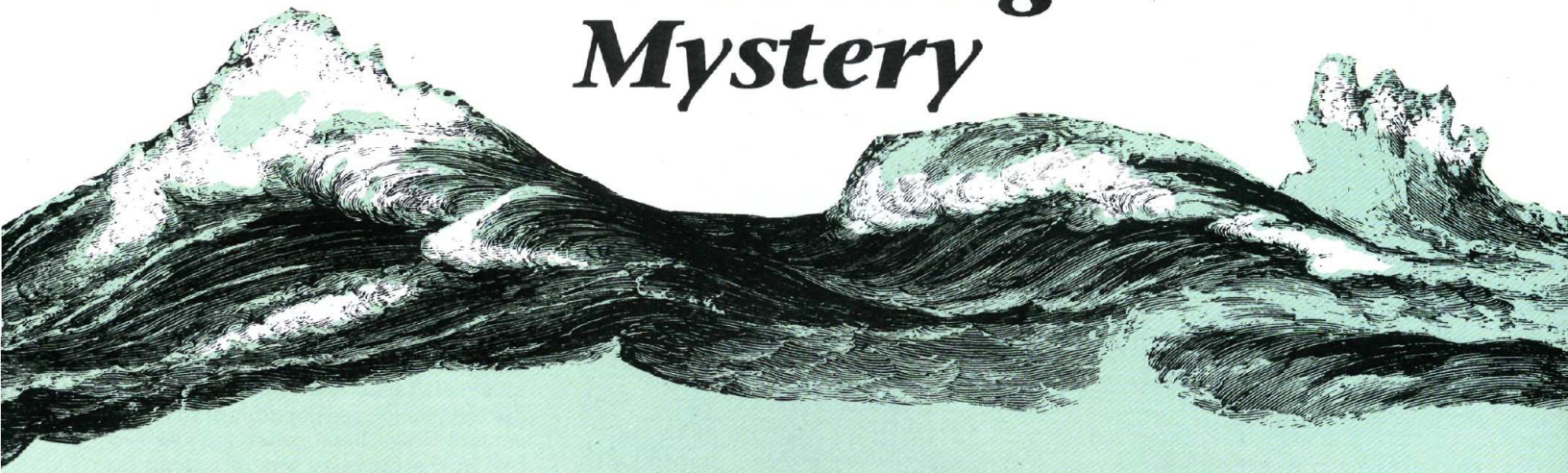
The amnesic shellfish poisoning mystery

Quilliam, Michael A.; Wright, Jeffrey L. C.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1021/ac00193a002
Analytical Chemistry, 61, 18, pp. 1053A-1060A, 1989-09-15

The Amnesic Shellfish Poisoning Mystery



Michael A. Quilliam and Jeffrey L. C. Wright
Atlantic Research Laboratory
National Research Council of Canada
1411 Oxford Street, Halifax
Nova Scotia, Canada B3H 3Z1

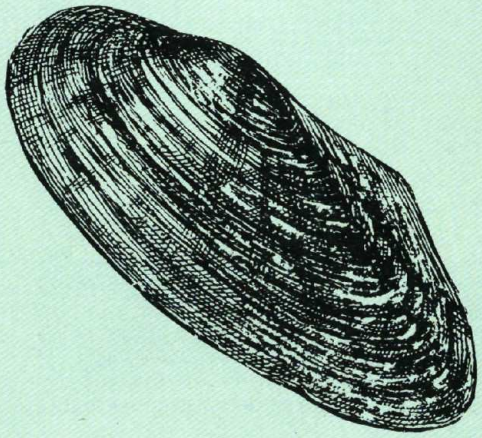
In late 1987 a mysterious and serious outbreak of food poisoning occurred in Canada. Symptoms of the poisoning included vomiting and diarrhea, followed in some cases by confusion, memory loss, disorientation, and coma. Three elderly patients died, and other victims still suffer from neurological problems. The term amnesic shellfish poisoning has been proposed for this clinical syndrome.

Epidemiologists from Health and Welfare Canada (HWC) attributed the illnesses to restaurant meals of cultured blue mussels (*Mytilus edulis* L.). Using the Association of Official Analytical Chemists' mouse bioassay for "red-tide" paralytic shellfish poison (*1*, *2*), HWC and Department of Fisheries and Oceans (DFO) scientists demonstrated that these mussels did contain toxic material and traced the problem to mussels harvested from a localized area of eastern Prince Edward Island. Intraperitoneal injections of acidic aqueous extracts of suspect mussels into mice caused death with some unusual neurotoxic symptoms very different from those of paralytic shellfish poison and other known toxins.

It was not known whether the toxic agent was a man-made pollutant or a natural toxin. The scientific detective story that unfolded was followed closely by a concerned Canadian public and made front-page newspaper headlines for several weeks. For health, political, and economic reasons, scientists in Canadian government laboratories were eager to solve the mystery quickly.

Initial methodologies

Several laboratories attempted analyses for certain targeted toxic substances such as heavy metals and organophosphorous pesticides. All of these analyses yielded negative results. Experiments also were conducted to compare the analytical "fingerprints"



of extracts of toxic and nontoxic control mussels. Any differences between the chromatograms or spectra of these extracts might be attributable to the toxic agent and allow for its identification. Methods used to generate such fingerprints included TLC, HPLC with a UV–vis diode array detector (DAD), GC/MS, and NMR spectrometry.

Some interesting differences were immediately obvious in the TLC and HPLC–DAD chromatograms of lipid-soluble fractions from digestive glands of toxic and control mussels. However, examination of the UV–vis spectra of the compounds giving rise to these differences revealed absorption bands at >400 nm that were characteristic of phytoplankton pigments. Indeed, the digestive glands of the toxic mussels were found to be engorged with a green plankton.

In retrospect these observations provided hints about differences in mussel diets and strongly suggested a natural toxin, but they gave no information about the nature of the toxin itself. Such an approach might have led eventually to detection and identification of the toxin, but it was soon established that lipid-soluble fractions were not toxic in the mouse bioassay. A more systematic approach was necessary.

On December 12, 1987, a team of scientists was assembled at the Atlantic Research Laboratory (ARL) of the National Research Council in Halifax,

Nova Scotia. This team consisted of all available chemists and marine biologists from ARL as well as some scientists from DFO, including personnel experienced with the mouse bioassay procedure. This team developed a strategy based on bioassay-directed separations and analyses (e.g., *3*) that led to the identification of the toxin on the afternoon of December 16, just 102 h after the start of the concerted investigation.

Bioassay-directed strategy

The general principles of the bioassay-directed approach used are summarized in Figure 1a. Extracts of both toxic and control mussels were taken in parallel through a series of preparative separation steps (Figure 1b). After each fractionation, the mouse bioassay was used quantitatively and qualitatively to determine which fraction(s) contained the toxin (or toxins if a mixture was present). Various chromatographic and spectroscopic techniques were also used to profile toxic fractions and the corresponding fractions from control samples. The objectives were to determine possible differences between toxic and control fractions that might correspond to the toxin(s) and to determine whether a fraction was simple enough to begin structure elucidation. Application of various spectroscopic methods to the purified toxin then provided clues about the structure of the compound, and relevant chemical literature was searched to determine if the toxin was a known compound.

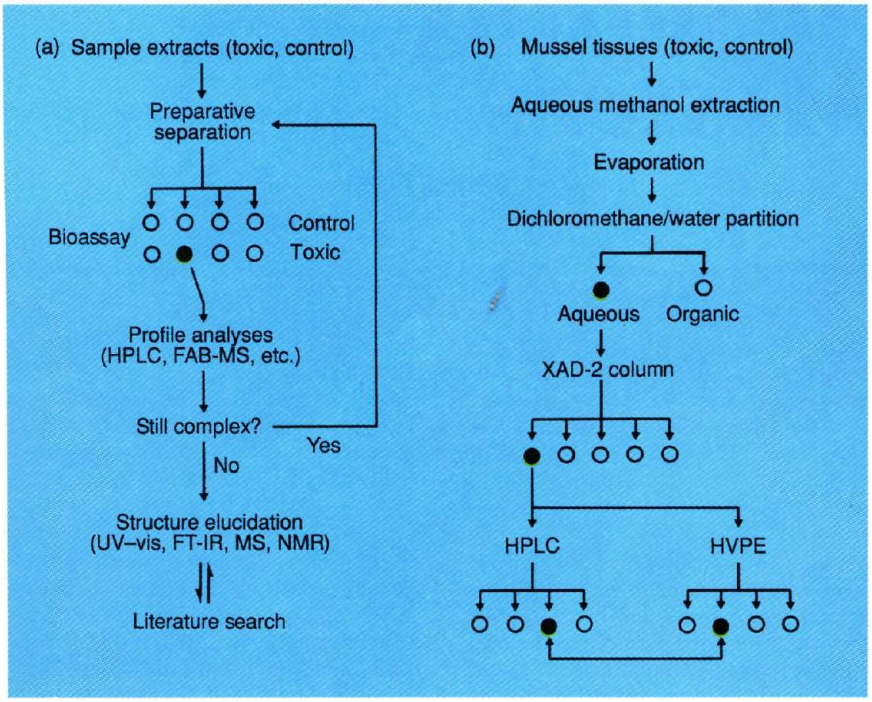


Figure 1. Flow charts illustrating (a) the general principles of the bioassay-directed analysis strategy and (b) the extraction and fractionation procedures used in the toxic mussel investigation.
The filled circles indicate a positive test in a mouse bioassay; open circles indicate a negative test.

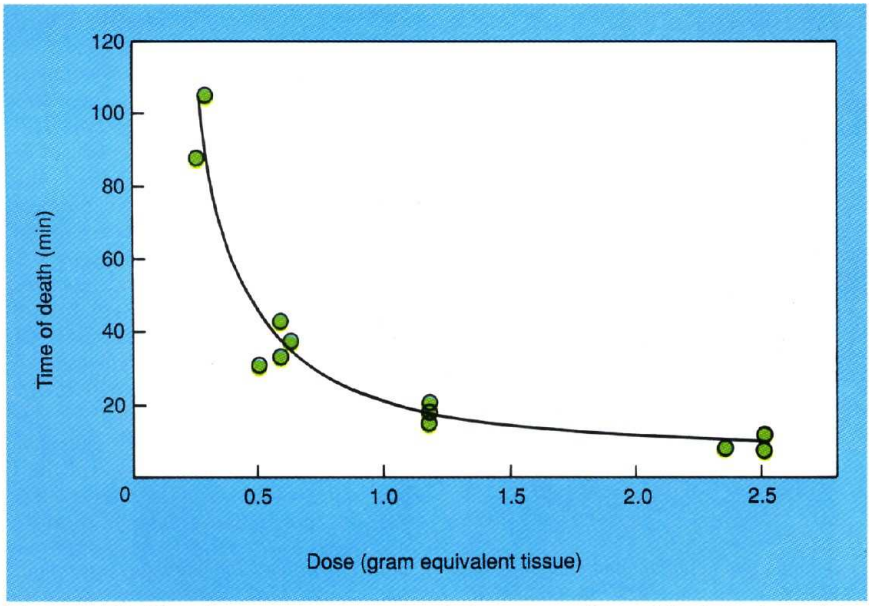


Figure 2. Working dose–response curve relating mouse time of death to the weight of mussel tissue equivalent in the injected dose.

To avoid large statistical fluctuations associated with sampling inhomogeneities, all experiments were conducted using large portions of toxic and control tissue homogenates. Dose control in the bioassay was of crucial importance for effective tracking and for establishing a toxicity balance. The toxic symptoms in mice (which included scratching, trembling, and death by asphyxiation) were quite different from those observed with known shellfish toxins, and it was essential to establish a dose–response curve for the bioassay. The working curve is shown in Figure 2. Note the narrow dynamic range, which implied that effective tracking of toxicity required that each bioassay correspond to material extracted from about 0.6 g of mussel tissue (the midpoint of the dynamic range). To determine the toxicity balance, the weight equivalent of mussel tissue extracted for each bioassay injection had to be known.

The preparative fractionation scheme used and the results of the mouse bioassay at each stage are presented in Figure 1b. A classical natural products extraction procedure using mild aqueous methanol at room temperature was selected for initial work in case the toxin was a labile compound. Later it was found that greater extraction efficiency could be achieved with more rigorous extraction methods. A partitioning between water and dichloromethane followed by XAD-2 column chromatography revealed that the toxin was water soluble. The final stage of the purification process was a dual approach invoking two separate fractionation procedures based on differ-

ent physicochemical properties. HPLC exploits differing partition ratios between a polar mobile phase and a nonpolar stationary phase, whereas high-voltage paper electrophoresis (HVPE) depends on different electrophoretic mobilities of charged species under a strong electric field. Thus the chances of erroneous identification of the toxin were minimized, especially by cross-checking toxic fractions from HPLC by HVPE and vice versa.

Both HPLC and HVPE also yielded informative profile analyses. A striking example of HPLC–DAD profile analysis comparing fractions of toxic and control mussel extracts is shown in Figure 3. The HPLC conditions were selected on the premise that the unknown toxic substance was a polar, ionizable compound such as a peptide. Chromatograms for absorption at 210 nm ± 10 nm indicated a peak at about 12 min (just after the tryptophan peak) for toxic but not for control XAD-2 fractions. Detection at 210 nm ± 10 nm was used initially to reveal all differences because most compounds absorb at shorter wavelengths. The complete three-dimensional representation of the HPLC–DAD data for the toxic sample at the appropriate time window is shown with the absorption spectrum taken at the peak maximum. The latter spectrum shows an absorption maximum at 242 nm (suggesting a conjugated system such as C=C–C=C). Reconstructed chromatograms for absorption at this wavelength are also shown to accentuate the toxic/control dichotomy. When these same XAD-2 fractions were analyzed using HVPE, a band running just behind glutamic acid but staining yellow rather than red with ninhydrin was observed in the toxic but not in the control extracts.

Furthermore, the HPLC–DAD suspect peak was collected and analyzed using HVPE, where it gave the same yellow band; the converse cross-check was also successful. Even more important was the finding that these fractions, collected from the two complementary separation techniques operated on a preparative scale, were shown to account for all of the toxicity within the reproducibility of the dose–response curve.

Spectroscopic strategy

While these highly encouraging results were being obtained, complementary profile analyses obtained for all toxic fractions by fast atom bombardment mass spectrometry (FAB-MS) showed that peaks at m/z 312 ($[M + H]^+$) in positive ion mode and at m/z 310 ($[M - H]^-$) in negative ion mode were increasing in prominence as the toxin was progressively purified. No significant corresponding signals arising from a compound of MW 311 were evident in

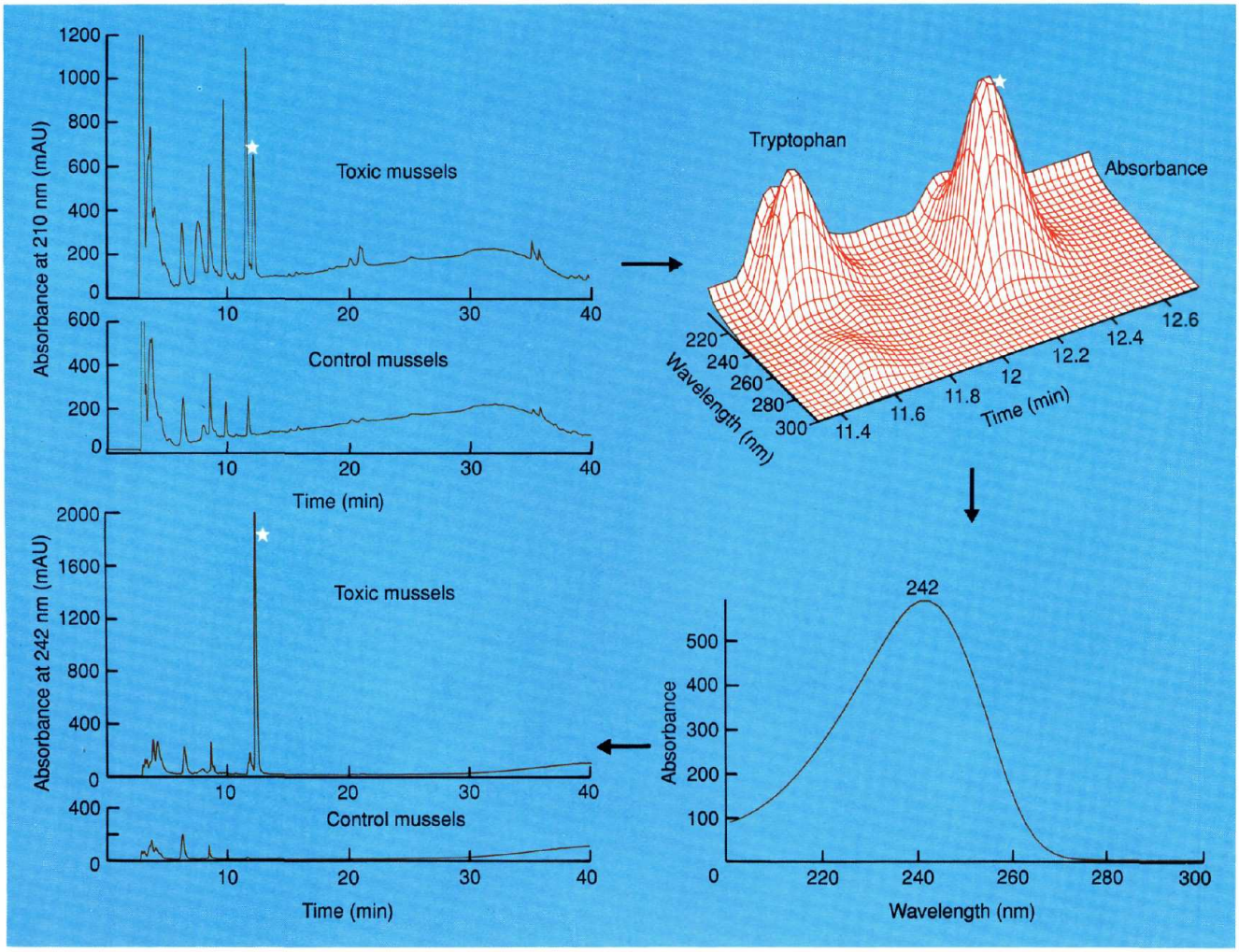
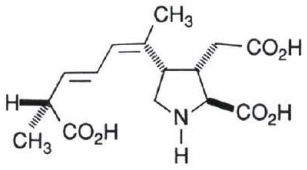


Figure 3. HPLC–DAD profiles of corresponding XAD-2 fractions from toxic and control mussels.

Chromatograms reconstructed from the DAD data at 242 nm show the differences more dramatically. Conditions: 25 cm × 4.6 mm i.d. Vydac 201TP column with 1.0 mL/min $CH_3CN/H_2O/CF_3COOH$; gradient elution from 5.0:94.9:0.1 to 99.9:0:0.1 over 40 min.

any fractions from control mussels. High-resolution MS (peak matching) on an XAD-2 fraction showed the positive ions at m/z 312 to have the composition $C_{15}H_{22}NO_6$. The fragmentation behavior of these ions (tandem MS, Figure 4a), through successive losses of 46 Da (HCOOH) and other small neutral species, eventually yielded an intense fragment at m/z 74. This is characteristic of protonated amino acids, as known previously from chemical ionization studies. In the meantime, FT-IR spectra of crude toxic fractions (Figure 4b) suggested the presence of >NH, —COOH, —CH=CH— (*trans*), and —CH_3 groups, and proton NMR spectra (Figure 4c) of concentrated toxic fractions showed signals characteristic of —CH=CH–CH=C—, CH_3–C=C—, and CH_3–CH< functionalities.

In case the toxin was a known compound, all information produced by the experiments was used as input data for a computerized literature search through the Chemical Abstracts and Registry files maintained by STN International (Columbus, OH). The list of possible compounds was reduced from thousands to one most likely candidate for the toxin—a tricarboxylic amino acid, domoic acid. Known pharmacological properties of this compound (*4–7*) are consistent with the neurotoxic symptoms caused by the affected mussels, although the compound had not previously been identified as a human intoxicant.



Domoic acid

Structure confirmation

At this stage it appeared likely that the toxin was either domoic acid or a closely related compound. Proof of chemical structure was provided by NMR, which required a sizable amount of purified material. Multiple HPLC injections had to be made overnight so that enough toxin could be collected to obtain a good-quality proton NMR spectrum. This also made it possible to acquire an improved FT-IR spectrum. The NMR spectrum was entirely consistent with a previously published spectrum of synthetically produced domoic acid (*8*) except for some slight variations later shown to reflect pH dependence of the ionic form of the compound. Subsequent work on highly purified mussel toxin has shown that 2D proton NMR, ^{13}C NMR, melting point, and optical rotation are all consistent with the known properties of domoic acid (*8*).

Toxicity

Although chromatographic separations of aqueous methanol extracts showed no other detectable toxins, it was still important to establish that domoic acid accounted for all of the toxicity in the original mussel tissue. In the days following, many hours of painstaking work established a toxicity balance. Figure 5 illustrates part of this effort as a dose–response curve for the bioassay, expressed by the amount of domoic acid injected (as determined by a high-speed HPLC assay [*9*]). The different data points plotted in Figure 5 reflect several toxic mussel samples extracted by different methods.

Domoic acid was first identified in crude extracts of certain seaweed found in Japan (*10*) and was used there as a folk medicine remedy for intestinal worm infestation. This fact argues against this compound as a toxin. However, when the high levels concentrated in the toxic mussels (up to 900 μg/g wet weight) are taken into account, the implication is that as much as 0.2–0.3 g may have been ingested and the difference between therapeutic and toxic doses is easily established.

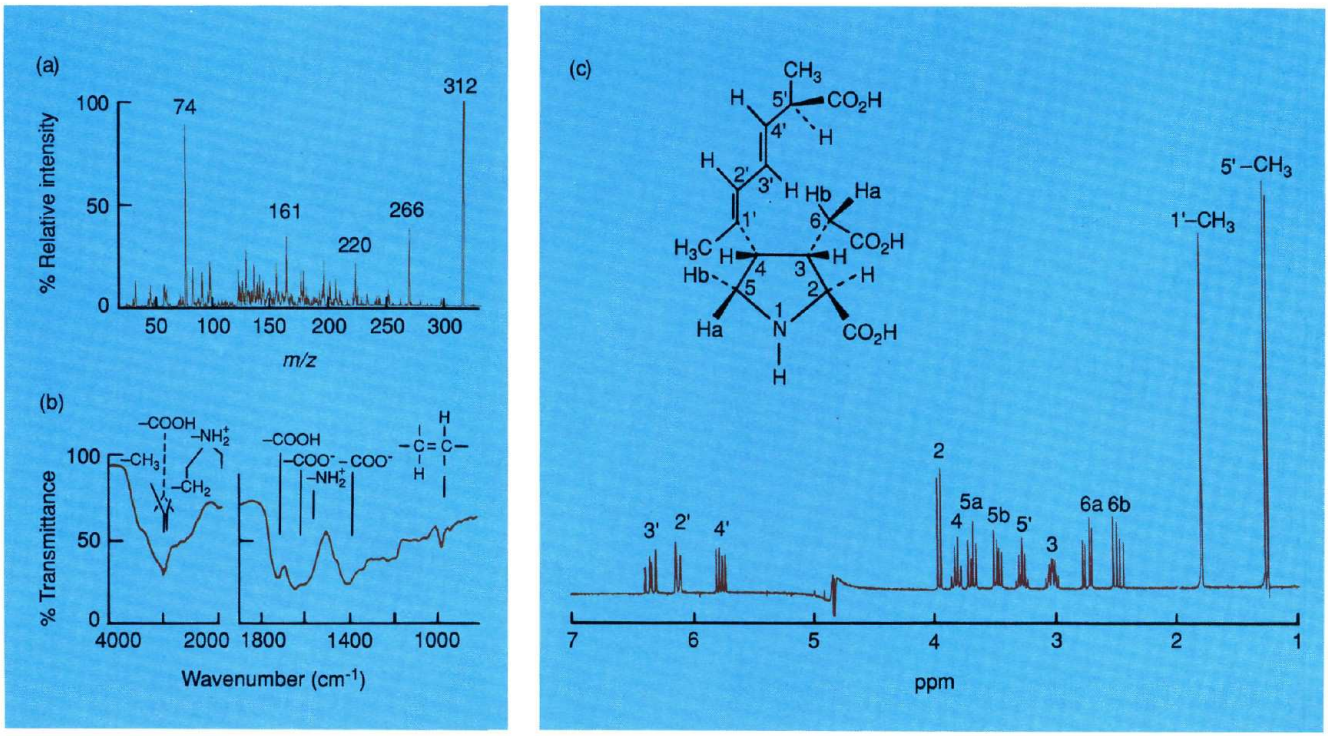


Figure 4. Spectroscopic data for the mussel toxin.

(a) MS/MS fragment ion spectrum of the m/z 312 ion in the positive ion FAB mass spectrum of a toxic XAD-2 fraction, (b) FT-IR spectrum of a thin film of the isolated toxin on CaF_2 window, and (c) 300 MHz proton NMR spectrum of purified toxin dissolved in D_2O.

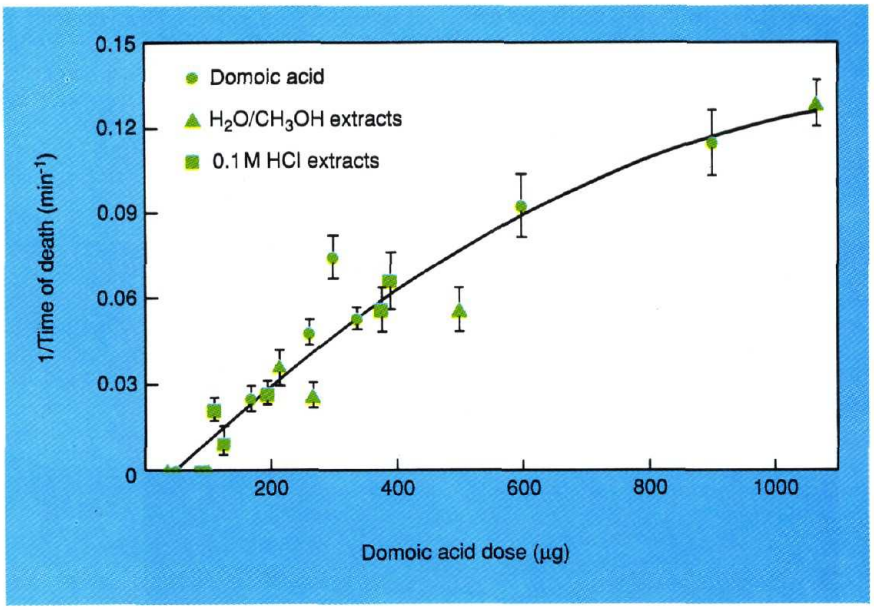
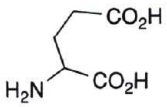


Figure 5. Dose–response curve, plotted as inverse of time of death (TOD) against dose of domoic acid (determined by HPLC analyses), for standard solutions and for different contaminated mussel extracts injected into the mouse.

Each point is the mean of 2–4 replicate measurements of TOD for one solution, and the error bars represent an estimate of the standard deviation. The curve is a second-order polynomial least-squares regression on all data points.

The neurotoxicity of domoic acid results from its effect as a potent glutamate agonist (*4–7*). Domoic acid can be considered to be a conformationally restricted form of glutamic acid that disrupts normal neurochemical transmission in the brain by binding to certain glutamate receptors of normal cells. This results in continuous stimulation of neurons and the eventual formation of lesions.



Glutamic acid

Retrospective

A detailed scientific account of the identification of the mussel toxin has been published (*11*), and a prolonged investigation into the source of the domoic acid was also conducted. The localization of domoic acid in the digestive system of the shellfish clearly pointed to a dietary origin for the toxin. As mentioned earlier, the digestive glands were engorged with plankton, the microscopic examination of which revealed large numbers of an unidentified diatom. Coincidentally, marine biologists patrolling the affected estuary observed a substantial uni-algal bloom of phytoplankton later identified as the diatom *Nitzschia pungens* forma *multiseries*. This work, which has been described in detail elsewhere (*12, 13*), also established unequivocally that domoic acid is produced as a secondary metabolite of the diatom.

This is the first report of a shellfish toxin from a marine diatom, and the implications for the shellfish industry are significant. The diatom *N. pungens* is widely distributed in the coastal waters of the Atlantic, Pacific, and Indian oceans, although production of domoic acid in all strains has not yet been demonstrated. It is known, however, that shellfish harvested from other locations on the eastern seaboard contain domoic acid (*9*).

The cause of the bloom of *N. pungens* cannot be answered with any degree of certainty. One possible explanation is that freshwater runoff from agricultural land caused stratification of the ocean layers and raised the nutrient level in the coastal waters at just the right time in the diatoms' lifecycle. In fact, there was a record-breaking storm with easterly winds on Sept. 7, 1987. It is of interest that a similar, but less dense, bloom of *N. pungens* occurred in the same area in late 1988. In this case, it was found that the rise and fall of cell density of *N. pungens* in the seawater accurately reflected that of domoic acid concentration in water and cultured mussel samples. Nevertheless, a DFO screening program implemented for domoic acid effectively protected the public and the rest of the shellfish industry during this repeat episode. Fortunately, shellfish contaminated with domoic acid clear themselves if kept in clean seawater, so the aquaculturist is not faced with a total loss if toxification of a crop occurs.

In retrospect, the speed with which the toxin was identified is attributable to several factors: its water solubility, which permitted discarding the entire lipid-soluble fraction; the high concentration in the contaminated mussels; the availability of an on-site bioassay that was rapid and reasonably precise; and the fact that the toxin turned out to be a compound that could readily be found in the chemical literature. More important than any of these factors, however, was the dedication and camaraderie of the entire team of scientists, technicians, and support staff who worked with minimal sleep until the mystery was solved.

This article is NRCC publication no. 30557

References

(1) *Official Methods of Analysis;* Association of Official Analytical Chemists: Arlington, VA, 1984; pp. 18.086–18.092.
(2) Adams, W. N.; Miescier, J. J. *J. Assoc. Off. Anal. Chem.* **1980,** *63,* 1336–43.
(3) Schuetzle, D.; Lewtas, J. *Anal. Chem.* **1986,** *58,* 1060 A–1075 A.
(4) Zaczek, R.; Coyle, J. T. *Neuropharmacology* **1982,** *21,* 15–26.
(5) Shinozaki, H.; Ishida, M. *Brain Res.* **1976,** *109,* 435–39.
(6) Biscoe, T. J.; Evans, R. H.; Headley, P. M.; Martin, M. R.; Watkins, J. C. *Nature* **1975,** *255,* 166–67.
(7) Takeuchi, H.; Watanabe, K.; Nomoto, K.; Ohfune, Y.; Takemoto, T. *Eur. J. Pharmacol.* **1984,** *102,* 325–32.
(8) Ohfune, Y.; Tomita, M. *J. Am. Chem. Soc.* **1982,** *104,* 3511–13.
(9) Quilliam, M. A.; Sim, P. G.; McCulloch, A. W.; McInnes, A. G. *Int. J. Environ. Anal. Chem.* **1989,** *36,* 139–54.
(10) Takemoto, T.; Daigo, K. *Chem. Pharm. Bull.* **1958,** *6,* 578–80.
(11) Wright, J.L.C.; Boyd, R. K.; deFreitas, A.S.W.; Falk, M.; Foxall, R. A.; Jamieson, W. D.; Laycock, M. V.; McCulloch, A. W.; McInnes, A. G.; Odense, P.; Pathak, V. P.; Quilliam, M. A.; Ragan, M. A.; Sim, P. G.; Thibault, P.; Walter, J. A.; Gilgan, M.; Richard, D.J.A.; Dewar, D. *Can. J. Chem.* **1989,** *67,* 481–90.
(12) Subba Rao, D. V.; Quilliam, M. A.; Pocklington, R. *Can. J. Fisheries Aq. Sci.* **1988,** *45,* 2076–79.
(13) Bates, S. S.; Bird, C. J.; deFreitas, A.S.W.; Foxall, R. A.; Gilgan, M.; Hanic, L. A.; Johnson, G. E.; McCulloch, A. W.; Odense, P.; Pocklington, R.; Quilliam, M. A.; Sim, P. G.; Smith, J. C.; Subba Rao, D. V.; Todd, E.C.D.; Walter, J. A.; Wright, J.L.C. *Can. J. Fisheries Aq. Sci.* **1989,** *46,* 1203–15.

Michael A. Quilliam, an associate research officer with the National Research Council of Canada, received his

Ph.D. in analytical chemistry from the University of Manitoba. After completing postdoctoral work at the Université de Montréal, Quilliam spent nine years as a faculty member in the Chemistry Department of McMaster University before joining ARL in 1987. His research interests include chromatography, spectroscopy, and the application of bioassay-directed analysis to solve problems in environmental toxicology.

Jeffrey L. C. Wright, a senior research officer with the National Research Council of Canada, was the leader of the mussel toxin team. He received a Ph.D. in bioorganic chemistry from Glasgow University. Wright's research interests include the discovery of bioactive compounds from marine organisms and the application of tandem mass spectrometry to distinguish stereoisomers.

Members of the mussel toxin team. From left to right, front row: P. F. Seto, J.L.C. Wright, P. G. Sim, A.S.W. deFreitas, C. Gillis, C. J. Bird, M. A. Quilliam, M. Falk; second row: M. A. Ragan, P. Odense, A. R. Taylor, A. M. Backman, C. A. Craft, G. K. McCully, P. LeBlanc, V. Pathak, W. D. Jamieson, D.J.A. Richard; third row: D. Robson, J. A. Walter, P. Thibault, M. V. Laycock, D. Tappen, R. K. Boyd, H. Watts, D. O'Neil, C. Vaughan, W. R. Hardstaff, M. Greenwell, A. G. McInnes, M. G. Flack, D. J. Embree, A. W. McCulloch, O. Henderson. Not shown: D. Brewer, D. Dewar, E. W. Dyer, R. A. Foxall, M. Gilgan, N. I. Lewis, M. McInerney-Northcott, J. L. McLachlan.