

***Chapter 7 Evaluation of toxicology
studies and discussion of potential
implications to public health from
consumption of shellfish contaminated
with PTX2-SAs***

7.1 INTRODUCTION

This chapter will summarize and evaluate findings of the toxicology studies in this thesis with relevance to public health issues from consumption of shellfish contaminated with PTX2-SAs. This evaluation will include a health risk assessment and a discussion of epidemiological evidence (anecdotal) of human exposure to PTX2-SAs by consumption of shellfish in Australia and New Zealand.

The safety evaluation of identified contaminants in food is a necessary and important process. Traditional foods are not considered to require the stringent testing that novel foods and newly identified food contaminants require based on their knowledge of long term use, but many traditional foods in these modern times are being prepared and processed in novel and different ways as our cultures converge and evolve, which has propagated the need to review the safety of even some traditional foods (Essers et al. 1998). 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 on 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.).

7.2 HAZARD IDENTIFICATION

According to the IPCS manual on principles for the assessment of risks to human health from chemicals (1999), hazard identification allows for the evaluation of evidence and the potential for an identified substance to cause harm to human health by addressing the main questions of A) whether a substance may pose a health hazard to humans from its inherent properties and B) under what circumstances is the hazard likely to cause adverse effects.

The PTX2-SAs are newly-recognized toxins being first identified in New Zealand shellfish in 1997 (Daiguji et al. 1998) and in marine phytoplankton samples in Ireland (James et al. 1999), and were subsequently categorized as potential new toxins present in shellfish foods. The structure of these toxins and associated background information can be found in chapter 1. 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, as reviewed in chapter 1. Some known incidents involving PTX2-SAs in Australia have been discussed in the

introduction to this thesis and additional incidents in New Zealand are mentioned in the Cawthron report No.750, New Zealand. The main incident of note in Australia occurred during December of 1997, when over 100 people living in the Eastern states of Australia were poisoned following consumption of pipis harvested off the NSW coastline. At the time of the incident, shellfish responsible for the poisoning were found to contain PTX2-SAs and only low levels of PTX-2 (Eaglesham et al. 2000) in the absence of any other known DSPs. Hence, the PTX2-SAs were considered the causative agent. Subsequently and during the course of this PhD, analytical techniques and knowledge on the algal toxins has developed and it is now realized that OA and other DSP toxins can exist as hydroxylated esters in shellfish that were previously going undetected by HPLC analysis when mouse bioassays were showing positive results, as discussed in chapters 1 and 3. From the results in chapter 3 on analysis of pipis involved in the 1997 NSW poisoning incident, it is not believed that the PTX2-SAs were the causative agent but more likely esters of OA that caused the observed clinical symptoms in consumers.

In vivo toxicology studies in this PhD included single oral and *i.p.* doses of PTX2-SAs (chapter 5). 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 HepG₂ cells to have cell cycle and gene expression changes with a dose of 800ng/mL (chapter 6). 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 was 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 PhD, 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.

7.3 ASSESSMENT OF TOXICITY

According to leading advisory bodies, a dose-response assessment is the process of determining the relationship between the dose of toxicant and the incidence and extent of an adverse health effect (IPCS 1999). It is especially important at this stage to determine if the toxicant in question has a threshold level, below which toxic effects are not found, or whether any level of exposure results in a harmful event leading to the classification of the substance as a mutagenic or carcinogenic compound.

In vitro studies showed HepG₂ cells to have gene expression changes with a dose of 800ng/mL PTX2-SAs. These gene expression changes included alterations in genes involved in cell cycle control and DNA repair processes, and many genes involved in lipid metabolism, genesis and transport were also altered.

In this thesis, The LOAEL for *in vivo* studies was considered to be 25µg/kg as changes were observed within cells of the intestinal tract 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 abnormalities were 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.

7.4 EXPOSURE ASSESSMENT

The possible routes of exposure to the PTX2-SAs in different scenarios include oral, dermal and inhalation. To elaborate, exposure could occur orally by consumption of contaminated shellfish or by swallowing algal bloom-contaminated seawater during recreational activities such as swimming and diving. Dermal exposure could occur when shelling or eating shellfish or by contact with algal blooms by recreational seawater users. Additionally, there is potential for recreational users of seawater to inhale toxins when, for example, water-skiing through algal blooms that may produce PTX2-SAs. Through all the potential routes of human exposure to PTX2-SAs, poisoning is only known to have occurred through the consumption of contaminated shellfish and the following assessment will only consider this mode of exposure.

Identification of an appropriate biomarker of exposure can aid in risk assessment, but despite a clearer understanding of the processes and mechanisms of toxicity for the PTX2-SAs being investigated in this PhD, a specific and useful biomarker of exposure to PTX2-SAs was not identified. Although, it would be interesting to compare the pattern of gene induction changes with the other DSPs in a future project to determine if a unique gene or group of genes were altered that could be utilized in an *in vitro* assay as a biomarker.

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 the 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 may have consumed approximately 150 µg of PTX-2SA during the meal. For an average 60kg person this equates to a dose of 2.5µg PTX-2SA/kg/bw. The amount of DSP-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 most *in vivo* toxicity studies discussed in chapter one 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 CTXs where humans are known to present with diarrhoea following intoxication whereas this has never been observed in laboratory animals when dosed with 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 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 G₂/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 toxicokinetic 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.

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

$$\text{GV} = \text{TDI} \times \text{bw} \times \text{P/C}$$

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:

X 10 for interspecies variation

X 10 for inter-individual variation

X 10 for less than a lifetime study

X 5 for a single dose study

X 5 for use of LOAEL instead of NOAEL

= a safety factor of 25000

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

$$\text{Therefore the GV} = 0.001\mu\text{g/kg} \times 60 \times (1/0.5) = 120\text{ng/kg of shellfish meat}$$

Scenario 2: If the a NOAEL is considered 1600µg/kg

X10 for interspecies variation

X 10 for inter-individual variation

X 10 for less than a lifetime study

X 5 for a single dose study

= a safety factor of 5000

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

$$\text{Therefore the GV} = 320\text{ng/kg} \times 60 \times (1/0.5) = 38\mu\text{g/kg of shellfish meat}$$

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

X 5 for a single dose study

= a safety factor of 5000

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

$$\text{Therefore the GV} = 1\mu\text{g/kg} \times 60 \times (1/0.5) = 120\mu\text{g/kg of shellfish meat}$$

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 YTX 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 in to context the extremity of some regulated guidelines on shellfish toxins. Currently shellfish containing 16µg OA equivalents/100g tissue is legislated as 'unsafe', they then demonstrated that YTX 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 tonnes 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 concentrations of PTX2-SAs. They were also asked to provide any other additional information such as any symptoms arising following consumption of contaminated pipis. The reporting forms were sent to farmers to report on shellfish harvested from 5 bays in Port Lincoln but responses for shellfish harvested from only 3 bays were received. These bays were Streaky Bay, Boston and Proper Bays. The collated information can be viewed in appendix 1. In summary, of all the batches consumed in the 1999-2002 period no illness was reported. Collated data on the occurrence and levels of PTX2-SAs in Australian shellfish analyzed at QHSS during this period is shown graphically in the appendix. In Australia the highest levels of PTX2-SAs in shellfish that have been analyzed by QHSS have not exceeded 2mg/Kg of shellfish meat (personal communication, Geoff Eaglesham, QHSS). The Cawthron report (no. 750, Mackenzie, 2002) provides a guide to the levels of PTXs found in NZ shellfish.

7.5 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.

7.5.1 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 (Cawthron 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 due to their association with others DSP's in shellfish and with application of the Precautionary Principle (Hart et al. 2003; Resnik 2003; Rogers 2003). The recommended guideline value of 160µg OA equivalents/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 with many smaller companies going out of business (Ken Lee, personal communication).

7.6 THE CONCLUDING STATEMENT

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*. 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 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 induce 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 G₂/M phase arrest in HepG₂ cells with no apoptosis detected up to 72 hours exposure. The results of the high density microarray have

helped to elucidate some of the underlying mechanisms involved in the toxicology of the PTX2-SAs. With all the 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.

Studies were restricted in number for this thesis caused by the limited quantity of purified PTX2-SAs available for toxicology investigations. 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.

The health risk assessment produced guideline values that are lower than the current recommend values, but when consideration of epidemiological evidence is assessed, PTX2-SAs cannot be considered as 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 levels of PTX2-SAs be monitored in recognition of the precautionary principle, 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 DSP and are a more considerable threat to human health than has been indicated by toxicology studies in this thesis.

7.7 FUTURE WORK

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 thesis 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-labelled toxins or with immunohistological 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 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.