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CONFIRMATION OF AN INCIDENT OF DIARRHETIC SHELLFISH POISONING IN EASTERN CANADA

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ABSTRACT

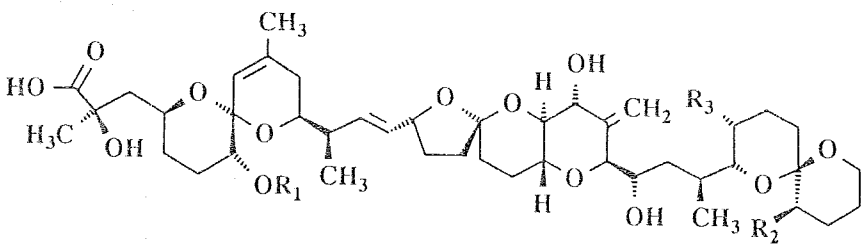
In August 1990, an incident of diarrhetic shellfish poisoning was suspected after 13 people developed gastroenteritis following the consumption of cultured mussels from Mahone Bay in Nova Scotia, Canada. Lipid extracts of mussel samples were found to be toxic in a mouse bioassay and analyses by liquid chromatography-mass spectrometry (LC-MS) established the presence of a high level of the DSP toxin, dinophysistoxin-1 (DTX-1). Conclusive proof of the toxin identity was provided by further experiments, including formation of chemical derivatives with the correct retention times in LC-MS and LC with fluorescence detection, accurate mass measurement using LSIMS mass spectrometry, and proton NMR spectroscopy. Analyses of survey samples showed that the incident was highly localized to one mussel growing lease and that by the end of August, the mussels had depurated all of their toxin load. The most toxic mussels, containing up to 100 µg of DTX-1 per 100 g edible tissue, were harvested on August 3. This suggests that a bloom of toxic plankton may have occurred in July and led to contamination of the mussels.

INTRODUCTION

Diarrhetic shellfish poisoning (DSP) is a severe gastrointestinal illness caused by the consumption of shellfish contaminated by certain species of dinoflagellates, including *Dinophysis* spp. and *Prorocentrum* spp. [1,2]. Okadaic acid (OA), its methyl homologue DTX-1, and its acylated derivative DTX-3 (see Fig. 1) are the principal toxins responsible for the diarrhetic symptoms, although other toxins such as the pectenotoxins and yessotoxins have also been implicated [1]. Most reports of DSP problems have come from Japan and Europe, although episodes of DSP-like illnesses have been documented on the east coast of the United States [3]. In some of the latter incidents, *Dinophysis* spp. or *Prorocentrum* spp. were associated with suspect shellfish, but chemical analyses were not performed. It is only recently that specific DSP toxins have been detected conclusively in natural populations of dinoflagellates from North American waters [4,5]. This paper reports on an incident of diarrhetic shellfish poisoning that occurred in Nova Scotia, Canada, in August 1990.

EXPERIMENTAL

Purified samples of OA and DTX-1, isolated from a laboratory culture of the dinoflagellate *Prorocentrum lima* grown at the NRC Institute for Marine Biosciences, were used to prepare accurate calibration solutions in dimethylformamide (Aldrich Chemical Co., Milwaukee, WI). HPLC grade acetonitrile was purchased from Anachemia (Lachine, PQ). A Milli-Q water purification system (Millipore Corp., Bedford, MA) was used to further purify glass distilled water. The derivatization reagents, 9-anthryldiazomethane (ADAM) and N-(9-acridinyl)-bromoacetamide (NABA), were prepared as described previously [5,6].



R_1	R_2	R_3	
H	H	CH_3	Okadaic Acid (OA)
H	CH_3	CH_3	Dinophysistoxin-1 (DTX-1)
Acyl	CH_3	CH_3	Dinophysistoxin-3 (DTX-3)

Figure 1. Structures of principal DSP toxins.

Mussel tissues were extracted by a procedure adapted from that described by Lee *et al.* [7]. An aliquot (4 g) of excised digestive glands was weighed into a glass culture tube with teflon-lined screw caps. Methanol (16 mL) was added and the mixture was homogenized thoroughly with a Polytron. After centrifugation, an aliquot (10.0 mL) of the supernatant fluid was transferred into a glass culture tube. Following addition of 2.0 mL of glass distilled water, the solution was extracted twice with 10 mL volumes of hexane by mixing thoroughly with a vortex mixer and centrifuging to separate the phases. The hexane extracts were discarded. Water (4.0 mL) and chloroform (16.0 mL) were added to the methanolic phase and mixed thoroughly with a vortex mixer. After separation of the phases with centrifugation, the lower chloroform phase was transferred into a round bottom flask. The chloroform extraction was repeated and the combined chloroform extracts were evaporated on a rotary evaporator using gentle heating (<50°C). The residue was redissolved in 0.1 mL methanol to give a solution with 20 g equivalents of tissue per mL. Solutions were stored in the freezer until analysis.

Derivatization with the ADAM reagent and silica column clean-up were performed as reported previously [5] with a method modified from that of Lee *et al.* [7]. Derivatization with the NABA reagent was performed according to Allenmark *et al.* [6]. HPLC analyses were performed on a Hewlett-Packard model 1090L instrument equipped with a binary DR5 solvent delivery system and an HP1046A fluorescence detector. Settings for the detector were 249 nm excitation and 407 nm emission protected by a 280 nm cut-off filter. Separations were performed at ambient temperature on a 250 x 4.6 mm I.D. Merck LiChrospher 100 RP-18 column with 1 mL/min acetonitrile/water (9:1).

Liquid chromatography-mass spectrometry (LC-MS) analyses were performed on a SCIEX API-III triple quadrupole mass spectrometer (SCIEX, Thornhill, Ontario) equipped with an ion-spray interface, as described previously [5]. Separations were performed on a microbore column (250 x 1 mm I.D.) packed with 5 µm Vydac 201TP stationary phase (Keystone Scientific, Bellafonte, PA) using a 1 µL injection volume. The mobile phase (50 µL/min) was aqueous acetonitrile with 0.1% trifluoroacetic acid (TFA) and a linear gradient of 40% to 100% acetonitrile over 20 min. The instrument was operated in either a full scan or a selected ion monitoring mode. Liquid-assisted secondary ion mass spectrometry (LSIMS) was performed on a ZAB-EQ instrument (VG Analytical, Manchester, UK) using a 25 keV Cs^+ ion beam and glycerol matrix. Proton NMR spectra were obtained with a Bruker MSL-300 spectrometer at 300 MHz.

RESULTS AND DISCUSSION

In early August 1990, at least 13 people developed symptoms of nausea, vomiting and diarrhea shortly after eating boiled or steamed mussels cultured locally in the Mahone Bay area in Nova Scotia. Most of the ill recovered within 24 hours but 3 people had symptoms lasting 3 to 4 days. Samples of leftover cooked mussels from a residence, raw mussels from a restaurant and samples from the local grower tested negative for PSP toxins and domoic acid. These samples also had acceptable bacterial counts. The presence of DSP toxins was suggested when intraperitoneal injections of lipid extracts killed mice overnight.

Analyses of methanolic extracts by ion-spray liquid chromatography-mass spectrometry (LC-MS) [5] using the SCIEX API-III technology quickly established that there was a high level of the DSP toxin, dinophysistoxin-1 (DTX-1), present in the suspect mussels and not in control mussels (Fig. 2). Interestingly, okadaic acid was not detected. The identification of DTX-1 was supported by a match of retention time and ion-spray mass spectrum (MH^+ ion at m/z 819) with those of a standard. Conclusive proof of the toxin identity was provided by further experiments, including formation of NABA and ADAM derivatives with the correct retention times in LC-MS and LC with fluorescence detection (Fig. 3), respectively, and preparative isolation of the toxin followed by accurate mass measurement by LSIMS mass spectrometry (Fig. 4) and proton NMR spectroscopy.

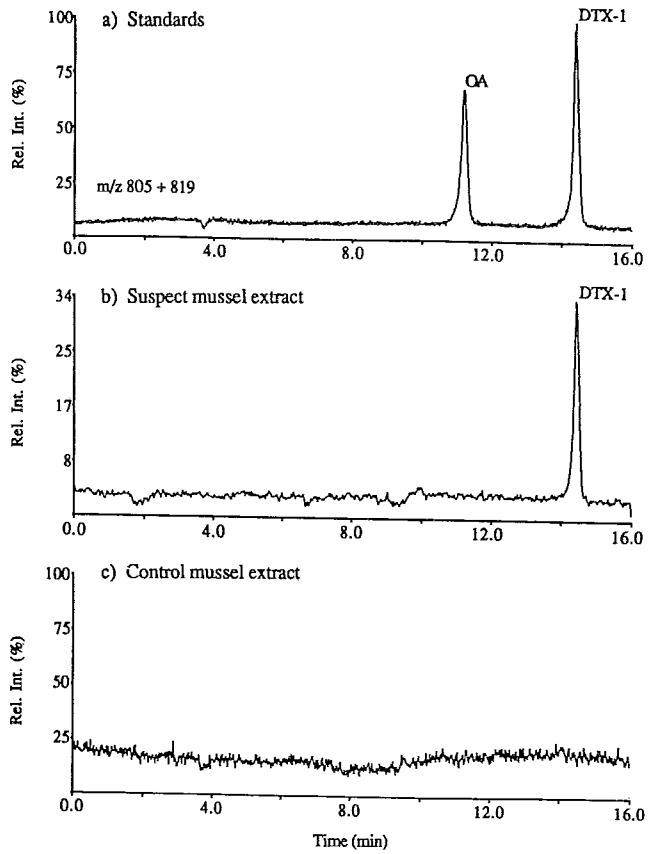


Figure 2. Ion-spray LC-MS analysis of DSP toxin standards (a) and the extracts of suspect (b) and control (c) mussel digestive glands.

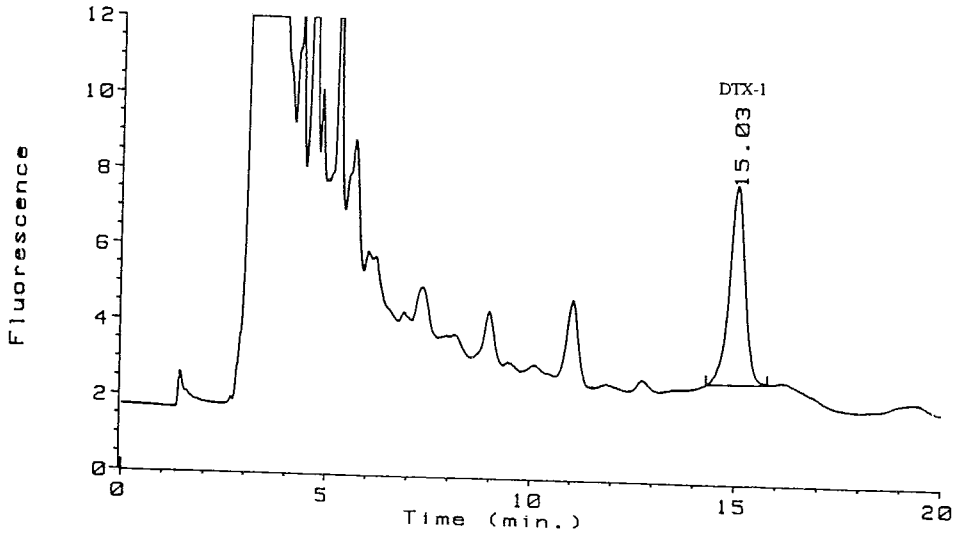


Figure 3. HPLC analysis of an extract of suspect mussel digestive glands using pre-column derivatization with anthryldiazomethane (ADAM) and fluorescence detection.

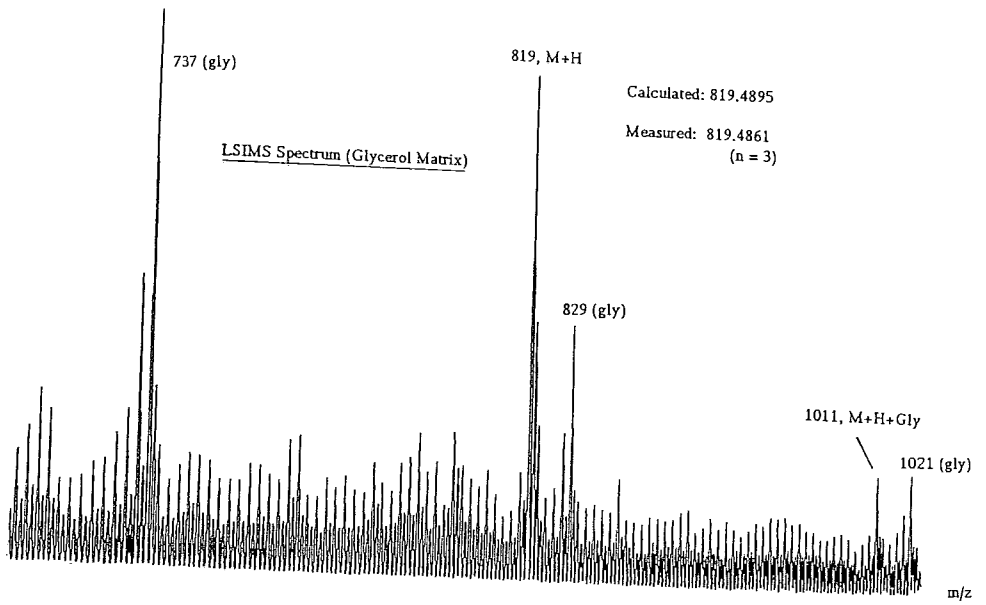


Figure 4. LSIMS mass spectrum of isolated toxin, showing results of accurate mass measurement.

Extraction and handling procedures were improved during the investigation, allowing the rapid quantitative analysis of survey samples by LC-MS. It was found that the incident was highly localized to one mussel growing lease and that by the end of August, the mussels had depurated all of their toxin load (see Fig. 5). The most toxic mussels, containing up to 100 µg per 100 g edible tissue (the legal level in Europe is 20 µg per 100 g), appear to have been harvested on August 3. This suggested that a bloom of toxic plankton may have occurred in July and led to contamination of the mussels.

Microscopic examination of toxic mussel digestive gland contents revealed remnants of a dinoflagellate identified as *Dinophysis norvegica*. This organism is known to produce both okadaic acid and DTX-1, although the ratio is known to vary, with documented instances (Norway) where only DTX-1 is present [2]. Control, non-toxic mussels showed no recognizable remnants of *Dinophysis*. Plankton tows from the waters adjacent to the affected area in mid-August did show several *Dinophysis* spp. (including *norvegica* as the dominant species) at low levels in the water column. LC-MS analysis of these plankton samples provided only a weak, unreliable signal for the toxin. Presumably, the bloom of plankton responsible for the mussel contamination had dissipated by the time of sampling.

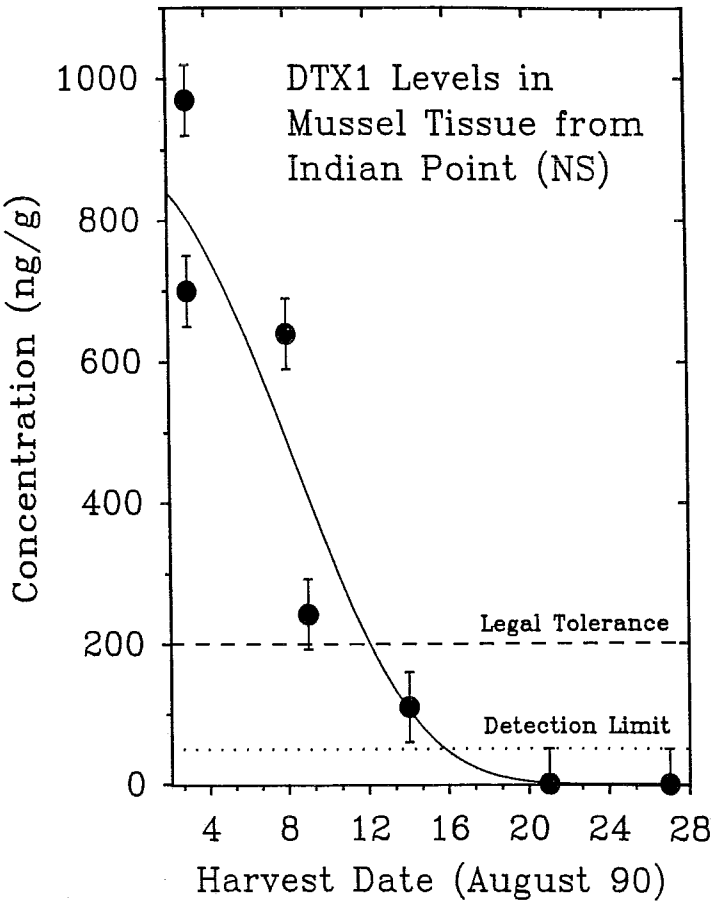


Figure 5. Quantitative results on mussel samples analyzed, showing the depuration of the toxin from the mussels over time.

Recent work with other species isolated from the mussel growing area in Mahone Bay after the incident revealed that a strain of *Prorocentrum lima* produces both okadaic acid and DTX-1 in culture [8]. It is possible that this benthic species was a source of the DSP toxin in the mussels. Indeed, in the summer of 1991, mussels in the same area were contaminated again, this time with both okadaic acid and DTX-1 [8]. Both *Dinophysis norvegica* and *Prorocentrum lima* were observed in the waters that summer also.

CONCLUSIONS

The toxin responsible for the August 1990 incident in Mahone Bay was established to be DTX-1. To our knowledge, this is the first case of diarrhetic shellfish poisoning in North America that has been proven through chemical analysis. The source of the toxin has not been established positively, but *Dinophysis norvegica* and *Prorocentrum lima* have been implicated by their presence in the water.

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