FRDC FINAL REPORT

Application of an in-vitro tissue culture assay for the determination of paralytic shellfish poison (Saxitoxin family) and comparison to the HPLC assay and the standard mouse bioassay.

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96/~~123~~ 323 **Application of an in-vitro tissue culture assay for the determination of paralytic shellfish poison (Saxitoxin family) and comparison to the HPLC assay and the standard mouse bioassay.**

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1. NON-TECHNICAL SUMMARY:

Paralytic shellfish poisoning (PSP) is a condition that can occur when humans consumer shellfish that have accumulated the causative algal biotoxin, saxitoxin. Saxitoxin, and a group of 18 or more similar analogues, are potent neurotoxins produced by a number of dinoflagellates that cause algal blooms or red tides (Hallegraeff 1993). PSP may result in human illness and in some cases, death. As the toxin is unable to be detected outside of the laboratory it poses a major health risk.

Laboratory detection of saxitoxin can be performed by a number of methods including mouse assay, high performance liquid chromatography (HPLC), radioimmunoassay or enzyme linked immunoassay (ELISA). As there are distinct disadvantages with all these techniques, initial investigations into an in-vitro tissue culture bioassay utilizing a mouse neuroblastoma cell line has been initiated.

The purpose of this study was to investigate the possible application of the tissue culture assay in Western Australia using techniques outlined in the scientific literature from overseas. Once the assay was established in the laboratory, saxitoxin levels in a selection of ten shellfish extracts were analyzed by the tissue culture assay and compared to results obtained by way of mouse bioassay and HPLC.

Results indicate that it would be possible to establish the cell bioassay technique in any tissue culture laboratory with the appropriate equipment and personnel skilled in tissue culture techniques. The investigation revealed that although it was possible to establish the assay, the results of the analysis of the shellfish extracts were inconsistent and lacked sensitivity, although results appeared to more consistent in shellfish extracts with lower level of toxin. In order to achieve repeatable results, further investigation must be undertaken to modify the Neuro-2a cell line to ensure that the number of sodium channel receptors on the cells is consistent and of a sufficiently high number.

KEYWORDS: Paralytic Shellfish Poisoning (PSP), saxitoxin, bioassay, shellfish.

2. BACKGROUND

Blooms of planktonic algae or 'red tides' have been noted since biblical times (White 1988). Although most of these blooms appear to be harmless and merely colour the water, some produce potent neurotoxins that can accumulate in filter feeding organisms such as shellfish. Human illnesses and deaths caused by ingestion of shellfish contaminated with algal neurotoxins have been widely reported around the world (Anderson *et al.*, 1985).

Of the 3000 species of dinoflagellates found worldwide, around 40 species have been implicated as producing toxins (Hallegraeff 1993) and many of these are capable of producing the alkyloid toxin saxitoxin (STX) that causes the condition known as 'paralytic shellfish poisoning' (PSP) in humans (Hallegraeff 1993). Saxitoxin, and a group of 18 or more chemically similar analogues, can accumulate in shellfish (Sullivan *et al.*, 1988: Hall *et al.*, 1990).. Contaminated shellfish cannot be identified visually and generally the toxins do not impart any taste or odour to the meat, so detection of toxins must be performed in the laboratory.

Detection and quantification of PSP toxins in shellfish meat in Australia is currently performed by the standard mouse bioassay, as sanctioned by the Association of Official Analytical Chemists (AOAC), or by High Performance Liquid Chromatography (HPLC). There are several disadvantages to both of these techniques. Although mouse bioassay has traditionally proved a fairly reliable assessment of the risks, pressure is mounting on the scientific community to eliminate live animal bioassays. This, combined with the fact that the assay requires the maintenance of a live animal colony, means that the technique may soon become unacceptable from an ethical point of view. The HPLC assay does provide a rapid, useful alternative to the mouse bioassay, but the equipment is very expensive and specialist knowledge is required to operate it. The other main drawback with HPLC is that the technique is specific for each analogue of the saxitoxin family and so suitable standards must be found for each of these analogues. When both these assays are critically assessed, it is obvious that an alternative method for PSP toxin detection needs to be made available.

One technique that has been investigated in the United Kingdom, the United States, Canada and Japan is an in-vitro tissue culture bioassay utilizing a mouse neuroblastoma cell line (Jellet *et al.*, 1992). Mouse neuroblastoma cells swell and eventually lyse in the presence of the chemical veratridine, that when combined with ouabain, increases sodium ion influx. Saxitoxin, if present, acts by blocking sodium channels and protects the cells from the action of the two chemicals. The cells will then remain morphologically intact and a direct correlation can be made between the amount of cell protection and the concentration of saxitoxin (Kogure *et al.*, 1988). The tissue culture assay can provide results identical to the mouse bioassay and is considerably more sensitive with a lower detection limit reported to be around 10ng STX equivalents per mL of extract (= 2.0 µg STX equivalents/100g shellfish tissue) (Jellet *et al.*, 1992).

The tissue culture bioassay proposed is relatively inexpensive to run, sensitive, utilizes readily available chemicals and is well within the scope of even modest tissue culture

facilities (Jellet *et al.*, 1992). It has been proposed that with slight modification, the technique could be automated to enable large numbers of samples to be screened at one time and reduces the reliance on live animals.

Subsequent to the commencement of this project, it was announced by a biotechnology company in Canada (Jellet Biotek Ltd.) that a commercial tissue culture bioassay kit was now available. An investigation of the usefulness of the kit was subsequently included in the project as a comparison to the assay under investigation.

3. NEED

1. Certain algal species produce potent neurotoxins that accumulate in shellfish. These toxins are capable of producing severe illness and death in consumers of shellfish contaminated with the toxins. As the contaminated shellfish cannot be identified visually and generally do not impart any taste or odour to the meat, there is a need for both wild harvested and farmed shellfish to be effectively screened for algal toxin contamination.

2. Most states in Australia that farm or harvest shellfish have now adopted shellfish Quality Assurance Programmes to monitor the safety of their product sold on both the domestic and international markets. Part of this Quality Assurance Programme is to monitor the presence of algal toxins in the shellfish meat and to apply restrictions and recall procedures on contaminated product if necessary. Toxin analysis is currently performed either by mouse bioassay or HPLC. As there are disadvantages with both these techniques, there is a need to investigate alternative methods of toxin detection that may be easier to perform, less expensive and more sensitive.

3. The in-vitro tissue culture bioassay outlined has been investigated and in use for research purposes in other shellfish producing countries around the world such as Japan, the United Kingdom, the United States and Canada. There is a need for this technique to be established in Australia to bring us in to line with the rest of the world, especially if the mouse bioassay is eventually phased out as an effective screening technique in the future.

4. OBJECTIVES

1. To investigate the usefulness of the in-vitro tissue culture bioassay to detect and quantify paralytic shellfish poisons (saxitoxin family) in shellfish.

2. To compare the results obtained by the tissue culture bioassay with those obtained by mouse bioassay and HPLC techniques.

3. To examine the commercially available tissue culture kit and compare the technique and relative cost to the laboratory based technique under investigation.

Please note: The Jellet commercial saxitoxin bioassay kit was not available for purchase at the time the application for funding was submitted in November 1995. The availability of kit was discovered by the authors in February 1997, 7 months after the commencement of the project. Due to the fact that no funding had been specifically requested, the project investigators were unable to purchase the kit and no laboratory assessment of the kit was undertaken. The authors were however, able to view a demonstration of the kit in Tasmania in April 1997. The assessment of the commercial kit is based on this demonstration and discussions with Dr Jellet.

5. METHODOLOGY

All tissue culture and saxitoxin bioassay methodology used during the course of the project is shown in the format of a Laboratory Manual at Appendix 1.

6. DETAILED RESULTS

6.1 Establishment of the mouse neuroblastoma cell line

The cell line Neuro-2a (CCL131) was purchased as vials of frozen cells from the American Type Culture Collection (ATCC) in the USA. Attempts to initially establish the cell line using Eagles Minimum Essential Media (EMEM) plus 10% foetal calf serum as the growth media was unsuccessful. The mouse neuroblastoma cell line Neuro-2a was initially slow to establish and in some cases the cells took over 5 days to reach confluency. Consequently the growth media was changed to RPMI plus 10% foetal calf serum. This media provided adequate growth and became know there after as the complete medium (CM).

It was difficult to achieve 100% confluency in the cell sheet as is normal with most cell lines, as the cells started to lift off and float at 80% confluency. Advice was sought from Dr Penny Truman from the Institute of Environmental Research in New Zealand who was also attempting to establish the saxitoxin bioassay and it became apparent, that not being able to achieve 100% confluency is a normal, although unpublished feature of this cell line.

Once the initial problems of finding a suitable growth medium, contamination and establishing the correct techniques for growing the cells were solved, the cell line was grown and multiplied in sufficient numbers to be stored in liquid nitrogen for future use. The cell line now reproduces rapidly, taking two days to reach maximum confluency and prefers to be split at a ratio of 1:3.

6.2 Develop the tissue culture assay using saxitoxin standards

Using the techniques outlined in the scientific literature (Jellett *et al* 1992 and Manger *et al* 1993), attempts were made to develop the tissue culture assay using saxitoxin standards. The published techniques varied greatly in the use of different chemicals for end point determination (i.e. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium

bromide; Thiazolyl blue (MTT), crystal violet), in the concentrations of the chemicals ouabain and veratridine and in the incubation times of the assay.

All of these parameters were examined and cellular morphology was used as an indicator of the assay success. It should be noted that the results obtained when assessing these parameters where of a qualitative, not quantitative nature and as such are not presented in this report.

Various parameters outlined in three distinct techniques (Jellet *et al* 1992, Manger *et al* 1993 and Hamasaki 1996), were utilized and combined to determine the resulting method for this project. For all three techniques and their combinations, the following factors were altered: ouabain/veratridine concentration, total volume of reagents in the assay well, removal of reagents after all stages, MTT concentration and incubation time, different solvents (dimethyl sulphoxide (DMSO), acidified isopropanol) and absorbance at which the plates were read. Later the repeatability of the method was trialed, different operators, the age of the ouabain and veratridine stocks, the number of days from passaging to seed the plates, incubation time from seeding plates to adding saxitoxin/ouabain etc., and the cell seeding density.

Initially, the Manger *et al,* (1993) method seemed promising, however the method outlined by Hamasaki *et al*, (1996) used a lower volume of reagents, and removed less cell content thereby reducing the errors incurred by variations in well volumes. This method was further refined and the method for the determination of the end point was altered to that of the Manger *et al*, (1993) technique with an MTT end point reaction, and later to that of Jellett *et al,* (1992) using crystal violet as the end point determination.

It was eventually established that a lesser volume for the reaction as described by Hamasaki *et al*, (1996) was essential to the success of the assay. It was also established that two possible end point reactions can be used; a 1/6 dilution in complete medium of a 5mg/ml MTT in PBS for 1 hour and dissolved in DMSO, or the crystal violet method as described by Jellett *et al,* (1992). As described in Jellet *et al*, (1992), plates were best read at an absorbance of 595nm.

Standard curves were constructed using the chemicals crystal violet and MTT as the end point determinate (Figures 1 and 2).

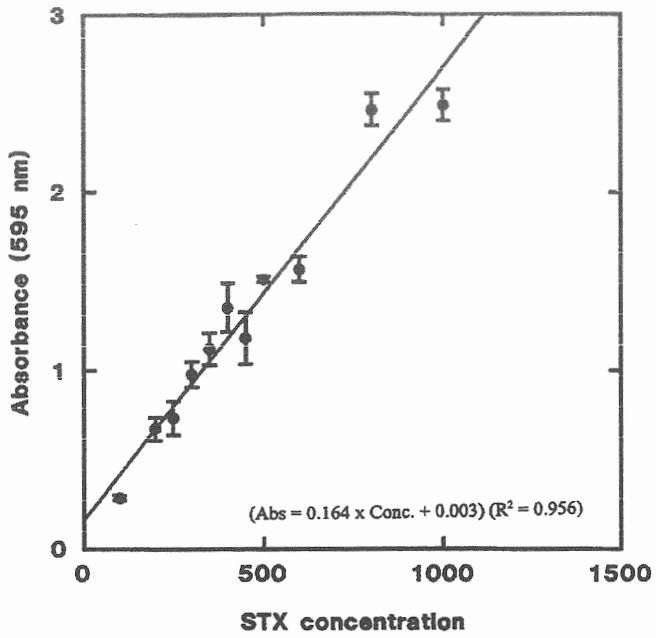

Figure 1: Saxitoxin standard curve using the chemical crystal violet as the end point determination. Mean saxitoxin concentration in pg/10µl (±SE).

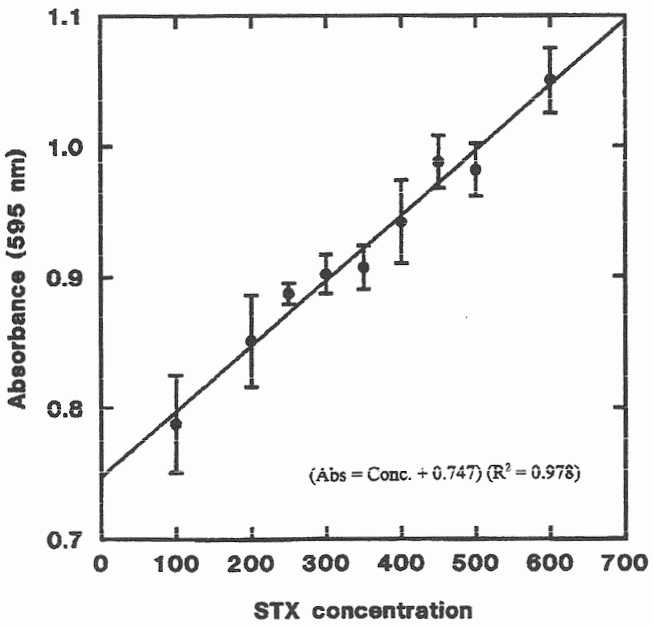

Figure 2: Saxitoxin standard curve using the chemical MTT as the end point determination. Mean saxitoxin concentration in pg/10µl (±SE).

The absorbance values obtained for saxitoxin levels of 100-1000 pg/10µl in the assay employing the use of crystal violet ranged from 0.290 to 2.490 respectively. The range of absorbances obtained for saxitoxin levels of 100-700 pg/10µl in the assay employing the use of MTT ranged from only 0.788 to 1.050 respectively. As the range of the absorbance values obtained using the crystal violet method was far greater, it was concluded that the use of crystal violet to determine the end point of the cell bioassay would be used for the analysis of the toxic shellfish extracts.

Results of this investigation show that the lowest concentration that may be detected by the cell based bioassay using crystal violet as the end point determinate is 50µg STX/100g shellfish meat. No quantitative results were obtained from saxitoxin standards below that concentration. It should be noted that the internationally accepted quarantine level for PSP is 80µg STX/100g shellfish meat.

The major difficulty experienced when initially attempting to establish the cell bioassay was the lack of consistency of the results obtained from the assays performed. Absorbance levels for saxitoxin standards were likely to vary from assay to assay quite markedly and consequently this affected the determination of toxin levels in the unknown extracts. An insight into this lack of consistency was gained when the investigators had the opportunity to witness a demonstration of a commercial cell bioassay currently being developed in Canada. Information with respect to this commercial kit and how it relates to the assay being developed in this project is explained later.

6.3 Analysis of shellfish extracts for saxitoxin levels

Samples of toxic shellfish extracts have been obtained from the Tasmanian Department of Health. The samples were filtered through a 22um filter and stored in aliquots for use in the assay. The samples were analyzed for the toxin levels once a suitable assay methodology was established that was capable of producing a reliable standard curve. A subsample of each toxic extract was then forwarded to the Department of Sea Resources in Hobart for comparative HPLC analysis and to the Institute of Medical and Veterinary Science in Adelaide for comparative mouse bioassay.

Extensive testing to determine the appropriate dilutions of the shellfish extracts to use in the assay was conducted. Figure 3 below shows the absorbances obtained for dilutions of between 1/8 to 1/128 of three toxic shellfish extracts.

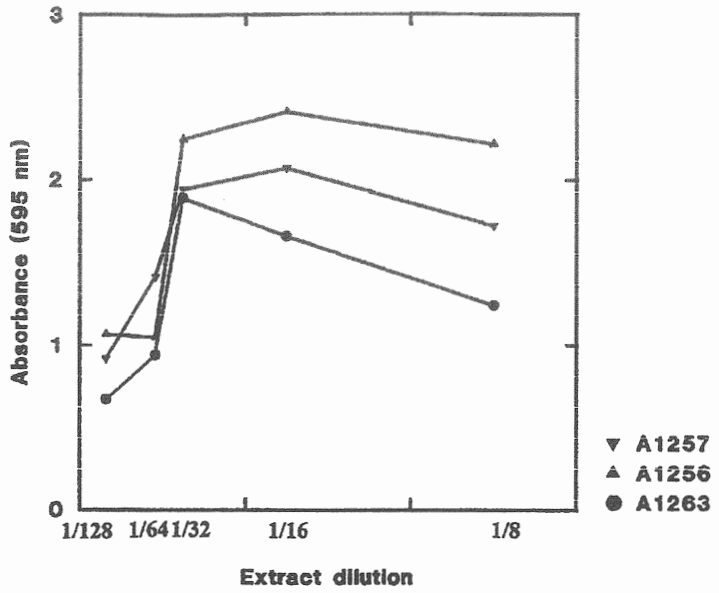

Figure 3: Mean absorbance readings of dilutions of three shellfish extracts. Crystal violet used for the end point determination.

It was shown that the application of neat, and in some cases1/2 or ¼ dilutions of shellfish extract on to the cell sheet markedly altered the results obtained. This may be in part due to the low pH of the acid extracted toxic shellfsih samples, or unknown inhibitory chemicals within the extract. It was concluded that using the absorbance readings from sample extracts diluted to below 1:16 to 1:32 to calculate overall toxin concentration of the original sample from the standard curve was more reliable. However, the use of more dilute shellfish extract to determine overall toxin level means that the assay may not be sensitive enough to detect toxin in samples containing low levels of saxitoxin.

6.4 Comparison of results obtained with mouse bioassay and HPLC techniques

Sample No.	Extract Details	HPLC assay	Mouse bioassay	Tissue culture assay Rep. 1	Tissue culture assay Rep. 2	Tissue culture assay Rep. 3	Tissue culture assay Rep. 4	Tissue culture assay Rep 5.	Tissue culture assay Mean ± SE
A1182	Mussels	210	1210	512	896	179	1203	1024	763 ± 185
A1185	Mussels	980	2308	947	666	460	1229	1408	942 ± 174
A1236	Mussels	1985	767	262	768	256	409	525	444 ± 95
A1244	Mussels	2102	754	186	691	154	211	192	287 ± 101
A1263	Mussels	289	350	217	243	77	147	384	213 ± 51
A1386	Mussels	97	92	141	176	64	51	120	110 ± 23
A1260	Mussels	60	52	93	51	72	58	112	77 ± 11
A1256	Mussels	533	738	512	365	307	230	435	370 ± 49
A1257	Oysters	240	215	166	102	102	211	-	145 ± 24
A1776	Mussels	20	40.5	30	13	16	19	28	21 ± 3

Table 1: Comparison of saxitoxin concentrations (µg STX/100g) in the 10 shellfish extracts analyzed by either HPLC, mouse bioassay or cell bioassay.

Table 1 shows a large discrepancy between the results obtained for the mouse bioassay as compared to HPLC results for those samples with a relatively high toxin level. An explanation for this discrepancy could be the fact that the contaminated shellfish samples used in this project were obtained from shellfish growing areas in Tasmania where blooms of PSP producing dinoflagellate *Gymnodinium catenatum* commonly occur (Hallegraeff *et al.* 1986). The toxin profile of *G. catenatum* is typically dominated by large amounts of the so-called 'c-toxin' group (Brown pers. Comm. 1997). At low levels (20-100 µg STX/100g meat), the toxin profile in contaminated shellfish extracts is almost exclusively c-toxins. These toxins that are in low potency, are easily separated and identifiable by HPLC making it relatively easy to obtain an accurate estimation of toxicity at low levels of PSP for *G. catenatum*. As the concentration of toxin in the shellfish increases there is an increase in the concentration of the more potent gonyautoxins and in particular the decarbamoyl gonyautoxins (GTX). There is the potential for some of these GTX compounds to coelute during HPLC seperation if the system is not correctly established. Because these toxins have significantly different toxicities, if during the integration process the toxins are not correctly identified, there is the potential for significant inaccuracy when attempting to analyse shellfish with high toxin levels by HPLC.

Cellular response to the various saxitoxin analogues is based on the selectivity of the voltage-activated sodium channel. The results obtained in a cell based bioassay are therefore more likely to correspond to the toxins relative potency in mammals. In light of this, and the discrepancy between some of the results of the HPLC assay, all results obtained in this investigation were compared to the mouse bioassay.

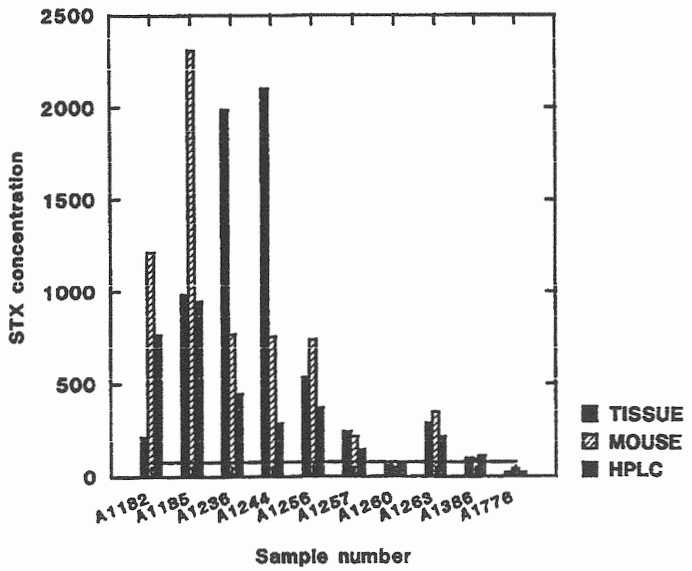

Figure 4: Comparison of saxitoxin concentration of 10 shellfish extracts analysed by mouse bioassay (IMVS Adelaide) or neuroblastoma cell bioassay.

The results of the cell bioassay analysis shown in Table 1 indicates the lack of consistency between the replicate assays and Figure 4 shows that in all but two extracts, the results obtained by cell bioassay analysis were lower than those obtained by the mouse bioassay.

6.5 Commercial assay kit

In April 1997, the project investigators were invited to the University of Tasmania in Hobart to view a demonstration of a commercial cell bioassay kit. Jellett Biotek is a biotechnology company based in Canada that is currently developing a commercial cell bioassay kit for the detection of PSP. The kit, the .Maritime In Vitro Shellfish Test (MIST) is a cell based biological toxicity assay which is very similar to the assay currently under investigation. The company produces three MIST kits; semi-quantitative, fully quantitative and a yes/no qualitative kit.

The cell bioassay utilizes precultured live neuroblastoma cells that have been suspended in a temperature dependent chemical to keep the cells in stasis until incubated at the required assay temperature. This allows the kits to be shipped from Canada to anywhere in the world with a one week shelf life. All kits contain pre-mixed reagents, necessary disposable equipment and comprehensive instructions.

Based on the information provided at the demonstration and the experience gained while attempting to establish this assay in the laboratory, several advantages and several disadvantages with the kit have been identified.

Advantages:

- The assay does not require specialist expertise in tissue culture techniques;
- It is consistent and 20 times more sensitive than the mouse bioassay (2µg STX eq./100g meat);
- The qualitative assay (Yes/No) can be performed by personnel with minimal laboratory experience, relying on visual assessment and can be conducted in the field.

Disadvantages:

- A plate reader is required to obtain quantitative results, therefore the assay must be performed in a laboratory;
- All assay kits require a 37°C incubator and a multichannel pipette (i.e. expensive to purchase) and the purchaser is required to perform all the necessary dilutions of the sample and reagents;

- For a 'one off' determination, the kits are expensive when compared to the assay under development and to the mouse bioassay. Cost at the time of the demonstration was A$267/plate on which four samples can be analyzed;

- Short shelf life;

- The assay kits are not readily available in Australia due to restrictions on importation imposed by the requirements of the Australian Quarantine and Inspection Services (AQIS);

- Shellfish extracts that contain tetrodotoxins or heavy metals will give a false positive reading (Jellet 1997 *pers. comm.*).

The most significant difference between this commercial kit and the assay currently under development is the neuroblastoma cell line. Over a period of 5 years, Jellet Biotek has selectively modified the cell line, by selecting cells that exhibit a greater number of sodium channels on a more consistent basis. This ensures that the assay produces consistent results at a sensitivity level much lower than can obtained on the normal unmodified neuroblastoma cell line.

6.6 Conclusions

Although difficulties were experienced with the initial establishment of the Neuro-2a cell line, the cell line is generally easy to culture and manipulate and once the correct techniques are established, produces consistent healthy monolayers.

Of all the assay techniques trialed, the best results were obtained using a slightly modified version of the crystal violet technique as outlined in Jellet et al (1992). The assay developed as a result of this project has a lower detection limit of 50µg STX/100g shellfish meat and the cost of the assay is estimated at A$78.00/ plate as compared to A$267.00/ plate for the commercial kit. The cell bioassay is not difficult to perform and could be attempted by any laboratory personnel with sufficient skills in tissue culture technique.

Results obtained on the unknown shellfish extracts when analyzed by the cell bioassay show an unacceptable lack of repeatability between the replicates and when compared to those results obtained using the mouse assay, show a lack of sensitivity.

This was the major difficulty experienced with the assay and it may be assumed is due to the fact that there would appear to be a marked variation in the number of sodium channel receptors in the cells at various times and under various culture conditions.

It was also demonstrated that the use of more concentrated shellfish extracts in the assay may produce an incorrect analysis due to the existence of inhibitory or complicating contaminants within the extract. It may be possible to remove these contaminants by way of clean up techniques using various molecular weight cut-off filters, however this was outside of the scope of this project.

Results obtained during the 12 months of this project indicate that the cell based bioassay using the neuroblastoma cell line show some promise for the detection of PSP toxins. Further investigation is required however, into the modification of the Neuro 2a cell line in order to increase the number and consistency of the sodium channel receptors.

7. BENEFITS

If the technique can eventually be proved suitable and is sanctioned by the Australian Shellfish Sanitation Advisory Committee, the shellfish farmers and harvesters will be provided with an inexpensive alternative technique to the standard mouse bioassay and HPLC assay. The health of domestic consumers of shellfish will be more adequately protected and Australia's reputation of supplying clean, green products to the international market will be maintained.

We have during the course of the investigation, been liaising closely with the Tasmanian Health Department and exchanging information and materials for comparison. The Tasmanian Health Department currently uses the HPLC technique to analyze shellfish extracts and have indicated their interest in the in-vitro assay as a cheaper and faster alternative. The results obtained in the course of this investigation of the comparison between the three techniques has highlighted a possible difficulty in the use of HPLC results alone for the detection of PSP toxins. The authors are unaware if results of this nature have been reported previously.

8. INTELLECTUAL PROPERTY

A full laboratory manual outlining the techniques used in this investigation has been produced as part of this final report. The manual will be invaluable to any laboratory who may wish to continue with the development of the tissue culture bioassay.

9. FURTHER DEVELOPMENT

- It has been concluded that to produce reliable repeatable results with the cell based bioassay, further work must occur to improve the cell line by selecting cultures that consistently and reliably produce large and stable numbers of sodium channel receptors.

- Further investigation is also required to develop clean-up techniques of toxic shellfish extract to remove inhibitory contaminants.

The investigators of this project have been approached by scientists from the Victorian Institute of Animal Science who have expressed an interest in continuing the development of this assay.

10. STAFF

Name	Position
Tina Thorne	Aquaculture Program Officer, Fisheries Western Australia
Heidi Cassell	Research Scientist, Fisheries Western Australia
Dr J.B. Jones	Senior Fish Pathologist, Fisheries Western Australia
Ray Brown	Research Officer, Health Department of Tasmania

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LABORATORY MANUAL

TISSUE CULTURE ASSAY FOR THE DETECTION

OF SAXITOXIN (PSP)

TABLE OF CONTENTS

1. INTRODUCTION

Blooms of planktonic algae or 'red tides' have been noted since biblical times. Although most of these blooms appear to be harmless and merely colour the water, some produce potent neurotoxins that can accumulate in filter feeding organisms such as shellfish. Human illnesses and deaths caused by ingestion of shellfish contaminated with algal neurotoxins have been widely reported around the world.

Around 20 species of dinoflagellates have been implicated as producing the alkaloid toxin saxitoxin (STX) that causes the condition known as 'paralytic shellfish poisoning' (PSP) in humans. Paralytic shellfish poisoning is the most common of shellfish poisonings. The characteristic symptoms of PSP can vary from headache, nausea and diarrhoea, to muscular paralysis, respiratory difficulty and death.

Saxitoxin, and a group of 18 or more chemically similar analogues, can accumulate in shellfish. Contaminated shellfish cannot be identified visually and generally the toxins do not impart any taste or odour to the meat, so detection of toxins must be performed in the laboratory.

2. DETECTION AND QUANTIFICATION OF STX - ASSAY TECHNIQUES

2.1 Standard Mouse Bioassay

The standard mouse bioassay, as sanctioned by the Association of Official Analytical Chemists (AOAC) is the method by which saxitoxins are currently analyzed. To assess toxin levels in this bioassay a shellfish extract is made, diluted accordingly and a 1ml aliquot is injected intraperitoneally into a test mouse. The biological effect on the mouse is used as an indicator to determine whether STX is present in the sample. The lowest detectable level of toxin is 40µg STX eq/100g wet weight of tissue. The mouse bioassay is rapid with results available within 24 hours, but tends to be non specific.

Although the mouse bioassay has traditionally proved a fairly reliable assay for saxitoxin and other algal biotoxins, significant pressure is mounting on the scientific community to eliminate live animal bioassays. This, combined with the fact that the assay requires the maintenance of a live animal colony, means that the technique may soon become obsolete and other assay alternatives are required.

2.2 High Performance Liquid Chromatography (HPLC)

HPLC for the detection for STX is the next most common technique utilized. It involves the alkaline oxidation of STX to fluorescent derivatives using periodic acid in sodium phosphate buffer, and their separation by HPLC. This method enables the quantification and resolution of individual toxin components [4].

The HPLC assay does provide a rapid, useful alternative to the mouse bioassay, but has several drawbacks including the fact that:

- the equipment is very expensive;
- specialist knowledge is required to operate it; and
- the technique is specific for each analogue of the saxitoxin family and so suitable standards must be found for each of these analogues.

When this assay and the mouse bioassay are assessed, it is obvious that an alternative method for PSP toxin detection needs to be made available in countries where shellfish are grown and consumed.

2.3 Cell Culture Bioassays

One technique that has been investigated in the United Kingdom, the United States, Canada and Japan is an in-vitro tissue culture bioassay utilizing a mouse neuroblastoma cell line [2]. Mouse neuroblastoma cells swell and eventually lyse in the presence of the chemical veratridine, which when combined with ouabain, increases sodium ion influx. Saxitoxin, if present, acts by blocking sodium channels and protects the cells from the action of the two chemicals. The cells will then remain morphologically intact and a direct correlation can be made between the amount of cell protection and the concentration of saxitoxin. It has been demonstrated that the tissue culture assay can provide results identical to the mouse bioassay and is considerably more sensitive with a lower detection limit reported to be around 10ng STX equivalents per mL of extract (= 2.0 µg STX equivalents/100g shellfish tissue).

3. REAGENTS REQUIRED

Tissue culture reagents	**Supplier**
Neuro 2a cell line, CCL 131	American Type Culture Collection, Washington USA
RPMI 1640 media powder	Australian Biosearch
Foetal calf serum (FCS)	Biosearch (Aust)
Antibiotic-antimycotic solution	Gibco (Aust)
Ethylenediaminetetra-acetic acid (dissodium salt)	Sigma (USA)
Crystal Violet 10%	Scott Scientific (WA)
Dimethyl sulfoxide (DMSO)	Sigma (Aust)
Ethanol (80%)	CSR Ltd (Aust)
Potassium dihydrogen phosphate (KH_2PO_4)	BDH (Aust)
Potassium Chloride (KCl)	Sigma Chemicals (USA)
Trypsin powder	Australian Biosearch
Sodium hydrogen phosphate (Na_2HPO_4)	Sigma Chemicals (USA)
Sodium Chloride (NaCl)	Sigma Chemicals (USA)
Sodium Bicarbonate ($NaHCO_3$)	Sigma Chemicals (USA)

Assay Reagents	**Supplier**
Saxitoxin dihydrochloride (STX) Reference standard	Food & Drug Administration (USA)
Glacial acetic acid solution (100%)	Allwest Scientific (Aust)
Hydrochloric Acid (HCl)	Rowe Scientific
MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) ($C_{18}H_{16}N_5SBr$)	Sigma (USA)
Phenol Red	Scot Scientific (Aust)
Ouabain ($C_{29}H_{44}O_{12}.8H_2O$)	Sigma (USA)
Veratridine ($C_{36}H_{51}NO_{11}$)	Sigma Chemicals (USA)

4. REAGENT RECIPES

4.1 Tissue Culture Reagents

- **RPMI 1640 Growth media**

To make 1 litre of media, add to the contents of one sachet of powder-
2g of $NaHCO_3$, and
950ml of distilled-dionised water, stir until dissolved.
Adjust to pH 7.2 and make up to 1L. Filter solution through a 0.22µm bottle top filter. Check sterility overnight, if sterile add 10ml of antibiotic/antimycotic solution (Gibco).

- **Complete medium (required for assay)**

Add 10% foetal calf serum to RPMI 1640 media (including antibiotic/antimycotic solution). Store at 4°C.

- **Trypsin Versene Solution (ATV)**

To make 1 litre of solution add;
8g of NaCl,
0.2g KH_2PO_4,
0.2g KCl,
1.15g Na_2HPO_4,
0.2g Ethylenediaminetetra-acetic acid (disodium salt) (versine) and
0.8g trypsin.

Add 2mls of 0.5% phenol red, make up to 1L in distilled-dionised water. Adjust to pH 7.4 with 40% KOH and filter through a 0.22µm filter to sterilise the solution. Aliquot and store in the freezer at -20°C.

- **Foetal Calf Serum**

Ensure that all foetal calf serum is heat inactivated prior to use. Test each batch to ensure compatibility with cell line by assessing cell growth during cultivation.

4.2 Assay Reagents

- **STX reference standard**

Saxitoxin reference standard (100mg/ml) can be obtained free of charge from the Washington Food and Drug Administration for research and monitoring purposes. The standard is diluted in 20% ethanol/distilled-dionised water at 1:1000 to make a 1000pg/10µl solution.

To obtain standard, contact - Dr Sherwood Hall, Ph.D.
Washington Seafood Laboratory
FDA HFS-426
200 C Street SW
Washington, DC 20204
USA

- **Ouabain (10mM stock solution)**

Weigh 72.9mg of ouabain powder into a 10mL volumetric flask and make up to 10mL with distilled-dionised water. Dissolve and filter the solution through a 0.22µm filter. Store at room temperature in the dark for one month.

- **Veratridine (1mM stock solution)**

Weigh 6.7mg of veratridine powder into a 10ml volumetric flask and make to 10mL with acidified (pH2). distilled-dionised water. Filter solution through a 0.22µm filter. Store at -20°C for up to a month, thaw only once.

- **Ouabain/Veratridine Solution**

This solution must be made fresh prior to each assay. 2.5 ml of the solution is required for one assay plate (i.e. 625µl of 10mM ouabain and 625µl of 1mM veratridine, and 1250 µl of complete media). Each 20µl aliquot of this mix contains 10µl of 5mM ouabain and 10µl of 0.5mM veratridine.

- **MTT (5mg/ml stock solution)**

Weigh 0.125g of MTT powder into a 25mL volumetric flask and make up to 25mL with phosphate buffered saline (PBS) pH7.4. Dissolve and filter through a 0.22µm filter. Store at 4°C in the dark.

- **Acetic Acid (33%)**

Dilute glacial acetic acid solution (99.9%) in distilled-dionised water at a ratio of 1:3.

- **Phosphate Buffered Saline (PBS) (x10 conc)**

Accurately weigh:

- 80g of sodium chloride (NaCl),
- 2g potassium chloride (KCl),
- 11.5g disodium hydrogen orthophosphate (Na_2HPO_4),
- 2g potassium dihydrogen phosphate (KH_2PO_4)

Dissolve in 800ml of distilled-dionised water, filter through a 0.22µm filter to sterilize and aliquote into 80mL amounts. The solution is stored at 4°C. To obtain a working

strength solution, each 80mL aliquot is made up to 1L with sterile distilled-deionised water.

5. EQUIPMENT REQUIRED

- Sterile cell culture flasks 75 cm^2
- Sterile cell culture 96 well flat bottom assay plates
- Sterile 'v' bottom trays for dilutions
- 0.22µm filter (for bottle top and syringes)
- Humidified incubator 37°C, 5% CO_2
- Microplate reader (with 595nm filter)
- Multichannel multistepper pipette 50-300 µl
- Single channel pipettes 5-20 µl, 0-200 µl and 0-1000 µl
- Sterile tips
- water bath
- centrifuge

6. METHODS

6.1. Cell Culture

- Cells and media

  - The cell line used is Neuro 2a mouse neuroblastoma cells - ATCC reference number 131-CCL.
  - Growth media is RPMI 1640 pH 7.2, with 10% foetal calf serum.
  - Freezing medium is RPMI 1640 with 10% DMSO.

- Retrieval of cells from liquid nitrogen storage

Rapidly thaw cells by immersing vial in a 37°C water bath. The vial is then swabbed with 70% alcohol and opened. Remove the cells using a pipette and resuspend in 15ml of growth media in a 75cm^2 flask. Incubate for 24 hours before changing media.

- Passaging cell line

The cell line grows best in 75cm^2 sterile culture flasks. To passage cell line, remove the media, rinse cell sheet once with 2ml ATV, then add a further 2ml ATV and leave on the cell sheet for 3 minutes at 37°C. Once cells have detached (check by microscopic examination), inactivate the trypsin solution with growth medium containing FCS. Gently mix and dispense into new flasks. The cells rapidly divide and the split ratio is 1:3, two to three times weekly. Incubate flasks at 37°C in a 5% CO_2 incubator with the lid of the flask in the vent position. The cell sheet does not reach more than a maximum confluency of 80%. The cell line is capable of multiplying 15 times in 7 days when subcultured correctly.

- Liquid nitrogen storage

To store cells into liquid nitrogen, viable cells in a flask have the medium removed, are rinsed once with ATV then further ATV is left on the cell sheet for 3 minutes at 37°C. Once cells have detached (check by microscopic examination), inactivate the ATV with growth medium containing FCS and centrifuge the cell suspension at 2000rpm for 10 minutes. Remove the growth media and resuspend in RPMI 1640 media containing 10% DMSO. The cells are aliquotted into ampoules (1 confluent flask = 3 ampoules), brought slowly down to temperature and stored in nitrogen.

- Seeding cells for an assay

Use a confluent flask of rapidly dividing cells preferably 2-3 days from passage. Trypsinise the cells in 2ml ATV then add 8ml growth media. Dilute the cells in growth media to a density of 1-1.5 x 10^5 cells/ml. Seed the cells onto the sterile 96 well tissue culture plates, 200µl well. Incubate plates for 24 hours prior to use in the STX assay.

6.2. Preparation of Shellfish Extract

Wash and shuck sufficient shellfish for 100g of whole wet tissue. Drain off excess liquor from the meat on a paper towel placed on a wire rack. Weigh 100g of whole meat into a pre-weighed mason jar and add 100ml of 1.0 N HCl. Homogenise meat for 30 seconds and weigh. Heat the sample in a 100°C water bath for 5 minutes, then remove the jar and allow to cool to room temperature. Reweigh jar. Adjust the pH of the sample to pH 3 with 5.0M NaOH and reweigh jar. Bring the weight of the sample up to 200g with distilled water, mix and allow to settle. Centrifuge sample liquid in 10ml centrifuge tubes at 5500 rpm for 5 minutes then remove the supernatant. Store the supernatant in the freezer until use.

6.3. Tissue Culture Bioassay for STX

6.3.1 Seeding of cells into plates

Actively growing Neuro 2a cells nearing confluency, are seeded into 96-well tissue culture plates at a cell density of 1 x 10^5 cells/ml (200µl of cell suspension/per well).

Incubate the plates for 24 hours at 37°C and 5% CO_2. Prior to the application of the reagents and samples, the cells should be actively growing in the wells but not confluent.

6.3.2 Dilution of samples

The appropriate dilutions of saxitoxin standard (2000pg/ul to 100pg/ul) and test samples (1:4 to 1:64) are prepared in separate 96-well 'v' bottomed plates and 10 ul from each well is transferred to the assay plate using a multichannel pipette when required.

After incubation, remove the media (2 flicks into the sink, them blot on sterile gauze)

6.3.3 Assay set up

A standard assay comprising of each saxitoxin standard in triplicate, five dilutions of two test samples in triplicate, cell controls and ouabain/veratridine controls is set up in the 96 well assay plate as follows;

	1	2	3	4	5	6	7	8	9	10	11	12
A STX std	2000	1000	800	600	500	450	400	350	300	250	200	100
B STX std	2000	1000	800	600	500	450	400	350	300	250	200	100
C STX std	2000	1000	800	600	500	450	400	350	300	250	200	100
D samples	o/v	1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64	o/v
E	o/v	1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64	o/v
F	o/v							sample	Two			o/v
G	o/v	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c
H												

Add fresh complete medium to the wells as follows:

100 µl to cell control wells
80 µl to ouabain/veratridine control wells
70 µl to standard control and sample wells

Add 10 µl of the diluted saxitoxin standard or diluted sample to the appropriate wells.
Add 20 µl of the ouabain/veratridine mix to all wells except the cell control wells.
Rock gently by hand to mix.
Incubate for 21-24 hours.

6.3.4 End point determination

CRYSTAL VIOLET METHOD

- Remove the media and reagents from the wells as described above
- Add 100 µl of PBS to each well to rinse and remove as described above
- Fix with 100 µl of 80% ethanol for 15 min
- Add 100 µl of crystal violet to each well for 5 min
- Rinse with tap water and flick to dry
- Leave overnight to completely dry
- Digest well contents with 100 µl of 33% acetic acid for 1hr
- Read on plate reader at 595nm ensuring that all plates are read at the same time interval.

MTT METHOD

- Remove the media and reagents from the wells as described above
- Add 60 µl of a 1:6 dilution of 5mg/ml MTT
- Incubate for 1 hour at 37°C
- Remove the MTT as described above
- Add 100 µl of DMSO
- Read on plate reader at 595nm ensuring that all plates are read at the same time interval.

6.4.5 Quantification of test samples

A standard curve of the absorbances gained from the saxitoxin standards is plotted and a line of best fit is applied. From the curve created for the saxitoxin standard the results from the unknown test samples can be read, taking into account the dilution factor.

It should be noted, that dilutions of between 1:2 and 1:16 of test samples gave a lower absorbance reading than dilutions above 1:16. This may be due to inhibitory chemicals within the sample preparation. As a consequence, all absorbance readings for test samples were recorded for dilutions greater than 1:16.

7. TROUBLE SHOOTING

- Cells

Ensure that prior to seeding the cells into 96 well plates for an assay that the cells are rapidly growing and are close to confluent. Do not use cells that have not been recently subcultured and do not use cultures that are forming clumps in the monolayer.

Cells for an assay must be actively growing, therefore if a new cell passage is retrieved from liquid nitrogen, passage the cells several times (at least one week of growth) prior to use, otherwise the cells will not be suitable.

- Removing media and reagents from wells

When removing well contents ensure that the flick motion is standardised as much as possible to avoid error.

- Addition of reagents

Minimise the time between the addition of the saxitoxin standards or diluted samples and the ouabain/veratridine mix.

Ensure that pipetting technique is as accurate as possible.

- Colour reaction

If attempting the technique using MTT as the end point determinant, it is important to ensure that no precipitate is lost thereby reduce errors that may occur through a reduction in the absorbance levels.

The length of time between addition of the DMSO and when the plates are read is not critical, but should be no more than 10 minutes and the time interval between addition of the reagents and the absorbances being read should be standardised as colour reaction increases with time.

8. COST OF ASSAY

Cells / media

(Prices for 1L)	
RPMI media	$ 4.30
Antibiotic/antimyotic solution	$ 2.00
Foetal Calf Serum	$ 37.00
0.22um syringe filter	$ 9.50
Sodium bicarbonate	$ 0.20
ATV	$ 0.20
Total media cost (per 100ml)	**$ 5.50**

Assay Reagents

STX standard	-
Veratridine	$ 3.60 / ml
Ouabain	$ 1.00 / ml
PBS	$ 0.30 / 10ml
80% Ethanol	$ 0.20 / 10ml
Crystal Violet	$ 0.40 / 10ml
33% Acetic Acid	$ 0.40 / 10ml
or	
MTT	$ 0.40 / 1ml
DMSO	$ 0.70 /10ml

Consumable equipment

Flask	$ 2.00
Plates (2 plates)	$ 3.40
Tips	$ 3.00
Total assay reagents and equipment cost	**$ 14.00**
Capital Depreciation (includes all capital items used in a tissue culture laboratory)	**$ 20.00**
Total labour (3hr x $20)	**$ 60**

TOTAL ASSAY COST	**no labour**	**$ 34**
	including labour	**$ 94**

(For 1 plate - 2 samples and std) (MTT and crystal violet endpoints equal in cost)