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Investigations into the Toxicology of Pectenotoxin-2-seco acid and 7-epi pectenotoxin 2-seco acid to aid in a health risk assessment for the consumption of shellfish contaminated with these shellfish toxins in Australia

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1.0 SUMMARY

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1.2 Objectives

Toxicology information is needed to 1) decipher the contribution of PTX2-SAs to the DSP syndrome, 2) determine their potential risk to human health, 3) to perform a health risk assessment for consumption of contaminated shellfish and 4) for guidance in the regulation of permitted toxin levels in shellfish that are sold for human consumption. Thus, the objectives for this project are as follows:

(i) Develop a robust method for extraction and purification of PTX2-SAs from shellfish and algae
(ii) Produce purified PTX2-SAs extracted from shellfish and algae for investigations
(iii) Conduct acute oral and *i.p.* studies in mice to determine the toxicopathology and body distribution of PTX2-SAs
(iv) Investigate mechanisms of toxicity of the PTX2-SAs with the use of a cDNA microarray technology and *in vitro* cell culture assays
(v) Perform a health risk assessment for the consumption of seafood contaminated with the PTX2-SAs to aid establishing guideline values for pectenotoxins in shellfish with respect to the 1997 Northern NSW poisoning incident.

1.3 Overview of project and outcomes

Purpose of Study: In December 1997 approximately 200 people were reported with severe diarrhoetic shellfish poisoning (DSP) in Northern New South Wales (Quilliam et al., 2000). Analysis of the shellfish associated with this incident revealed relatively high pectenotoxin-2-seco acid (PTX2-SA) concentrations (approx. 300μg/kg shellfish meat), with only trace amounts of pectenotoxin-2 (PTX2) and okadaic acid (OA). Following this incident, PTX2-SAs were considered a health threat and guidelines were implemented in the absence of toxicological data, which has caused a great economic burden to shellfish industries around the globe, in particular to Australia, New Zealand and Ireland. Such regulation created in the absence of scientific data demonstrated the need to determine the toxicology of PTX2-SA in

commercial shellfish. Thus a comprehensive study on the toxicology and possible health implications of the PTX2-SAs in Australian shellfish was conducted.

Methodology: PTX2-SAs were isolated in several batches from shellfish (pipis, oysters and mussels) and from algal bloom samples. Toxin extraction was conducted with several purification stages and chemical analysis was performed with high-pressure liquid chromatography coupled to a tandem mass spectrometer (HPLC-MS/MS). Acute dosing studies with mice included toxicopathology investigations with light microscopy and electron microscopy, in addition to toxin distribution and DNA damage studies by detection of malon-dialdehyde in mouse urine. *In vitro* studies with HepG2 cells included cytotoxicity assays, cell cycle investigations using flow cytometry and gene expression profiling of cells exposed to PTX2-SA employing microarray technology. Anecdotal evidence of human exposure to high levels of PTX2-SAs by shellfish consumption was collected for discussion in a health risk assessment.

Results and discussion: Acute pathology studies demonstrated that the PTX2-SAs do not cause the characteristic symptoms or lesions associated with DSP toxins. No diarrhoea was observed at any dose level in mice and no deaths occurred up to the maximum dosing level of 1.6mg/kg PTX2-SA. Only one batch of PTX2-SA extract produced toxic lesions characteristic of a DSP toxin (batch 1-pilot study) but after follow up studies, it was determined that this first batch of shellfish most likely contained an additional unidentified shellfish toxin or contaminant that co-extracted with PTX2-SAs during toxin isolation and purification procedures. This finding highlighted the importance of supporting the inclusion of the mice bioassay in procedures for shellfish toxin testing to enable detection of new toxins, and also highlighted the importance of toxin purification for toxicology studies. A significant rise in malondialdehyde excretion was observed within 24 hours of dosing mice, indicating that the PTX2-SAs cause oxidative damage by lipid peroxidation *in vivo*.

In vitro studies showed HepG$_2$ cells to have cell cycle and gene expression changes within 24 hours of a dose of 800ng PTX2-SAs per mL of media. Cell cycle arrest was observed at the G$_2$/M checkpoint and gene expression changes included alterations of genes involved in cell cycle control, lipid metabolism and transport, lipid genesis and trace metal transport. Many genes involved in DNA repair processes were moderated at the 24 hour point, but as no apoptosis was observed up to 72 hours it is a promising indication that any DNA damage that may have been caused by the administration of PTX2-SAs was not lethal, and was able to be repaired.

In light of the information provided by toxicology investigations in this study, with particular reference to evidence of *in vivo* lipid peroxidation by raised levels of MDA in mouse urine, and changes in cell cycle distribution and gene expression in a cultured human cell line, it is concluded that there is potential for these toxins to induce biological changes in mammalian cells *in vivo* and *in vitro*, and hence potential for PTX2-SAs to cause health effects in humans when consumed. Further to this statement, it should be noted that these studies were acute studies only, and it has not been established if these observed changes could result in disease or whether they could be repaired or returned within normal limits without the manifestation of illness or disease occurring.

During the course of this three-year study, developments in techniques for shellfish toxin identification within our laboratories have revealed that the shellfish responsible for the 1997 NSW poisoning incident contained significant concentrations of okadaic acid acyl esters that were not detected at the time of the NSW incident. Although reportedly less toxic than

okadaic acid itself, the OA ester concentrations present were likely sufficient to cause the observed symptoms. It is also theorized that these esters could be hydrolyzed in the human gastro-intestinal tract to release okadaic acid. In the light of this new evidence and with no pathology lesions or diarrhectic symptoms being observed in PTX2-SA dosing studies with mice, we now believe these OA acyl esters to be the causative agent in the 1997 NSW DSP incident and not the PTX2-SAs.

Utilizing the acute toxicology information in this thesis, a health risk assessment for consumption of PTX2-SA contaminated shellfish was performed. This risk assessment, employing numerous safety factors essential for an incomplete data set, produced guideline values that are lower than the current recommend concentrations. To date, there has been no solid evidence that PTX2-SAs cause illness in humans – all documented incidents involving the PTX2-SAs have also included other DSP contaminants that are known to cause human illness. Pathology has not unequivocally been demonstrated in animal studies and thus, in consideration of the epidemiological evidence, PTX2-SAs cannot be considered as high a risk to public health as was previously thought. For the reasons discussed above, and weighing up risk-benefit considerations of the economic burden the current guideline values are causing to shellfish industries around the globe, it is recommended that PTX2-SAs no longer be regulated with other DSPs until such a time that toxicological or epidemiological evidence can prove the PTX2-SAs are a more considerable threat to human health than has been shown though toxicology investigations undertaken in this study.

OUTCOMES ACHIEVED

This study has produced a substantial amount of acute toxicology data and has provided a good basis for future chronic toxicology investigations with the PTX2-SAs for regulatory purposes. The work of this PhD study has contributed to the establishment of appropriate regulatory guidelines for levels of PTX2-SAs in commercial shellfish in Australia. Organizations that have already reviewed and utilized information from this thesis include the South Australian Shellfish Quality Assurance Program and the Crawthron Institute on behalf of the Marlborough Sounds Shellfish Quality Programme, New Zealand.

The PhD thesis from which this report is summarised from will available in full from the Australian Digital Theses Program, which can be accessed via the Griffith University web site www.gu.edu.au and is anticipated to be accessible in full by the end of 2003.

KEYWORDS: **Pectenotoxin-2-seco acid, diarrhoetic shellfish toxin, DSP, public health toxicology.**

2.0 ACKNOWLEDGMENTS

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3.0 BACKGROUND

The epidemiology of shellfish toxins around the world and their risk to human health have been well documented (Viviani 1992; Ledoux and Fremy 1994; Soames-Mraci 1995; Aune 1996; Desenclos 1996; Okada and Niwa 1998; Burgess and Shaw 2001). A significant number of human poisonings occur each year worldwide through the consumption of fish and shellfish contaminated with a variety of marine toxins, with a mortality rate of approximately 15% (Hallegraeff *et al.* 1995). Several different classes of shellfish poisoning exist that are caused by the build-up of algal toxins within marine animals. Incidences of poisoning related to marine algal toxins come under the main categories of diarrhetic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), amnesic shellfish poisoning (ASP) and ciguatera (CTX), depending upon the toxins and the symptoms that they cause.

The DSPs are polyether compounds, are found worldwide and pose a particular problem for the length of time the toxins remain active within the shellfish (Yasumoto *et al.* 1985; Edebo *et al.* 1988; Pillet and Houvenaghel 1995; Bauder *et al.* 2001; Svensson 2003). Additionally, shellfish can become sufficiently toxic to cause poisoning by efficient filtering of seawater containing sparse toxic dinoflagellate populations (200 cells/L or less) (Luckas 1992). Investigations have been conducted to determine post-harvesting methods of reducing the toxicity of DSPs contained in shellfish. It was found that boiling shellfish in a slightly alkaline medium with bicarbonate salts caused detoxification percentages from 24 to 79% in toxic mussels (Vieites and Leira 1995), but cooking shellfish in this manner is not desirable and thus could not be a commercial or regulatory procedure. Thus, with the high morbidity caused and worldwide distribution, DSPs present a serious problem for shellfish industries and to public health.

DSP is characterized by an acute gastrointestinal disturbance following the consumption of shellfish contaminated with either, or a combination of, okadaic acid (OA) (Edebo *et al.* 1988; Edebo *et al.* 1988; Cohen *et al.* 1990; Draisci *et al.* 1998), hydroxylated esters of OA (Norte

et al. 1994; Fernandez *et al.* 1996; Windust *et al.* 1997; Hu *et al.* 1999; Vale *et al.* 1999; MacKenzie *et al.* 2002; Vale and Sampayo 2002; Vale and Sampayo 2002); dinophysistoxins (DTX) (Lee *et al.* 1987; Yanagi *et al.* 1989; Carmody *et al.* 1996, Amano, 1986; James *et al.* 1999), pectenotoxins (PTX) (Yasumoto *et al.* 1985; Hamano *et al.* 1986; Ishige *et al.* 1988; Draisci *et al.* 1996; Daiguji *et al.* 1998; Burgess and Shaw 2001), yessotoxins (YTX) (Terao *et al.* 1990; Yasumoto and Murata 1993; Ogino *et al.* 1997; Aune *et al.* 2002) or azaspiracids (AZA) (Daiguji *et al.* 1998; Ofuji *et al.* 1999; Quilliam 1999; Draisci *et al.* 2000,Yasumoto, 2001).

The classification of PTXs, YTXs and AZAs with the DSPs occurred due to the symptoms induced by these toxins resembling those of the OA class, with the additional fact that these toxins can often be found in combination with known diarrhoetic toxins in shellfish. PTXs, YTXs and AZAs have not been confirmed to be diarrhoea-causing and thus their classification is under review. Moreover, despite PTX2-SAs being currently classified with the DSPs in Australia, there is little evidence to support this characterization and thus there is debate over their classification with DSP causing toxins (Burgess and Shaw, 2001).

Oral ingestion of the DSP toxins can lead to the gastrointestinal disturbances of acute diarrhoea, nausea, vomiting and abdominal pain, with symptoms often beginning within 30 minutes of consumption of contaminated shellfish. No human mortalities to date have been reported in the literature from any cases of DSP poisoning, although there has been considerable morbidity resulting in hospitalization. Additionally, research has shown that PTX-1 is hepatotoxic (TERAO et al. 1986; ZHOU et al. 1994) and PTX-2 shows potent cytotoxicity to human lung, colon and breast cancer cell (JUNG et al. 1995). Certain DSPs, for example OA, have been found to be potent protein phosphatase inhibitors (LUU et al. 1993; FESSARD et al. 1994) and cause neurodegeneration (Arias *et al.* 1993). Protein phosphatase inhibitors are associated with tumour promotion and it may be possible that individuals exposed to shellfish contaminated with certain DSPs have an increased risk of developing cancer.

DSP toxins are produced by several of the *Dinophysis* species including *Dinophysis acuta, D. fortii, D. acuminata, D. norvegica, D. mitra* (Lee *et al.* 1989; Yasumoto and Murata 1990), and *D. caudata* (Eaglesham *et al.* 2000), in addition to being produced by benthic species such as *Prorocentrum lima* (Pillet and Houvenaghel 1995; Pillet *et al.* 1995). DSP toxins have been detected in both axenic and nonaxenic batch cultures of *Prorocentrum* species thus indicating that production of toxins by the algae themselves occurs and does not support the theory that a symboiant bacteria is exclusively producing the toxins (Kodama 1988; Prokic *et al.* 1998), this issue has been thoroughly reviewed by Quilliam (Quilliam 1999). In various species of algae an intraspecies variation and a seasonal variation in the toxin content and toxin profile is seen (Lee *et al.* 1989). Lee et al (1989) postulated that these variations may account for the different toxin profiles seen in algae collected from different countries and noted that toxin production may be influenced by environmental factors rather than solely differences in genetic composition (Lee *et al.* 1989). Additonally, DSP toxin synthesis has been found to be restricted to daylight periods and coupled to G_2 and S phase cell cycle events (Pan *et al.* 1999). The biological function of DSP production in dinoflagellates is not currently understood but investigations have reported that OA may serve a purpose as a specific mitogenic factor for *P. lima* and was able to inhibit the growth of another species thus providing the OA-producing *P. lima* with a competitive advantage (Aguilera *et al.* 1997).

Pectenotoxins are a group of toxins associated with DSP and isolated from algae commonly known to produce other DSPs such as OA and DTX-1. Examples of such algae include the dinoflagellates *Dinophysis acuta* (James *et al.* 1999; Suzuki *et al.* 2001), *D. fortii,* (Lee *et al.*

1989; Draisci *et al.* 1996; Suzuki *et al.* 1998; Vale and Sampayo 2002), *D. acuminata* (Burgess 2003, PhD thesis, Griffith University) and *D. caudata* (Burgess 2003, PhD thesis, Griffith University and Geoff Eaglesham, QHSS oral communication). Although classified with the DSPs, as the PTXs are often found in combination with other DSPs in shellfish, there is debate over whether these toxins should be classified as DSP toxins. Some groups have found mild diarrhetic effects caused by the administration of PTXs (Ishige *et al.* 1988) while others have found no such evidence (Terao *et al.* 1986; Edebo *et al.* 1988). Additionally, many DSPs have been found to be potent phosphatase inhibitors, but PTX-1 and PTX-6 were found to be inactive against PP-1 and PP2A (Luu *et al.* 1993) and current research indicates that the PTX2-SAs do not to cause protein phosphatase inhibition (personal communication, Luis M. Botana López Univ. Santiago de Compostela, Lugo-Spain).

Pecteonotoxin-2 was first identified in Europe in the Northern Adratic Sea but it was then known to be more abundant in Japan (Yasumoto *et al.* 1985; Draisci *et al.* 1996). Investigation of the chemistry of PTX's has facilitated the elucidation of new structures and there has been rapid progression in the identification of new analogues. It is believed that several of the pectenotoxins are derived from a parent pectenotoxin, where the parent molecule is metabolized within the scallops to form other pectenotoxin analogues (Yasumoto and Murata 1990; Sasaki *et al.* 1997; Daiguji *et al.* 1998; Sasaki *et al.* 1998; James *et al.* 1999; Suzuki *et al.* 2000; Vale and Sampayo 2002).

In December of 1997 there was a DSP poisoning incident in northern NSW, where over 100 people living in the Eastern states of Australia were poisoned following consumption of pipis (*Donax delatoides*) harvested off the coastline of Northern NSW (Quilliam et al., 2000). This particular incident included 56 cases of hospitalisation in NSW alone. At the time of the incident the shellfish responsible for the poisoning were found to contain relatively high levels of PTX-2SA with low levels of PTX-2 and OA. The levels of PTX-2 and OA were considered too low to be the cause of the DSP symptoms seen, and thus the PTX2-SAs were thought to contribute as the causative agent to the symptoms observed in the victims.

The Australian DSP incident of 1997 demonstrated the need to determine the toxicology of these associated PTX2-SAs in commercial shellfish. The implementation of guidelines for PTX2-SA levels in shellfish produced in the absence of toxicological data has caused a great economic burden on the Australian shellfish industry. Thus, in response to this incident, a comprehensive toxicological study was initiated to investigate the possible pathological and mechanistic actions of the PTX2-SAs present in Australian shellfish and provide information to aid in the establishment of more appropriate guidelines.

4.0 NEED

The need for the research is demonstrated by the lack of a suitable guideline value for pectenotoxins in shellfish that has been based on scientific data. To produce these guidelines toxicological data needs to be produced and currently there is a lack of knowledge for PTX2-SA metabolism and mechanisms of toxicity. There is currently no oral toxicology data on the PTX2-SAs and thus their impact on human health cannot be assessed. There have been no published works of their metabolism in mammals or humans, and their chronic effects on health are unknown.

5.0 OBJECTIVES

A comprehensive study has been undertaken in which the aims are to

1. Develop a robust method for extraction and purification of PTX2-SAs from shellfish and algae
2. Produce purified PTX-SAs extracted from shellfish and marine algae for investigations
3. Conduct acute oral and *i.p.* studies in mice to determine the toxicopathology and body distribution of PTX2-SAs
4. Investigate mechanisms of toxicity of the PTX2-SAs with the use of a cDNA microarray technology and *in vitro* cell culture assays
5. Perform a health risk assessment for the consumption of seafood contaminated with the PTX2-SAs to aid establishing guideline values for pectenotoxins in shellfish with respect to the 1997 Northern NSW poisoning incident.

This study will provide a greater understanding of the metabolism and mechanisms of toxicity for the PTXs and provide information useful in the clinical assessment of DSP poisoned patients and also provide information to be used in the setting of guideline values for pectenotoxins in seafood.

6.0 METHODS

6.1 Safety

For this project all procedures were conducted in a manner of Good Laboratory Practice (GLP), in accordance with Material Safety Data Sheet (MSDS) guidelines and all procedures involving animals conformed with the 'Australian code of practice for the care and use of animals for scientific purposes' (NHMRC 1997).

6.2 Toxin extraction

The PTXs in this study were extracted from pipis (*Donax deltoides*) collected from Ballina, NSW and oysters and mussels collected near Port Lincoln, SA, or from *Dinophysis caudata* bloom (plankton net) samples collected off the coast of Ballina, NSW. The toxins were extracted into methanol with sonication and centrifugation followed by cleanup stages employing organic partitioning, column chromatography with sephadex LH-20, preparative HPLC and analytical HPLC separation with a mobile phase of 70:30% acetonitrile/water with 2mM ammonium formate and 0.01% formic acid. Toxin composition and concentrations were determined by HPLC-MS. The HPLC-MS confirmed that there was no okadaic acid, DTX-1, DTX-2, DTX-3 or PTX-2 in the samples.

6.3 Stability studies

The pectenotoxins are known to be unstable after isolation from algae or shellfish (Sakaki et al., 1998) and during the process of extracting and purifying PTXs for this project the percentage composition of the seco-acids in stocks altered during different stages of purification. These changes or loss of toxins seemed to occur randomly and thus investigations were undertaken to try and identify which physicochemical factors caused changes in the composition of the seco-acids in the stock toxin mixtures. Investigations were conducted on the stability of the seco-acids in water, in a mobile phase of acetonitrile and water, in different environments of temperature (freeze-thawing, 37°C, boiling to evaporation, and storage at room temperature or freezing), by drying under nitrogen, and exposure to UV light. Investigations were also conducted on the effect of biological fluids from mice such as blood and gastric juices (*in vitro*). The effect of citrate and lithium heparin on the seco acids were also investigated, as these chemicals are used as blood preservatives in toxicology investigations.

All chemical studies were conducted with 3 toxin samples A, B and C (Table 1). These stocks were extracted and purified from *D. caudata* bloom samples. Toxin sample A was composed of PTX-2 only. Toxin samples B and C were composed of PTX2-SA and 7-epi-PTX2-SA mixtures with no PTX-2. These stocks were used for test samples and for controls. All experimental samples were performed in triplicate with controls.

Table 1. Stock toxin samples A, B and C concentrations determined by HPLC-MS.

Original	Stock A		Stock B		Stock C	
	μg/mL	% Distribution	μg/mL	% Distribution	μg/mL	% Distribution
PTX 2 SA	0	0	23	76	4.5	30
7-epi-PTX-2 SA	0	0	7	24	11	70
PTX-2	33	100	0	0	0	0

6.4 Hydrolysis reaction for OA acyl esters

Standard analysis for DSP toxins in the past has not included an alkaline hydrolysis reaction to detect OA acyl esters, which have been documented to contribute to incidences of DSP in Portugal (Vale et al., 2000, Vale et al., 2002). In light of this evidence it was necessary to determine if the pipis from the NSW poisoning incident of 1997 contained these OA acyl esters. Pipis (*Donax delatoides*) for this investigation were supplied by Gustaaf Hallegraeff, university of Tasmania, who had been keeping shellfish from the NSW poisoning incident stored frozen. The samples arrived to our laboratories frozen and with official documentation from the NSW Health Department.

The method to detect OA acyl esters involved homogenising 100g of shellfish tissue; triplicate samples (4g) of homogenate were mixed with 16mLs of 90% methanol and homogenised again at high speed. Samples were centrifuged and the supernatant was transferred to a 30mL glass tube. Two mLs of supernatant was mixed with a small volume of 2.5N sodium hydroxide and samples were heated at 75°C for 45 minutes. Samples were cooled, neutralised with 2N HCl and filtered prior to analysis. The remaining methanolic extract was cleaned with hexane washes and also analysed for toxins.

6.5 Toxicology studies

Investigations have included *in vivo* toxicopathology studies, distribution studies, urinary biomarker detection of lipid peroxidation (malondialdehyde), and *in vitro* assays employing flow cytometry and cDNA microarray technology.

6.5.1 Aims

1. To determine the main target organs for PTX-2-SA and 7-epi-PTX2-SA and observe the pathology that is caused by these toxins when ingested via the oral and *i.p* routes.
2. To determine if a single dose affects the behaviour of the mice
3. To compare the oral and *i.p* toxicology for the seco acids with the toxicology findings for PTX-2
4. To provide a reasonable estimate of a NOAEL or LOAEL.

6.5.2 Mice

Female C57/BL/6J and SJL/J mice (circa 20g) were randomly distributed into groups and acclimatised for 14 days. The temperature was controlled at 22±2°C with a 12h light/dark cycle. Rodent food pellets and tap water were provided ad libitum. Following all doses, mice were observed continuously for 6 hours and then periodically until termination. Daily weight gain/loss, food and water consumption was monitored. Urine and stools were collected and were either extracted for toxin analysis or analysed for specific urinary biomarkers. Mice were killed by carbon dioxide overdose at 3, 6, 12 and 24 hours or at 14 days post dose. Liver, kidney, entire GIT, lung and spleen tissues were taken at autopsy and fixed in either 3% glutaraldehyde for electron microscopy (EM) or in 10% formalin for light microscopy and processed for histological analysis with haematoxylin and eosin staining. The GIT was fixed inside and out to ensure good structural preservation. The entire liver was removed, washed in saline, blotted and weighed.

6.5.3 Preparation of total toxin for dosing

A known concentration of toxin stock was diluted in a calculated volume of milli Q water to produce the target dose concentrations of PTX2-SA. Doses were prepared immediately prior

to administration as stability studies in our laboratory showed the conversion of PTX2-SA to 7-epi-PTX2-SA begins to occur after 10 minutes in water. The dose, at a volume of 0.5 mLs, was drawn into a syringe and the dose was administered by a single gavage or *i.p* injection. Control mice were dosed with toxin vehicle alone.

The dose concentrations were confirmed with LC-MS. Use of the LC-MS also confirmed that there was no okadaic acid, DTX-1, DTX-2 or PTX-2 in the samples. Examinations of an alkaline hydrolysate showed that there were no significant levels of DTX-3 present (for example, 1.8 ng/kg in the dose given to each animal in the 875µg/kg study).

6.5.4 Stool and urine.

Extractions: Stools and urine were collected in separate containers within the metabolism chambers. The stool samples were ground with a mortar and pestle. Then the stool samples and the urine samples were extracted separately with 10mLs methanol and put on a rolling mixer for 24 hours. Samples were centrifuged, mixed with 15 mLs hexane, partitioned and separated, and then mixed with an equal volume of chloroform, with partitioning and separation. The samples were then rotary evaporated to dryness and taken up in 2 mLs of methanol. PTX concentrations were determined with the use of HPLC-MS.

The urinary biomarker malondialdehyde (MDA) was investigated to detect for oxidative lipid damage. Urine was collected following dose at the times specified in table 2. Urine preservatives were added and samples were stored in the freezer until analysis.

2.4.5.2 Measurment of malondialdehyde in mouse urine

Reactive oxygen species are known to be involved in many disease processes involving lipid peroxidation of cell membranes (Kehrer, 1993). Malondialdehyde, detected in tissues and in urine is known to be a biomarker of such damage (Mihara and Uchiyama 1978; Bagchi *et al.* 1993; Chaudhary *et al.* 1994; Draper *et al.* 2000). Urinary MDA was measured in mouse urine to determine if the PTX2-SAs and PTX2 caused lipid peroxidation *in vivo* following dosage with PTX2-SAs and PTX2. Urine was collected following dosage at the times specified in Table 2. Urine preservatives of 0.1% BHT and 1% desferal were added and samples were stored in the freezer until analysis.

All chemicals for MDA analysis were purchased from Ajax chemicals (Australia) and all reagents and stock solutions were prepared as previously described (Beljean and Bruna 1988). The method of urinary MDA measurement employed by this project is a gas-liquid chromatography (GLC/MS) method developed by Beljean-leymarie and Bruna, (1988). MDA results were normalized against the ratio of urinary creatinine to protein and compared to control mice urine that were administered toxin vehicle alone.

2.4.5.3 Urinary creatinine and protein measurement

Analysis of urinary creatinine and protein were assayed for all samples being processed for urinary MDA. Analysis of urinary creatinine and protein was provided as a service by the Veterinary Pathology Department at the University of Queensland.

6.5.5 Tissue extractions

Blood was taken into citrate by heart puncture. All tissues for distribution studies were taken at necropsy weighed and immediately placed in an aliquot of methanol. Samples were homogenised in a tissue homogeniser and extracted for toxins in the same manner as shellfish

tissue with the exception that once the chloroform extracts were evaporated under nitrogen the samples were taken up in a 300µl of methanol for HPLC analysis.

6.5.6 Summary of in vivo investigations.

All studies were single dose investigations. Studies were carried out with oral and *i.p* routes of dosing to enable comparison of observed toxicology of the PTX2-SAs, if any existed, with that of published toxicity information for other DSP toxins.

Table 2

Study	Toxin	Dose µg/Kg	Exposure time
Oral	PTX2-SA (mix of 2SA and 7-epi-2SA)	25, 190, 340, 500, 875, 1280	3h, 6h, 12h, 24h, 14 days 3h, 6h and 12h
	PTX2-SA only 7-epi-PTX2-SA only SA-unidentified 'early peak	1600 600 660	3h, 6h 3h, 6h 3h, 6h
	PTX-2 only	320	3h, 6h, 24h
IP	PTX2-SA (mix of 2SA and 7-epi-2SA) PTX-2	250, 640 300	3h, 6h, 24h 3h, 6h, 24h
Distribution studies oral and ip	PTX2-SA (mix of 2SA and 7-epi-2SA) PTX-2	300 300	30min, 1h, 6h, 12h, 24h 1h
MDA detection in urine oral and ip	PTX2-SA (mix of 2SA and 7-epi-2-SA PTX-2	25, 750 650	2 x 24h collection 2 x 24h collection

6.6 *In vitro* toxicology: microarray and cell cytotoxicity studies

6.6.1 Introduction

With considerations of reduction of animals for toxicology investigations it was decided to perform *in vitro* toxicity tests with the PTX2-SAs. PTXs have been found to be potently cytotoxic to various human cancer cell lines (Jung et al. 1995). For this reason the effect of PTX2-SAs on cell cycle distribution and gene expression were investigated utilizing a permanent human cell line with cDNA microarray technology and flow cytometry.

Microarray techniques, performed with RNA from both *in vivo* and *in vitro* sources, have been shown to be a very useful and informative tool in toxicological investigations (Burczynski *et al.* 2000; Hegde *et al.* 2000; Liu *et al.* 2001; Fang *et al.* 2002). Use of these techniques may help elucidate toxic mechanisms involved in their potential cytotoxicity and predict any chronic toxicity that could be caused by the PTX2-SAs.

For the cell cycle and microarray study, it was firstly required to determine what dose and duration of PTX2-SAs to give the cell line. Thus, an *in vitro* cytotoxicity study was

conducted to determine the lethal concentration that caused death in 50% and 30% of cells (LC50 and LC30). The results for the cytotoxicity and cell cycle investigations are discussed comparatively with the microarray data.

6.6.2 Toxin Extraction And Preparation.

Cytotoxicity studies were conducted with eight PTX2-SA extracts. These included PTX2 extracted from algae, and seven samples containing individual PTX2-SA isomers extracted and purified from pipis and oysters. A toxin batch of 50% PTX2-SA and 50% 7-epi-PTX2-SA, from algae only was used for cell cycle and microarray investigations. All doses were prepared in the relevant media immediately prior to dosing as stability studies have shown the conversion of PTX2-SA to 7-epi-PTX2-SA begins to occur after 10 minutes in aqueous solutions. Control cells were dosed with 0.1% methanol in DMEM.

6.6.3 Cell Line And Animals

Human liver cells (HepG2) were selected as the cell line of choice for studies, as reported i*n vivo* and *in vitro* investigations have shown various PTXs to be hepatotoxic by causing rapid necrosis of hepatocytes (Terao *et al.* 1986; Ishige *et al.* 1988; Aune 1989; Zhou *et al.* 1994; Mi and Young 1997; Fladmark *et al*. 1998). In addition, distribution studies conducted during this PhD had revealed that PTX2-SAs can be detected in the mouse liver within 30 minutes following oral gavage.

The HepG2 cell line used for these experiments were sub-cultured from established cultures held in the Applied Research Centre for Genomic Technology within The Department of Chemistry and Biology of The City University of Hong Kong, SAR, P.R. China.

6.6.4 Maintenance Of $HepG_2$ Cells

The cells were maintained in $80cm^2$ flasks in DMEM supplemented with 10% heat-inactivated foetal bovine serum and 1% antibiotics at 37ºC in a 5% CO_2/95% air humidified atmosphere. For sub-culturing, cells were washed twice with 10mLs 0.01M PBS and detached from cell culture by exposure to a 5% trypsin/EDTA solution for 5 minutes at 37ºC. Following this incubation, the flask was gently tapped to dislodge cultures and cells were observed under a microscope to ensure detachment had occurred. The trypsination was stopped by adding sufficient media (15mL) to dilute the trypsin. Viable cells were counted with a heamocytometer using trypan blue exclusion staining (Kaltenbach *et al.* 1958). Cells were then either sub-cultured into separate flasks, seeded into 96 well plates for cytotoxicity studies, 6 well plates for flow cytometry cell cycle studies or into culture dishes for microarray studies using appropriate cell densities. The flasks or culture plates were then sealed and incubated at 37ºC for 24 hr prior to the addition of toxins for investigation.

6.6.5 Methods Employed For Investigations Into The Effects Of Cell Cycle Distribution

Toxin dose preparation.

PTX2-SAs from toxin batch 2 (see 7.1) were used for cell cycle studies and only one dose of the toxin was chosen for cell cycle studies due to the limitation of toxin availability. This dose was determined from the results of the cytotoxicity study for $HepG_2$ cells and was the concentration producing 30% cell death (LC30) at 96 hours in $HepG_2$ cells (800ng/mL). The investigations were conducted at the earlier, minimally cytotoxic time point of 24 hours. A calculated volume of toxin stock was dried under nitrogen and re-suspended in 0.1% methanol in DMEM to produce a final concentration of 800ng/mL of PTX2-SAs with sonication for 3 minutes. Control cells were dosed with 0.1% methanol in DMEM.

Cell culture

Viable cells were counted with a haemocytometer using trypan blue exclusion staining and seeded into 6 well plates using cell densities of 4 $X10^5$ cells/mL (12 hour incubations), 3 $X10^5$ cells/mL (24 hour incubations), 2 $X10^5$ cells/mL (72 hour incubations). Cells were left to attach to the plates overnight at 37ºC. The following day, 2mL of media/ toxin preparation of 800ng/mL (freshly prepared) was added to each of the treatment wells, with control cells being dosed with 0.1% methanol in media (2 mL). Cells were incubated for 12, 24 or 72 hours.

Cell cycle analysis by flow cytometry

Following the timed treatment period with PTX2-SAs, cells were removed from the incubator and observed under the light microscope for changes in cell morphology. Media was transferred from the wells to labelled FCS tubes and cells were detached from the plates with 500uL of trypsin/EDTA. Once the cells were detached from the plate the trypsin/EDTA was diluted with media and a pipette was used to ensure cells were well separated. The media was transferred by pipette to the respective FCS tubes and cells were collected by centrifugation (100xg for 5 min). The supernatant was decanted into storage tubes for further analysis and the pellet was re-suspended gently in 1mL of hypotonic propidium iodide (30μg/mL) solution (prepared in 0.1% sodium citrate treated with 0.1% Triton X-100 and 20μg/mL DNase-free RNase) for DNA staining. The samples were mixed by pipette to ensure good dispersion of the pellet and placed on ice. Triplicate samples of stained cells were analysed for cell cycle changes within the populations of G_0/G_1, S, and G_2/M immediately with a FCS Calibur flow cytometer. Data was analysed by Modfit^{TM} LT (version 2.0) cycle distribution software and statistical analysis was performed with a paired, two-tailed Student's t-test.

6.6.6 Methodology for the investigation of changes in gene expression in $HepG_2$ cells exposed to seco acids with the use of a high density cDNA microarray.

Cell culture and toxin preparation

When cell cultures had reached confluency, cells were detached from culture flasks with trypsin/EDTA and plated into 8 culture dishes (10cm diameter). Cells were left to attach to the plates and ensure confluency for 24 hours at 37°C. A dose of 800ng/mL PTX2-SAs was chosen for the microarray to maintain consistency with the cell cycle studies and was prepared by evaporating 559 μL stock under nitrogen, re-suspending in 30uL methanol and diluting in 30mL media with sonication for 3 minutes. Media was removed from the culture dishes and 7.5 mL of dosed media was then added to each plate delivering 800ng/mL of seco acids to the cells. The control dishes were dosed with 7.5mL of 0.1% methanol in media. All dishes were returned to the 37°C incubator for 24 hours. At 24 hours morphology was observed under the light microscope prior to RNA extraction. Photographs of changes in morphology were not taken of the cells in the microarray study as this would involve having the cells out of the incubator for a sustained period and may cause the microarray to be altered with the inducement of early stress genes. Cells at confluency in 10cm dished were known in the laboratory to yield at least 150ug RNA (the required amount for a microarray) and confluency was reached at 24 hours post dosing with the cell density that was plated.

RNA extraction

RNA was extracted as per the manufactures guidelines for Trizol with minor modifications. The media for the 4 control and 4 treatments dishes was poured away and dishes were blotted on tissue to remove residual media. The cells were washed with serum-free media to remove all traces of FBS which reacts with the Trizol reagent. The waste media was decanted, dishes were blotted on tissue and 1mL of Trizol was added to each dish with swirling. The Trizol/cell mixtures were transferred to two polyprolene tubes - one for the treatment RNA and one for the control RNA. A further 1 mL of Trizol was added to the culture dishes to wash any traces of remaining cells and this was added to the respective polypropylene tubes. The RNA samples were then centrifuged (9460xg) for 12 minutes at 4°C. The supernatant was carefully removed with a pipette to produce several 1 mL aliquots in labelled microcentrifuge tubes. To the 1 mL of supernatant, 220uL of chloroform was added and mixed by vigorous inversion to cause phase separation of the RNA and DNA layers. The tubes were centrifuged (15,000xg) for 15 minutes at 4°C.

The aqueous layer (approx. 500uL) was removed with a pipette and mixed with 500uL of ice-cold isopropanol. The samples were mixed by vigorous inversion and placed in a freezer (-20°C) for 20 minutes to precipitate the RNA. The samples were then centrifuged at approx. 10,000xg at 4°C for 10 minutes. The isopropanol was poured off and the pellet was washed with 1mL of ice cold ethanol (75%), tapping the tube gently to dislodge (but not dissolve) the pellet. The samples were once again centrifuged (approx. 10,000xg) at 4°C for 10 minutes. The ethanol was poured off and remaining ethanol was evaporated from the samples at RT. Any remaining water was removed with a pipette. The samples were taken up in 20uL of prewarmed (55°C) RNA storage solution. The samples were vortexed for a few seconds, tap spinned in a centrifuge to ensure all the sample was at the bottom of the tubes and incubated at 55°C for 5 minutes. Samples were vortexed and tap spinned again. Aliquots of the treated and the control samples in storage solution were combined (total yield approx. 90μL) and samples were again vortexed and tap centrifuged prior to RNA quantification with the RNA biophotometer which used milli Q water as the blank. The control and treated RNA samples were diluted 1 in 100 with milli Q water for quantification.

Gel electrolphoresis.

Gel electrophoresis was performed to ensure the RNA was of sufficient quality and purity for hybridization in the microarray. A 0.1% agarose gel was made up and left to set whilst the samples for electrophoresis were prepared. The 1:100 dilution quantified with a biophotomer was used for electrophoresis. A minimum of 200ng of RNA is required for electrophoresis and thus the following volumes of loading dye and RNA samples were mixed on parafilm.

	Control	Test
Loading Dye μL	2	2
Sample μL	5	3
Milli.Q water μL	3	5
Total vol. for loading on to gel (μl)	8	8

The electrophoresis tank was filled with 0.5x TE buffer and the samples were loaded on the gel with a pipette immediately after being mixed. The gel was run with 80-100 volts for approximately 30 minutes. Visualization was by fluorescence photography with a Gel Doc 1000 coupled to Molecular Analyst (version 1.5) with an integration time of 0.70 seconds.

The RNA samples were then diluted to produce 50 ±3 μg/mL aliquots. The diluted samples were incubated at 55°C for 10 minutes, tap spun and re-quantified to ensure the RNA concentration in the aliquots were approximately 50μg/mL. Aliquots were then combined to produce samples of 30μL which is equivalent to 150μg/mL RNA (sufficient for 1 array).

Direct incorporation labelling for total RNA

This methodology used total RNA as the input to the labelling reaction instead of the standard mRNA. The following labelling reaction was done separately for both the control samples and for the test samples. All work was done in an RNAse free environment inside a tissue culture hood and all prepared reagents and tubes were autoclaved at 121°C for 90 minutes.

1. RT labelling reaction

Thirty microliters of extracted RNA was mixed with 8μL of oligo dT primer and incubated at 70°C in a thermal PCR controller for 10 minutes. The samples were then chilled on ice for 10 minutes. During this time the RT reaction mix was made up for cDNA synthesis. Each component of the reaction mix was added in the following order with the SSII RT being added immediately before use of the reaction mix:

Component	**Volume μL (sufficient for two reactions)**
RNAse inhibitor	2
5X Buffer	33.6
10X low T dNTPs	16.8
DTT (0.1M)	16.8
SSII RT	8.4

After 10 minutes on ice the RNA samples were mixed with 38.4μL of RT reaction mix. Then 4μL of Cy3 and Cy5 dUTP was added to the control and to the treatment samples respectively. Cy3 dUTP is red in colour and Cy5 dUTP is blue in colour. The dyes were added, mixed with a pipette and tap centrifuged to ensure all the reaction mix was collected in the bottom of the reaction tube. The samples and controls were then incubated at 42°C for 2.5 hours. Every hour of the incubation the samples were briefly mixed with gentle tapping of the tubes then replaced in the incubator.

2. Hydrolysis

Twenty microlitres of a 0.5M NaOH and 100mM EDTA mixture was added to each reaction vial to stop the cDNA synthesis. Samples were mixed by vortex and incubated at 65°C for 10 minutes. Samples were neutralized by adding 20μL of 0.5M HCL in 1M tris buffer (pH 7.4) and stored at –20°C until the clean up stage.

3. Clean up stage

This stage was performed to purify the cDNA and decrease the background DNA present. Keeping the samples in low light conditions, the three Cy3 and three Cy5 reactions were paired in triplicate Microcon YM-30 tubes. These tubes contain filters that filter the reaction mix containing small 'background' fragments of cDNA and retained the test cDNA on their filter. The three tubes were centrifuged at 13,000 RPM for 3 minutes. The Microcon 30 concentrators were then filled with 500uL of 1 X TE buffer (pH8.0) and recentrifuged at 13,000rpm for 10 minutes to wash the retained cDNA. This wash step was repeated a further 2 times, each time discarding the waste buffer. The probe solution was eluted from the filter by inverting the filter in the tube (carefully) and re-centrifuging at 2000 rpm for 1 min.

4. Hybridisation.

The following blocking reaction mix was made and added to 40uL of the prepared cDNA.

Component	**Volume μL**
Poly dA (8mg/mL)	2
Yeast tRNA (4mg/mL)	2
CoT-1 DNA (10mg/mL	2
20X SSC	6
10% SDS	0.4

The volume of hybridized sample required for a microarray glass slide (9984 genes, 6cm x 2cm hybridization area) was approximately 60μL. The tubes containing the cleaned hydrolysis products were wrapped in parafilm and aluminium foil and the probes were denatured at 100°C for 2 minutes, then left to cool at room temperature. Samples were then spun briefly and applied to the microarray slide. The microarray was placed in a humid box on an oscillating mixer for 1 hour before being placed in an incubator for 16 hours at 65°C. Following incubation, the slides were processed though the following washing steps in 50 mL conical tubes at 37°C:

- The chip was soaked in 40mL of 2X SSC, 0.1% SDS solution for several minutes in a shaking water bath until the cover slip fell off from the chip. The chips were then transferred to new 2X SSC/ 0.1% SDS and incubated for 15 minutes in the shaking water bath. This step was repeated once.
- The chips were removed from the X2 wash and transferred to 0.02X SSC and incubated in the shaking water bath for 15 minutes, this step was repeated a further 2 times.

Finally the slides were transferred to a new 50 mL Corning centrifuge tube with a Kimwipe in the bottom and were spun (1000xg) for 3 minutes to remove all washing solutions.

6. Scanning and data analysis

After spinning the slides were immediately scanned with GenePix 4000 and analysis was performed with GenePix Pro 3.0 analysis software.

7.0 RESULTS AND DISCUSSION

7.1 Toxin Extraction

Table 3. A summary of extraction sources for different batches of the PTX2-SAs.

	Shellfish	Source
Batch 1	Pipis Oysters Mussels	Ballina Consignment 1 SA Consignment 1 SA Consignment 1
Batch 2	Algal bloom samples	Ballina
Batch 3	Pipis	Ballina Consignment 1
Batch 4	Oysters	SA Consignment 2

Stock batches of PTXs were monitored every few months to ensure they maintained their concentrations and composition of SAs. Table 4 below shows how one batch of toxins that were extracted from shellfish did change its composition and concentration during the course of this project.

Table 4

Batch 1	PTX2-SA	7-epi-PTX2-SA	Total
Month 1	127µg	85µg	212µg
Month 2	45µg	107µg	152µg

This change in distribution of seco acids has not been able to be explained by the results of the chemical stability studies as no significant degradation for the environmental factors that were thought may affect such a change, such as repeated freeze-thawing, were found. It is thought that this particular batch (1) contained an additional component, which eluted with the PTX2-SAs during extraction, and affected the physical stability of the batch.

7.2 Stability studies

Stabilities studies showed that PTX2-SAs were affected by exposure to water, citrate, lithium heparin, blood and gastric juices, boiling and being left dry on glass for 24 hours. PTX2 was converted to seco acids in small quantities by exposure to blood, gastrointestinal juices and mobile phase. PTX2 appeared to be destroyed by boiling to evaporation as it was neither detectible nor converted to seco acids following boiling of the test samples. No significant difference in toxin composition or concentration was observed for other test conditions such as exposure to UV light and other parameters as detailed in the methodology.

7.3 Hydrolysis reaction for OA acyl esters

Our laboratories were supplied with pipis from the 1997 NSW poisoning incident and following a hydrolysis reaction it was possible to see that the pipis from the 1997 NSW poisoning incident contained significant concentrations of OA acyl esters that were not detected at the time of the incident. Results showed that the pipis contained approximately 1µg/g pipis flesh of OA acyl esters. Although reportedly less toxic than okadaic acid itself, the concentrations of OA acyl esters present may have been sufficient to cause the observed symptoms. With the varying and extreme pH environments in the gastro-intestinal tract, it is theorized that these esters could be hydrolyzed in the human gastro-intestinal tract to release okadaic acid. In the light of this new evidence it is reasonable to assume that these OA acyl esters were the most likely the causative agent of the diarrhectic symptoms reported in the

1997 NSW DSP incident and not the PTX2-SAs. There have been official reports of OA acyl ester involvement in DSP poisoning incidents in Portugal (Vale *et al.* 1999; Vale and Sampayo 2002; Vale and Sampayo 2002). Alkaline hydrolysis of shellfish samples revealing high levels of OA have also been reported for shellfish in New Zealand (MacKenzie *et al.* 2002) and in Spain (Morono *et al.* 2002). Additionally, an OA diol-ester has been shown to be almost as toxic as OA in a diatom bioassay (Windust *et al.* 1997). Hydrolysis of shellfish samples suspected of containing DSP toxins is now routinely performed in QHSS as a consequence of this information and the reported poisoning incidents involving OA esters (G. Eaglesham, personal communication).

7.4 *In Vivo* Toxicology Results

7.4.1 Pathology studies

Acute oral dosing

None of the characteristic lesions for DSP toxins were observed in the mice following PTX2-SA dosing. No mice died within the time periods indicated and no diarrhoea was observed in any studies.

Only batch 1 of PTX2-SA toxin extractions demonstrated any significant pathological findings in mice. These pathological findings were consistent with a gastrological irritant causing severe necrosis of microvilli in the duodenum and severe haemorrhage of the glandular stomach. This toxicity was only observed with 'batch 1' toxins used in the pilot study investigations. Batch 1 PTX2-SAs were extracted from pipis and coupled to an oyster and mussel extract from South Australia – the extracts of the separate shellfish were combined to keep a consistency in stock PTX2-SAs for toxicology purposes. All follow up studies with PTX2-SA extracts from shellfish and algal sources (batches 2-4) have induced no significant pathology whatsoever. Analysis of this first batch of toxins has not revealed any other known DSP toxins such as OA, OA derivatives such as acyl esters or DTXs, and contained no PTX-2. A large number of pipis from the same consignment as batch 1 were re-extracted and no other known shellfish toxins, including gymnodidium and yessotoxin, could be detected from the pipis, and thus it is believed the causative agent was most likely present in the extract of mussels and oysters from SA, of which the entire consignment was used in the first extraction. At this point in time the causative agent of the lesions and changes in behaviour seen in the mice orally administered with batch 1 PTX2-SAs has not been identified. All the studies were conducted to GLP and we do not believe this to have been caused by a laboratory-derived contaminant in the toxin vehicle or from glassware as no lesions were observed in control animals. Laboratory investigation is continuing on this issue. This finding demonstrates the requirement to maintain and support regulatory inclusion of the mouse bioassay in testing of shellfish toxins to identify 'un-known ' and 'novel' shellfish toxins, and also highlights the importance of toxin purification processes.

Acute *i.p.* dosing

No mice died during the observed time periods for any of the toxin preparations. Pathological observations seen at the LM level included sub-capsular haemorrhage damage to the liver with PTX2 doses but no pathology was observed with PTX2-SAs.

EM investigations with low PTX2-SA doses did find changes in the basal lamina of cells in the villi of the duodenum. The significance of these changes has not been fully explored and it was not determined if these changes would correcte themselves over time - usually a 24h

cycle leads to a renewal of surface villi within the intestinal tract. Mitochondria were also altered – indicating of changes in oxidative phosphorylation were occurring and hyperplastic RER was also observed.

7.4.2 Distribution studies

The target organs where the PTX2-SAs were detected included the liver and GIT tissue for both oral and *i.p* dosing. It was found that the PTX2-SAs could be detected in the GIT contents within 30 minutes of administration via *i.p* – therefore a significant amount is excreted rapidly into the GIT unchanged.

7.4.3 Malondialdehyde studies

MDA investigations have been performed with both oral and *i.p* dosing. Dosage of PTX2-SAs caused a significant pertubation of MDA excretion in the urine indicative of lipid peroxidation action *in vivo*. The MDA levels were much lower in the controls than any of the PTX treatment groups and should be considered a valid finding. Another DSP, OA, has been found to cause oxidative lipid peroxidation *in vivo* (Matias *et al.* 1999), in cultured human intestinal cells (caco-2) (Traore *et al.* 2000; Creppy *et al.* 2002), and in a renal cell line (vero) (Matias and Creppy 1996; Traore *et al.* 1999). The results of MDA analysis with PTX2-SAs were further investigated with a cDNA microarray where many genes involving lipid metabolism were altered compared to controls (7.5.3). Lipid peroxidation has been previously associated with numerous disease processes and tumourigenesis (Kehrer 1993; Chaudhary *et al.* 1994; Bachowski *et al.* 1998). Further investigation employing chronic or repeat dose studies are recommended to determine if this lipid peroxidation caused by PTX2-SAs leads to a state of pathogenesis *in vivo*.

7.5 *In Vitro Cell* Cytotoxicity And Microarray Studies

7.5.1 Cytotoxicity of PTX2-SA on $HepG_2$ cells

Toxin vehicle

The mean optical density (O.D.) of control $HepG_2$ cells was compared to the O.D. of $HepG_2$ cells that had been exposed to serial dilutions of toxin vehicle (methanol) in media over a 24 or 96 hour period. It was found that concentrations of methanol less than 0.2% in media did not cause any significant change in cell viability when assessed with the students t-test.

A Prism data analysis tool was used to determine LC30 and LC50 values for 24 and 96 hr PTX2-SA exposures, these values are shown in Table 3.

Table 3

PTX2-SA ng/mL		
Exposure	LC30	LC50
24 hours	2200	Not possible to determine at 800ng/mL
96 hours	922	1225

In gene studies, it is generally known that any quantity of a toxicant or chemical stimulant will cause a perturbation of gene expression. For the purpose of the microarray investigation it was desirable to harvest as much RNA from living cells as possible. To ensure this was possible, a dose was chosen that would cause the least amount of cell death within the chosen exposure time and for this reason it was decided to dose approximately one third to a half of

the estimated LC30 determined from the 24 hour cytotoxicity studies. This concentration was estimated to be 800ng/mL using a 'Prism' data analysis tool. To complete this study it would be preferential, and recommended, to have a range of doses and exposure times to have more information of dose response indices, but this was not possible with limited toxin availability.

6.5.1 Cytotoxicity On $HepG_2$ Cells And Isolated Rat Hepatocytes

Morphological observations in $HepG_2$ cells following doses of PTX2SAs included the development of a speckled appearance of tiny invaginated grooves on the cell surface, and expressed pin-head morphology - a term used where the cells become more spherical and detached from the culture plate. This term has previously been used to describe $HepG_2$ cells treated with DSP toxins (Flanagan *et al.* 2001). In addition to the above morphology described for PTX2-SAs, $HepG_2$ cells dosed with 10µg/mL PTX2 developed blebs on their cell surface. The toxic effects of other DSPs (OA, DTX-1, PTX-1 and YTX) have been investigated with the use of isolated hepatocytes (Aune 1989; Aune *et al.* 1998; Fladmark *et al.* 1998). The study by Aune (1988) described morphological changes in hepatocytes similar to those found in this study. Morphological changes described by Aune were found to be induced by all the DSPs listed above with OA being the most toxic causing blebs to appear on the surface of cells, as was found for PTX2 in this study. The cell rounding and blebbing caused by OA has also been described as an effect on mammalian fibroblast cell lines where a two-stage process was observed with cells first becoming square-shaped then proceeded to become round with 'micro-blebs' on their cell surface when dosed (Fessard *et al.* 1994; Fessard *et al.* 1996)

7.5.2 Investigations into the effects of cell cycle distribution

The results showed a shift of PTX2-SA treated cells arrested in G_2/M phase compared to controls and also showed there was no significant apoptosis induced up to 72 hours following a dose of 800ng/mL PTX2-SAs. In cytotoxicity assays, cell cycle arrest at the G_1 or G_2/M checkpoint is considered a response to DNA damage and allows for repair of DNA prior to entering S phase (DNA synthesis) or mitosis respectively (Freshney 1994). Although no apoptosis was detected in this PhD study, such arrest is thought to precede the onset of apoptosis if the cell is unable to repair extensive DNA damage (Meikrantz *et al.* 1998). Additionally, cellular arrest in the G_2/M check point can be caused by disruption of the formation of microtubules as inhibition of microtubule polymerization is known to affect the ability of cells to enter mitosis (Barrientos and Moraes 1999). It was not determined whether the G_2/M arrest was reversible or irreversible in this study with the PTX2-SAs. Further investigation, including longer incubation periods and higher doses, will be required to determine if PTX2-SAs induce apoptosis in human cell lines. Other DSPs have been shown to cause cytotoxicity to cells. For example, OA has previously been shown to cause an S phase arrhest in caco-2 cells (Traore *et al.* 2001). *In vivo* investigations have been carried out with the related toxins PTX1 and PTX2 that have shown them to be hepatotoxic by causing rapid necrosis of hepatocytes (Terao *et al.* 1986; Ishige *et al.* 1988; Aune 1989; Zhou *et al.* 1994; Mi and Young 1997). *In vitro* studies with PTX1 showed induction of apoptosis in rat and salmon hepatocytes (Fladmark *et al.* 1998); cell death was found to be caused by apoptosis rather than by necrosis, this being determined by the lack of trypan blue uptake and the chromatin condensation typical of apoptosis. In vitro studies with the parent molecule of the PTX2-SAs, PTX2, found this toxin to be potently cytotoxic to various human cancer cell lines (Jung *et al.* 1995). This study with the DNA-cleavage and the rat plasma membrane assay showed that PTX-2 neither exerts its cytotoxic activity by blocking DNA synthesis nor by blocking reduction-oxidation processes within the cell membrane. PTX2-SAs have been reported as not being cytotoxic (Daiguji *et al.* 1998; Yasumoto 2001), but neither of these

publications described the methods employed for cytotoxic investigation nor presented results, but merely stated the information without evidence.

The mechanisms and cause of G_2/M arrest observed in this study following exposure to PTX2-SAs were investigated with the aid of a high density cDNA microarray of gene expression in $HepG_2$ cells exposed to 800ng/mL PTX2-SAs for 24 hours. Several genes that have been associated with cell cycle regulation were altered. Examples of up-regulated genes that have been associated with cell cycle regulation included CDC42 (Lamarche *et al.* 1996; Roux *et al.* 1996; Philips *et al.* 2000; Royal *et al.* 2000; Assoian and Schwartz 2001), CDK9 (Grana *et al.* 1994), MAP-1 (Tan *et al.* 2001), Apol-3 (Horrevoets *et al.* 1999) and RDX (Wilgenbus *et al.* 1993). Examples of down regulated genes in this category included ASNS that codes for asparagine which is critical for cellular processes and known to be induced with nutrient depletion (Kilberg and Barbosa 2002; Leung and Kilberg 2002) and BZAP45 which is a regulator of transcription at the G1/S phase (Mitra *et al.* 2001). The cell cycle is regulated by the interaction of many molecules. Key among these are the cyclins that drive the stages of the cell cycle though their interactions with other cellular molecules. Cyclins combine with cyclin dependent kinases (cdks) to form activated kinases that phosphorylate targets leading to cell cycle regulation. Cdk inhibitors act to keep the cell cycle from progressing until all repairs to damaged DNA have been completed. A breakdown in the regulation of this cycle can lead to out of control growth and contribute to tumor formation. The CDC42 protein is involved in the P38 MAPK and RAS signaling pathway, which is one of the controlling mechanisms in cell cycle regulation.

CDK9 has been shown to be involved with phosphorylation during HIV type-1 transcription (Zhou *et al.* 2000). Some of the up-regulated genes mentioned above, for example, RDX and MAP-1 are also known to be involved in apoptosis. Radixin is a cytoskeletal protein and has other functions such as a role in morphogenesis and cell growth as (Wilgenbus *et al.* 1993) and MAP-1 has been shown to be involved with apoptosis by its interaction with BAX protein leading to mediation of caspase-dependent apoptosis (Tan *et al.* 2001). IER3 is an early response anti-apoptosis gene and was down regulated in microarray studies. IER3 inhibits apoptosis though its interaction with Fas, p53 or tumour necrosis factor alpha and is known to be involved in cell growth and maintenance (Im *et al.* 2002; Im *et al.* 2002). As mentioned above, ASNS is involved in several metabolic pathways including alaninine and aspartate metabolism, in addition to other processes such as nitrogen metabolism and porphyrin and chlorophyll metabolism. ASNS is known to block progression of the cell cycle though G1 phase when nutrients become depleted. So even though we can see there was no G1 phase arrest evident at 24 hours from the cell cycle studies, the microarray results show genes were already altered by 24 hours that by 72 hours had affected regulation at the G1 phase.

Other genes that maybe implicated in processes related to cell cycle regulation include genes for transcription factors and protein synthesis. The finding of genes involved in transcription ties in with the observation of hyperplastic RER in EM studies. Genes in this category that were up-regulated included genes coding for ribosomal protein synthesis RPL31, RPS24, RPS27A, RPS27L and genes down regulated included EIF2S1 (Ernst *et al.* 1987), EIF4A2, which is an RNA-dependent ATPase with helicase activity (Kyono *et al.* 2002) and FRAP1 (Brown *et al.* 1994; Lench *et al.* 1997) that is involved in the coding of DNA repair proteins and cause the inhibition of G1 progression. Other down regulated genes in this category included HDAC2 involved in the activation of the oestrogen receptor (Laherty *et al.* 1998), MRPL19, a mitochondrial ribosomal protein (Kenmochi *et al.* 2001; O'Brien 2002), RPLP0 (aka 364B) whose association with oestradiol is thought to be required for regulation of the

oestrogen receptor (Saceda *et al.* 1998), TCEA1 (Ito *et al.* 2000), the IFNGR2 gene and USP9X which is a specific cysteine protease for removing ubiquitin from conjugated protein substrates (D'Andrea and Pellman 1998). The IFN gamma receptor is a transducer for interferons that play a role in several cellular processes, phosphorylation of which signals transcriptional events. Mutations in the IFNGR2 gene have been associated with disease (Nabeshima 1998; Doffinger *et al.* 2002). FRAP1 specifically is known to be involved in the inhibition of cell cycle though the G1 phase (Lench *et al.* 1997). As this gene along with several others known to inhibit the G1 phase were down regulated at the 24 hour point, it is a possible indication of a response by the cell to signal for continuation the cell cycle following DNA repair that may have already taken place.

Another gene that has been indicated to be involved in the cell cycle process was the down regulated gene coding for the cytoskeleton protein and chaperonin CCT7 (Won *et al.* 1998). Genes involved in cellular communication were also down regulated and include CALM2 that codes for the calcium binding protein calmodulin (Toutenhoofd *et al.* 1998), CANX also known for its calcium binding and chaperone functions (Tjoelker *et al.* 1994), GNAQ - a G protein (Magovcevic *et al.* 1995) involved in protein Kinase C activation, PCTK1 which is a protein kinase related to cdc28 (Okuda *et al.* 1992; Okuda *et al.* 1994), PHKG2 another phosphorylase kinase (van Beurden *et al.* 1997), SLC25A4 which is involved in the electron transfer in mitochondrion by carrying ADP/ATP (De Marcos Lousa *et al.* 2002), YWHAZ that may participate in activating the Ras oncogene (Tommerup and Leffers 1996) and the previously mentioned FRAP1, which is involved in cellular communication by its interaction with mTor, which is essential for signaling hepatocyte proliferation (Coutant *et al.* 2002).

7.5.3 cDNA microarray

Results of the microarray showed there were 19 up-regulated genes and 41 down-regulated genes consistent to each of triplicate microarrays. The expression of several genes involved in cell cycle regulation were altered compared to controls in the cDNA microarray, as discussed in 7.5.2. The second major group of genes affected were those involved in lipid genesis, metabolism and transport. Genes involved in this process that were found to be up-regulated included APOL3 involved in lipoprotein homeostasis (Horrevoets *et al.* 1999), CYP7B1 involved in bile acid synthesis (Setchell *et al.* 1998), and PPARG which is involved in fatty acid storage and adipogenesis (Tontonoz *et al.* 1994; Tontonoz *et al.* 1998), and is thought to be activated by oxidized low density lipoproteins (Nagy *et al.* 1998). Activators of PPARG have been shown to enhance colon polyp formation and may contribute to cancer formation (Saez *et al.* 1998).

19 genes were up regulated	**41 genes were down regulated**
1. Apolipoprotein l, 3 2. Atp synthase, h+ transporting, mitochondrial f0 complex, subunit e 3. Cell division cycle 42 (gtp binding protein, 25kd) 4. Complement component 4 binding protein, beta 5. Cyclin-dependent kinase 9 (cdc2-related kinase) 6. Cytochrome p450, subfamily viib (oxysterol 7 alpha-hydroxylase), polypeptide 1 7. Homo sapiens, clone mgc:4459 image:2960564, mrna, complete cds 8. Hypothetical protein mgc3207 9. Methyl-cpg binding domain protein 3 10. Modulator of apoptosis 1 11. Peroxisome proliferative activated receptor, gamma 12. Putative selenocysteine lyase 13. Radixin 14. Rho gtpase activating protein 8 15. Ribosomal protein l31 16. Ribosomal protein s24 17. Ribosomal protein s27a 18. Ribosomal protein s27-like 19. Thioredoxin	1. Alpha-fetoprotein 2. Annexin a7 3. Asparagine synthetase 4. Atp synthase, h+ transporting, mitochondrial f0 complex, subunit c (subunit 9) isoform 3 5. Basic leucine-zipper protein bzap45 6. Bystin-like 7. Calmodulin 2 (phosphorylase kinase, delta) 8. Calnexin 9. Ceruloplasmin (ferroxidase) 10. Chaperonin containing tcp1, subunit 7 (eta) 11. Cop9 subunit 6 (mov34 homolog, 34 kd) 12. Enolase 1, (alpha) 13. Eukaryotic translation initiation factor 2, subunit 1 (alpha, 35kd) 14. Eukaryotic translation initiation factor 4a, isoform 2 15. Fk506 binding protein 12-rapamycin associated protein 1 16. Guanine nucleotide binding protein (g protein), q polypeptide 17. Histone deacetylase 2 18. Homo sapiens clone 23763 unknown mrna, partial cds 19. Immediate early response 3 20. Interferon gamma receptor 2 (interferon gamma transducer 1) 21. Kiaa0101 gene product 22. Mitochondrial ribosomal protein l19 23. Myosin, light polypeptide, regulatory, non-sarcomeric (20kd) 24. Nadh dehydrogenase (ubiquinone) fe-s protein 5 (15kd) (nadh-coenzyme q reductase) 25. Paternally expressed 10 26. Pctaire protein kinase 1 27. Peptidylprolyl isomerase f (cyclophilin f) 28. Phosphatidylserine synthase 1 29. Phospholipase a2, group ib (pancreas) 30. Phosphorylase kinase, gamma 2 (testis) 31. Proteasome (prosome, macropain) 26s subunit, atpase, 1 32. Protein tyrosine phosphatase, receptor type, c-associated protein 33. Ribosomal protein l29 34. Ribosomal protein, large, p0 35. Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4 36. Transcription elongation factor a (sii), 1 37. Translation initiation factor if2 38. Trinucleotide repeat containing 11 (thr-associated protein, 230 kd subunit) 39. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide 40. Ubiquitin specific protease 9, x chromosome (fat facets-like drosophila) 41. Udp glycosyltransferase 2 family, polypeptide b17

Those genes linked to lipid metabolism that were down regulated included AFP, which is involved in foetal hepatogenesis and expressed in cancerous liver cells or during liver regeneration (Jin *et al.* 1998; Spear 1999), ENO1 which is involved in glycolysis and

gluconeogenesis and is known as a transcriptional repressor that interacts with c-myc (Feo *et al.* 2000) and is also involved in phenylalanine, tyrosine and tryptophan biosynthesis. Also down regulated was PLA2G1B, which catalyses the hydrolysis of glycerolphospholipids to produce free fatty acids and is also involved in calcium homeostasis (Yagami *et al.* 2002; Yagami *et al.* 2003), and finally PTDSS1, the functions of which has been reviewed for mammalian cells (Kuge and Nishijima 1997) stating it is involved in glycerolipid metabolism, aminophosphonate metabolism, sphingophospholipid biosynthesis and porphyrin and chlorophyll metabolism.

The list of genes involved in lipid metabolism and transportation are especially interesting when correlated to the finding of the changes in MDA levels in mouse urine as a marker of oxidative lipid peroxidation. It is also interesting to note that certain marine algal extracts, for example caulerpenyne (Bitou *et al.* 1999) are well known to modulate lipid metabolism when added to the diet, and as a result have become a very popular in alternative methods of weight management with dietary supplementation of marine algal extracts in many herbal remedies available commercially (Brudnak 2002). Many substances that cause lipid peroxidation are also known as Peroxisome proliferators (Tontonoz *et al.* 1994; Nagy *et al.* 1998; Nilakantan *et al.* 1998; Saez *et al.* 1998; Tontonoz *et al.* 1998; Lee *et al.* 2000), and it would be interesting to investigate if peroxisome numbers increase in hepatocytes following repeat-doses of PTX2-SAs. It is important to identify substances in food that are potential peroxisome proliferators as many substances that cause peroxisome proliferation have been shown to cause hepatocellular tumours (ECETOC 1992).

7.6 Evaluation of toxicology studies and discussion of potential implications to public health from consumption of shellfish contaminated with PTX2-SAs

The health related issues of algal toxin contamination of seafoods and the problems associated with risk assessment have been addressed, emphasizing issues of lack on knowledge of biomarkers and other indicators of exposure to toxins and insufficient toxicology data (Van Dolah 2000; Van Dolah *et al.* 2001).

The risk assessment process usually consists of 4 main stages that include health hazard identification, toxicological data review and dose-response assessment, exposure assessment, and risk characterization and management (IPCS 1999.)

7.6.1. Hazard identification

PTX2-SAs were categorized as potential new toxins present in shellfish foods when first identified in New Zealand shellfish in 1997 (Daiguji *et al.* 1998) and in marine phytoplankton samples in Ireland (James *et al.* 1999). The biggest influence of characterizing these new toxins as a potential threat to human health was their association with DSP incidents involving other DSTs such as OA and DTXs, and also for being known as hydrolysis products of PTX2, which was already identified as a potential hazard to human health

In vivo toxicology studies in this PhD included single oral and *i.p.* doses of PTX2-SAs. No toxicology was observed at the LM level up to the maximum dosing level of 1.6mg/kg PTX2-SA. Changes were observed at the ultra-structural level with the use of EM and these changes could be seen at 25µg/kg in mice. Lipid peroxidation was found to occur at doses of 25µg/g and above – this figure should not be assumed to be the LOAEL as lower doses were not given to mice. No functional manifestations like significant changes in weight were observed and no neoplastic or carcinogenic observations could be made in these acute short-term *in vivo* studies. The significance of the observed changes in terms of disease cannot be evaluated without sub-chronic or repeat-dose studies as renewal of the intestinal lining usually occurs within a 24 hour period.

In vitro studies showed $HepG_2$ cells to have cell cycle and gene expression changes with a dose of 800ng/mL. Many genes involved in DNA repair were moderated at the 24 hour point, but as no apoptosis was observed up to 72 hours, it is a promising indication that any DNA damage that may have been caused by the administration of PTX2-SAs was not lethal and able to be repaired. The results of these cell cycle and cDNA array studies, in terms of human health risk assessment, imply there could be a potential for human effects with consumption of PTX2-SA in shellfish, but further *in vitro* and *in vivo* studies are required to predict the likelihood of any potential chronic and carcinogenic activity of the PTX2-SAs following the consumption of contaminated seafoods over a sustained period of time.

In light of the information provided by toxicology investigations in this study, with reference to evidence of *in vivo* lipid peroxidation by raised levels of MDA in mouse urine, and changes in cell cycle distribution and gene expression in a cultured human cell line, it is concluded that there is potential for PTX2-SAs to cause health effects in humans. Further to this statement, it should be noted that these studies were acute studies only, and it has not been established if these observed changes could result in disease or whether they could be repaired or returned within normal limits without the manifestation of illness or disease occurring. For this reason, different scenarios will be investigated in the health risk assessment for discussion purposes.

The LOAEL for *in vivo* studies was considered to be 25µg/kg as changes were observed within cells of the intestinal tract with EM and levels of MDA in urine were significantly raised in mouse urine at that concentration. A NOAEL could not be determined in these studies as 25µg/kg was the lowest dose administered to mice. Both of these endpoints were findings from acute studies, observed within 24 hours or less of dosing. It is worthwhile to note that mice permitted to live to 14 days post a dose of 25µg/kg did not present with any pathology at the LM level. Further to this, no histopathology was observed at the LM level up to the maximum dosing level of 1.6mg/kg PTX2-SA. It is known that concentrations of up to 5mg/kg were administered to mice, by *i.p.* and *p.o.*, with no pathological findings being found (Towers et al, personal communication. It is possible that the observed changes at 25µg/kg could return to normal without the manifestation of disease or illness occurring and therefore it could be considered that a NOAEL of 1.6mg/kg or 5mg/kg (*p.o.*) (Towers et al. personal communication) could be incorporated in the calculations for a guideline value.

Nothing is currently known of the chronic toxicology of PTX2-SAs and thus their potential implications to public health in the long term cannot determined. The toxicology investigations in this thesis were acute studies, and it has not been established if the observed changes could be repaired or returned within normal limits without the manifestation of illness or disease occurring.

7.6.2 Exposure assessment

The potential routes of human exposure to PTX2-SAs include oral (contaminated shellfish consumption and swallowing contaminated sea water when swimming), dermal (swimming in algal blooms or when shelling contaminated shellfish), and inhalation (sea spray containing algal blooms, for example when water skiing). Though all these possible routes of exposure the main risk to human health by exposure to PTX2-SAs is from the consumption of contaminated shellfish and the following assessment will only consider this mode of exposure.

Without the benefit of a biomarker to measure human exposure to PTX2-SAs, anecdotal evidence of shellfish consumption and known levels of PTX2-SAs in shellfish can be evaluated. By analysis of the pipis from the 1997 poisoning incident in NSW, the amount of toxin consumed by a patient was estimated. It was found that the pipis contained approximately 300µg PTX2-SAs/kg of pipis flesh. The average meal consists of approximately 0.5 kg of pipis. Therefore, each person consumed approximately 150 µg of PTX-2SA during the meal. For an average 60 kg person this equates to a dose of 2.5µg PTX-2SA/kg/bw. The amount of DTX-1 sufficient to induce gastroenteritis in a human is 32µg when eaten (Yasumoto *et al.* 1985). This figure is considerably lower than the doses employed for many of the *in vivo* toxicity studies that have been performed for various DSP toxins. In light of this information the question should be posed as to why the oral toxicity studies are conducted with such high doses in mice? Additionally, it has been found that the oral toxicity of OA is 25-50 times lower than that seen from *i.p.* dosing (Aune *et al.* 1998). Thus, the question is raised "Are humans more sensitive to DSP toxins than mice?" These issues may relate to differences in metabolism for the DSPs or other physiological processes such as absorption mechanisms and simply to differences between intestinal surface area to body weight ratios. A good example of such a difference is that of intoxication with CTX'S where humans are known to present with diarrhoea following intoxication whereas this has never been observed in laboratory animals when dosed with ciguatoxins (CTXs) (Richard

Lewis, personal communication). These factors of inter-species and inter-individual differences for safety evaluation are usually accounted for by the application of uncertainty factors. Uncertainty factors are usually based on criteria set out by the IPCS and IOMC upon a review of all the available data, but contemporary thinking is that these standard safety factors can be modified and reduced on the basis of the completeness and relevance of the data set on toxicity information for a substance (Renwick 1993; Renwick and Lazarus 1998; Renwick 1999; Renwick 1999; IPCS 2001). With review of several papers on the criteria for such judgment of uncertainty factors it is realized that the studies in this thesis do not conform to the criteria recommended in Renwick, (1993) and thus the standard factors of 100 must be applied. If the PTX2-SAs had been shown to be carcinogenic and a NOAEL not determined, then mechanistic and toxicokinetic information could be generated and used to assess the type of safety factors employed for genotoxic or non-genotoxic carcinogens (ECETOC 1996; Greim 2003). Such factoring was also not applicable to this study with the PTX2-SAs as no apoptosis was observed in cell cycle studies, and it is assumed that any DNA damage that may have occurred was able to be repaired, an assumption made with observation of the G_2/M cell cycle arrest and alteration of expression for genes involved in DNA repair. Of course this can only be an assumption at this stage of risk assessment, as repeat-dose exposure to PTX2-SAs has not been investigated.

It has been proposed that the traditional safety factors applied to contaminants and inherent chemicals in food can be reduced in light of known mechanistic, toxicokinetic and epidemiological data of the substance being assessed (Renwick 1993; Preston 1996; Essers *et al.* 1998; Dybing *et al.* 2002) and this strategy was adopted by the WHO in 1994 and thus has also been incorporated as recommended criteria for adjustment factors by many regulatory and advisory bodies for the assessment of contaminants in food (ECETOC 1995; Joint FAO/WHO Expert Committee on Food Additives. Meeting (57th : 2001 : Rome Italy) *et al.* 2002). Distribution studies were conducted to see where PTX2-SA could be detected in the mouse, but it was not within the realms of this PhD to be able to identify metabolites or conduct autoradiographical or immunohistochemical distribution studies, and thus toxico-kinetic observations could not be made. Thus, the data set from studies in this PhD is considered incomplete and therefore such uncertainty factors cannot be reduced, most notably by the absence of toxicokinetic parameters. Toxicodynamic and toxicokinetic data is not necessary to define a NOAEL, but is essential if a factor other than 100 is to be applied to a NOAEL for risk assessment (Renwick 1993; Meek *et al.* 2002; Meek *et al.* 2003). The applicability to human risk assessment with use of human cells for *in vitro* studies could enable some reduction in uncertainty factors if dose ranges and exposure times could produce a dose response observation.

$$\textbf{TDI} = \frac{\text{LOAEL}}{\text{Uncertainty factor}}$$

$$GV = TDI \times bw \times P/C$$

GV	= guideline value
TDI	= tolerable daily intake
bw	= body weight (assuming an average weight of 60kg)
P	= proportion of daily intake assigned to food, which would be 1 for this assessment
C	= amount consumed (in a normal meal is approximately 0.5kg)

Scenario 1: if the LOAEL of 25µg/kg applies to a health risk assessment then single oral dosing safety factors according to IPCS, 1999 are calculated thus:
X10 for interspecies variation
X 10 for inter-individual variation
X 10 for less than a lifetime study
X5 for a single dose study
X5 for use of LOAEL instead of NOAEL
= a safety factor of 25000

Therefore the TDI $\frac{25\mu g/kg}{25000}$ = 0.001µg/kg/day

Therefore the GV = 0.001µg/kg x 60 x (1/0.5) = 120ng/kg of shellfish meat

Scenario 2: is a NOAEL is considered 1600µg/kg
X10 for interspecies variation
X 10 for inter-individual variation
X 10 for less than a lifetime study
X5 for a single dose study
= a safety factor of 5000

Therefore the TDI = $\frac{1600mg/kg}{5000}$ = 320ng/kg/day

Therefore the GV = 320ng/kg x 60 x (1/0.5) = 38µg/kg of shellfish meat

Scenario 3 if the NOAEL is 5mg/kg *p.o.*
X10 for interspecies variation
X 10 for inter-individual variation
X 10 for less than a lifetime study
X5 for a single dose study
= a safety factor of 5000

Therefore the TDI = $\frac{5mg/kg}{5000}$ = 1µg/kg/day

Therefore the GV = 1µg/kg x 60 x (1/0.5) = 120µg/kg of shellfish meat

The figures calculated are all below the current recommended guideline value of 160µg/kg of shellfish meat. In considering this calculated value it should be noted that the above determined NOAEL concentrations were the highest doses given to mice in these studies, and not simply the highest doses where no effects were seen. Therefore, it should be considered that the NOAEL could in fact be much higher. Regretfully higher doses could not be performed due to lack of purified toxin availability, but such determination with repeat-dose

chronic studies is the ultimate resource for an appropriate health risk assessment. Further to this, the LOAEL in scenario 1 was the lowest dose given for *in vivo* studies and it should also be considered that the LOAEL figure stated could be lower.

In communications by Towers et al. (personal communication, 2000) on yessotoxin the authors were comparing the oral and *i.p.* toxicity and theorizing on the quantities of shellfish that would need to be consumed for a mouse or a human to suffer toxicity to aid putting into context the extremity of some regulated guidelines on shellfish toxins. Currently shellfish containing 16μg OA eqiv/100g tissue is legislated as 'unsafe', and Towers et al. then demonstrated that yessotoxin is two fold more toxic than OA upon *i.p.* injection and thus shellfish containing 8μg YTX/100g would fail the mouse bioassay and would be considered unsafe. They went on to theorize that a 20g mouse would need to eat 13.5 kg of unsafe shellfish to obtain a sufficient YTX dose to cause lethality and that a 100kg man would need to eat 6.8 tones of said shellfish in one sitting to receive the equivalent dose of YTX.

Anecdotal evidence of consumption of shellfish contaminated with PTX2-SAs between the years 1999-2002 was collected by Ken Lee of the South Australian shellfish quality assurance program. This evidence was collected in retrospect by asking shellfish farmers to fill in tables of estimated numbers of shellfish eaten and known levels of PTX2-SAs. They were also asked to provide any other additional information such as any symptoms arising. The reporting forms were sent to farmers to report on shellfish harvested from 5 bays in Port Lincoln but responses for shellfish harvested from 3 bays were received. These bays were Streaky Bay, Boston and Proper Bays. In summary, of all the batches consumed in the 1999-2002 period no illness was reported following consumption of contaminated shellfish. In Australia the highest levels of PTX2-SAs in shellfish that have been analysed by QHSS have not exceeded 2mg/Kg of shellfish meat (personal communication, Geoff Eaglesham, QHSS). The Crawthron report (no. 750, Mackenzie, 2002) provides a guide to the levels of PTXs found in NZ shellfish.

7.6.3 Health risk characterization and management

Risk characterization is an evaluation of scientifically collected information to estimate the extent of risk to health taking into consideration the level of uncertainty that may exist and the estimates of human risk under relevant exposure scenarios (IPCS 1999.).

In light of the risk assessment producing a guideline value that was calculated to be lower than the current recommended guideline value, a risk:benefit analysis for the consumption of contaminated shellfish should be performed considering the fact that there is no hard evidence that a human poisoning event has taken place involving only PTX2-SAs.

7.6.4 Putting the hazard into context

To date, there has been no solid evidence that PTX2-SAs cause illness in humans – all documented incidents involving the PTX2-SAs have also included other DSP contaminants. Pathology has not unequivocally been observed in animal studies. Therefore, the *in vivo* dosing concentrations could be put into the context of human consumption of shellfish. Thus, in the absence of safety factors, if extrapolation of doses were to be calculated for a 60kg person and correlated to the maximum recorded levels of PTX2-SAs in Australian shellfish, known to be 2mg/kg shellfish meat (QHSS) and in New Zealand shellfish at 4.1mg/kg shellfish meat (Crawthron report), then the following could be calculated:

Scenario 1: 25µg/kg LOAEL
25µg/kg X 60kg = 1500µg of toxin would be required to be consumed in one meal to cause the observed changes seen in mice. Therefore, in Australia a person would have to consume approximately 750g of contaminated shellfish to be exposed to the same dose, and in NZ would need only consume 365g of shellfish meat.

Scenario 2: 1.6mg/kg NOAEL
1.6mg/kg X 60kg = 96mg of toxin would be required to be consumed in one meal to cause the observed changes seen in mice. Therefore, in Australia a person would have to consume approximately 48kg of contaminated shellfish to be exposed to the same dose, and in NZ would need consume approximately 23 kg of shellfish meat.

Scenario 3: 5mg/kg (NZ study) NOAEL
5mg/kg X 60kg = 300mg of toxin would be required to be consumed in one meal to cause the observed changes seen in mice. Therefore, in Australia a person would have to consume approximately 150kg of contaminated shellfish to be exposed to the same dose, and in NZ a person would need to consume approximately 73kg of shellfish meat in one sitting!

Additionally, it should also be considered that although a TDI was calculated and incorporated into the guideline value calculations in the previous section, the average person would not eat a meal of shellfish a day, and in reality may only eat shellfish on a rare occasion.

The PTX2-SAs are currently categorized and regulated with other DST's and the recommended guideline value of 160µg OA equiv./kg of shellfish meat has resulted in a crippling of the South Australian shellfish industry by the mandatory closure of many bays to shellfish harvesting for 15 of the past 24 months. This tight regulation has caused a great economic burden to shellfish farmers around Australia with many smaller companies going out of business (Ken Lee, personal communication)

8.0 LIMITING FACTORS/ CRITIQUE

Availability of purified PTX2-SA extracts has been the greatest limitation of laboratory experimentation and toxicology studies, for example a sub-chronic multiple dosing study was not possible due to lack of available toxin. This issue could possibly be over come with either the development of technology and understanding for *Dinophysis* cell culture or for extracting large quantities of PTX2 and chemically converting the toxin to the PTX2-SAs.

Availability of purified toxin extracts has been the greatest limitation of toxicology studies. For this reason studies were only conducted with female mice and thus it is undetermined if pathology may be different in male mice. Additionally, a multiple dosing study could not be conducted, and it was not possible to have duplicate or triplicate numbers of animals for some studies, thus for the greater part, statistical analysis of observations could not be performed. Use of other staining techniques would have been beneficial for pathology observations and it would have been interesting to be able to determine the structures of metabolic conjugates of the PTX2-SAs in the excreted faeces. This last point of interest however, would amount to a PhD project in its own right.

9.0 BENEFITS

This project has helped remove some of the uncertainty in a health risk assessment and we believe this information could be used by the FRDC to compile a recommendation to the regulatory authorities for the setting of more appropriate guidelines for permitted concentrations of the PTX2-SAs in Australian shellfish.

10.0 FURTHER DEVELOPMENT

Further toxicological studies are required with the PTX2-SAs that need to be performed in accordance with OECD guidelines. We are aware that a research group in New Zealand are undertaking this task. It is suggested that sub-chronic repeat-dose studies be performed.

It is recommended that the information from this project be compiled with the results of the New Zealand study on the PTX2-SAs and that a committee such as ASQAAC form a working group to progress the extension of this information to the industry and report to ANZFAA to petition to have the current guidelines altered in light of the new information on the toxicology of PTX2-SAs.

Despite ongoing international research into the production of algal toxins and their accumulation in shellfish, the reason for production of toxins and much of their toxicological action is not understood at this time. It is clear that toxic algal blooms and associated algal toxin identification is on the increase around the world. Thus, it is important that shellfish poisons and their related syndromes must continue to be monitored, researched and regulated to ensure the safety and health of shellfish consumers.

While toxicological investigations in this project have highlighted acute studies in mice dosed with PTX2-SAs, it is hoped that the findings provide guidance for remaining toxicology work that is required and that is pertinent to the risk evaluation of PTX2-SAs in shellfish for human consumption.

Firstly, it is necessary to conduct repeat-dose investigations over a period of time to ascertain any chronic toxicity or pathological finding that may arise from long term exposure to PTX2-SAs. The causation of lipid peroxidation *in vivo* detected by raised levels of MDA in the urine of dosed mice is one of the key findings for concern for consumption of PTX2-SAs. This issue should be further investigated with DNA damage studies both *in vitro* and *in vivo*. Low dose studies employing EM for observation of changes in the intestinal and liver tissues is important to elucidate if the observed changes continue to progress after 24 hours exposure, and to see if PTX2-SAs are peroxisome proliferators. It would be advantageous to conduct distribution studies with the production of radio-labeled toxins or with immuno-histological techniques to identify any additional target organs and to enable identification and tracking of any metabolites of the PTX2-SAs as they are ingested and excreted.

Finally, it is fundamental to DSP regulation that research be undertaken to assess the effects of consuming PTX2-SAs in combination with several of the DSP toxins in one meal, as often occurs in the real-life situation where shellfish can be contaminated with a combination of DSP toxins.

11.0 PLANNED OUTCOMES

Knowledge gained will essentially relate to provision of toxicological data on pectenotoxins that can be interpreted in terms of a human health risk assessment. This will ultimately result in the development of more appropriate guideline values for this group of toxins in Australian shellfish. It is planned to communicate results to the shellfish industry (such as the FRDC and ASQAAC) and hope such organisations can liase with state government departments of agriculture and fisheries in terms of development of the guidelines.

The main benefit of the work will relate to the fact that currently no guidelines based on scientific data exist for these toxins in shellfish harvested and sold in Australia. The provision of quantitative guidelines for the toxins will enable the industry to precisely determine the suitability of the product for commercial sale and consumption through analysis for toxins in shellfish before harvest. This will in turn avoid the possibility of litigation from persons becoming ill through consumption of contaminated shellfish and it will also avoid the wastage that occurs when shellfish products are re-called during an outbreak of diarrhoetic shellfish poisoning.

12.0 CONCLUSION

The toxicology studies in this thesis have shown there is potential for these toxins to induce biological changes in mammalian cells *in vivo* and *in vitro*. Nothing is known of the chronic toxicology of PTX2-SAs or other PTXs and their potential implications to public health in the long term and has not been determined. This information is needed to perform an appropriate health risk assessment for consumption of contaminated shellfish and hence for guidance in the regulation of toxin levels in shellfish that are sold for human consumption.

Despite severe pathology being identified in the pilot study, follow up studies could not replicate the pathology seen in the pilot study with different consignments of shellfish extract. No behavioral changes were of note and little consistent pathology could be seen at the LM level following PTX2-SA dosing. At the EM level, changes were seen within the terminal web, RER and mitochondria following PTX2-SA dosing. The significance of these changes cannot be fully evaluated without follow up studies with increasing doses and exposure periods to determine if such changes are repairable over time or cause permanent changes.

The LOEL for this study is considered 25 μg/kg, determined by the observed changes noted following EM studies and MDA analysis in urine, but further metabolism, absorption and distribution investigations of these toxins will be required to determine the NOEL to aid in a health risk assessment for the consumption of shellfish contaminated with PTX2-SA and 7-epi-PTX2-SA.

PTX2-SAs extracted from algae have been shown to cause G_2/M phase arrest in $HepG_2$ cells with no apoptosis detected up to 72 hours exposure. The results of the high density microarray has helped to elucidate some of the underlying mechanisms involved in the toxicology of the PTX2-SAs. With all these interactions of various genes involved in cell cycle regulation and other cellular processes it would appear that DNA damage may have been caused by the effects of PTX2-SA and this should be further investigated with the use of various tests such as the detection of 8-hydroxy-2'-deoxyguanosine in urine or tissues for oxidative damage to DNA (Renner *et al.* 2000; Halliwell 2002), or staining techniques for various indicators of DNA damage, for example, micronuclei formation to investigate

genotoxicity as has been demonstrated with OA (Carvalho Pinto-Silva *et al.* 2003). The second most significant observation was the number of genes associated with lipid metabolism or genesis that were altered. This is especially interesting when correlated with the finding of increased levels of MDA in the urine of mice treated with PTX2-SAs indicating that lipid peroxidation damage had occurred. The significance of these alterations should be further investigated to evaluate if the changes are pathological over a period of time.

Many genes involved in DNA repair were moderated at the 24 hour point, but as no apoptosis was observed up to 72 hours it is a promising indication that any DNA damage that may have been caused by the administration of PTX2-SAs was able to be repaired and thus cells were not going into apoptosis. The results of these cell cycle and cDNA array studies, in terms of human health risk assessment, imply there could be a potential for human effects with consumption of PTX2-SA in shellfish, but further *in vitro* and *in vivo* studies are required to predict the likelihood of any potential chronic and carcinogenic activity of the PTX2-SAs following the consumption of contaminated seafoods over a sustained period of time.

Although the health risk assessment produced guideline values that are lower than the current recommend values, when consideration of epidemiological evidence is assessed, PTX2-SAs cannot be considered a high a risk to public health as previously thought with no real evidence to support the assumption that PTX2-SAs caused human illness. For these reasons and the reality of the economic burden the current guideline values are causing to shellfish industries around the globe, it is recommended that PTX2-SAs be monitored but no longer regulated as tightly with other DSPs until such a time that toxicological or epidemiological evidence can prove that the PTX2-SAs are a more considerable threat to human health than has been indicated by toxicology studies in this thesis.

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14.0 APPENDIX

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