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Cell Bioassay for Paralytic Shellfish Poisoning (PSP): Comparison with Postcolumn Derivatization Liquid Chromatographic Analysis and Application to the Monitoring of PSP in Shellfish

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We performed a neuroblastoma cell (Neuro2a) culture assay modified slightly from a method reported previously to provide a simple and sensitive evaluation of paralytic shellfish poisoning (PSP) toxicity in shellfish. The cell bioassay was just as sensitive for C-toxins as for gonyautoxins. The sensitivity of our cell bioassay was 4 times that of the current standard mouse bioassay. Using the cell bioassay, we evaluated PSP toxicity in 361 shellfish samples collected from Mikawa Bay and Ise Bay, Aichi Prefecture, Japan, from April 1999−March 2002. The results were compared with those obtained in a postcolumn derivatization liquid chromatographic analysis. PSP toxins were detected in 236/361 samples by both assays, and there was a fairly good correlation ($r = 0.9001$, $n = 236$, $p < 0.001$) between the results from the two assays. We applied this cell bioassay when short-necked clams in the bay turned poisonous in 2001. The chronological changes in PSP toxicity in the short-necked clams were analyzed and compared with those of the cell density of poisonous plankton (*Alexandrium tamarense*) occurring in the bay. The PSP toxicity in shellfish peaked 2 weeks after the cell density reached a maximum. We recommend using the cell bioassay for routine monitoring of PSP toxicity in shellfish living in natural marine environments.

KEYWORDS: Cell bioassay; paralytic shellfish poisoning; monitoring; LC analysis

INTRODUCTION

Saxitoxins and related analogues (*1*), such as those associated with paralytic shellfish poisoning (PSP), are known to inhibit nerve−muscle transmission by blocking the sodium channels in the excitable membrane and cause a lethal toxicity through breathing muscle paralysis (*2*). PSP toxins are a group of carbamate alkaloid neurotoxins, which are either non-sulfated (saxitoxins; STX), singly sulfated (gonyautoxins; GTX), or doubly sulfated (*N*-sulfocarbamoyl-11-hydroxysulfate toxins; C-toxins). These PSP toxins are originally produced by dinoflagellates such as *Alexandrium tamarense* and *Alexandrium catenella* but accumulate in shellfish through ingestion of the dinoflagellates during blooms.

An increase in the PSP toxicity in the short-necked clam (*Tapes philippinarum*) in Mikawa Bay, Aichi Prefecture, Japan, was observed in 1991, approximately 1 week after the density of *A. tamarense* reached a maximum (*3*). The major PSP toxins in short-necked clams in the bay were C-toxins (*3*). In 2001, an elevated PSP toxicity was again observed in the short-necked clam as will be described in the present study. The monitoring of PSP toxicity in shellfish is therefore widely acknowledged as important for public health.

The mouse bioassay has been mainly used to evaluate PSP toxicity and has been adopted as the standard in many countries (*4*). Recently, the pressure to limit the use of animals is increasing, and the restriction of the use of experimental animals requires the development of alternative methods that are not dependent upon animal toxicity. The liquid chromatographic (LC) analysis is a sensitive and reliable method, because it can detect individual PSP toxins but requires prolonged analytical periods of 3 days and suffers from a global shortage of analytical standards for the individual toxins found in the STX family.

A number of cell-based assays have been developed for the detection of PSP toxins with sodium-channel-blocking activity (*5*−*8*). Their most outstanding characteristics are that they do not use animals and can evaluate the total PSP toxicity with a single set of assays. The mechanism whereby cell toxicity correlates to sodium channel blocking in nerve cells is that PSP toxins function as antagonist of voltage-gated sodium channel active toxins *in vitro* (*2*). In the present study, we evaluated the PSP toxicity in 361 shellfish samples collected from Mikawa Bay and Ise Bay, Aichi Prefecture, Japan, using the cell bioassay with a slight modification of the method reported previously. Our approach incorporated a colorimetric method based on the ability of metabolically active cells to reduce a tetrazolium compound, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt,

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DOJINDO, Kumamoto, Japan) to a dark yellow water-soluble formazan product (*9*). WST-8 is much more sensitive than other water-soluble salts (XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide; MTS, 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), and the assay procedure is simple, unlike MTT, which requires formazan to be dissolved. The results of our cell bioassay were compared with those obtained by LC analysis. A total of 10 PSP toxins (STX, neo-STX, GTX1,2,3,4, decarbamoyl GTX2,3, C1, and C2) were analyzed by LC, and they are the most important PSP toxins in our country. We also studied the feasibility of applying this cell bioassay to PSP-monitoring programs in shellfish living in natural marine environments.

MATERIALS AND METHODS

Test Samples. A Total of 361 shellfish samples: short-necked clams (*Tapes philippinarum*), 352; purple Washington clams (*Saxidomus purpuratus*), 3; oysters (*Crassostrea gigas*), 2; cockles (*Fulvia mutica*),1; ark shells (*Scapharca broughtonii*), 1; round clams (*Mactra chinensis*), 1; and Japanese geoduck (*Panopea japonica*), 1 were collected from Mikawa Bay and Ise Bay between April 1999 and March 2002.

PSP Standards. The gonyautoxin mixture (GTX I−IV) (178 mouse units (MU)/mL = 35.6 μg saxitoxin /mL, Wako Pure Chemical, Osaka, Japan) was diluted for cell bioassays to five different concentrations: 35.6, 7.1, 1.4, 0.28, 0.056 MU/mL, with 50 mM CH_3COOH. A total of 10 purified PSP toxins (C-toxins, C1 and C2; gonyautoxin (GTX) group, GTX1, 2, 3, and 4; decarbamoylGTX2 and 3; and saxitoxin (STX) group, STX and neo-STX) were provided by the Japan Food Research Laboratories (Tokyo, Japan).

Extraction and Refinement. Shellfish samples were taken out of the shell, and all of the tissue was used for analysis following extraction according to the method used in the standard mouse bioassay (*4*). Aqueous extracts as diluted 2-fold with 0.1 N HCl were stored at −20 °C. For the cell bioassay, the stored extracts were thawed and filtered with a membrane filter having a pore size of 0.22 μm (Millipore Co., Milford, MA). If necessary, the refined samples were diluted to 1:5 and 1:25 with 50 mM CH_3COOH. For the LC analysis, the same thawed extracts were passed through a column of Sep-Pak C-18 (Waters Co., Milford, MA) and filtered with an ultrafiltration kit, Ultra-free C3-LGC (Millipore Co.) (*10*).

Neuroblastoma Cell (Neuro2a) Culture and Bioassay. The cell culture and bioassay were performed according to the method of Manger et al. (*7*) with the modifications noted below. Briefly, mouse neuroblastoma cells (Neuro2a, ATCC CCL-131; Dainihonseiyaku, Osaka, Japan) were grown in RPMI 1640 (Invitrogen Corp., Carlsbad, CA) medium supplemented with 5% fetal bovine serum, 10% horse serum, 50 units/mL penicillin G, and 50 μg/mL streptomycin sulfate (Invitrogen Corp.). For the bioassay, each well of a 96-well microplate (Invitrogen Corp.) was inoculated with 200 μL of a suspension of 10^5-10^6 cells/mL in the above growth medium. After the incubation of tissue culture dishes for 24 h at 37 °C with 5% CO_2, the medium was removed and 5 μL of refined sample extracts or standard PSP toxins together with 5 μL each of 10 mM ouabain (Sigma Chemical Co., St. Louis, MO) and 1 mM veratridine (Sigma Chemical Co.) and 85 μL of growth medium were added to the test wells for 16 h of incubation. Each plate contained 12 samples together with the standards at 6 different concentrations (35.6, 7.1, 1.4, 0.28, 0.056, and 0 MU/mL). Samples and standards were assayed in replicates of 3 or 4. The outer ring of wells in each plate received 200 μL of growth medium. After incubation, the medium was removed and replaced with 90 μL of RPMI without supplements and 10 μL of WST-8 reagent. After another incubation of ca. 3 h, the viability of the cells in each well was assessed at 450 nm with a reference at 600 nm using an automated multiwell-scanning spectrophotometer (MPR-A4i, TOSOH, Tokyo, Japan).

Postcolumn Derivatization Liquid Chromatography. All of the shellfish samples refined as described above were applied according to the method of Oshima et al. (*10*). LC was performed using ion-pair chromatography and postcolumn derivatization analyses. The system was comprised of a pump (LC10ATvp, Shimadzu, Kyoto, Japan) with a sample auto-injector (SIL-10ADvp, Shimadzu), a reversed-phase column (InertsilC8-5, 4.6 × 150 mm, GL Science, Tokyo, Japan), a reagent pump (LC100, Yokogawa, Tokyo, Japan), a water bath (Thermominder EX, Taitec, Saitama, Japan), a fluorescence detector (RF-10AXL, Shimadzu), and a recorder (C-R6A, Shimadzu). Aliquots of each sample were subjected to analyses of the C-toxins, the GTX group, and the STX group. Mobile phases containing 2 mM tetrabutylammonium in acetate buffer at pH 6.0 for the analysis of the C-toxins, 2 mM sodium 1-heptanesulfonate in 10 mM ammonium phosphate buffer at pH 7.1 for the GTX group, and 2 mM sodium 1-heptanesulfonate in 30 mM ammonium phosphate buffer at pH 7.1, acetonitrile (100 + 5), for the STX group were used. For the calculation of the toxicity from the LC chromatograms, the following values of specific toxicity (mouse unit; MU/μmol) were used: GTX1(2468), GTX2(892), GTX3(1584), GTX4(1803), dcGTX2(1617), dcGTX3(1872), C1(15), C2(239), STX(2483), and neo-STX(2295).

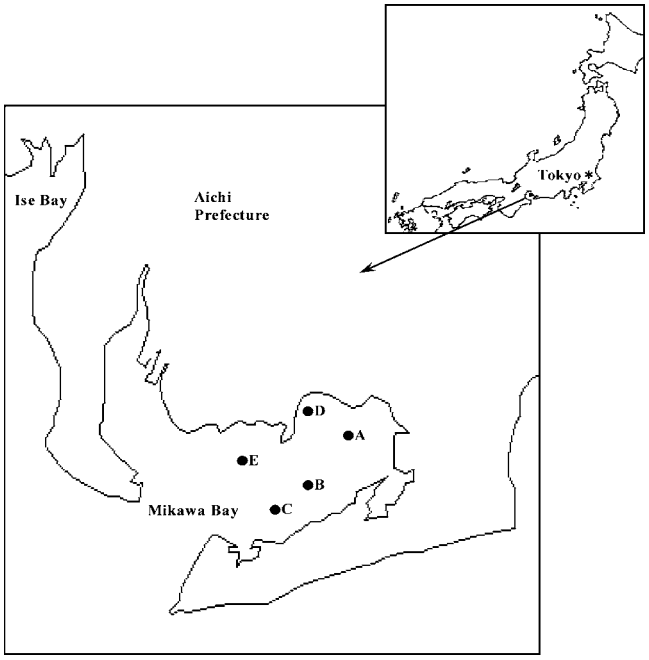

Figure 1. Representative areas where dinoflagellates and shellfish samples were collected in Mikawa Bay during January–March, 2001. (A–E) Area where *A. tamarense* was collected. (D and E) Area where shellfish samples were collected.

Count of Cell Density (Cells/mL) of *A. tamarense*. About 250 mL of seawater was collected in a plastic bottle using a cloth bucket from the collection areas in Mikawa Bay (**Figure 1**) between January 31 and March 27, 2001. The collected sample was gently mixed, and then l mL was placed on the cell-count board (number 5608-C, Rigou, Tokyo, Japan) with a boundary line using an FINN-pipet (Labsystems, Helsinki, Finland). The total cell number of *A. tamarense* was counted using an optical microscope. In the same way, triplicate samples were measured, and the cell density (cells/mL) of *A. tamarense* was calculated as the mean.

RESULTS AND DISCUSSION

We first examined the sensitivity of the cell bioassay for a mixture of C-toxins (C1 and C2), major toxins in shellfish samples in Mikawa Bay, and a mixture of gonyautoxins (GTX 1, 2, 3, and 4). As shown in **Figure 2**, the cell bioassay is just as sensitive to C-toxins as to gonyautoxins when the dose is adjusted to MU/mL. Both curves show a dose-dependent increase in neuroblastoma cell viability and are linear between 0.28 and 7.1 MU/mL. The detection limit for both groups of toxins is estimated to be 0.28 MU/mL (equivalent to 0.56 MU/g

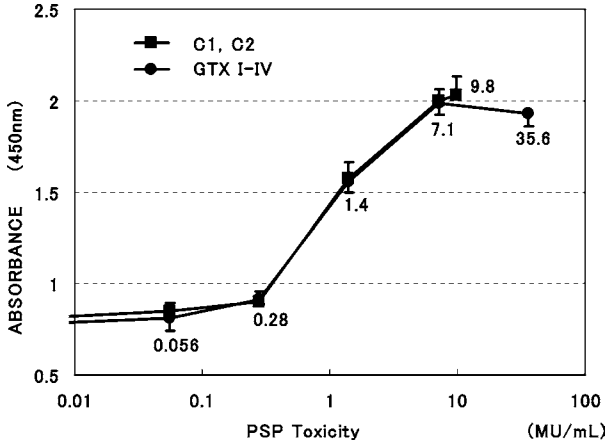

Figure 2. Dose–response curves for purified C-toxins (C1 and C2) and gonyautoxin mixture (GTX I–IV) in the cell bioassay. Both curves are shown to cause a dose-dependent increase in the viability of neuroblastoma cells and be linear between 0.28 and 7.1 MU/mL. Values represent the mean of four replicates. The error bars indicate SD.

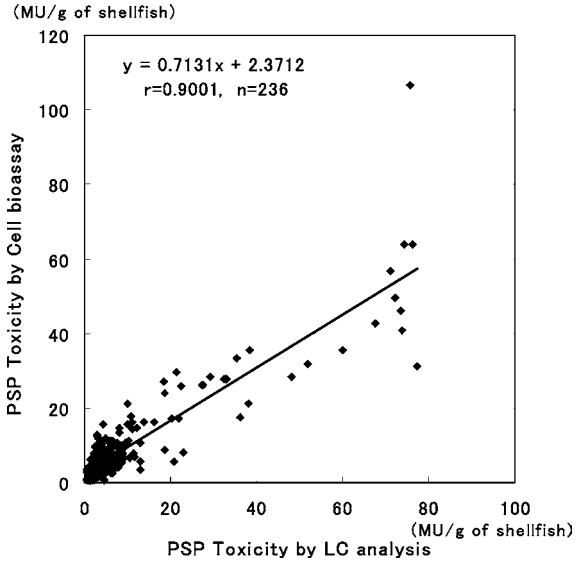

Figure 3. Correlation between the 236 results from the cell bioassay and LC analysis for the shellfish extracts in which PSP toxicity was beyond 0.56 MU/g of shellfish in both assays.

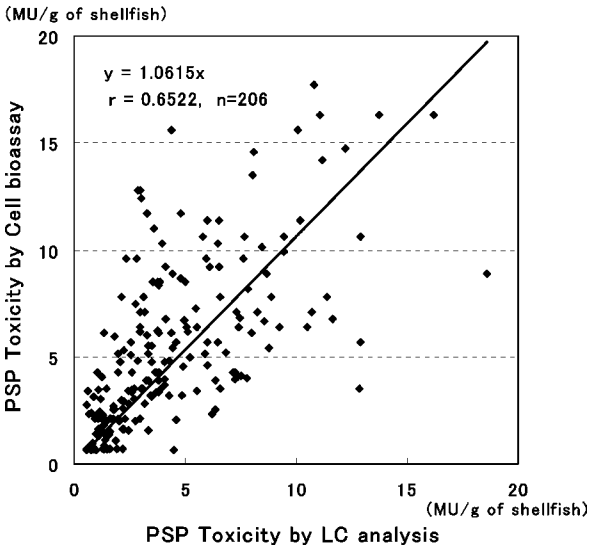

Figure 4. Correlation between the 206 results from the cell bioassay and LC analysis for the shellfish extracts in which PSP toxicity was between 0.56 and 20 MU/g of shellfish in both assays.

of shellfish). The detection limit of the cell bioassay is 4 times lower than that of the current standard mouse bioassay (1.75 MU/g of shellfish, equivalent to ca. 35 μg of saxitoxin/100 g of shellfish). Because the cell bioassay shows the same sensitivity in measuring C-toxins and gonyautoxins and the only commercially available PSP toxins is the gonyautoxin mixture (GTX I−IV) containing gonyautoxin 1, 2, 3, and 4, we used the gonyautoxin mixture (GTX I−IV) as a standard material in the subsequent experiments.

The PSP toxicity in the 361 shellfish samples collected from Mikawa Bay and Ise Bay in 1999−2002 was evaluated using this cell bioassay and LC. From the cell bioassay, 240 samples showed PSP toxicity above the detection limit (0.56 MU/g of shellfish) and 121 samples showed PSP toxicity below it. Among these 240 samples, 236 were also shown to have PSP toxicity beyond 0.56 MU/g of shellfish by the LC analysis. With regard to these 236 samples, the coefficient of the correlation between the results from the cell bioassay and LC analysis ($r = 0.9001$) was highly significant ($p < 0.001$). The cell bioassay gave a slightly lower value than the LC analysis, as shown by the slope of the regression line (0.7131) (**Figure 3**). This tendency was marked at the higher PSP toxicity level, that beyond 20 MU/g of shellfish by LC analysis. This discrepancy remains to be elucidated. On the other hand, for the results of the 206/236 (87.3%) extracts with less than 20 MU/g of shellfish by both assays, the slope of the regression line forced through the origin was close to 1 (1.0615) (**Figure 4**). Even though the slope is close to 1, the spread in the data is large. This might be the effect of a differing measurement system. The significance in application of the cell bioassay is that it can evaluate the total PSP toxicity in shellfish samples with a single set of assays, and the bioassay is a simple, fast, and economical method compared to the LC analysis.

Table 1 shows representative data from an LC analysis on the components of the PSP toxins in the several kinds of shellfish collected from Mikawa Bay in 2001. The short-necked clam samples ($n = 10$) contained 88.4 mol % C-toxins (C1 and C2). Other shellfish such as oysters, ark shells, cockles, and Japanese geoduck also contained a high proportion of C-toxins (86.5−95.9 mol %). On the other hand, purple Washington and round clams contained less C-toxins (0−40.4 mol %) and more toxins of the GTX group, especially decarbamoyl-GTX2 and decarbamoyl-GTX3 (56.1−100 mol %). These results indicate that the major PSP toxins in the shellfish tested were C-toxins and that our cell bioassay is effective for monitoring the PSP toxicity when C-toxins are the primary agent.

Figure 5 illustrates the chronological changes of the PSP toxicity in the short-necked clams and the cell density of *A. tamarense* at D in Mikawa Bay (**Figure 1**) when an elevated PSP toxicity in the shellfish in the bay was observed during January−March, 2001. The PSP toxicity in the shellfish peaked at 35.5 MU/g of shellfish on February 28, 2 weeks after the cell density reached a maximum (138 cells/mL) on February 13, and had decreased to less than the regulatory limit in Japan (4 MU/g of shellfish, equivalent to 80 μg of saxitoxin/100 g of shellfish) on March 17, about 3 weeks after the cell density decreased to less than 10 cells/mL on February 23. It has been reported that the period between the occurrence of poisonous plankton and the appearance of PSP toxicity in shellfish is influenced by various factors such as the amount of poisonous plankton, the seawater temperature, and the kind and size of

Table 1. Components of the PSP Toxins in Several Kinds of Shellfish Collected from Mikawa Bay in 2001

		short-necked clam (10)[a]		oyster (2)		ark shell (1)		cockle (1)	
group	PSP toxin	(μmol/L)	(mol %)	(μmol/L)	(mol %)	(μmol/L)	(mol %)	(μmol/L)	(mol %)
C	C1	18.11	88.4[b]	5.16	86.5	0.11	92.6	0.27	92.3
	C2	31.19		5.71		0.11		0.48	
STX	neoSTX	0.31	0.9[c]	0.05	1.9	nd[e]	0	nd	0
	STX	0.19		0.19		nd		nd	
	GTX4	0.17		0.05		nd		nd	
	GTX1	nd		nd		nd		nd	
GTX	dcGTX3	0.70	10.7[d]	nd	11.6	nd	7.4	nd	7.7
	dcGTX2	0.26		0.07		nd		nd	
	GTX3	3.41		0.80		0.02		0.06	
	GTX2	1.41		0.53		nd		nd	
	total	35.10	100	12.57	100	0.24	100	0.82	100

		Japanese geoduck (1)		purple Washington clam (3)		round clam (1)	
group	PSP toxin	(μmol/L)	(mol %)	(μmol/L)	(mol %)	(μmol/L)	(mol %)
C	C1	0.53	95.9	2.36	40.4	nd	0
	C2	0.60		1.76		nd	
STX	neoSTX	nd	0	0.25	3.6	nd	0
	STX	nd		0.11		nd	
	GTX4	nd		0.02		nd	
	GTX1	nd		nd		nd	
GTX	dcGTX3	nd	4.1	3.06	56.1	0.11	100
	dcGTX2	nd		2.03		0.02	
	GTX3	0.02		0.38		nd	
	GTX2	0.03		0.22		nd	
	total	1.18	100	10.19	100	0.13	100

[a] The number of test samples. [b,c,d] The total proportion on a molar basis of each PSP group in the shellfish samples. [e] Not detected.

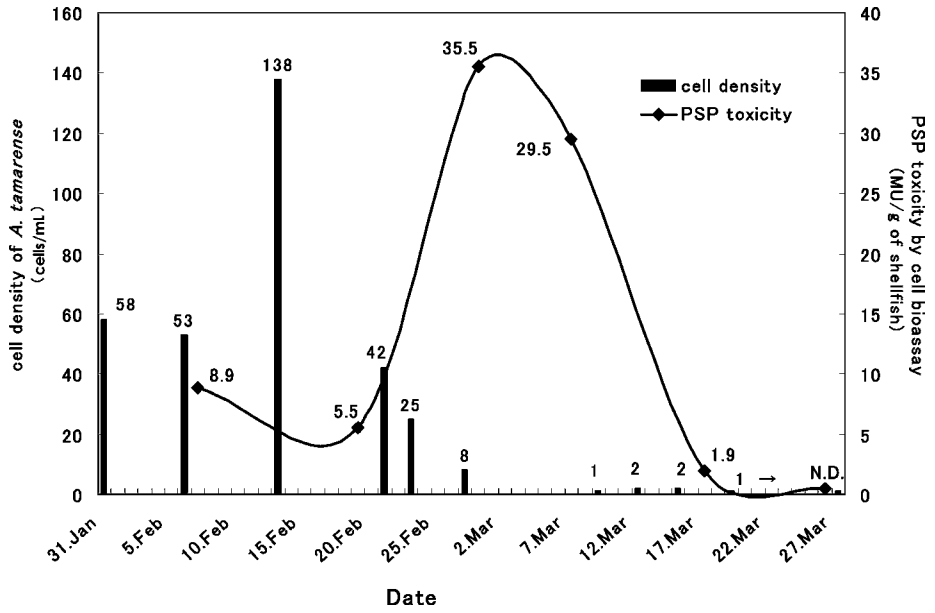

Figure 5. Chronological changes of the PSP toxicity in the short-necked clams and the cell density of *A. tamarense* at D in Mikawa Bay (**Figure 1**) during January–March, 2001.

the shellfish (*15*, *16*). The monitoring of PSP toxicity in shellfish using the cell bioassay should therefore be carried out in the early stages of plankton blooms.

One of the advantages of the cell bioassay is that it can analyze 12 samples at a time, and it takes only 1 day to complete the entire procedure. On the other hand, the LC analysis takes 3 days because it requires three different mobile phases for the C-toxins, the GTX group, and the STX group, respectively. Another advantage of the cell bioassay is that it can be used for the monitoring of PSP toxicity at levels lower than the detection limit of the current standard mouse bioassay. The mouse bioassay standardized by the Association of Official Analytical Chemists is commonly used to quantitatively determine the PSP toxicities of shellfish in many countries (*4*). We believe that the cell bioassay will serve as a complementary or alternative procedure to conventional animal testing. We recommend using the cell bioassay for routine monitoring of PSP toxicity in shellfish living in natural marine environments.

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