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TOXIN PRODUCTION OF *ALEXANDRIUM MINUTUM* (DINOPHYCEAE) FROM THE BAY OF PLENTY, NEW ZEALAND

F. HOE CHANG,[1] DONALD M. ANDERSON,[2] DAVID M. KULIS[2] and DESMOND G. TILL[3]

[1]National Institute of Water and Atmospheric Research Ltd, P.O. Box 14901, Kilbirnie, Wellington, New Zealand; [2]Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, U.S.A.; and [3]New Zealand Communicable Disease Centre, ESR, Porirua, New Zealand

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F. H. Chang, D. M. Anderson, D. M. Kulis and D. G. Till. Toxin production of *Alexandrium minutum* (Dinophyceae) from the Bay of Plenty, New Zealand. *Toxicon* **35,** 393–409, 1997.—Paralytic shellfish toxins of two clonal cultures of *Alexandrium minutum* isolated during the 1993 toxic shellfish events in the Bay of Plenty, New Zealand, were analyzed using high-performance liquid chromatography. Toxin composition profiles of both cultures showed neosaxitoxin (> 65 mole%) as the principal toxin, with saxitoxin and gonyautoxins (GTX1–4) as minor components. Neither C-toxins (C1–4) nor GTX5–6 were detectable in the two isolates. Bay of Plenty isolates of *A. minutum* have a unique toxin profile not found in any other isolates of this species that have been characterized. This weakens the hypothesis that *A. minutum* was recently introduced to New Zealand waters by ballast water or other long-distance transport mechanisms, and argues instead that the species was endemic to the area, but not noticed in the past. The average toxicity of the cultures was 8.8 and 11.0 pg saxitoxin equiv. $cell^{-1}$ with acetic acid or HCl extraction, respectively. These are at the high end of the range of toxicity reported for *A. minutum* strains from around the world, and on a cell volume basis are comparable to the most toxic strains of the *Alexandrium tamarense* group. The toxin profile of *A. minutum* most closely matches that of mussels and to a lesser degree tuatua harvested from the Bay during the 1993 outbreak, but is quite different from the profile measured in scallops and pipi. Plausible mechanisms for bioconversion of the ingested algal toxins within the latter two shellfish species can be proposed, but it seems more likely that either other strains of *A. minutum* or other saxitoxin-producing dinoflagellates were ingested by those shellfish. This study established that *A. minutum* from the Bay of Plenty contains saxitoxins, has a unique toxin composition compared to all other isolates of this species, and was responsible for at least part of the PSP toxicity measured in shellfish during the 1993 outbreak. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

New Zealand is a country with rich shellfish resources and a vigorous shellfish aquaculture industry that until recently were free from any threat from marine biotoxins of algal origin. In early 1993 on the north-east coast of North Island, however, more than 180 people were reported to have suffered shellfish poisoning along with numerous instances of respiratory irritation from airborne toxin in sea spray (Chang, 1993, 1994; Chang *et al.*, 1995; Bates, 1993; Mackenzie *et al.*, 1995). The toxicity was eventually characterized as neurotoxic shellfish poisoning (NSP), linked to new brevetoxin-like compounds (Ishida *et al.*, 1994, 1995; Morohashi *et al.*, 1995). *Gymnodinium* cf. *breve*, identified during the toxic shellfish outbreaks, was suggested to be the causative organism of the NSP (Chang, 1995). Simultaneously, toxins responsible for paralytic shellfish poisoning were detected in shellfish from those same areas at levels above the regulatory threshold of 80 μg STX equiv. 100 g^{-1} shellfish meat (Chang *et al.*, 1995). As the levels of PSP toxins detected in most commercial shellfish (mussels, scallops, oysters and pipi) were not very high (ranging from 100 to 400 μg STX equiv. 100 g^{-1}), PSP did not represent a serious human health threat at that time.

At the height of toxicity episode, two *Alexandrium minutum* clones were isolated from the Bay of Plenty and successfully cultured. Both tested positive for PSP using mouse bioassay (Chang, 1994). The detection of PSP toxins in commercial shellfish, and the subsequent positive PSP mouse tests on the clonal cultures prompted the present investigation into the nature of the toxins produced by this species. Attempts were also made to compare toxin composition profiles of the cultures with profiles from several species of shellfish collected during the toxicity episode. Given the speculation that toxic dinoflagellates have been introduced to some regions as a result of ballast water transport of resting cysts (e.g. Hallegraeff and Bolch, 1992), the toxin profiles of our *A. minutum* cultures were also compared to profiles of other strains of the species isolated from Australia, France, Portugal, Taiwan, New Zealand and Spain.

MATERIALS AND METHODS

Cultures

During the shellfish toxicity episodes in early January 1993, two clonal cultures of *A. minutum* (clones AMABOP006 and AMABOP014) were developed from water samples taken at Pio's Ocean Beach and Tauranga Harbour, western Bay of Plenty, New Zealand (Fig. 1). These unialgal, clonal isolates were maintained in f/2 medium (Guillard and Ryther, 1962), at 18°C in a constant-temperature room, at 80 $\mu E\ m^{-2}\ sec^{-1}$ of cool-white fluorescent light under a 14:10 hr light:dark regime.

For taxonomic study, cells of *A. minutum*, either collected directly from the Bay of Plenty, or cultured from water samples taken from the same region, were examined using a Nikon Diaphot inverted light microscope. Specimens were then prepared for scanning electron microscopy by fixing in 1% glutaraldehyde/2% OsO_4. Cells were collected on 25 mm diameter Nuplepore filters (2 μm pore size). Portions of these filters were dehydrated in increasing concentrations of acetone, critical-point-dried, sputter-coated with gold–palladium, and examined with a Philips 505 scanning electron microscope. All the samples collected for phytoplankton enumeration from the Bay of Plenty were preserved in 1% Lugol's iodine solution (Chang *et al.*, 1995) and counted following a 24 hr period using an inverted light microscope (Utermöhl, 1958).

Cultures used for toxin analysis were grown in duplicate 25 ml volumes of f/2 medium made with 0.2 μm filtered Vineyard Sound seawater (31 psu salinity). The f/2 medium was modified by adding H_2SeO_3 and reducing the concentration of $CuSO_4{\cdot}5H_20$ to a final concentration of 10^{-8} M. Cultures were incubated at 20°C on a 14:10 hr light:dark cycle (c. 250 $\mu E\ m^{-2}\ sec^{-1}$ irradiance provided by cool-white fluorescent bulbs). Growth was monitored on a Turner model 10 AU fluorometer. All cell count samples were enumerated with a Sedgwick Rafter counting chamber, with at least 400 cells counted for each sample.

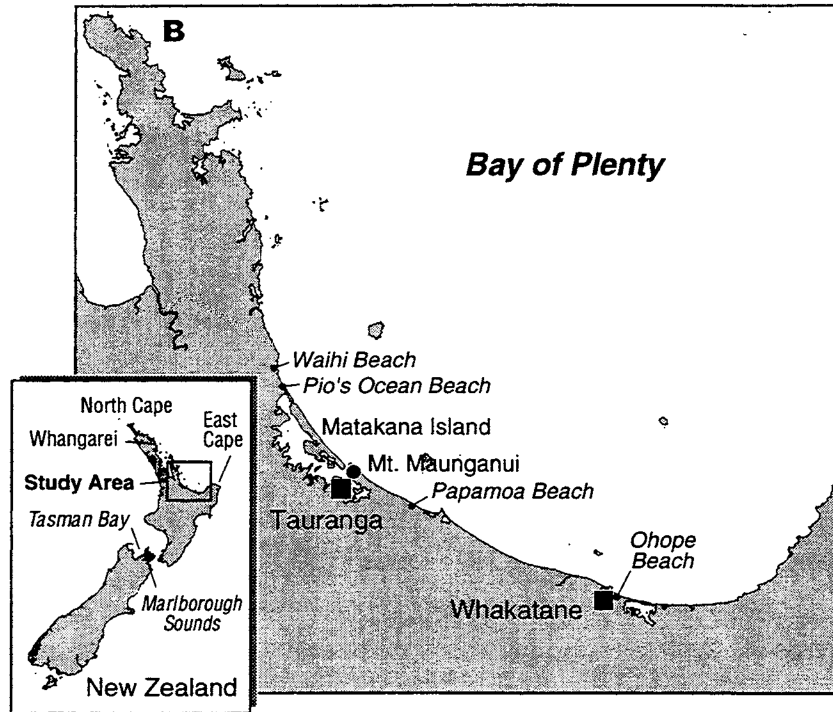

Fig. 1. Study area in the Bay of Plenty, North Island, New Zealand.

Toxin extraction

Upon reaching mid-exponential growth, cultures were harvested for cell density and saxitoxin determinations as follows. The cultures were thoroughly mixed by hand, and two 1.5 ml cell count samples were removed and preserved with Utermöhl's solution (Utermöhl, 1958). A 15 ml sample for toxin extraction was also removed. The toxin sample was placed in a siliconized, polypropylene centrifuge tube and centrifuged (5000 *g*, 5 min, 23°C), the supernatant aspirated, and 300 μl of either 0.5 M acetic acid (HOAc) or 0.1 M hydrochloric acid (HCl) was added to the cell pellet. The pellets were resuspended in the acid by gentle pipetting, and a second set of 25 μl cell count samples were removed and preserved in 1 ml filtered seawater with Utermöhl's fixative. (The reasons for taking cell count samples at two different times during this process are given below.) Toxin extraction was completed by sonifying the cell suspensions with a Branson sonic cell disruptor for 20 sec at a setting of 6 A while the samples were in an ice-water bath. The HCl extracts were hydrolyzed by placing the tightly sealed 15 ml tubes in a boiling water bath. Once the temperature of the extract had reached 97°C the samples were heated for an additional 5 min to hydrolyze the *N*-sulfocarbamoyl toxins to their carbamate derivatives.

Four different shellfish samples were collected from the Bay of Plenty during the 1993 outbreak. Samples of *Paphies* sp. (a clam called tuatua), *Perna canaliculus* (mussel), and *Pecten novaezelandiae* (scallop) were collected at three different locations along the western shore of the Bay of Plenty (Table 1). Additional samples of clams known as pipis (*Paphies australis*) were collected approximately 70 km to the east of the other shellfish sampling sites (Table 1, Fig. 1).

Table 1. Dates and sample sites of shellfish collection in the Bay of Plenty, New Zealand

Species	Date	Sample	Water temperature (°C)
Pipi	27 Jan 1993	Ohope Beach (east end)	16
Mussel	9 Feb 1993	Mussel Rock (Mt Maunganui)	17
Tuatua	16 Feb 1993	Papamoa Beach	19
Scallop	2 Mar 1993	Tauranga Harbour	22

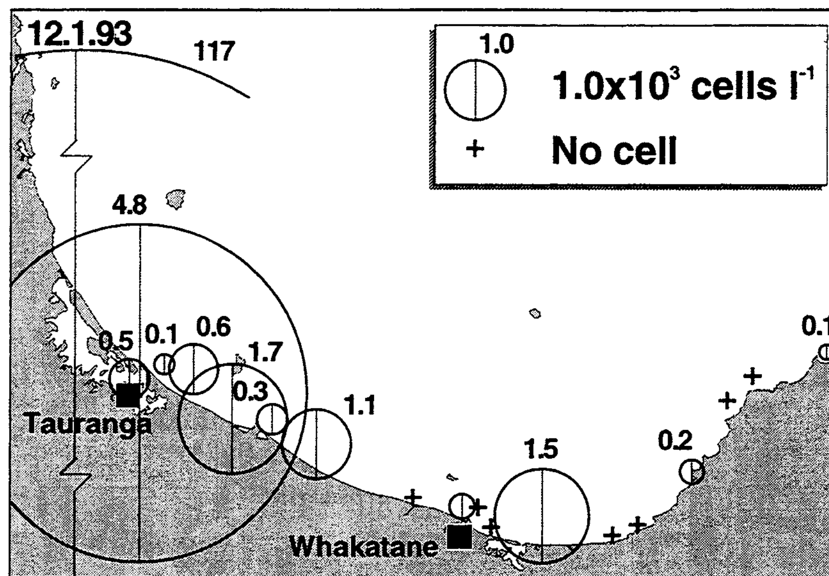

Fig. 2. Surface distribution of *A. minutum* in nearshore waters around the Bay of Plenty on 12 January 1993.
Concentrations are denoted by the size of the circle centered at each sampling station.

Shellfish were processed by blending 100 g of meat from each sample with 100 ml of 0.1 N HCl for 2 min, according to the standard Association of Official Analytical Chemists (AOAC; Hungerford, 1995) method. The mixture was centrifuged and the supernatants were refrigerated and shipped to Woods Hole on ice via an express courier. All of the samples were stored frozen at −20°C prior to analysis by high-performance liquid chromatography (HPLC), at which time the extracts were thawed and centrifuged as described previously. Shellfish extract supernatant was passed through a Millipore, Sep-Pak C18 light cartridge following the manufacturer's recommendations.

Toxin analysis

The purified shellfish extract and the algal extract (100 μl each) were loaded into separate auto-sampler vials, and analyzed for the saxitoxins by HPLC using the three-step isocratic elution method of Oshima *et al.* (1989a), following the modifications of Anderson *et al.* (1990). Toxin composition profiles were determined from analyses of two separate samples and external standard solutions, supplied by Y. Oshima, were run before sample analysis and after every fourth sample. Abbreviations used throughout this text are: STX, saxitoxin; NEO, neosaxitoxin; GTX1,4, gonyautoxins 1 and 4; GTX 2,3, gonyautoxins 2 and 3; GTX5, gonyautoxin 5 (or B1) (Hall, S., Ph.D. Thesis, University of Alsaska, 1982); GTX6, gonyautoxin 6 (or B2); C1,2, toxins C1 and C2; C3,C4, toxins C3 and C4; dc, decarbamoyl. The terms GTX1,4, GTX2,3, C1,C2 and C3,C4 are used to represent the pooled concentrations of two toxins to account for possible epimerization. Toxicities (in STX equivalent $cell^{-1}$) were calculated from the molar composition data using individual potencies (Oshima *et al.*, 1982). The specific toxicities of the individual toxins (μg STX equiv. $\mu mole^{-1}$) were as follows: C1, 3.45; C2, 55.0; C3, 7.6; C4, 32.9; GTX1, 567.6; GTX2, 205.2; GTX3, 364.3; GTX4, 414.7; GTX5, 36.8; dcGTX2, 371.9; dcGTX3, 430.6; NEO, 527.9; dcSTX, 293.0; STX, 571.1.

RESULTS

Bloom details

During the January 1993 toxicity episode in the Bay of Plenty, *A. minutum* was present throughout the entire bay (Fig. 2). On 12 January 1993, the highest concentration (up to 1.17×10^5 cells $liter^{-1}$) was centered around Pio's Ocean Beach, on the western side of the bay. *Scrippsiella trochoidea* (25×10^3 cells $liter^{-1}$) was the most abundant non-toxic

dinoflagellate species recorded, but diatoms (e.g. *Lauderia annulata*, *Pseudonitzschia* and *Nitzschia* spp. and *Leptocylindrus danicus*), as a group, were generally more abundant than dinoflagellates. A small number of *Pseudonitzschia pungens* forma *pungens* found at the time was cultured and tested negative for domoic acid. From mid-January to March 1993, routine shellfish biotoxin monitoring from this area using mouse bioassay showed low to moderate levels of PSP (80–412 μg STX equiv. 100 g^{-1}) in mussels (*P. canaliculus*), tuatuas (*Paphies* spp.), pipis (*P. australis*) and scallops (*P. novaezelandiae*). *Gymnodinium* cf. *breve* was also found in the same area from 27 January onwards, but generally in much lower cell concentrations than *A. minutum* (surface concentration up to 8.8×10^3 cells $liter^{-1}$) (Chang *et al.*, in press).

Taxonomy

Cells of *A. minutum* Halim (Figs 3 and 4) are subspherical and oval in shape, and occur as single cells or rarely in pairs, in both cultures and field samples. Cells of this species are usually quite small, 15–25 μm long and 14–22 μm wide. Both the cingulum and sulcus are deeply excavated [Fig. 3(A)]. The cingulum is displaced from 1 to 1.5 times its width, while the sulcus extends to the antapex. The first apical plate (1′) has a conspicuous, small ventral pore (Vp) on its right anterior margin [Fig. 3(B)], and is often in direct contact with the apical pore complex (Po) [Fig. 3(C)]. The sixth precingular plate (6″) is narrow. The anterior sulcal plate (Sa) is approximately as long as it is wide with a straight or almost straight anterior margin, while the posterior sulcal plate (Sp) is almost always symmetrical [Fig. 3(D)].

Sample processing

With typical centrifugation methods used to collect *A. tamarense* cultures for toxin extraction (e.g. Anderson *et al.*, 1994), it was not possible to concentrate completely the *A. minutum* cells used in this study. In standard plastic centrifuge tubes, *A. minutum* cells adhered to the tube walls, therefore siliconized tubes were used to process the sample. This prevented cell adhesion on the tube walls and thorough recovery of the culture was accomplished. To assure that the toxin content of the cultured cells was properly quantified, cell count samples were taken in two different ways as previously described. Unfortunately, there was a large discrepancy between the cell concentrations determined from the two types of samples. Consistent values were seen in the duplicate samples for each counting method, but values for the raw culture counts taken prior to processing were 2.5 to 3.5 times higher than the corresponding counts of the cell slurry after centrifugation and concentration. This introduced considerable uncertainty into the toxin content calculations.

Toxin composition: cultures

Toxin composition (in mole%) of the two *A. minutum* isolates following toxin extraction with mild acetic acid was generally similar, although isolate AMABOP006 contained half as much STX than AMABOP014, which was correspondingly lower in NEO [Table 1, Fig. 5(A)]. For convenience in comparisons with the shellfish extracts, an average of the two cultures is given in the table, in addition to the individual toxin profile of each isolate. Only six toxin components (GTX1–4, NEO and STX) were detected by HPLC, with NEO clearly dominant (> 65 mole%). Neither C-toxins (C1–4) nor GTX5–6 were detectable

in either isolate. Among the four gonyautoxins detected, GTX1,4 was about as abundant as GTX2,3, at approximately 7% and 6%, respectively.

As expected given the absence of sulfamate toxins, hydrolysis during extraction with hot HCl did not change the relative abundance of the different toxins significantly, although

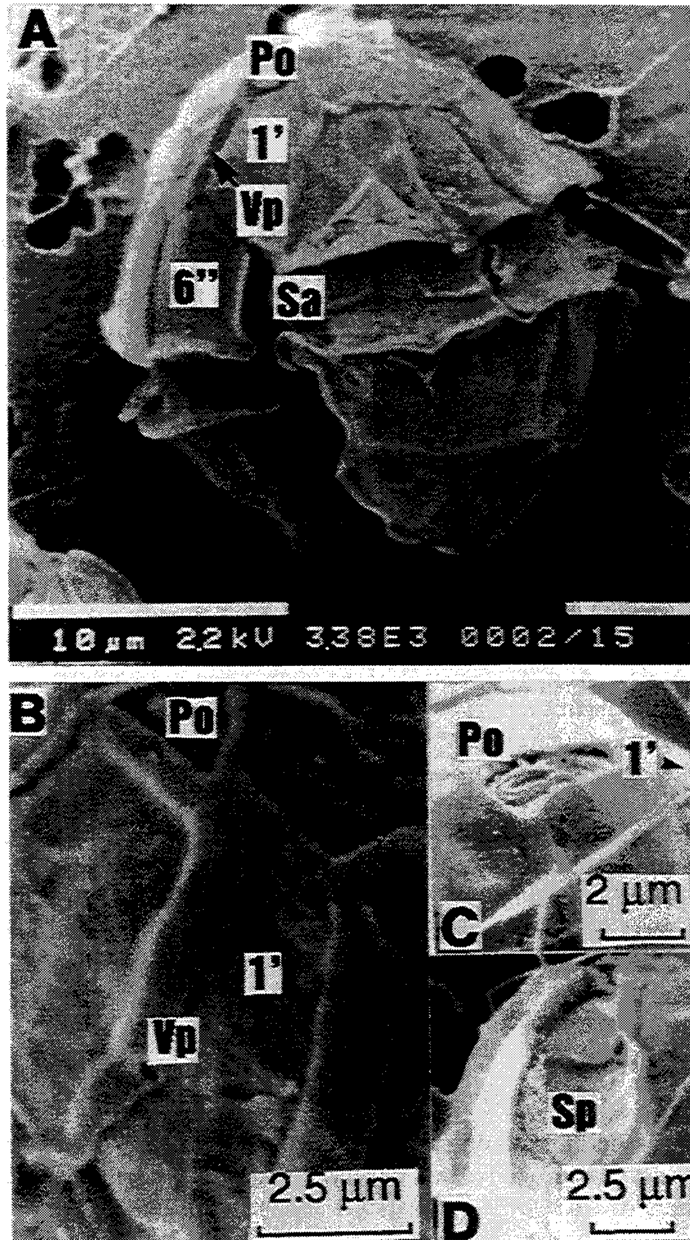

Fig. 3. *Alexandrium minutum* Halim from the Bay of Plenty.
(A) Ventral view, showing the first apical plate (1′), sixth precingular plate (6″), and comma-shaped apical pore (Po). (B) Ventral pore (Vp) on the right anterior margin of the first apical plate (1′). (C) Comma-shaped apical pore (Po). (D) Posterior sulcal plate (Sp).

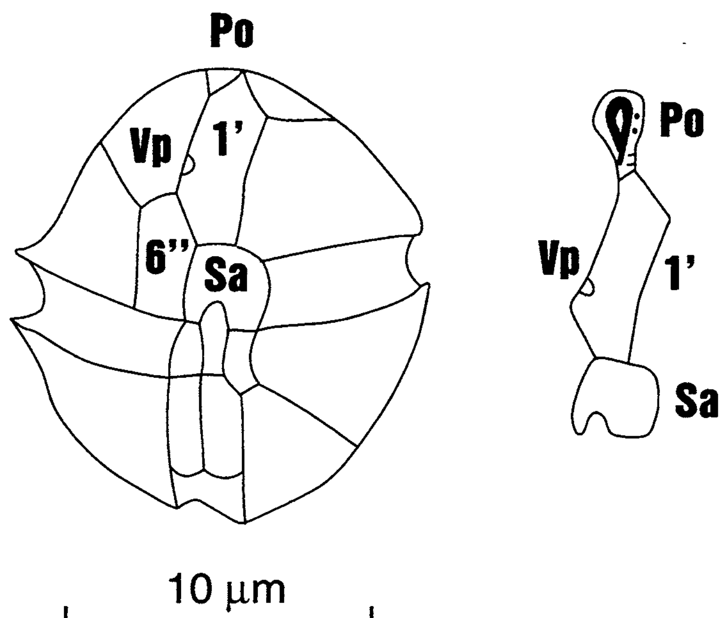

Fig. 4. Diagrammatic summary of ventral view of plate tabulation of *A. minutum* from the Bay of Plenty, New Zealand, including details of the apical pore plate (Po), the first apical plate (1′), the ventral pore (Vp), and the anterior sulcal plate (Sa).

an approximate 20% increase in the number of moles of NEO and thus the total toxin content was observed with both isolates. Trace quantities of dcGTX3 and dcSTX were measured in the acid-hydrolyzed sample of clone AMABOP014, but not when that isolate was extracted with mild acid.

Toxin content: cultures

When the moles of each toxin were converted to STX equivalents to provide a measure of the potency of each strain, isolate AMABOP006 contained 11.6 pg STX equiv. cell^{-1} with acetic acid extraction, increasing about 20% to 13.9 with acid hydrolysis (Table 2). Isolate AMABOP014 also increased from 6.0 to 8.0 pg STX equiv. cell^{-1} with hydrolysis. Both of these increases were due to a rise in the abundance of NEO, but not as a consequence of chemical conversions of saxitoxin derivatives, since all other toxins were unchanged in their molar toxin content. The authors have no explanation for this increase, especially since GTX6, the sulfamate derivative that would hydrolyze to NEO, was not detected in the acetic acid extract by HPLC and only a very small amount was detected by non-quantitative capillary isotachophoresis/ capillary zone electrophoresis/ electrospray mass spectrometry (CITP/CZE/ESMS) (Thiebault, P., personal communication). The presence of high NEO concentrations in both the acetic and hydrochloric acid extracts was also confirmed by CITP/CZE/ESMS. The average toxin contents of the cultures were 8.8 and 11.0 pg STX equiv. cell^{-1} with acetic acid or HCl extraction, respectively.

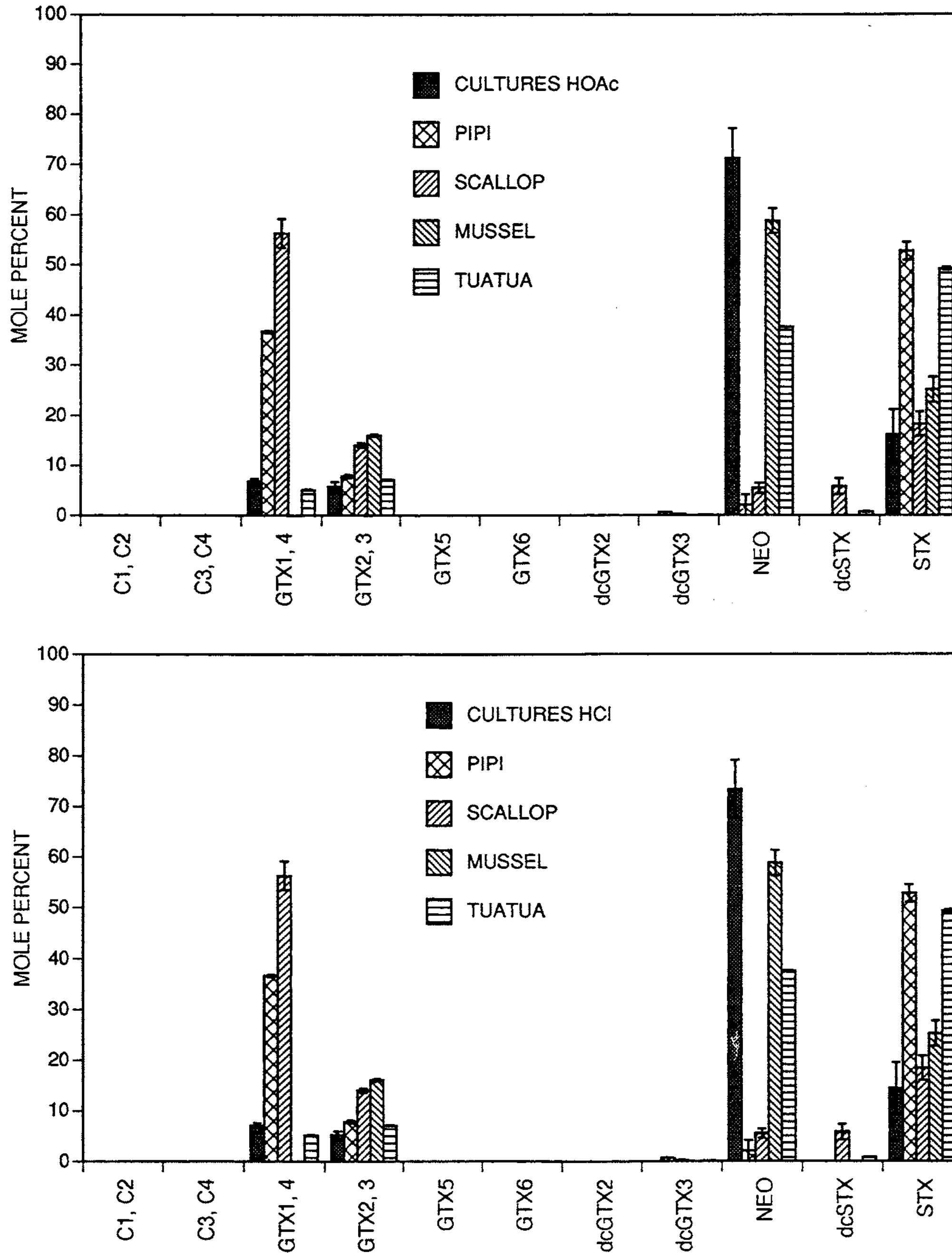

Fig. 5. Comparison of the toxin composition of shellfish and cultures of *A. minutum* from the Bay of Plenty.
(A) Cultures (average of two strains) extracted in mild 0.25 N HOAc (final concentration), compared to scallop, pipi, tuatua and mussel extracts using the AOAC (Hungerford, 1995) method in hot 0.05 N HCl (final concentration). (B) Cultures (average of two strains) extracted in hot 0.05 N HCl (final concentration) to mimic the AOAC (Hungerford, 1995) procedure, compared to scallop, pipi, tuatua and mussel extracts using the same methods. Error bars denote $\pm$ 1 S.D.

Table 2. HPLC analysis of New Zealand *A. minutum* extracts

Sample		GTX1	GTX2	GTX3	GTX4	dcGTX3	NEO	dcSTX	STX	Total
Culture					AMABOPO06, acetic acid extract					
	Average fmole/cell	0.47	0.05	0.83	1.24	UD	17.19	UD	2.49	22.27
	(S.D.)	(0.05)	(0.01)	(0.07)	(0.00)		(0.66)		(0.21)	(0.84)
	Average fgSTX/cell	264.38	10.57	304.10	512.18	UD	9075.10	UD	1423.97	11,590.29
	(S.D.)	(26.38)	(2.47)	(24.01)	(2.00)		(349.80)		(121.30)	(469.00)
	Average mole%	2.09	0.23	3.76	5.55	0.00	77.19	0.00	11.17	100.00
	(S.D.)	(0.13)	(0.06)	(0.44)	(0.23)		(0.07)		(0.53)	
	Average combined epimer pair %*	7.64	4.00			0.00	77.19	0.00	11.17	100.00
	(S.D.)	(0.26)	(0.44)				(0.07)		(0.53)	
Culture					AMABOPO06, hydrochloric acid extract					
	Average fmole/cell	0.40	0.05	0.95	1.70	UD	21.12	UD	2.47	26.69
	(S.D.)	(0.07)	(0.04)	(0.24)	(0.40)		(4.15)		(0.38)	(5.20)
	Average fgSTX/cell	225.28	9.31	347.58	706.73	UD	11,146.51	UD	1411.48	13,846.89
	(S.D.)	(40.41)	(7.55)	(88.97)	(165.58)		(2188.68)		(215.62)	(2691.71)
	Average mole%	1.5	0.2	3.5	6.3	0.00	79.1	0.00	9.3	100.0
	(S.D.)	(0.02)	(0.18)	(0.23)	(0.26)		(0.12)		(0.41)	
	Average combined epimer pair %*	7.8	3.7			0.00	79.1	0.00	9.3	100.0
	(S.D.)	(0.26)	(0.29)				(0.12)		(0.41)	
Culture					AMABOPO14, acetic acid extract					
	Average fmole/cell	0.24	0.12	0.76	0.45	UD	7.58	UD	2.46	11.61
	(S.D.)	(0.05)	(0.01)	(0.08)	(0.03)		(0.50)		(0.12)	(0.80)
	Average fgSTX/cell	135.79	24.26	277.94	186.80	UD	4000.02	UD	1407.50	6032.31
	(S.D.)	(28.25)	(2.85)	(29.89)	(13.70)		(264.43)		(66.86)	(405.97)
	Average mole%	2.04	1.01	6.55	3.88	0.00	65.26	0.00	21.25	100.00
	(S.D.)	(0.29)	(0.05)	(0.26)	(0.02)		(0.16)		(0.45)	
	Average combined epimer pair %*	5.92	7.57			0.00	65.26	0.00	21.25	100.00
	(S.D.)	(0.29)	(0.26)				(0.16)		(0.45)	
Culture					AMABOPO14, hydrochloric acid extract					
	Average fmole/cell	0.23	0.13	0.89	0.73	UD	10.38	UD	2.99	15.35
	(S.D.)	(0.03)	(0.01)	(0.01)	(0.03)		(0.38)		(0.14)	(0.44)
	Average fgSTX/cell	131.62	27.05	322.83	303.20	UD	5478.41	UD	1707.60	7970.72
	(S.D.)	(14.00)	(2.73)	(2.45)	(14.20)		(201.69)		(78.67)	(246.99)
	Average mole%	1.52	0.86	5.78	4.77	0.00	67.60	0.00	19.47	100.00
	(S.D.)	(0.20)	(0.11)	(0.21)	(0.36)		(0.55)		(0.34)	
	Average combined epimer pair %*	6.29	6.64			0.00	67.60	0.00	19.47	100.00
	(S.D.)	(0.41)	(0.24)				(0.55)		(0.34)	
Cultures						Average acetic acid extract				
	Average mole%	2.06	0.62	5.16	4.72	0.00	71.23	0.00	16.21	100.00
	(S.D.)	(0.22)	(0.39)	(1.44)	(0.85)		(5.96)		(5.06)	
	Average combined epimer pair %*	6.78	5.78				71.23	0.00	16.21	100.00
	(S.D.)	(0.88)	(1.49)			0.00	(5.96)		(5.06)	
Cultures						Average hydrochloric acid extract				
	Average mole%	1.50	0.53	4.65	5.55	0.00	73.35	0.00	14.40	100.00
	(S.D.)	(0.15)	(0.36)	(1.15)	(0.84)		(5.76)		(5.08)	
	Average combined epimer pair %*	7.06	5.19			0.00	73.35	0.00	14.40	100.00
	(S.D.)	(0.85)	(1.20)				(5.76)		(5.08)	

UD, Undetectable.
*Epimer pair toxins are: GTX1 + GTX4 and GTX2 + GTX3.

Toxin composition: shellfish

Four shellfish samples (pipi, tuatua, scallop and mussel) were analyzed by HPLC for PSP toxins. The four extracts exhibited two types of toxin profile, one of which was similar to the toxin profile of *A. minutum* cultures. In both the hydrolyzed *A. minutum* culture extracts and the mussel extracts, NEO was the dominant toxin (73% and 59%, respectively, Tables 2 and 3). STX was ranked second (14% for the cultures and 25% for the mussels) in these samples. The tuatua sample also had a high percentage of NEO (38%), but STX was the dominant toxin at 49%. In the cultures and in the mussel and tuatua samples, lower percentages of GTX1,4 and GTX2,3 were observed ($\leq 16\%$ for any epimer pair). A second toxin composition profile was seen in the pipi and scallop samples, which had high concentrations of the GTX1,4 epimer pair (37% and 56%, respectively)

Table 3. HPLC analysis of New Zealand shellfish extracts

Sample		GTX1	GTX2	GTX3	GTX4	dcGTX3	NEO	dcSTX	STX	Total
Pipi	µgSTX eq./100 g shellfish meat	UD	4.72	1.89	54.90	1.03	3.91	UD	108.85	175.31
	(S.D.)		(0.29)	(0.15)	(1.74)	(0.00)	(3.91)		(1.48)	(3.73)
	Average mole%	0.00	6.37	1.44	36.68	0.66	2.05	UD	52.80	100.00
	(S.D.)		(0.52)	(0.14)	(0.43)	(0.01)	(2.01)		(1.77)	
	Average combined epimer pair %*	36.68	7.81			0.66	2.05	UD	52.80	100.00
	(S.D.)	(0.43)	(0.53)			(0.01)	(2.01)		(1.77)	
Scallop	µgSTX eq./100 g shellfish meat	UD	2.87	8.05	60.17	0.17	7.39	4.37	27.01	110.04
	(S.D.)		(0.12)	(0.33)	(10.66)	(0.17)	(0.74)	(0.85)	(1.54)	(7.90)
	Average mole%	0.00	5.43	8.57	56.28	0.16	5.43	5.78	18.35	100.00
	(S.D.)		(0.65)	(0.30)	(5.77)	(0.14)	(0.96)	(1.57)	(2.44)	
	Average combined epimer pair %*	56.28	14.00			0.16	5.43	5.78	18.35	100.00
	(S.D.)	(5.77)	(0.71)			(0.14)	(0.96)	(1.57)	(2.44)	
Mussel	µgSTX eq./100 g shellfish meat	UD	1.93	0.84	UD	UD	22.64	0.00	10.51	35.92
	(S.D.)		(0.08)	(0.11)			(0.48)	(0.00)	(1.71)	(2.38)
	Average mole%	0.00	12.88	3.15	0.00	0.00	58.77	0.00	25.21	100.00
	(S.D.)		(0.26)	(0.21)			(2.48)	(0.00)	(2.53)	
	Average combined epimer pair %*	0.00	16.03		0.00	0.00	58.77	0.00	25.21	100.00
	(S.D.)		(0.34)				(2.48)	(0.00)	(2.53)	
Tuatua	µgSTX eq./100 g shellfish meat	23.44	20.33	10.82	21.52	0.95	361.52	4.07	511.93	954.59
	(S.D.)	(1.08)	(1.33)	(0.04)	(0.12)	(0.17)	(4.38)	(0.56)	(4.11)	(11.55)
	Average mole%	2.27	5.45	1.63	2.85	0.12	37.64	0.76	49.27	100.00
	(S.D.)	(0.07)	(0.28)	(0.02)	(0.06)	(0.02)	(0.08)	(0.09)	(0.31)	
	Average combined epimer pair %*	5.12	7.08			0.12	37.64	0.76	49.27	100.00
	(S.D.)	(0.09)	(0.28)			(0.02)	(0.08)	(0.09)	(0.31)	

UD, Undetectable.
*Epimer pair toxins are: GTX1 + GTX4 and GTX2 + GTX3.

and STX (53% and 18%, respectively), and lower amounts of GTX2,3 and NEO ($\leq$ 14%).

Only the tuatua sample, at 955 μg STX equiv. 100 g^{-1} of shellfish meat, was significantly above the quarantine levels of 80 μg STX equiv. 100 g^{-1}. None of the other shellfish samples was especially toxic, ranging from 175 to a low of 36 μg STX equiv. 100 g^{-1} in the pipi and mussel samples, respectively. Results obtained by HPLC analysis and those obtained by mouse bioassay were generally comparable, although the HPLC results were always higher: pipi 175 vs 38; mussel 36 vs 33; tuatua 955 vs 279; and scallop 110 vs 54 μg STX equiv. 100 g^{-1}.

DISCUSSION

This study clearly established that *A. minutum* from the Bay of Plenty is toxic, and was responsible for at least part of the PSP toxicity measured in commercial shellfish during the 1993 toxic shellfish events. The toxin profile of *A. minutum* closely matches that of mussels and is similar to the tuatua harvested from the Bay during the outbreak, but is quite different from the profile measured in scallops and pipi. Bioconversion pathways for accumulated algal toxins within shellfish can be implicated in these differences, but it appears more likely that either other strains of *A. minutum* or other saxitoxin-producing dinoflagellates were ingested by two of the species of shellfish. The Bay of Plenty *A. minutum* toxin composition is also quite different from that of other isolates of this species from sites throughout the world. This weakens the argument that *A. minutum* was introduced to New Zealand via ballast water or some other long-distance transport mechanism, as there is no identified source population with the corresponding toxin profile. It seems more likely that the species has long been resident in New Zealand coastal waters, but was detected only recently as a result of bioassays associated with a concurrent outbreak of a different type of shellfish toxicity. These and other aspects of this study are discussed in more detail below.

Toxin composition comparisons, shellfish vs cultures

The two Bay of Plenty isolates of *A. minutum* showed very similar toxin composition profiles to each other, characterized by an unusually high proportion of NEO ($>$ 65 mole%), and minor proportions of STX and GTX1–4 (Table 2). The isolates differed by about 55% from each other in toxin content, expressed either as fmoles of total toxins per cell or as STX equiv. per cell. This may reflect real, genetic differences between isolates, slight differences in the stage of growth at harvest, or the accuracy of cell counts, which proved problematic with this species (see below). Both isolates showed an increase in the relative abundance of NEO following hydrolysis, which elevated their toxin content. Ordinarily, this would suggest that the strong acid conditions of the AOAC bioassay are needed to reveal the total potential toxicity of these *A. minutum* isolates (and therefore the shellfish which consume them), but this conclusion can be questioned since there were no sulfamated STX derivatives present which would have been hydrolyzed to their more potent carbamate forms. At this stage, we have no explanation for the increase in NEO following hydrolysis.

Similarities were observed when culture toxin profiles (following acid hydrolysis) were compared to extracts from mussels and to a lesser extent tuatua from the 1993 outbreak [Fig. 5(B)]. There were, however, significant differences with the other two shellfish types

collected from the same embayment (scallops and pipi), which were dominated by GTX1,4 (scallops) and STX (pipi) but contained very little NEO. A series of biochemical pathways has been proposed to explain differences in PSP toxin composition between dinoflagellates and the shellfish that have ingested them (e.g. Lee *et al.*, 1992; Cembella *et al.*, 1993), and Cembella *et al.* (1994) discuss in detail the partitioning in tissues and the transformations that occur specifically within scallops and surfclams. In the latter reference, these authors documented a decrease in NEO and a corresponding increase in STX in *Placopectin magellanicus* that had consumed toxic *Alexandrium* species. A similar conversion was also reported by Fix-Wichmann *et al.* (1981) who examined the digestive gland of the same species of scallop. This reductive loss of the N-1 hydroxy moiety of NEO affected by marine bacteria or natural reductants such as glutathione and cysteine could explain the toxin composition profile of the tuatua, which was lower in NEO and higher in STX, but not of the scallop and pipi, which were low in NEO but high in GTX1,4 and STX [Fig. 5(B)]. In the latter instance, an *N*-sulfotransferase enzyme is needed for the conversion of NEO to GTX1,4. Thus far, such enzymes have been identified in the dinoflagellates *Alexandrium tamarense* and *Gymnodinium catenatum* (Oshima, 1995) but not yet in shellfish. A minor amount of GTX1,4 was detected in our *A. minutum* cultures, so sulfotransferases are likely to be present in this species as well. As discussed by Cembella *et al.* (1993), toxin conversion through the action of these enzymes may be significant during the early stages of digestion in shellfish viscera while the dinoflagellate cells remain intact and metabolically active. It is thus possible to link the different patterns of toxicity seen in all of the shellfish to the *A. minutum* cultures we have characterized, but the linkage to the scallops and pipi is the weakest.

It should be noted that the *A. minutum* 'bloom' peaked on 12 January 1993 (Chang *et al.*, in press) and all of the shellfish samples were collected during the decline of the bloom. Pipi samples, collected 15 days after the peak of the bloom, did not have a toxin profile resembling that of the two *A. minutum* isolates. This probably reflects the fact that they were collected from Ohope Beach which is far removed from the area where the *A. minutum* cells were isolated (Fig. 1). In contrast, mussel and tuatua were collected near the origin of the cultures, on 9 and 16 February, respectively. Even though this is approximately 1 month after the height of the bloom, the toxin profiles of these samples corresponded well with the profiles of the *A. minutum* cultures that were tested.

Scallop samples, like the mussel and tuatua, were also collected relative close to the origin of the cultures, but almost 2 months after the bloom peak. The toxin profiles of the scallops were not well matched to the toxin profile of the cultures. The same is true of tuatua and mussel samples collected from the Bay of Plenty in early 1993 by Hannah *et al.* (1993). In addition to not having a toxin profile similar to our cultures, these samples were different from one another and from all of the shellfish samples assayed in our study. This supports the view that there may be other saxitoxin-producing organisms in the Bay of Plenty area, either different species or different strains of the same species, each with unique toxin profiles. This possibility is supported by the presence of low concentrations of *A. tamarense* found in the Bay of Plenty in 1993 (Chang, 1995). The Hannah *et al.* (1993) tuatua were dominated by STX (36 mole%), with GTX2,3 (26%) and GTX1,4 (24%), whereas their mussel sample contained predominantly GTX2,3 (76%). Note also that the tuatua and mussel samples in Hannah's study contained 326.4 and 27.7 μgSTX equiv. 100 g^{-1} shellfish meat, respectively, whereas the tuatua and mussel samples in this study contained 954.6 and 35.9 μgSTX equiv. 100 g^{-1} shellfish meat, respectively. Mussels are

known to accumulate and depurate toxins quickly and it is possible that the tuatua clam does not.

Comparisons with other A. minutum *strains*

There are several ways in which to compare *A. minutum* isolates from different parts of the world. Looking first to toxin content (Table 4), there is considerable variability among isolates, with strains from Taiwan being the most toxic (80–184 fmoles cell^{-1}, or 20.6–47.0 pg STX equiv. cell^{-1}), and those from Portugal and Australia being less potent (0.37 and 0.8 pg STX cell^{-1}, respectively). Values in Table 4, however, should be used cautiously, as several were calculated from plots rather than tables and the values may not represent the same stage of batch culture growth for all isolates. Nevertheless, it is apparent that the Bay of Plenty isolates examined in the present study are at the high end of the toxicity range for *A. minutum*.

Compared to other *Alexandrium* species, Bay of Plenty *A. minutum* can be considered quite toxic on a cell volume basis. For example, Cembella *et al.* (1988) report values of 20–32 pg STX equiv. cell^{-1} for the most potent isolates of the *A. tamarense* group found along the north-eastern coasts of the United States and Canada, a range approximately double that for the Bay of Plenty *A. minutum* (Tables 2 and 3). *Alexandrium tamarense* has a cell volume that is two to three times larger than *A. minutum*, so toxicity per unit volume is comparable.

In this study, the toxin content of *A. minutum* cultures determined from cells enumerated either before or after centrifugation proved to be highly variable. When cells were counted before and after centrifugation and extraction with mild acid, the counts differed by more than 2–3-fold, the pelleted cells being less abundant. The reasons for this discrepancy are unclear, as siliconized centrifuge tubes were used, and the walls of those tubes were devoid of cell accumulations, as was the supernatant above the cell pellet. Cell lysis probably occurred during the centrifugation process or perhaps after the acid was added to the cell pellet. If cells did lyse in the acid, then toxin would be retained and measured, but the cell counts from that pellet would be too low, giving an abnormally high toxin content. In that circumstance, the precentrifugation cell counts would be more appropriate for determining toxin per cell values. If, however, some cells lysed during centrifugation, then released toxin was probably aspirated away with the cell pellet supernatant. The pellet cell counts would then be most appropriate for toxin content calculations. There is, unfortunately, no way of knowing with certainty which of these approaches is correct. However, if cell pellet counts are used to calculate the toxicity of our isolates, the resulting levels are anomalously high (e.g. 42–68 fmoles cell^{-1}) compared to most other *A. minutum* isolates in Table 4. These levels are also four to seven times higher in toxicity than the most toxic *A. tamarense* isolates (Cembella *et al.*, 1988) when compared on a toxin per unit cell volume basis. Furthermore, the cultures used in our study were isolated from a bloom in which *A. minutum* cell concentrations were high but which caused only mild to moderate toxicity in shellfish. For these reasons, the decision was made to use the cell counts prior to centrifugation in the calculations of toxin content. Given this apparent propensity for *A. minutum* cells to lyse during processing (which has not been reported previously for other *Alexandrium* species), it seems worthwhile to re-examine the methods used to count cells and measure their toxicity in the other studies summarized in Table 4. This may help to explain the more than two orders of magnitude variability in toxin content observed among isolates. It is possible that harvesting the cultures by filtration (Franco and

Table 4. Comparison of *A. minutum* toxicity

Location (culture)	Toxin content (fmole/cell)	Potency (fgSTX/cell)	Dominant toxins (mole%) C1,C2	GTX1,4	GTX2,3	NEO	STX	Reference
New Zealand, Bay of Plenty (AMABOP006)	22.27	11,590	0.0	7.6	4.0	77.2	11.2	This paper
New Zealand, Bay of Plenty (AMABOP014)	11.61	6032	0.0	5.9	7.6	65.3	21.3	This paper
New Zealand, Marlborough Sounds (Anakoha A)	7.09	2360	0.0	23.0	36.0	13.0	28.0	Mackenzie, L. (personal communication)
New Zealand, Marlborough Sounds (Anakoha B)	4.86	1850	0.0	25.0	25.0	19.0	31.0	
New Zealand, Tasman Bay (Croisilles A)	5.15	2280	0.0	51.0	9.0	22.0	18.0	
New Zealand, Whangarei (NEPCC508)	2.76	880	0.0	96.4	2.9	0.0	0.7	Cembella *et al.* (1987)
Australia, Port River (AMAD 06)	2.54	1000	0.0	73.5	26.5	0.0	0.0	Hallegraeff *et al.* (1991)
Australia, Port River (AMAD 15)	2.57	1000	0.0	82.9	17.1	0.0	0.0	
Taiwan (AmTK1)	184.40	47,000	0.0	94.6	5.4	0.0	0.0	Chou, H. (personal communication)
Taiwan (AmTK2)	79.50	20,600	0.0	99.6	0.4	0.0	0.0	
Portugal, Laguna Obidos (NEPCC253)	1.16	370	0.0	97.4	2.6	0.0	0.0	Cembella *et al.* (1987)
Spain, Ria de Vigo (AL-1V)	18.00	6413	0.0	98.0	2.0	0.0	0.0	Franco *et al.* (1994)
Spain, Ria de Vigo (AL-2V)	2.00	715	0.0	98.0	2.0	0.0	0.0	
Spain, Ria de Vigo (AL-3V)	4.00	1383	0.0	94.0	6.0	0.0	0.0	
France, Morlay Bay (AM89BM)	2.34	386	44.0	0.0	56.0	0.0	0.0	Ledoux, M. (personal communication)

Fernàndez, 1993) might be a reasonable alternative to centrifugation, but the concerns of cell lysis are still valid.

Another way to compare *A. minutum* isolates is through their toxin composition profiles, as has been done, for example, for other toxic *Alexandrium* species (Cembella *et al.*, 1987; Anderson *et al.*, 1994). A striking difference emerges from this analysis, as the Bay of Plenty *A. minutum* do not resemble any other isolate characterized to date (Oshima *et al.*, 1989b; Sournia *et al.*, 1991; Hallegraeff *et al.*, 1991; Ledoux *et al.*, 1993; Franco *et al.*, 1994) (Table 4). Strains from Taiwan, Australia, Portugal and Spain all contain mostly GTX1,4 toxins, as does a strain which has been described as *Alexandrium angustitabulatum* Taylor and Cassie (Balech, 1995) isolated from a bloom in Whangarei, New Zealand, in 1983. An isolate from Morlay Bay, France, contained only GTX2,3 and C1,2. The most similar profiles are those of several *A. minutum* from Marlborough Sounds and Tasman Bay, on the northern tip of South Island, New Zealand, which did contain some NEO (18%), but had comparable amounts of GTX1,4 and GTX2,3 and STX.

These results are not surprising, as toxin composition is known to vary widely among different *Alexandrium* isolates, even if they conform to the same morphotype (Oshima *et al.*, 1982; Cembella *et al.*, 1987; Noguchi *et al.*, 1990; Anderson *et al.*, 1994). There is thus no doubt that the Bay of Plenty *A. minutum* are genetically distinct from all other isolates of that species that have been analyzed to date. They are also quite different from strains of the same species or of a closely related species from New Zealand. Based on these findings, it is difficult to argue that *A. minutum* was recently introduced to Bay of Plenty waters through human activities or natural currents, as has been proposed for other toxic dinoflagellates (e.g. Hallegraeff and Bolch, 1992). It is always possible that the putative 'source' population exists elsewhere but has not yet been identified and characterized with respect to its toxins, but it seems more probable that *A. minutum* has been endemic to the Bay of Plenty for many years, perhaps causing low but undetected levels of toxicity in shellfish. No PSP monitoring was conducted in the area prior to the 1993 outbreak. The discovery of *A. minutum* in 1993 would then have been a fortuitous result of the investigations surrounding a different but concurrent shellfish toxicity episode linked to brevetoxin-like compounds from another dinoflagellate (Chang, 1995; Chang *et al.*, 1995; Ishida *et al.*, 1994, 1995; Morohashi *et al.*, 1995). Now that the potential for PSP toxicity has been verified, vigilant monitoring is required to protect both the health of the public and the shellfish industry. More work is also needed to reconcile the differences between some of the shellfish extracts and the cultures, and perhaps to identify other saxitoxin-producing strains or organisms in the region.

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