysfunction and muscle paralysis. One child's illness rapidly progressed to respiratory paralysis and death. Several algae blooms occurred in 1996, 1997 and 1998, but no PSP cases were reported because contaminated areas were rapidly closed to avoid toxic shellfish consumption.

Here, we report the detection and the spectroscopic characterization of PSP and DSP toxins extracted from cockles collected in the Tetouan region (Figure 1) from May 1995 to November 1996.

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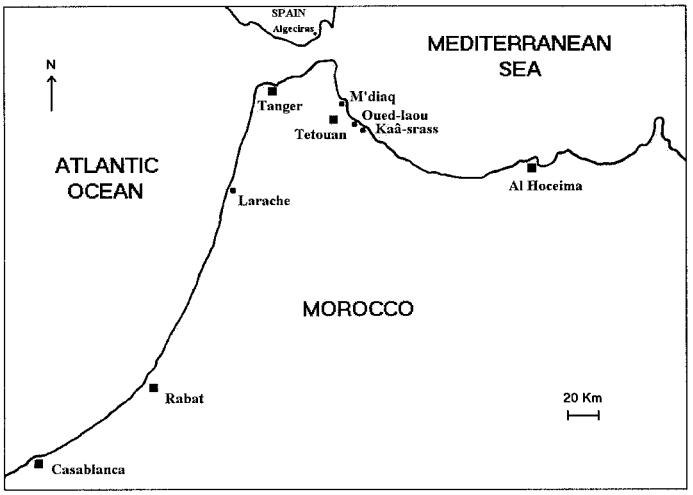

Figure 1. Geographical card of the north of Morocco.

MATERIALS AND METHODS

Cockle (*Acanthocardia tuberculata*) specimens used in this study were collected from three stations (M'diaq, Oued-laou, Kaâ-srass) in the Tetouan region of the Mediterranean coast (Figure 1) on November 1994, May 1995, October 1995, May 1996 and November 1996. The cockles represent in this region approximately 80 % of the bivalves. Sampling is done at broad coasts (10 - 15 m depth): we rake the bottom using a rake and bring back the samples using a net. The cockle tissues were kept at – 30 °C until use.

PSP (gonyautoxins, saxitoxin, neosaxitoxin) and DSP (okadaïc acid) standards were obtained from Diagnostic Chemical Limited Co. (Charlottetown, Prince Edward Island, Canada).

The AOAC extraction method for PSP (Holliningworth and Wekell 1990) was used. Cockle meat (100 g) were blended with 100 mL of HCl (0.1 *N*) and boiled for 5 min. The volume of the mixture was brought to 200 mL with distilled water, stirred and centrifuged at 3000 × g for 10 min. The recuperated supernatant (water-soluble extract) was analyzed to evaluate the toxicity levels.

The methanol method for extraction of DSP (Vernoux et al. 1985), a modification of the method of Yasumoto et al. (1976), was applied. Minced cockles were extracted with three volumes of methanol at room temperature (25 ± 0.5 °C). The methanolic extract was filtered through a Büchner and the filtrate was evaporated to dryness. The residue was partitioned between methanol (80 %) and light petroleum (20 %). The methanol-soluble material was further partitioned between diethyl ether (75 %) and ethanol (25 %). The ether extract was evaporated to dryness, yielding the lipid-soluble residue (LSR). Butanol was added to the

remaining ethanolic phase and then the butanol extract was evaporated to dryness, yielding the water-soluble residue (WSR).

Swiss albino mice (20 ± 2 g) were injected intraperitoneally in order to evaluate the toxicity of the samples. For the AOAC method, the toxicity is expressed as PSP in mouse units (MU) per 100g of meat. For the methanol method, both the WSR and the LSR were emulsified in 1 % Tween 60 at 37 °C and then injected intraperitoneally into mice (Vernoux et al. 1982). In this case, the toxin content was expressed as the minimum lethal dose (MLD) per gram of animal.

The most toxic lipid-soluble fraction was called F3, this fraction was obtained from the lipid-soluble extract of the cockles collected on November 1994 after partial purification using a chromatography on Silica-gel 40 – 63 μm (elution: chloroform-methanol 9:1, v/v), chromatography on Florisil 60 – 100 mesh (elution: ethyl acetate-methanol 9:1, v/v) and gel filtration on column filled with Sephadex LH20 (elution: ethanol-water 96:4, v/v) at a constant flow of 0.5 ml/min and at room temperature (25 ± 0.5 °C) (Tagmouti-Talha 1987; Vernoux and Tagmouti-Talha 1989; Vernoux et al. 1994). F3 was selected for analysis of DSP.

Water-soluble toxin was analysed by HPLC according to the method of Oshima et al. (1984). The toxins were separated on Develosil C8-5 column (Nomura Chemicals) by isocratic elution with two different mobile phases: **(a)** 2 m*M* 1-heptasulfonate sodium salt in 10 m*M* ammonium phosphate buffer (pH 7.1) for gonyautoxins 1-6 (GTX1-4, B1, B2) and **(b)** mobile phase (a) plus acetonitrile (5:100, v/v) for the saxitoxin (STX), neosaxitoxin (NEO) group. The elution was carried out at a constant flow of 0.4 mL/min. Toxins in the eluate from the column were continuously oxidized by heating with periodate, and the resultant fluorescent compounds were detected by a fluoromonitor after acidifying the reaction mixture with acetic acid.

Lipid-soluble toxin was analyzed by HPLC coupled to mass spectrometry according to the method of Lee et al. (1989). The mass spectrometric experiments were performed on a Sciex API III (Thornhill) triple quadrupole mass spectrometer equipped with an atmospheric pressure ionization (API) source operated in the ionspray mode.

The protein phosphatase test consisted of a colorimetric measurement of phosphatase inhibition, which used an artificial substrate (paranitrophenyl phosphate [pNPP]) and a semi-purified protein phosphatase PP2A extracted from a rabbit muscle (Cohen et al. 1988; Simon and Vernoux 1994). Inhibition of the catalytic subunit of protein phosphatase (PP2A) in the presence of the toxin (a standard okadaïc acid or F3 toxic extract) is followed spectrophotometrically at 405 nm (Bialojan and Takai 1988; Simon and Vernoux 1994). Okadaïc acid standards (0.1 to 5 ng) were prepared in 10 μL of 40 m*M* Tris/HCl buffer (pH 8.5) containing 20 m*M* KCl and 30 m*M* $MgCl_2$ and added to the reaction mixture. The phosphatase inhibition activity of PP2A was measured at different concentrations of okadaïc acid (calibration curve) and at different dilutions of F3 extract.

The HEP G2 cell line was established from a human hepatoblastoma (Knowles et al. 1980) and the cells were cultured, according to the method of Borenfreund et al. (1990), on HAM F12 medium containing 10 % of foetal vow serum and 4 μg/L of gentamicine. The neutral red uptake (NRU) test, which measures cell viability, was then performed (Borenfreund and Puerner 1985). The NRU was estimated from the fluorescence at 540 nm in cells exposed to a dilution series of okadaïc acid or the F3 extract. Cells were incubated for 24 hr at 37 °C in a humidified 5 % CO_2 incubator. At different times during the incubation, with or without extracts, cells were carefully observed under an inverted phase contrast microscope. For the NRU assay, data were expressed as fluorescence unit at 540 nm (FU_{540}). The 50 % control NRU inhibitory concentrations (IC_{50}) of the okadaïc acid and the F3 extract were estimated from FU_{540} values.

RESULTS AND DISCUSSION

The toxicity levels of cockles from the Tetouan region, during the study period, were 4000, 1731, 1952, 1880 and 2043 MU in November 1994, May 1995, October 1995, May 1996 and November 1996, respectively. Toxicity levels for all the samples were higher than the public health safety threshold (400 MU/100g of meat) throughout this period.

In all cases, the cockle extracts prepared using AOAC standard methods caused rapid death of the mouse, preceded by PSP symptoms (MLD = 0.076 mg of extract per gram of mouse). In control experiment with uncontaminated cockle extract no death of mouse occurred. Furthermore, the HPLC chromatogram showed a large number of PSP toxins, including gonyautoxin V (B1), gonyautoxin VIII (C), gonyautoxin I (GTX1), gonyautoxin II (GTX2), gonyautoxin III (GTX3), gonyautoxin IV (GTX4), neosaxitoxin (NEO) and saxitoxin (STX) (Figure 2). The peaks eluted between C and GTX4 and between GTX3 and GTX2 were not identified. The big peak eluted before STX might be assigned to decarbamoylsaxitoxin (dc-STX).

Among the lipid-soluble extracts, only the fraction (F3) produced muscular atony and total paralysis of the mouse immediately after injection (MLD = 0.83 mg of F3 per gram of mouse). The F3 fraction was analysed by mass spectroscopy coupled to HPLC. The results showed the presence of DSP toxins (Figure 3). The spectrum showed two major peaks with molecular weights of 820.5 and 842.5 daltons (Figure 3). The first peak represent dinophysistoxin I (DTX1) + $2H^+$ and the second peak (842.5) represent probably DTX1 + Na^+. The spectrum from another fraction contained two peaks, one probably corresponding to okadaïc acid and the second, with a molecular weight of 1193.8 daltons, was not identified (Figure 4). This compound might be an important precursor to other DSP toxin. Therefore, this toxin was always not detected on LSR, probably an enzymatic hydrolysis that gives DSP toxin with a lower molecular weight.

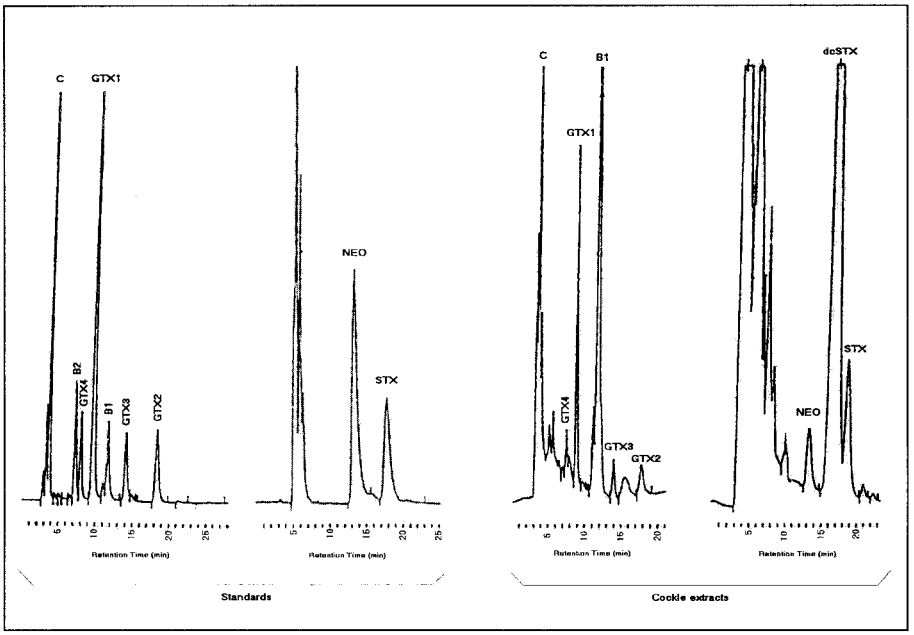

Figure 2. HPLC analysis of cockles extract from the algae bloom of Nov. 1994.
(B1: Gonyautoxin V) (B2: Gonyautoxin VI) (C: Gonyautoxin VIII) (GTX1: Gonyautoxin I)
(GTX2: Gonyautoxin II) (GTX3: Gonyautoxin III) (GTX4: Gonyautoxin IV)
(NEO: Neosaxitoxin) (dc-STX: Decarbamoylsaxitoxin) (STX: Saxitoxin)

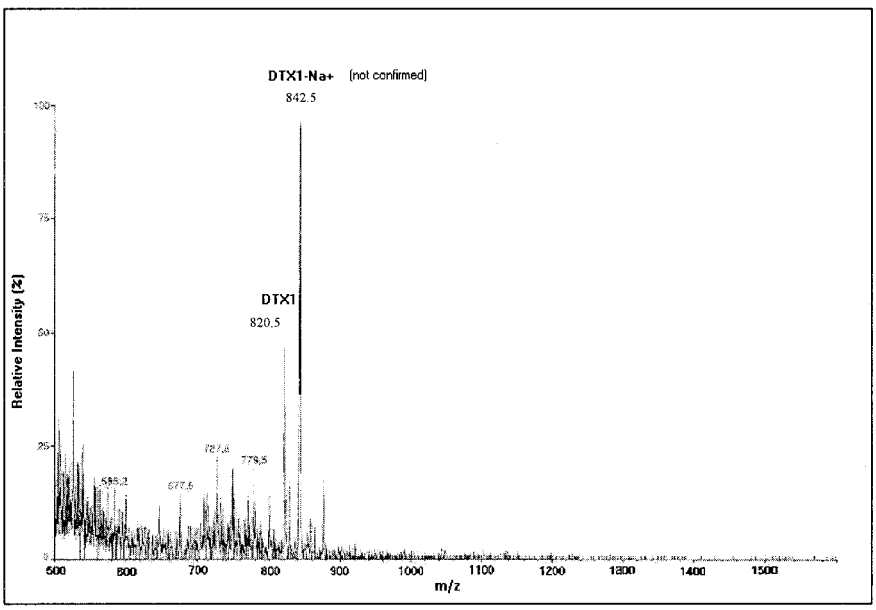

Figure 3. The mass spectrum coupled to HPLC of the F3 fraction.
(DTX1: Dinophysistoxin I)

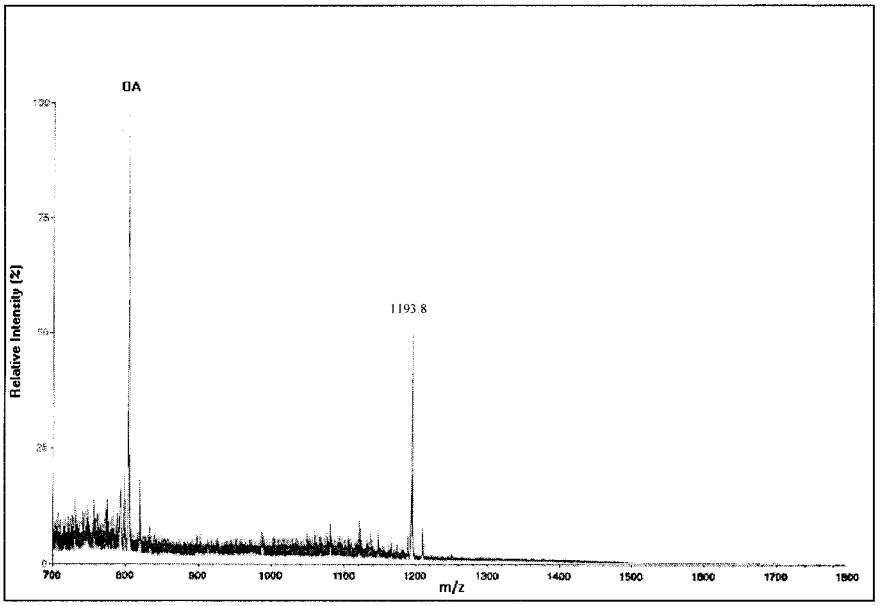

Figure 4. The mass spectrum coupled to HPLC of one fraction of the DSP extract.
(OA: Okadaïc acid)

Phytoplankton analysis showed that *Gymnodinium catenatum* (PSP), *Gonyaulax tamarins* (PSP), *Alexandrium minutum* (PSP), *Pyrodonium sp.* (PSP), *Dinophysis caudata* (DSP) and *Dinophysis sp.* (DSP) were present, which were consistent with the presence of the corresponding toxins (*Benhissoune 1998, personal communication*).

A calibration curve was established by measuring the inhibition of PP2A at various concentrations of okadaïc acid (Figure 5). In the linear portion of the curve (OA concentration < 0.5 ng/mL), the PP2A activity decreases linearly with increasing concentrations of OA. The protein phosphatase activity was also measured at different dilutions of F3 fraction (Figure 5) and compared to the OA results. The F3 curve appeared to fit the calibration curve quite well. These results confirm the presence of okadaïc acid or compounds which similarly inhibit phosphatase activity, in the F3 extract.

Morphological damage to cells was observed 1 hr after exposure to F3 fraction and was initially characterized by membrane blebbing. As the concentration of F3 increased, the number of affected cells increased, giving totally round-cell features (Amzil et al. 1992). Previous studies on okadaïc acid have described membrane blebbing (Aune et al. 1991; Fessard et al. 1994). Recently, it was demonstrated that okadaïc acid is an ionophore and can complex with Na^+, K^+, Mg^{2+} and Ca^{2+} ions (Blaghen et al. 1997) ; this action probably has an effect on membrane permeability.

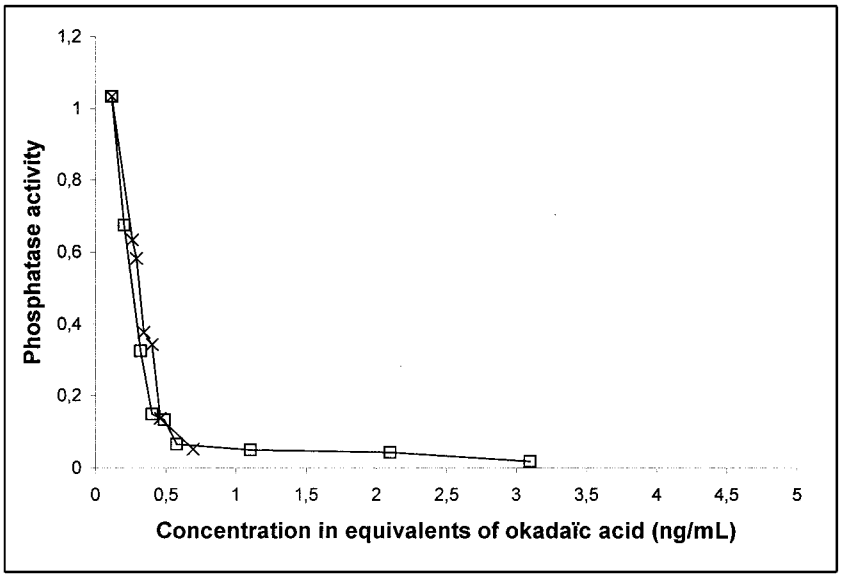

Figure 5. Inhibition of PP2A activity from okadaïc acid (OA: -□-) and the lipid-soluble toxic fraction (F3: -×-).

The dose-response curves were obtained by measuring cell viability (fluorescence unit) at different okadaïc acid and F3 concentrations (Figure 6 and Figure 7) and after 24 hr exposure of NRU.

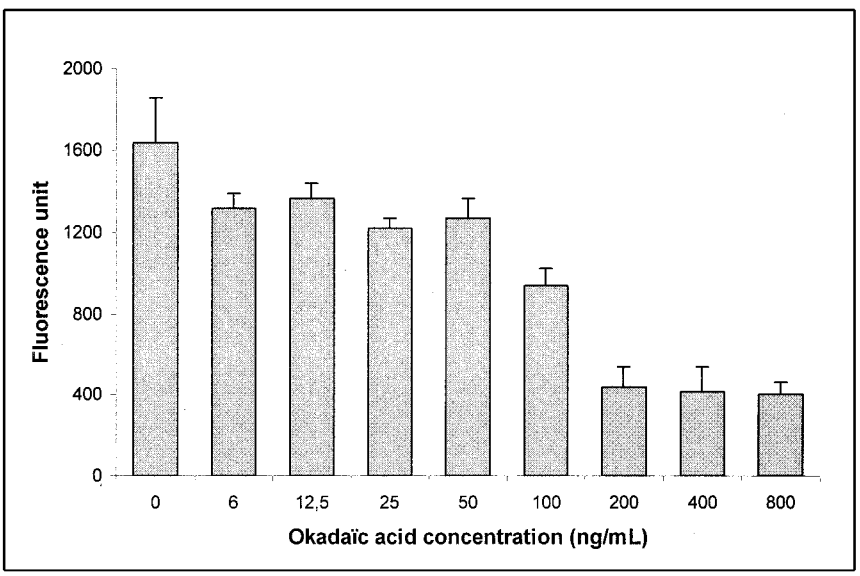

Figure 6. Cell viability using the okadaïc acid after 24 hr of incubation.

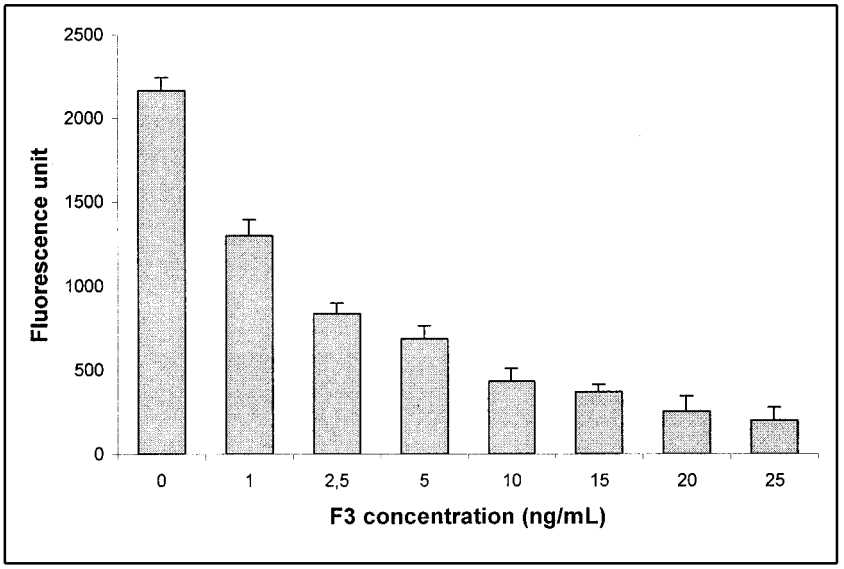

Figure 7. Cell viability using the F3 toxic extract after 24 hr of incubation.

Cell viability (fluorescence unit) decreased more rapidly for F3 than for okadaïc acid. The 50 % inhibitory concentration (IC $_{50}$) against HEP G2 hepatocyts for okadaïc acid and F3 were 112 ng/mL and 4 ng/mL, respectively. It has been observed that F3 extract damages HEP G2 cells more than okadaïc acid. This suggests the presence of one component with a higher toxicity or the presence of different toxins in F3 extract which could act synergically.

Our studies have shown a greater toxicity than in the past of cockles from the Tetouan region between November 1994 and November 1996. These results indicate the presence of both PSP (saxitoxin and its derivatives) and DSP toxins. We have used a cytotoxicity test and enzymatic bioassay as valuable tools for studying marine phycotoxins and to determine toxicity of unknown natural extracts. To obtain purified DSP toxins, HPLC techniques will be applied. The structures of the toxins will be determined using homo and heteronuclear NMR experiments. These are currently in progress.

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