PART II

ASSESSMENT OF THE RISK OF BIOTOXINS IN BIVALVE MOLLUSCS

Azaspiracids

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1. BACKGROUND INFORMATION[1]

The syndrome that later was named azaspiracid poisoning (AZP) was detected for the first time in 1995 among consumers in the Netherlands after eating blue mussels from Ireland. The symptoms were similar to those of diarrhoeic shellfish poisoning (DSP), but the concentration of the DSP toxins was low. Subsequently, the azaspiracid (AZA) toxin group was discovered. Thus far, AZAs have only been detected in Europe. The European Union (EU) has set a regulatory level of 0.16 mg/kg with mouse bioassay (MBA) as the reference method. However, an MBA protocol with adequate specificity or detectability has not been validated. Current testing is based on preliminary liquid chromatography with mass spectrometry detection (LC-MS) methods using a limited supply of AZA-1 reference standard.

2. ORIGINS AND CHEMICAL DATA

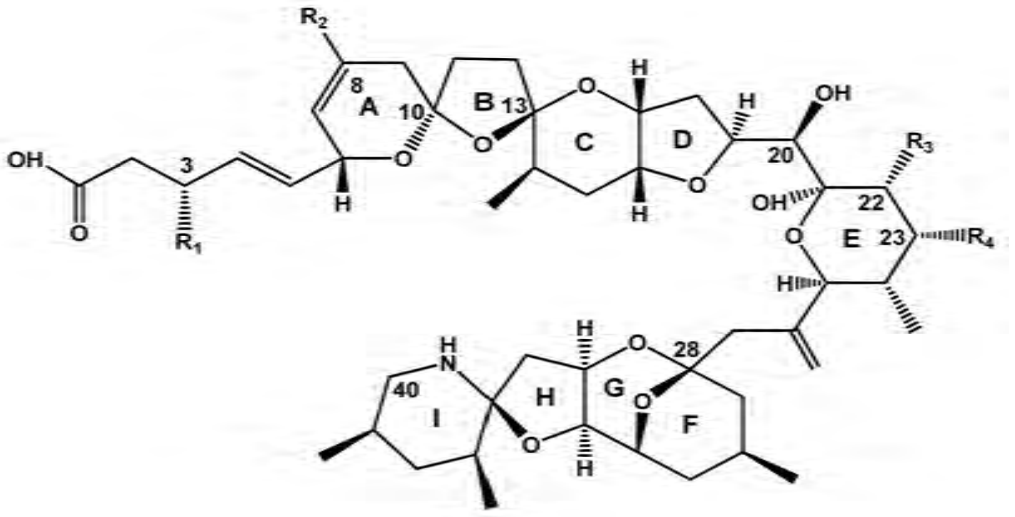
Azaspiracids are nitrogen-containing polyether toxins with a unique spiral ring assembly, a cyclic amine and a carboxylic acid, and were first detected in mussels (*Mytilus edulis*) in Ireland in 1995. Currently, 20 different congeners have been identified (Satake *et al.*, 1998b; Ofuji *et al.*, 1999a, 2001, James *et al.*, 2003, Rehmann, Hess and Quilliam, 2008); however, toxicological information is only available for AZA- to -5 (Satake *et al.*, 1998b; Ito *et al.*, 1998, 2000, 2002; Ofuji *et al.*, 1999a, 2001). Compared with the other analogues, AZA-1, -2 and -3 are the major contributors to the overall toxic equivalents found in shellfish, both because of their high concentration and because of their comparatively high toxicity (Figure 1).

Recently, AZA-1, -2 and -3 were identified within the heterotrophic dinoflagellate, Protoperidinium spp. (Yasumoto, 2001). However, Krock *et al.* (2009) and Krock, Tillmann and Cembella (2009) found a small dinoflagellate in the North Sea to produce AZA in culture, and thus it is presumed that this organism or related species were also responsible for production of AZA in Ireland and other countries where AZAs have been reported.

As described by Twiner *et al.* (2005), AZAs have been detected in bivalve species other than mussels, including oysters (*Crassostrea gigas*, *Ostrea edulis*), scallops (*Pecten maximus*), clams (*Tapes phillipinarium*), cockles (*Cardium edule*) and razor fish (*Ensis siliqua*) (Hess *et al.*, 2003; Furey *et al.*, 2003). Several countries, including France, Ireland, Italy, the Netherlands, Norway, Spain and the United Kingdom of Great Britain and Northern Ireland, have documented either cases of AZA intoxications and/or contaminated shellfish (Satake *et al.*, 1998a). Although the human symptoms resembled those of DSP, the illness was subsequently named AZP (Ofuji *et al.*, 1999a) once AZA was identified as a novel toxin (Satake *et al.*, 1998b).

[1] Corresponds to the "Background Information" section of the Expert Consultation Report.

FIGURE 1
Azaspiracids: AZA-1 (R1,2,4 = H; R3 = CH3), AZA-2 (R1,4 = H; R2,3 = CH3)



Notes: The initial structure proposed by Satake *et al.* (1998b) was corrected by Nicolaou *et al.* (2004a, 2004b). The corrected structure is shown.

3. BIOLOGICAL DATA

3.1 Biochemical aspects

3.1.1 Absorption, distribution and excretion/toxicokinetics

No data reported.

3.1.2 Biotransformation

No information on pathways of AZA metabolism in animals has been reported.

3.1.3 Effects on enzymes and other biochemical parameters/mechanism of action

At present, there are virtually no data on the mechanism of action, although based on the studies that Roman *et al.* (2002) performed, some information on targets on cellular level became available. They report on potential cellular targets of AZA-1, which causes diarrhoeic and neurotoxic symptoms and whose mechanism of action is unknown. In excitable neuroblastoma cells, the systems studied were membrane potential, F–actin levels and mitochondrial membrane potential. While AZA-1 did not modify mitochondrial activity, it did decrease F–actin concentration. These results indicate that the toxin does not have an apoptotic effect but uses F–actin for some of its effects. Therefore, cytoskeleton seems to be an important cellular target for AZA-1 effect. AZA-1 did not induce any modification in membrane potential, which does not support for neurotoxic effects. In human lymphocytes, cAMP, cytosolic calcium and cytosolic pH (pHi) levels were also studied. AZA-1 increased cytosolic calcium and cAMP levels, and did not affect pHi. Cytosolic calcium increase seemed to be dependent on both the release of calcium from intracellular Ca^{2+} increase pools and the influx from extracellular media through Ni^{2+}-blockable channels. AZA-1 induced Ca^{2+} increase is negatively modulated by agents that regulate protein kinase C (PKC) activation, protein phosphatases 1 and 2A (PP1 and PP2A) inhibition and cAMP increase. The effect of AZA-1 on cAMP is not extracellularly Ca^{2+} dependent and unsensitive to OA (Roman *et al.*, 2002).

AZAs were cytotoxic to P388 cells but to KB cells the potency was much less prominent (EU/SANCO, 2001). AZA did not inhibit PP2A. It was noted that *in vitro*

studies performed in human cells from healthy donors suggest that the threshold for AZA analogues to modify cellular function would be 24 µg/kg for a 60 kg person.

3.1.3.1 Effect of AZA on DNA fragmentation

In vivo administration of AZA was studied to see whether it causes deoxyribonucleic acid (DNA) fragmentation. Mice organs (ICR male 4 week) were stained with apoptotic peroxidase *in situ* apoptosis kit after p.o. treated with AZA. By this, the livers showed apoptosis in all examined cases (300 µg/kg: 1, 2 and 4 hours, and 600 µg/kg: 4, 18 and 24 hours), but not in the lung and kidney.

3.1.3.2 Effect of AZA-1 on an in vitro *model of GI permeability*

The human colonic cell line Caco-2 was used to assess the impact of AZA-1 on intestinal barrier function. Barrier function was measured by transepithelial electrical resistance (TEER). TEER works by measuring the rate of flux of ions across the paracellular pathway as an electrical resistance. Disruption of the paracellular barrier is a contributing factor to increased fluid secretion in diarrhoea. AZA-1 was found to decrease TEER in a dose-dependent fashion over time. A significant decrease was observed at 5 nM at 24 hours (Ryan, Hess and Ryan, 2004). This functional assay may be developed as a possible *in vitro* assay for AZA.

3.1.3.3 Cytotoxic and cytoskeletal effects of AZA-1 on mammalian cell lines

Initial investigations have shown that AZA-1 is differentially cytotoxic to several different cell types as determined by the MTS assay, which measures mitochondrial activity. Calculated EC_{50} values for the Jurkat cell line (lymphocyte T cells) were 3.4, 1.1 and 0.9 nM for 24, 48 and 72 hour exposures, respectively. The effect of AZA-1 on membrane integrity was tested by measuring the release of a cytosolic enzyme, glucose-6-phosphate dehyrogenase (G6PD), from Jurkat cells. Significant elevations in G6PD activity were detected in the extracellular medium for AZA-1 exposed cells, with preliminary EC_{50} values of 0.2 and 0.07 nM for 24 and 48 hours of exposures. AZA-1 was also reported to be capable of rearranging cellular F-actin in Jurkat cells. This was apparent with the concurrent loss of pseudopodia and cytoplasmic extensions that function in mobility and chemotaxis prior to cytotoxicity (Twiner *et al.*, 2005).

3.2 Toxicological studies

3.2.1 Acute toxicity

Oral studies

Acute oral studies with AZA in mice were performed. AZA was extracted from mussels collected in Killary Harbour, Ireland, in February 1996. During the course of toxin purification, the major toxin was concentrated in a lipid fraction coded Killary Toxin-3 (KT3) (as cited in Ito *et al.*, 2000). By oral administration (by gavage) of 60 µl of this KT3 fraction, mice did not show any clinical changes during 24 hours. At autopsy after 4 hours, active secretion of fluid from the ileum and debris of necrotizing epithelial cells from upper portion of the villi were observed in the lumen (SEM), and after 8 hours, erosion of the villi from the top resulted in the shortened villi, and prominent accumulation of fluid was observed accompanying edema in the lamina propria. Then, after 24 hours, these changes were not observed but epithelial cells of adjacent villi were fused to each other (Ito *et al.*, 1998).

Male ICR mice receiving orally by gavage a single dose of 500, 600 or 700 µg purified AZA/kg b.w. did not show any behavioural changes within 4 hours. Number of survivors after 24 hours were 0/2, 3/6 and 1/2 at 500 (8 weeks old), 600 (5 weeks old) and 700 µg/kg b.w. (5 weeks old), respectively. At 600 and 700 µg/kg b.w., diarrhoea and b.w. decrease were observed within 24 hours.

At single oral doses of 300–700 µg/kg b.w., AZA caused dose-dependent changes in small intestines (necrotic atrophy in the lamina propria of the villi) and in lymphoid tissues, such as thymus, spleen and Peyer's patches. In the spleen, the number of non-granulocytes was reduced and damage to both T and B lymphocytes occurred. In addition, liver weight increased, colour of the liver changed from dark red to pinkish red and fatty changes in the liver were observed. AZA did not cause prominent changes in the stomach mucosa, but the appearance of many degenerating cells was observed in the large intestine. The pancreas appeared to loose zymogen granules locally, but cells were not injured. Histopathological damage to other organs (kidney, heart and lung) was not observed. The acute morphological changes in the mouse, induced by AZA, were distinctly different from those of OA (Ito *et al.*, 2000).

In the latest experiments from Ito *et al.* (2002), a total of 18 four-week-old mice, 5 six-week-old mice and 2 five-month-old mice were used to produce severe injuries and then to observe recovery. Four dose levels: 250, 300, 350 and 450 µg AZA (more purified extract from blue mussels at Killary Harbour and Arranmore Island in Ireland) /kg b.w. (dissolved in 50 percent ethanol) were given orally to five groups. Ten mice that survived the initial treatment received a second treatment on day three. Nine mice that survived the second treatment were killed between days 7 and 90 after treatment. Thirteen control mice were used. The highest dose of 450 µg/kg b.w. caused death in 11/16 treated (four-week-old) mice. Two out of two 6-week-old mice and another two out of two 5-month-old mice, receiving 300 and 250 µg/kg b.w., respectively, also died. Of ten mice that survived the first treatment, one died after the second treatment with 350 µg/kg b.w. Slow recoveries were revealed after oral administration of 300, 350 and 450 µg/kg b.w. Erosions and shortened villi in the stomach and the small intestine persisted for more than 3 months; edema, bleeding and infiltration of cells in the alveolar wall of the lung for 56 days; fatty changes in the liver for 20 days; and necrosis of lymphocytes in the thymus and spleen for 10 days. Thus, the lowest oral dose of 250 µg AZA/kg b.w. appeared to be lethal in mice in this study.

It has to be noted that the partially purified Killary Toxin-3 (KT3) toxin caused much more severe intestinal fluid accumulation and histological damage to the pancreas than the more purified toxin used in the studies of Ito *et al.* (2000). There may be several unknown analogues of AZA present in the crude fraction. It should also be mentioned that the difference between the mouse lethalities by oral and intraperitoneal (i.p.) administration was much less significant with AZA than with other phycotoxins (Ito *et al.*, 2000).

Intraperitoneal studies

Mice exposed to AZA by i.p. react differently than those exposed to other shellfish toxins. After i.p. dosing of the partially purified KT3 to male ddY mice, the animals became sluggish, sat still in corners and showed progressive paralysis and laboured breathing. No diarrhoea was observed. At low doses, the animals died 2–3 days after dosing. The minimal lethal dose was reported to be 150 µg/kg b.w. (Satake *et al.*, 1998a). Ito *et al.* (1998) injected 10 µl of the partially purified KT3 i.p. to 10 male ICR mice (age 3 weeks). All animals showed inactivity and general weakness and died within 24 hours. Morphological changes caused by KT3 were distinctly different from those induced by DSP, paralytic shellfish poisoning (PSP) or amnesic shellfish poisoning (ASP) toxins. The main target organs of KT3 were liver, spleen, pancreas, thymus and digestive tract. In contrast, those of DSP toxins are the digestive tract, of PSP toxins the central nervous system (CNS) and of ASP toxins the brain. The target site of KT3 was the small intestine, where villi degenerated from the top. At the histopathological level, parenchym cells of the pancreas and hepatocytes, which contain numerous rough endoplasmic reticula, were preferentially affected and it is probable that KT3 inhibits protein synthesis.

Satake *et al.* (1998b) reported an i.p. lethal dose of purified AZA to mice of 200 µg/kg b.w. (Table 1). The i.p. lethal doses for AZA-2 and -3 to mice were 110 and 140 µg/kg b.w., respectively, (Ofuji *et al.*, 1999a) and for AZA-4 and -5 approximately 470 and less than 1 000 µg/kg b.w., respectively (Ofuji *et al.*, 2001).

TABLE 1
Lethal dose of AZA-1 in mice

Species age	Sex	Route	Lethal dose (µg/kg b.w.)	References
Mouse ICR 4–5 week	male	oral	>450	Ito *et al.*, 2002
Mouse ICR 5 month	male	oral	>250	Ito *et al.*, 2002
Mouse ddY	male	i.p.	200	Satake *et al.*, 1998a, 1998b

3.2.2 Short-term toxicity

Repeated dose toxicity

Oral studies

Oral doses of 50, 20, 5 and 1 µg AZA/kg b.w. were given twice a week, up to 40 times, within 145 days, to 4 groups of 10, 10, 5 and 6 mice (4 weeks old), respectively. Nineteen control mice were used. Nine mice out of ten at 50 µg/kg b.w. and three out of ten at 20 µg/kg b.w. became so weak (inactivity and weight loss) that they were sacrificed before being treated 40 times (mainly after 30 treatments). Interstitial pneumonia and shortened small intestinal villi were observed. At 5 and 1 µg/kg b.w., no mortality was seen. The mice that survived 40 treatments were kept for up to 3 months after withdrawal. No fatty changes in the liver, previously seen at acute or lethal oral doses, were observed. At 50 µg/kg b.w., a lung tumour was seen in 1/10 mice dosed 32 times. At 20 µg/kg b.w., a lung tumour was observed in 1/10 mice dosed 36 times and in 2 additional mice after withdrawal. In addition, hyperplasia of epithelial cells in the stomach was seen in 6/10 mice at 20 µg/kg b.w. At 5 µg/kg, all 5 mice showed erosion of small intestine (possibly attributed to unhealed injuries rather than late effects developed during withdrawal period). At 1 µg/kg, 1 out of 6 mice developed hyperplastic nodules in the liver and 2 mice out of 6 showed mitosis in liver (Ito *et al.*, 2002).

TABLE 2
Short-term toxicity of AZA-1 in mice

Species Strain, sex, age	No.	Route	Dose µg/kg b.w.	Doses given (days)	Effects	References
Mouse ICR male 4w~	10/10	p.o. 2 times/w	50	17~40 50~145)	Death Stomach and intestines (gas, erosion), Lung (inflammation)	Ito *et al.*, 2002
Mouse ICR male 4w~	3/10 9/20	p.o. 2 times/w	20	30~36 (102~127) 12~20 (35~68)	Death Stomach and intestines (gas, erosion), Lung (inflammation)	Ito *et al.*, 2002 Ito, unpublished

For assessing possible chronic human health effects, studies involving repeated oral administration of AZA-1 are most desirable.

As seen in the Table 2, even less than 1/10 of the lethal dose became a lethal dose when given to mice repeatedly. At the 50 µg/kg dose level, AZA-1 killed 10/10 mice with up to 40 doses given, 3/10 mice died at 20 µg/kg dose level after up to 30~36 doses given (Ito *et al.*, 2000) or 9/20 mice after 20 doses given (data unpublished). For doses of 10, 5 or 1 µg/kg, lethal case was not seen by 40 doses given (Ito, unpublished).

Mice that died during the exposure study showed commonly decreased b.w., ballooning and lucent gastrointestinal (GI) organs containing a lot of gas. Pathological changes were observed in multiple organs: lung (interstitial inflammation and congestion), stomach (erosion), small intestine (shortened villi, edema and atrophic lamina propria) and liver (some cases – single or focal necrosis, small inflammation, mitosis or congestion). The reason for gas accumulation was assumed to be poor absorption from degenerated intestine, and low circulation by weakness, and low expiration of CO_2 by the injured lung. With 20 µg/kg, 7 survived mice after 40 gavages were observed for 1 month (n=2), 2 months (n=2) and 3 months (n=3). Hyperplasia of the stomach appeared in 6/10 mice in this group. Four lung tumours appeared from two groups; one from 50 µg/kg x32, and three from 20 µg/kg x36, x40+2 month and x40+3 month, but the appearance rate was unclear (4/20<) (Ito *et al.*, 2002).

3.2.3 Long-term toxicity/carcinogenicity

To examine potential carcinogenicity of AZA-1, another experiment was conducted on 95 mice using repeat doses. Group (1) 20 µg/kg-2 per week (n=20, 40 dosages), (2) 20 µg/kg-2/w (n=10, 33 dosages), (3) 5 µg/kg-2/w (n=22, 40 dosages), (4) 10 µg/kg-1/w (n=23, 20 dosages), (5) 5 µg/kg-1/w (n=20), and control (n=52).The dose levels in Groups (1) and (2) were changed depending on their condition as follows: (20 µg/kg x17+10 µg/kg x23) and (20 µg/kg x13+15 µg/kg x5+10 µg/kg x5); then each mouse that survived up to 20 weeks was dosed (1) 19.2, (2) 19.57, (3) 8.24, (4) 7.66 and (5) 4.34 µg/kg, respectively. Sixty-six mice were sacrificed at 8 months according to the chronic cases, but no tumour was observed among these mice. Among the residual 20 mice, comprising 10 mice of Group (2), 6 mice of (3) and 4 mice of (4), five tumours appeared at up to 1 year. These five tumours contained two malignant lymphoma and three lung tumours (1: adeno-carcinoma and 2: epithelial type tumours), and one lung tumour from a total of 71 control mice (8M+12M: n=39+32) (Ito, unpublished). Thus, it is statistically difficult to demonstrate carcinogenicity, but tumour appearance is at least 7.1 percent from two experiments (9/126), as the total number of treated mice was 126, with no control mice developing tumours during the same period.

Because ICR mice show a relatively high ratio of spontaneous tumours in the lung, liver and whole body (21.1, 17.2 and 7.5 percent) at 2 years old (Brayton, 2007), AZA may possibly be either tumorigenic itself or a promoter to early appearance.

Multiple lymphatic nodules in the lung were observed in 10 out of 27 mice at 8 months from Groups (1) and (3); this phenomenon might contribute in some way to clarifying a possible mechanism of tumorigenesis.

3.2.4. Genotoxicity

No data on the possible genotoxic effects of AZAs have been reported.

3.2.5 Reproductive toxicity

Microinjection of AZA-1 caused dose-dependent effects on heart rate, growth rate, hatching success and viability in Japanese medaka *Oryzias latipes* embryos. Within 4 days of exposure to doses of ≥ 40 pg/egg of AZA-1, substantial retardation in development was observed as reduced somatic growth and yolk absorption, and delayed onset of circulation and blood pigmentation. Embryos treated with ≥ 20 pg/egg AZA-1 had slower heart rates (bradycardia) for the 9 day in ovo period followed by reduced hatching success. The studies demonstrate that AZA-1 is a potent teratogen to finfish (Colman *et al.*, 2004).

3.3 Observations in domestic animals/veterinary toxicology

No data on the possible effects of AZAs in domestic animals have been reported.

3.4 Observations in humans

In November 1995, at least eight people in the Netherlands became ill after eating mussels (*Mytilus edulis*) cultivated at Killary Harbour, Ireland. Although human symptoms such as nausea, vomiting, severe diarrhoea and stomach cramps were similar to those of DSP, contaminations of the major DSP toxins OA and dinophysistoxins (DTXs) were very low. These observations prompted the investigators to explore the causative toxin in the mussels for structural studies. After chemical analytical research, the investigators identified and quantified AZA (Satake *et al.*, 1998a, 1998b). Based on these results, the toxicity of the mussels was estimated to be 0.15 MU/g (equivalent to 0.6 µg AZA/g) (EU/SANCO, 2001). A higher toxin content of 1.4 µg AZAs/g of meat (0.4 MU/g of meat) was reported by Ofuji *et al.* (1999b). Human toxicity was seen between 6.7 (5 percent confidence level) and 24.8 (95 percent confidence level) micrograms per person with a mean value of 15 µg/person. However, new data on the heat stability of AZA suggest that it is not appropriate to take into account a reduction in AZA concentration because of heating. Therefore, the recalculated range of the LOAEL is 23–86 µg/person with a mean value of 51.7 µg/person (EU/SANCO, 2001).

4. ANALYTICAL METHODS

4.1 General

European Union legislation (Council Directive 91/492) requires each member State involved in shellfish harvesting to have a national marine biotoxin monitoring programme to monitor shellfish harvesting areas for the presence of toxins produced by several different species of marine phytoplankton.

MBA with acetone extraction, followed by liquid/liquid partition with diethyl ether, can be used to detect AZAs. The rat bioassay can also be used for detection of AZAs with a diarrhoeic response in any of three rats considered a positive result.

Methods based on liquid chromatography (LC) coupled to mass spectrometry (LC-MS) are used as alternative or complementary methods to the biological testing method, i.e. MBA.

European Union guidelines state that the total quantity of AZA must not (measured in the whole body or any part edible separately) exceed 160 µg AZA equivalents/kg.

4.2 *In vivo* assays

DSP mouse bioassay (MBA)

Mussel extracts are injected intraperitoneally in mice as is done for the DSP MBA. The results suggest that AZAs can be extracted with acetone from raw meat because of increased solubility by the presence of water and lipids in the meat (EU/SANCO, 2001). The AZA response is characterized by hopping, scratching and progressing paralysis that is atypical for DSP (Flanagan *et al.*, 2001; Satake *et al.*, 1998a). The shortest time for mouse death was 35 minutes (at 6 times of the lethal dose) and the longest was 30 hours and 46 minutes (EU/SANCO, 2001). The test should be considered semi-quantitative at best, with a detection limit of about 4 µg in a 20 g mouse (lethality of 200 µg/kg bodyweight). Mice showed large variation in susceptibility depending on individuals and age. In an MBA using 24-hour observation, the lethality in mice translates to a detectable concentration of about 160 µg of AZA-1 equivalent per kilogram of shellfish flesh. To the authors' knowledge, neither intralaboratory nor interlaboratory validation of this method has been carried out for AZAs.

Rat bioassay

This assay is based on diarrhoea induction in rats. The (starved) animals are fed with suspect shellfish tissue (mixed into the diet) and observed during 16 hours for signs of diarrhoea, consistency of the faeces and food refusal. The method is at best

qualitative for AZAs because no reports are available on the concentrations that will induce a certain response. The test is still used routinely in the Netherlands and is an officially allowed procedure in EU legislation. To the authors' knowledge, neither intralaboratory nor interlaboratory validation of this method has been carried out for AZAs.

4.3 *In vitro* assays

Cell morphology assays

The development of alternative diagnostic strategies for the detection of phycotoxin contamination in shellfish is driven by scientific, ethical and financial concerns.

To address this, an assay has been developed based upon the cytopathological responses of cultured mammalian cells to phycotoxins. The primary response of these cells to any OA family of toxins is to "round up" and lose their distinctive morphology, within 3 hours, yet they remain about 90 percent viable for up to 48 hours. AZA positive samples, when applied to this system, do not cause the "rounding up" effect on cultured cells. Instead, the cellular viability, as measured by an MTT assay, drops to less than 10 percent of the viability of control cells after 18–24 hours. Combination of cell morphology observation at 3 hours with 24-hour viability measurement enables the detection of both OA type toxins and AZA in shellfish (Flanagan *et al.*, 2001).

Transepithelial electrical resistance (TEER) assays

As the main symptoms of AZA toxicity in humans are GI disturbances, the human colon cell line, Caco-2, was selected for studies by Ryan *et al.* (2004), because of their ability to form tight junctions and generate TEER. When Caco-2 cells are grown on microporous membranes, they form an intact monolayer similar to the *in vivo* GI tract. The intactness of the monolayer can be measured as the TEER. The TEER reflects the barrier function of the GI cells. Exposure of highly confluent cells to AZAs showed significant reduction in the TEER. This assay has proved to be sensitive for detection of AZA; however, full validation in matrix has yet to be carried out. It should be noted that, while the TEER assay may be an appropriate functional assay, it is not specific to AZAs but is also influenced by other toxins present, e.g. OA.

Both *in vivo* and *in vitro* assays are relatively lengthy because they require 16–24-hour observations (mouse and rat bioassay) or 24-hour exposure (morphology and TEER assays).

To the authors' knowledge, neither intralaboratory nor interlaboratory validation of these functional methods for AZAs has been carried out.

4.4 Biochemical assays

No biochemical assays have been developed yet. However, at least two groups are currently working on immunoassays (Norwegian Veterinary Institute, Oslo, and National Diagnostic Centre Ireland, Galway). Because of the number of isomers and homologues present, this technique would be most appropriate for detection in a screening scenario.

4.5 Chemical analytical methods

AZAs do not have ultraviolet (UV) absorbance maxima above 210 nm. Therefore, UV methods are not possible on the underivatized analyte. Derivatization of the AZA molecule has not yet been achieved, although two groups have worked on this item (Philipp Hess, personal communication, 2011). Similarly, fluorescence methods are not available because of the lack of a derivatization method.

Liquid chromatography-mass spectrometry (LC-MS)

The first LC-MS quantitative determination method reported for AZAs was based on selected ion monitoring (SIM) detection (Ofuji *et al.*, 1999b), with one ion per compound and external calibration. Linearity was checked over a relatively wide concentration range (from 50 pg to 100 ng). The recovery data were acceptable; however, only the parent ion was monitored in this method, thereby limiting the confirmatory character of this method.

An ion-trap LC-MS^n method for AZAs was presented by James *et al.* (2001). A microliquid chromatography-tandem mass spectrometry method (micro-LC-MS/MS) was developed for the determination of AZAs (Draisci *et al.*, 2000). The method reported focused on the identification of AZAs, so in fact it had a qualitative accent. Eventually, the aim was formulated as "…to investigate the suitability of LC-MS and LC-MS-MS in order to unambiguously detect AZA in shellfish." By applying SIM on the ions corresponding to the protonated molecules only, the most sensitive form of detection was obtained (maximum intensities). Using the collision-induced dissociation (CID) MS-MS capabilities of the tripleQ, a selected reaction monitoring (SRM) method was developed, resulting also in quantitative data. Good linearity (r^2>0.999) was observed for a small concentration range (0.1 –1 µg/ml), while the detection limit was approximately 20 ng of AZA per gram of whole mussel. In conclusion, the developed method provided very selective and specific data. However, as stated by the authors, a "full validation was hampered by the lack of availability of the AZA standard necessary for recovery experiments".

Quilliam, Hess and Dell'Aversano (2001) reported the integration of the analysis of AZA-1 –3 into a multitoxin method, also incorporating DA, OA, DTXs, PTXs and spirolides. This method enables the analysis of marker compounds from six groups of toxins in a single LC-MS run, thereby significantly speeding up methodology for screening. A variation of this method, analysing OA, DTXs and AZA-1-3, has been in use in Ireland since 2001 and has the added advantage of the use of triple quadrupole MS-MS, which enables added identification through the monitoring of two fragment ions for each of the three AZAs (Hess *et al.*, 2001, 2003). Similar to other reports, this study also found very good sensitivity for AZAs, with a detection limit of about 0.2 ng/ml (= 2 pg on column), which translates into a determination limit of 0.01 µg/g of shellfish flesh, 16-fold lower than the current EU limit.

Lehane *et al.* (2002) reported the development of a liquid chromatography-electrospray ionization with mass spectrometry (LC-ESI-$MS)^n$ method for the determination of the three most prevalent AZA toxins (AZA-1-3), as well as the isometric hydroxylated analogues (AZA-4-5). They demonstrated that LC-multiple tandem MS resulted in more sensitive analysis than LC-single-MS, which suggests "that the reduction in background noise in MS^n is more dramatic than the decline in analyte signal." Although the authors state they have developed a method that requires minimal sample preparation steps, total sample preparation will most probably require the major part of total analysis time.

Next to the article just mentioned, the same research group reported a comparison of solid-phase extraction (SPE) methods for the determination of AZAs in shellfish by the LC-ESI-MS^n method of Lehane (Moroney *et al.*, 2002). Good recovery and reproducibility data were obtained for one diol SPE cartridge and two C_{18} SPE cartridge types. As they state: "the efficient SPE methods presented here for sample preparation should prove more useful in the development of alternative analytical methods for AZP toxins in shellfish." This fits well with their earlier statement: "Sample preparation for the determination of phycotoxins in shellfish can be problematic due, in part, to an extensive variation in the toxic content." The same group reported the same method development in a different journal (Furey *et al.*, 2002). Extensive linearity studies for the determination in shellfish extracts are worth mentioning: Rather good results were

obtained for a concentration range over two decades, which is typically sufficient for regulatory control. An application based on the method just mentioned was reported by the same group (James *et al.*, 2002a, 2002b). The report shows LC-MS^3 spectra of AZA-1-3 both for standards and mussel extracts.

Brombacher, Edmonds and Volmer (2002) have reported a comprehensive study of the mass spectrometric behaviour of AZAs using three types of mass spectrometry: triple quadrupole, ion trap and quadrupole-time-of-flight hybrid mass spectrometry. This study is confirmed by the findings of other reports and unpublished work, indicating that the mass spectrometric behaviour is less dependent on the manufacturer of a mass spectrometer (differences in source design, etc.) than on the type of mass spectrometry (ion trap vs triple quad or Q-TOF). The same group also reported a very rapid analysis of AZAs using monolithic columns (Volmer, Brombacher and Whitehead, 2002), a technique that may be applied for a number of toxin groups and lead to yet speedier analysis of the large number of lipophilic toxins potentially present in a single sample.

An interlaboratory study of an LC-MS method for determination of AZA-1 (and other lipophilic toxins) was carried out in shellfish extracts (Holland and McNabb, 2003). The eight participating laboratories generally obtained consistent sets of data for the broad group of toxins down to low levels (< nag/ml, equivalent to 0.05 mg/kg). The method could reliably detect AZA-1 and a range of other toxins. However, the results were not sufficient to meet Codex requirements for a quantitative method to enforce Codex standards.

As a consequence of limited availability of standards for AZAs for interlaboratory studies, single laboratory validation according to the harmonized guideline (IUPAC/AOAC/ISO) is under way in a number of Codex Member States (Ireland, Norway, the United Kingdom, and others). The LC-MS approach continues to evolve and improve for quantification of marine biotoxins and is increasingly being employed in developed countries for marine biotoxin analyses. International initiatives will most probably lead to interlaboratory validation according to internationally accepted protocols, as soon as certified standards are available.

5. LEVELS AND PATTERNS OF CONTAMINATION OF BIVALVE MOLLUSCS

5.1 Occurrence of AZA in Europe

Although previously the heterotroph *Protoperidinium crassipes* had been associated with AZAs, recent studies including a statistical review of Irish monitoring data (Moran *et al.*, 2007) could not corroborate this hypothesis. Instead, a small dinoflagellate was discovered to produce AZAs in the field and in culture, *Azadinium spinosum* (Krock *et al.*, 2009; Tillmann *et al.*, 2009). Because of its small size (5 by 15 µm) and its fragility to iodine-based fixing agents, this organism may not have been identified in other regions. However, it is noted that *A. spinosum* has also been found in Danish waters (Krock, Tillmann and Cembella, 2009b). In addition, AZAs have been reported to occur in shellfish from France, Morocco, Portugal and the United Kingdom (Amzil *et al.*, 2008; EFSA, 2008; Taleb *et al.*, 2006; Vale, Bire and Hess, 2008). Finally, AZA-2, a major analogue of AZA-1 also in Irish shellfish, has most recently been discovered in a sponge collected from Japanese waters (Ueoka *et al.*, 2009). Therefore, a significantly wider geographical distribution than initially proposed must be assumed.

Ireland

In November 1995, at least eight people in the Netherlands became ill after eating mussels (*Mytilus edulis*), cultivated at Killary Harbour, Ireland (McMahon and Silke, 1996; Satake *et al.*, 1998a). A toxin then called Killary Toxin-3 or KT3 was detected. Satake *et al.* (1998b) elucidated the structure of KT3 and called the toxin AZA. Mussels collected in February 1996 showed a toxin content of 0.15 MU/g (=0.6 µg AZA/g) (EU/SANCO, 2001).

Since 1996, mussels produced in Ireland have been linked to several AZP incidents (in 1997, cases of contamination recurred in the Arranmore Island region of Donegal, northwest Ireland, and repeatedly caused human intoxication in other European countries). Although no known toxic phytoplankton were observed in cultivation areas after these intoxications, it is probable that AZP toxins were produced by marine dinoflagellates (James *et al.*, 2002a).

Mussels collected at Killary Harbour on 23 April 1996 (5 months after the incident) contained 1.14 µg AZA/g of meat, 0.23 µg AZA-2/g of meat and 0.06 µg AZA-3/g of meat (total AZAs 1.4 µg/g of meat). Mussels collected at Arronmore Island on 3 November 1997 (1–2 months after the incident) contained 0.865 µg AZA/g of whole mussel meat (including [HP]), 0.25 µg AZA-2/g and 0.24 µg AZA-3/g (total AZAs 1.36 µg/g). Results of MBA revealed 0.4 MU/g of meat (Ofuji *et al.*, 1999b). Anderson *et al.,* (2001) reported that the maximum AZA content in shellfish during the Arranmore Island incident was 10.7 µg/g of HP.

In November 1997, James *et al.,* (2000) detected 2.21 µg AZAs/g in raw whole meat of mussels.

After the initial intoxication in Arranmore Island and Killary Harbour, the toxin persisted for a further 7–8 months. Oysters seem to be just as susceptible as mussels to intoxication by AZP toxins (Table 3) (James *et al.*, 2000).

TABLE 3
Levels of AZAs in mussels and oysters from Ireland

Location in Ireland	Date	Total AZAs µg/100 g (mussel)	Total AZAs µg/100 g (oyster)
County Cork	November 1998	70	70
County Cork	February 2000	10	20
Bruckless, Co. Donegal	November 1999	10	30

This susceptibility has not been confirmed, as a more recent study shows a clear difference between AZA-contamination in oysters compared with mussels (Hess *et al.*, 2003).

5.2 Food consumption and dietary intake estimates

EU/SANCO (2001) stated that, based on poisoning incidents in Ireland, levels of AZAs causing human intoxication were calculated to be between 6.7 and 24.9 µg. These figures included a reduction in AZA content because of heating of the mussels. New data on heat stability have revealed that this reduction of the toxin content because of heating was not justified. Therefore, the recalculated range of the LOAEL appeared to be between 23 and 86 µg per person assuming a maximum consumption of 100 g shellfish/meal. EU/SANCO (2001) applied a safety factor of 3 to convert the lowest observable adverse effect level (LOAEL) to a no observable effect level (NOAEL). Based on an intake level of a maximum of 100 g shellfish meat/meal, and the lowest LOAEL divided by three, EU/SANCO (2001) stated that an allowance level of 8 µg AZAs/100 g of shellfish should result in no appreciable risk for human health. To allow for detection by MBA, a level of 16 µg/100 g was proposed.

However, at a shellfish consumption of 300 g/meal, a person will already be consuming an amount of AZAs equal to the LOAEL in humans.

Ofuji *et al.* (1999b) reported a level for total AZAs in raw mussel meat in poisoning incidents of 1.4 µg/g of meat. At a consumption of 100–300 g/meal, this means an intake of 140–420 µg AZAs/person. As these figures represent an effect level (LOAEL), usually a factor of ten is used for calculation of an NOAEL. This means that the NOAEL is 14–42 µg per person assuming a consumption of 100–300 g shellfish meat/meal. As a consequence, the allowance level in shellfish meat has to be 14 µg/100 g. It has to be noted that no factor of ten was applied to the NOAEL for intraspecies differences (variation in the human population).

5.3 Effects of processing

AZAs are heat stable and resistant to processing.

6. DOSE RESPONSE ANALYSIS AND ESTIMATION OF CARCINOGENIC RISK

No data at present.

7. EVALUATION[2]

7.1 Toxicology of azaspiracids and regulatory limits

Absorption, distribution, metabolism and excretion

No data are available.

Mechanism of action

No data are available.

Toxicity in animals

Preliminary experiments indicate that AZA-1, administered once or twice by gavage at dose levels of 250–450 µg/kg b.w., caused death in some mice and serious GI, pulmonary and hepatic effects that persisted for a prolonged period in those that survived.

In a preliminary long-term experiment, repeated administration once or twice a week by gavage of 20 µg/kg b.w. for 10–20 weeks caused death in some mice, and doses of 5–20 µg/kg b.w. caused a statistically insignificant increased incidence of lung tumours at 1 year in survivors. Because the strain of mouse used in this experiment normally has a high background incidence of pulmonary as well as hepatic tumours, these results may indicate that AZA is carcinogenic, or more probably, that it is a tumour promoter. No genotoxicity data are available and no definitive conclusions regarding relevance to humans can be drawn.

No oral toxicity data are available on AZA analogues, but on the basis of i.p. studies in mice, it would appear that AZA-2 and -3 are somewhat more toxic than AZA-1, and AZA-4 and -5 are less toxic.

Observations in humans

Limited data in humans indicate an LOAEL between 23 and 86 µg/person for acute GI effects.

[2] It must be pointed out that, as a result of the Expert Consultation, the evaluation section in the report differed from the draft chapter. The Evaluation Section of the present "Background Document" corresponds to the Expert Consultation Report.

Evaluation

The 2004 Expert Consultation established a provisional acute reference dose (ARfD) of 0.04 µg/kg b.w., based on the LOAEL of 23 µg per person in humans and a b.w. of 60 kg, using a tenfold safety factor to take into consideration the small number of people involved.

The Expert Consultation found that because of insufficient data on the chronic effects of AZA, no tolerable daily intake (TDI) could be established.

The consumption of 250 or 380 g shellfish meat by adults would lead to a derived guidance level of 0.0096 or 0.0063 mg/kg, respectively.

Gaps in the data

The preliminary studies, in which AZA was administered by gavage, indicate the possibility of severe and prolonged toxic effects at low doses. Administration by gavage may, however, have contributed to the observed severe erosive effects in the GI tract. Repeat studies involving administration of the test material by feeding are urgently required.

To establish a TDI, data on long-term carcinogenicity and genotoxicity, and reproductive toxicity are needed. Information on absorption, excretion and metabolism is also required.

7.2 Analytical methodology

Available methods

In vivo bioassays

Mouse or rat bioassays can detect AZAs with a limit of detection (LOD) of about 0.16 mg/kg, but there are potential interferences from other lipophilic toxins. Further method development and validation is required, particularly to achieve lower LODs.

Instrumental methods

AZAs lack a chromophore for LC-UV determination, and conditions for fluorescence derivatization have not been established. However, LC-MS has shown great promise as a highly specific and sensitive technique for detection of AZAs. One multitoxin protocol (McNabb, Selwood and Holland, 2005) has been subjected to a full within-laboratory validation (four shellfish species) and a limited interlaboratory study. The limit of quantitation (LOQ) for this method was 0.05 mg/kg but lower limits would be readily achievable, which will be necessary to enforce the proposed levels.

Recommendation for choice of Reference Method (Type II)

Because an LC-MS method is the best available option, a collaborative study of a multitoxin method that includes AZAs should be conducted to fully meet Type II criteria. However, applicability of the technique is currently limited by the lack of certified analytical standards.

Management of analytical results

Analytical data for all methods should be expressed as milligrams of AZA-1 equivalents per kilogram of whole flesh, using toxicity equivalence factors (TEFs) for AZA-2 and AZA-3. Other analogues are considered of low relevance.

Standards and reference materials

Lack of reference materials and standards is a severe limitation to research, development, method validation and management of AZA contamination. Codex should encourage Member States to participate and fund initiatives such as those of the National Research Council of Canada (NRC), Halifax, to develop standards and certified reference materials (CRMs) for AZAs.

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Brevetoxins

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1. BACKGROUND INFORMATION

Neurotoxic shellfish poisoning (NSP) is caused by consumption of molluscan bivalves that have accumulated brevetoxins (BTXs) from marine dinoflagellates of the genus *Karenia*. Recent evidence suggests that several marine rhaphidophytes (e.g. *Chattonella* spp.) also produce BTXs and are potential contributors to NSP. Until 1987, NSP was considered to be endemic to the North American Gulf of Mexico, where "red tides" had been reported as early as 1844. Recent history has shown that BTX-producing algae, and consequently NSP, have expanded well beyond the confines of the Gulf of Mexico. In 1987, a major Florida *K. brevis* bloom was dispersed by the Gulf Stream northward into waters of North Carolina, the United States of America, where 48 persons became ill with NSP. *Karenia brevis* has since continued to be observed in North Carolina waters. In 1992/1993, more than 180 persons became ill in the first recorded outbreak of NSP in New Zealand. The algal species responsible for this outbreak was not determined nor the circumstances surrounding its presence. An extensive review concluded that *K. mikimotoi* was the most likely causative agent but that at least three other suspect species were present at the time (Todd, 2002). Confirmed occurrences of NSP appear to be limited to the North American Gulf of Mexico, the southeast coast of the United States of America, and the New Zealand Hauraki Gulf region. However, the discovery of new BTX-producing algae and the apparent trend towards expansion of harmful algal bloom distribution suggest an emerging public health hazard in other regions of the globe.

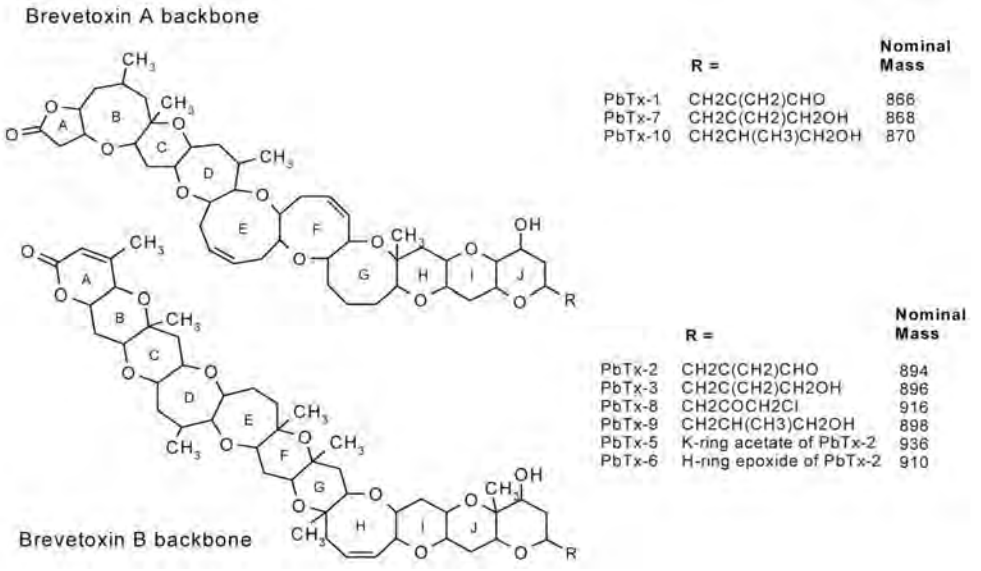
2. BREVETOXIN ORIGINS AND OCCURRENCE IN MOLLUSCAN SHELLFISH

2.1 Brevetoxin production by marine algae

2.1.1 Production by Karenia *spp.*

The NSP toxins, called BTXs, are cyclic polyether natural products produced by a few species of marine dinoflagellates (e.g. *K. brevis*) and raphidophytes (e.g. *Chattonella* sp.). Most of what is known about the BTXs derives from many years of research on *K. brevis* (syns. *Gymnodinium breve*, *Ptychodiscus brevis*). *K. brevis* produces at least nine BTX congeners, grouped according to their backbone structures (types A and B; Figure 1). Principal A-type BTXs are PbTx-1 and PbTx-7, and the principal B-types are PbTx-2, PbTx-3, and PbTx-9. PbTx-2 is the most abundant congener from *K. brevis*, while PbTx-1 is the most potent (Landsberg, 2002). In their natural state, and as accumulated in molluscan bivalves, the BTXs are tasteless, odourless, and heat and acid stabile.

FIGURE 1
Chemical structures of type A and B brevetoxins



Source: Plakas *et al.*, 2004.

2.1.2 Production of brevetoxins (BTXs) by Chattonella *spp.*

BTX production by raphidophyte species is a recent discovery and much remains to be learned of their toxin profiles. BTX-like toxins are produced by four algal species belonging to the class Raphidophyceae (raphidophytes). Three neurotoxic compounds were isolated from *Chattonella antiqua* cultures, CaTx-I, CaTx-II and CaTx-III, which appeared to correspond to BTXs PbTx-2, PbTx-3 and oxidized PbTx-2. Five neurotoxic components were tentatively identified from cultures of the red-tide-producing species *Fibrocapsa japonica*, FjTx-I, FjTx-II, FjTx-IIIa, FjTx-IIIb and FjTx-IV. These neurotoxic components corresponded to PbTx-1, PbTx-2, PbTx-9, and PbTx-3 and oxidized PbTx-2, respectively. In 1995, an unusual large-scale red tide of *Heterosigma akashiwo* occurred in Kagoshima Bay, Japan, causing massive fish kills. Four neurotoxic components, HaTx-I, HaTx-IIa, HaTx-IIb and HaTx-III, corresponding to PbTx-2, PbTx-9, PbTx-3 and oxidized PbTx-2, respectively, were isolated. Four neurotoxic components were isolated from *Chattonella marina* and were identified to be PbTx-2, PbTx-3, PbTx-9 and oxidized PbTx-2.

2.2 Brevetoxin metabolites in bivalve molluscs

2.2.1 Bivalve metabolites of brevetoxins

Until recently, NSP toxins in shellfish were presumed to reflect only those toxins produced by algae, unmodified as they accumulate in shellfish. Evidence to suggest metabolism of PbTx in bivalve molluscs first appeared in chemical studies of New Zealand shellfish after the 1992–93 outbreak of NSP (Ishida *et al.*, 1995, 1996, 2004a; Morohashi *et al.*, 1995, 1999; Murata *et al.*, 1998). Further evidence came from studies in the Eastern oyster (*Crassostrea virginica*) following a 1996 Gulf of Mexico *K. brevis* bloom (Dickey *et al.*, 1999) and from a related NSP outbreak investigation (Poli *et al.*, 2000). Subsequently, metabolism of BTXs was demonstrated in Eastern oyster through controlled laboratory exposures to *K. brevis* and its purified toxins (Plakas *et al.*, 2002, 2004; Wang *et al.*, 2004). Results from these investigations suggest that metabolites of PbTx contribute to shellfish toxicity, which is under-represented in the traditional protocol for diethyl ether extraction and MBA.

Several metabolites of PbTx were identified in molluscan shellfish following the New Zealand bloom. Ishida *et al.* (1995) isolated a C-42 *N*-taurine conjugate of PbTx-2 (named BTX-B1) in the cockle *Austrovenus stutchburyi*. Minimum lethal dose (MLD) of BTX-B1 was 0.05 mg/kg i.p. in mice. Murata *et al.* (1998) found an oxidized (sulphoxide) *S*-cysteine conjugate (named BTX-B2) in the greenshell mussel *Perna canaliculus*, and proposed biosynthetic routes from either PbTx-2 or -3. The MLD of BTX-B2 in mice was 0.306 mg/kg i.p. Morohashi *et al.* (1995) described fatty acid esters of PbTx-2 in *P. canaliculus* formed by cleavage of ring D, esterification with palmitic or myristic acids, and oxidation of the terminal aldehyde, which they named BTX-B3. These derivatives, in which the backbone structure is disrupted, were apparently non-toxic (at 0.3 mg/kg i.p.) to mice. Morohashi *et al.* (1999) also isolated *N*-myristoyl- and *N*-palmitoyl conjugates of BTX-B2 (named BTX-B4) in *P. canaliculus*. BTX-B4 was threefold more toxic than BTX-B2 (cysteine-PbTx-B sulphoxide) and comparable in potency to PbTx-3, accounting for two-thirds of the total mouse toxicity in mussel tissue extracts (Morohashi *et al.*, 1999). Ishida *et al.* (1996) identified an oxidative metabolite of PbTx-2 in the cockle (*Austrovenus stutchburyi*), which they named BTX-B5. The Pacific oyster *Crassostrea gigas* yielded only PbTx-2 and PbTx-3 (Ishida *et al.*, 1996).

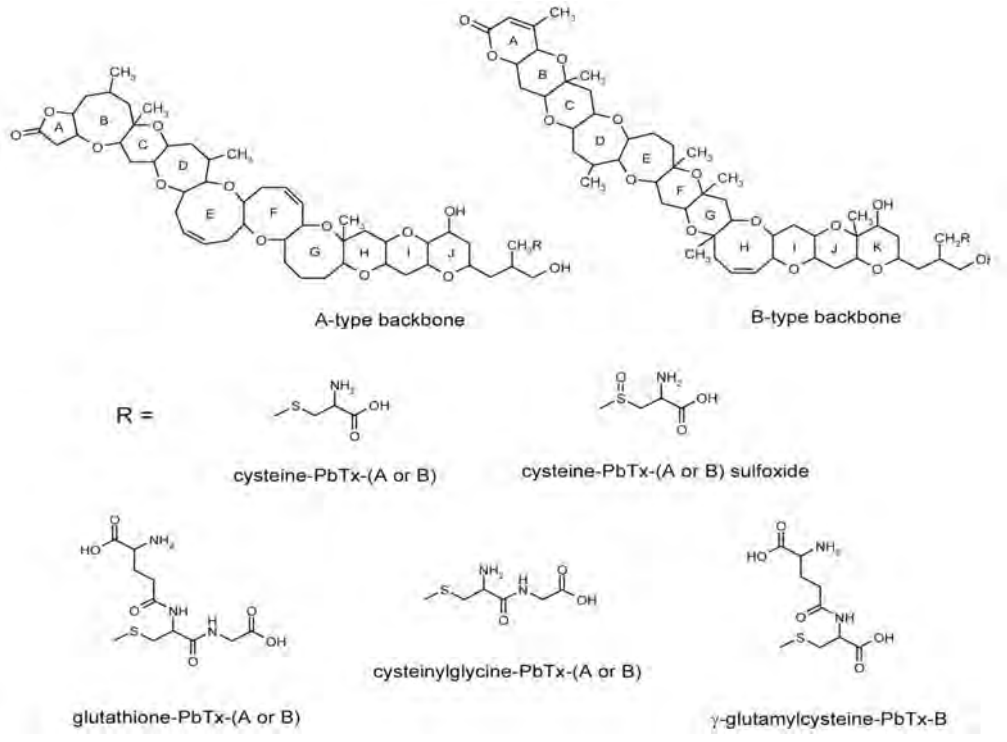
An equally complex profile of PbTx metabolites, including some of those found in New Zealand shellfish, was identified in the Eastern oyster (*Crassostrea virginica*) after the 1996 United States Gulf of Mexico *K. brevis* bloom. The extensive metabolism of PbTx-2, including its reduction to PbTx-3 and PbTx-9, was demonstrated in studies with pure toxin (Plakas *et al.*, 2002; Wang *et al.*, 2004). Cysteine and oxidized cysteine conjugates of PbTx-1 (named cysteine-PbTx-A and cysteine-PbTx-A sulphoxide) and PbTx-2 (named cysteine-PbTx-B and cysteine-PbTx-B sulphoxide) were identified as the more prominent ions in the PbTx metabolite profile, by LC/MS (Plakas *et al.*, 2002, 2004; Wang *et al.*, 2004). Cysteine-PbTx-B sulphoxide is the same compound isolated previously from Greenshell mussel (BTX-B2) by Murata *et al.* (1998). Wang *et al.* (2004) also identified glutathione and related di-peptide (γ-glutamylcysteine and cysteinylglycine) conjugates, with A- and B-type backbone structures, where similar reaction through the sulphhydryl group of the cysteine residue was indicated. Structures of these metabolites are presented in Figure 2.

Other BTX derivatives identified in Eastern oyster include the oxidized form of PbTx-2, in which the terminal aldehyde group is replaced with a carboxylic acid group (Wang *et al.*, 2004). This is the same as BTX-B5 identified in the cockle (Ishida *et al.*, 1996). Only trace levels of the corresponding oxidized form of PbTx-1 were found in the oyster (Plakas *et al.*, 2004). Wang *et al.* (2004) also described hydrolysis products in Eastern oyster in which the lactone ring (A ring) of A- and B-type BTX backbone structures is opened. These include the open A-ring forms of cysteine-PbTx-A and cysteine-PbTx-B. Amino acid-PbTx conjugates react with fatty acids through amide linkage to form a series of fatty acid-amino acid-PbTx conjugates (Wang *et al.*, 2004). Most abundant were *N*-hexadecanoyl-cysteine-PbTx-B and its sulphoxide, and *N*-tetradecanoyl-cysteine-PbTx-B and its sulphoxide. The sulphoxide forms were structurally consistent with the BTX-B4 metabolites identified by Morohashi *et al.* (1999) in Greenshell mussel. These fatty acid conjugates were major contributors to the composite cytotoxicity in extracts of *K. brevis*-exposed oysters.

In general, conjugates with B-type BTX backbone structure were in much higher abundance than those of A-type in Eastern oyster (Wang *et al.*, 2004). This is consistent with the relative abundances of their respective parent algal toxins PbTx-2 and PbTx-1 found in *K. brevis* field samples and cultures from the United States Gulf of Mexico region. The BTX metabolite profile observed in field-exposed oysters was confirmed in oysters exposed to *K. brevis* cultures in the laboratory (Plakas *et al.*, 2002, 2004;

Wang *et al*., 2004). Comparisons with New Zealand shellfish data indicate some species specificity in the molluscan metabolism of BTXs.

FIGURE 2
Structures of the cysteine (and peptide) adducts of BTX as identified in the Eastern oyster by LC-MS(/MS)



Source: Plakas *et al*., 2004.

2.2.2 Uptake and elimination

Research efforts to identify PbTx metabolites in molluscan bivalves, as summarized above, are very recent. Similarly, detailed studies of uptake and elimination of PbTx and its metabolites in bivalves are only now appearing in the scientific literature. Dickey *et al*. (1999) reported that the PbTx-contaminated Eastern oyster retained toxicity by MBA for up to 75 days after dissipation of a *K. brevis* bloom in the United States Gulf of Mexico. In controlled studies exposing oysters to pure BTXs (PbTx-3 and PbTx-2), Plakas *et al*. (2002) determined that PbTx-3 was rapidly accumulated and eliminated unaltered within two weeks after exposure. PbTx-2 was also rapidly accumulated but was extensively metabolized. A portion of the PbTx-2 was immediately reduced to PbTx-3 and eliminated within two weeks, as was the case for direct PbTx-3 exposure. Other metabolites (i.e. cysteine conjugates) were eliminated slowly from the oyster, persisting throughout an eight-week depuration period. In a more recent six-month depuration study, Plakas *et al*. (2004) examined the elimination of PbTx and metabolites in Eastern oyster following controlled exposures to *K. brevis* cultures. Slow depuration of BTX metabolites was confirmed through measurement of specific metabolites by using LC-MS and through measurement of composite toxin by *in vitro* assays (i.e. receptor binding, cytotoxicity, and enzyme-linked immunosorbent assay [ELISA]). Potential LC-MS determinants of PbTx exposure and toxin content were identified. Results of this comprehensive elimination study are detailed below.

2.2.2.1 Parent polyether brevetoxins (PbTxs) and reduction products

In oysters exposed to *K. brevis* cultures in the laboratory, parent algal BTXs PbTx-1 and PbTx-2 were not detectable by LC-MS. Both possess an unsaturated aldehyde in their tail regions, conferring high reactivity. This finding was consistent with earlier studies of oysters exposed in the laboratory to pure BTX PbTx-2 (Plakas *et al*., 2002) and in the field to *K. brevis* blooms (Poli *et al*., 2000; Plakas *et al*., 2002). PbTx-3 and PbTx-9, which are found in *K. brevis* but are also products of reductive metabolism of PbTx-2 (Plakas *et al*., 2002; Wang *et al*., 2004), were found in appreciable levels, but eliminated largely within two weeks of depuration. PbTx-7 and PbTx-10, the reduced forms of PbTx-1, were non-detectable.

2.2.2.2 Oxidation products

Oxidized PbTx-2 was found at lower levels than those of the reductive metabolites in Eastern oyster, but declined at a similar rate, and was not detectable at one week after exposure. Only trace levels of the corresponding oxidized form of PbTx-1 were found under these conditions. In a study of New Zealand shellfish (i.e. cockle, Greenshell mussel), the oxidized PbTx-2 compound (BTX-B5) was also eliminated rapidly (Ishida *et al*., 2004b). The study considered BTX-B5, together with PbTx-3, good markers of shellfish neurotoxicity following *K. brevis* blooms.

2.2.2.3 Amino acid (and peptide) adducts

Cysteine adducts of PbTx-1 and PbTx-2 are the most prominent ions in the PbTx metabolite profile in Eastern oyster, by LC-MS. Cysteine adducts of PbTx-1 and PbTx-2 are also very persistent in the oyster, and were detectable for up to six months after exposure to *K. brevis* (Figure 2). Elimination half-lives were as follows: cysteine-PbTx-B, 8.2 weeks; cysteine-PbTx-B sulphoxide, 10.5 weeks; cysteine-PbTx-A, 8.7 weeks; and cysteine-PbTx-A sulphoxide, 10.9 weeks. The sulphoxide forms comprised on average 26 percent of the total cysteine conjugates for A- and B-type BTXs. However, sulphoxidation was recognized as an artefact of sample preparation and storage (Plakas *et al*., 2002; Wang *et al*., 2004), and the extent to which it occurs in the oyster as a product of BTX metabolism is unknown. Based on this study, and those described, the S-cysteine (and its sulphoxide) adduct(s) is an excellent marker for BTX exposure in the Eastern oyster, based on its high abundance and persistent nature.

Of the peptide adducts (Figure 2), glutathione-PbTx-B was found in the highest relative abundance. Levels of this metabolite were initially comparable with those of the cysteine adducts, but declined rapidly and were not measurable beyond two weeks of depuration. Glutathione-PbTx-A was in much lower abundance initially and non-detectable at one week. Similarly, cysteinylglycine and γ-glutamylcysteine conjugates were in relatively low abundance, non-detectable at two weeks after dosing. Elimination of glutathione and related di-peptide conjugates was considerably more rapid than that of the cysteine conjugates, and comparable to that of PbTx-3 and PbTx-9.

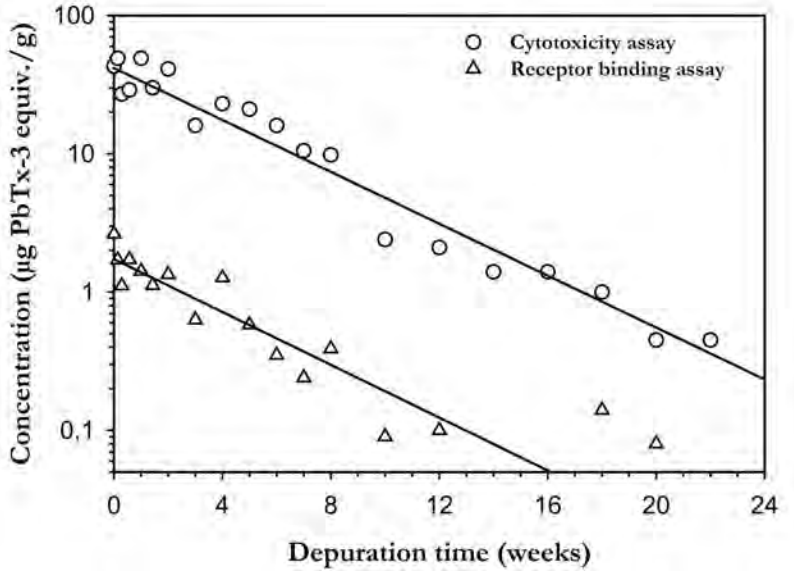
2.2.2.4 Hydrolysis products

Hydrolysis products, as identified by Wang *et al*. (2004), include the open A-ring forms of cysteine-PbTx-A and cysteine-PbTx–B. These open A-ring cysteine adducts were as persistent in the oyster as their closed-ring counterparts, but unstable in extracts, and their elimination was not described. As expected, open A-ring forms of the reactive parent BTXs PbTx-1 or PbTx-2 were not found in the oyster, while that of the A-type BTX PbTx-7 was found. Open A-ring forms of BTXs (including PbTx-1 and PbTx-2) are found in *K. brevis* culture, but have not been studied quantitatively, nor conditions of their formation (in culture).

2.2.2.5 Fatty acid-amino acid-PbTx adducts

Fatty acid-amino acid-PbTx conjugates give relatively weak signals by LC-MS, compared with the cysteine conjugates. In the elimination study, these metabolites persisted at low levels for up to ten weeks of depuration. With low signal: noise and variable responses, no attempt was made to model their elimination. Because of their diverse nature and relatively low abundance, fatty acid conjugates may prove less useful LC-MS markers for BTX exposure or total toxin content in Eastern oyster, while their overall contribution to toxicity is possibly substantial.

FIGURE 3
Elimination of composite toxin



Notes: As measured by cytotoxicity and receptor binding assays, in the oyster after exposure to *K. brevis*.
Source: Plakas *et al*., 2004.

2.2.2.6 Measurement of composite toxin

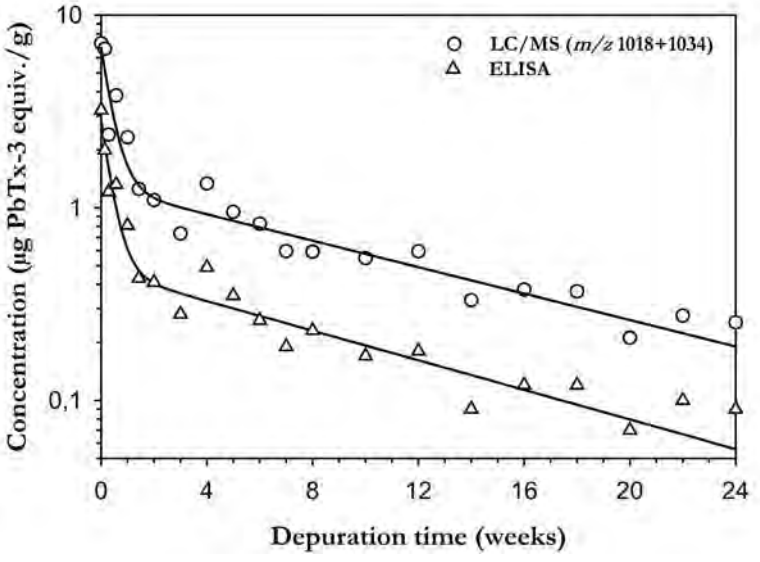
Elimination of composite toxin in the oyster was determined by *in vitro* assay. Toxin measurements by cytotoxicity assay were compared with those of receptor binding assay (RBA), as these assays are functionally based, where binding of BTXs to the pharmacologic receptor (voltage-gated sodium channel) is the recognition element (Van Dolah and Ramsdell, 2001). Both assays respond to BTX metabolites in the oyster (Plakas *et al*., 2002, 2004; Wang *et al*., 2004). Composite toxin measurements by either assay reflect a slow elimination of BTXs in the oyster following *K. brevis* exposure, with toxin activity detectable for 5–6 months after dosing (Figure 3). Elimination half-lives for composite toxin as determined by cytotoxicity and RBAs were 3.2 and 3.0 weeks, respectively. While slopes of the elimination curves were nearly the same, numerically cytotoxicity values were on average thirtyfold greater than those of the RBA, as measured against PbTx-3 standard. Similar ratios in assay values were found previously (Dickey *et al*., 1999). Differences in responses were attributable to metabolites less polar than PbTx-3 (i.e. fatty acid conjugates), for reasons unknown.

Rate of elimination of composite toxin was also measured by ELISA. ELISA, as performed here, incorporated antibody directed against BTXs of B-type backbone structure (specifically, H-K ring region). ELISA data were correlated closely with LC-MS data when plotted against the more abundant B-type BTX metabolites, cysteine-PbTx-B and cysteine-PbTx-B sulphoxide (Figure 4). Elimination half-lives for composite toxin by ELISA and for the summed LC-MS value were

7.8 and 7.9 weeks, respectively. ELISA and RBA are considered viable alternatives to MBA in screening toxic oysters after *K. brevis* blooms (Dickey *et al.*, 2004). However, the data illustrate that ELISA and RBAs can diverge in their measurements because of BTX metabolites. Based on the elimination curves, the most persistent metabolites (i.e. cysteine conjugates) appear inherently less toxic (by *in vitro* assay) compared with PbTx-3. Differences in responses of *in vitro* assays to BTX metabolites, compared with PbTx-3 standard, were also noted previously (Poli *et al.*, 2000).

Murata *et al.* (1998) found BTX-B2 (cysteine-PbTx-B sulphoxide) with one-third the cytotoxicity of PbTx-3. Differences in assay responses to PbTx and metabolites may be particularly apparent under chronic or multiple exposure conditions, where accumulation of the cysteine conjugate would be expected.

FIGURE 4
Elimination of composite toxin



Notes: As measured by ELISA (B-type BTXs), versus the sum of the B-type cysteine adducts (m/z 1018 and 1034), as determined by LC-MS, in the oyster after exposure to *K. brevis*.
Source: Plakas *et al.*, 2004.

3. BIOLOGICAL DATA IN MAMMALS

3.1 Biochemical aspects

3.1.1 Absorption, distribution, excretion and biotransformation

In the toadfish model (*Opsanus beta*), Kennedy *et al.* (1992) found that radiolabelled PbTx-3 was rapidly distributed within 1 hour of intravenous (i.v) administration (40.2 percent muscle, 18.5 percent intestine and 12.4 percent liver); after 96 hours, levels in the liver remained constant, but those in bile, kidney and skin increased, with a variety of metabolites detected.

Cattet and Geraci (1993) orally administered sublethal doses (18.6 µg/kg) of PbTx-3 in rats, and found wide distribution to all organs, with the highest concentrations in the liver up to 8 days after exposure. Ingested PbTx-3 was eliminated approximately equally in urine and faeces. Male F344 rats received a single oral dose of H^3-labelled PbTx-3 and were killed after 6, 12, 24, 48, 96 or 192 hours. Tissues were collected and analysed for radioactivity. Another group of animals received a bolus dose of H^3-PbTx-3 orally; urine and faeces were collected at 24-hour intervals for a period of 7 days. PbTx-3 distributed widely to all organs and concentrations decreased gradually with time. The highest PbTx-3 level was found in the liver at all sampling

times. Based also on the intravenous (i.v.) studies described below, it can be concluded that the liver received PbTx-3 from the portal as well as the hepatic circulation, and so continued to accumulate PbTx-3. Seven days after administration of the oral bolus dose approximately 80 percent of the dose was excreted via urine and faeces, with equivalent amounts in each. However, during the first 48 hours, more PbTx-3 was cleared through the faeces, whereas afterwards, most toxin was cleared through urine (Cattet and Geraci, 1993; FAO, 2004).

With i.v. administration of PbTx-3 in rats, Poli *et al.* (1990a, 1990b) found that approximately 90 percent of the administered radioactive tracer was cleared within one minute from the circulation. I.V. studies in male Sprague-Dawley rats with H^3-labelled PbTx-3 showed a rapid clearance of PbTx-3 from bloodstream (<10 percent remained after 1 minute) and distribution to the liver (18 percent of the dose after 30 minutes), skeletal muscle (70 percent of the dose after 30 minutes) and gastrointestinal (GI) tract (8 percent of the dose after 30 minutes) ($T_{½}$ distribution phase approximately 30 seconds). Furthermore, radiolabelling distributed to the skeletal muscle (70 percent), liver (19 percent), and intestine (8 percent) with little activity found in the heart, kidneys, lungs, spleen, testes or brain. Elimination over a 24-hour period was primarily through the faeces. The parent compound was present in the skeletal muscle, but several metabolites of PbTx-3 excreted in the bile were found in the faeces. Thin layer chromatography (TLC) of urine and faeces indicated biotransformation to several more polar compounds. By day 6, 14.4 percent of radioactivity had been excreted in urine and 75.1 percent in faeces, with 9.0 percent remaining in carcass, suggesting biliary excretion as an important route of elimination (FAO, 2004).

To evaluate BTX toxicokinetics from acute exposure up to 7 days, Benson, Tischler and Baden (1999) dosed 12-week-old male F344/Crl BR rats with a single exposure of 6.6 µg/kg PbTx-3 through intratracheal instillation. More than 80 percent of the PbTx-3 was rapidly cleared from the lung and distributed by the blood throughout the body, particularly the skeletal muscle, intestines and liver with low but constant amounts present in blood, brain and fat. Approximately 20 percent of the toxin was retained in the lung, liver and kidneys for up to 7 days. The majority of the PbTx-3 was excreted within 48 hours after exposure, with twice as much excreted in the faeces as in the urine. The results of this study suggest that the potential health effects associated with inhaled BTXs might extend beyond the transient respiratory irritation seen in humans exposed to sea-spray during red tides (Benson, Tischler and Baden, 1999; FAO, 2004).

Studies with isolated perfused livers and isolated hepatocytes confirmed the liver as site of metabolism and biliary excretion as an important route of toxin elimination. PbTx-3 was excreted into bile as parent toxin plus four more-polar metabolites, one of which appeared to be an epoxide derivative. Whether this compound corresponded to PbTx-6 (C27-28 epoxidized PbTx-2), to the corresponding epoxide of PbTx-3 or to another structure is unknown (Poli *et al.*, 1990a, 1990b; van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004).

The *in vitro* percutaneous penetration of ^{3}H-labelled PbTx-3 in human and guinea-pig skin was examined and the effects of three vehicles (water, methanol and dimethylsulphoxide [DMSO]) were compared. Epidermal surfaces with PbTx-3 in water were occluded for the entire duration (48 hours) of the experiment in order to reduce evaporation. Epidermal surfaces with PbTx-3 in methanol or DMSO were exposed to ambient conditions (incubation of diffusion cells at 36 °C). Total penetration through the isolated human skin was 0.43, 0.14 and 1.53 percent of the dose with water, methanol and DMSO as vehicle, respectively. Total penetration through guinea-pig skin was 1.5, 3.4 and 10.1 percent of the dose with water, methanol and DMSO as vehicle, respectively. Penetration through guinea-pig skin was significantly faster than through human skin with methanol and DMSO as vehicles. Analysis of the receptor

fluid indicated that >80 percent of radioactivity was associated with unchanged PbTx-3 (Kemppainen *et al*., 1989; FAO, 2004).

Dermal penetration and distribution of ^{3}H-labelled PbTx-3 into pig skin (0.3-0.4 µg/cm^{2} of skin) was studied in *in vivo* and *in vitro* studies. DMSO was used as vehicle. In the *in vivo* studies, the application site was covered with a non-occlusive protective patch. In the *in vitro* studies, the epidermal surfaces were exposed to ambient air (22 °C). *In vivo* studies revealed a mean cutaneous absorption of 11.5 percent of the administered dose during 48 hours of topical application (calculated by dividing percentage of dose excreted following topical administration by percentage of dose excreted following subcutaneous [s.c.] administration and multiplying by 100). In *in vitro* studies, mean cutaneous absorption during 48 hours after application was 1.6 percent (based on accumulation of radioactivity in receptor fluid) or 9.9 percent (based on receptor fluid and dermis). Penetration through the epidermis into the dermis was rapid; maximal dermal accumulation was seen at 4 hours (9.1 percent *in vivo* and 18 percent *in vitro*). At 24 hours, the amount in the dermis decreased to 2.3 and 15 percent *in vivo* and *in vitro*, respectively. In the *in vitro* study, >95 percent of radioactivity in the receptor fluid was unchanged PbTx-3 (Kemppainen *et al*., 1991).

3.1.2 Effects on enzymes and other biochemical parameters

BTXs as potent mixtures are characterized by a multiplicity of congeners that change in relative concentrations to one another with bloom growth stage *in situ* and age of the red-tide culture in the laboratory, and the presence and absence of antagonists and metabolites. PbTx-1 and PbTx-2 remain the structural backbones of all natural BTXs and their active and inactive metabolites. The newly identified natural antagonists, brevenals, although polyether molecules, are neither derivatives nor metabolites. Their structures represent totally novel polyethers with the remarkable property of acting as antagonists or reversing agents for effects caused by the BTXs. Subtle changes in the conformational preference of derivative BTXs induce a significant change in the gross shape of the molecule, which is in part believed to be responsible for the loss of binding affinity and toxicity (Rein *et al*., 1994). Brevenals are known to displace BTXs in RBAs; the nature of which is presumed to be competitive, but this is a phenomenon that has yet to be demonstrated conclusively (Bourdelais *et al*., 2003, 2004). Like the BTXs, multiple forms of these biologically active materials exist (A. Bourdelais, personal communication).

Compared with STXs, which block Na^{+} ion influx, BTXs specifically induce a channel-mediated Na^{+} ion influx (Baden and Trainer, 1993). Although evidence suggests that BTXs affect mammalian cortical synaptosomes and neuromuscular preparations and possibly mast cells (Gallagher and Shinnick-Gallagher, 1980; Risk *et al*., 1982; Kirkpatrick *et al*., 2004), all of the effects associated with BTXs result from the substantial and persistent depolarization of nerve membranes (Wu and Narahashi, 1988). This depolarization alters the membrane properties of excitable cell types in ways that enhance the inward flow of Na^{+} ions into the cell; this current can be blocked by external application of tetrodotoxin.

The BTXs act by binding at site 5 in a 1:1 stoichiometry to the α-subunit associated with voltage-sensitive sodium channels (Rein *et al*., 1994). The toxin appears to produce its sensory symptoms by transforming fast sodium channels into slower ones, resulting in persistent activation and repetitive firing (Watters *et al*., 1995; FAO, 2004; Baden, 1983; Purkerson, Baden and Fieber, 1999). Recent work by Purkerson, Baden and Fieber (1999) and others using electrophysiology studies of single sodium channel of rat CNS cells suggest that PbTx-3 may cause hyperexcitability, as well as inhibitory effects, in the intact brain (Apland, Adler and Sheridan, 1993; Templeton, Poli and Solon, 1989; Templeton, Poli and LeClaire, 1989). As a consequence of their lipid solubility, these toxins are expected to pass easily through cell membranes

including the blood brain barrier (BBB), as well as buccal mucosa and skin (Mehta, Kemppainen and Stafford, 1991; Kemppainen *et al*., 1991; Apland, Adler and Sheridan, 1993). Further, Rein *et al*. (1994) and Jeglitsch *et al*. (1998) demonstrated that specific derivatization of specific loci on the active BTX molecule results in a demonstrated reduction in peculiar aspects of BTX action. BTXs induce four separate effects on the voltage-sensitive sodium channel (interchangeable in literature with voltage-gated sodium channel): shifting of the activation potential to more negative values, making normally closed channels favour an open configuration; a prolongation of mean open time so that once channels are open they remain so longer; an inhibition of inactivation so that channels cannot reach the inactivated state to allow allosteric reversion to the closed state; and certain derivatives induce multiple subconductance states that result in aberrant gating kinetics. Each of these aspects of normal toxic action occurs as a result of a demonstrated chemical modification. The conclusion that can be extracted from these findings is that the overall human symptomatology, as well as potency, will likely change depending on the complement of toxin derivatives present (including the antagonists that will reduce potency).

The respiratory problems associated with the inhalation of aerosolized Florida red-tide toxins are believed to result from the opening of sodium channels of nerve cell membranes by the BTXs (Baden and Mende, 1982; Baden and Trainer, 1993; Asai *et al*., 1982; Borison, Ellis and McCarthy, 1980; Franz and LeClaire, 1989; Baden, 1989). These effects can be blocked by atropine (muscarinic blocker) as well as tetrodotoxin (sodium channel blocker), but not by the interruption of vagal nerve stimulation or by diaphragm dissection in experimental animals (Baden and Mende, 1982; Gallagher and Shinnick-Gallagher, 1980; Asai *et al*., 1982; Trainer *et al*., 1991; Baden, 1989; Tsai, Chou and Chen, 1991; Watanabe, Lockey and Krzanowski, 1988). In isolated canine tracheal smooth muscle, neostigmine, an acetylcholinesterase inhibitor, potentiated the BTX-induced contraction; mepyramine, phentolamine, methysergide and chlorisondamine did not affect the contraction (Asai *et al*., 1982). In isolated human bronchial smooth muscle, Shimoda *et al*. (1988) found similar results as well as attenuation by verapamil (calcium and sodium channel blocker). Therefore, BTX produces contraction of the lower airway smooth muscle by stimulation of the cholinergic nerve fibre sodium channels with acetylcholine release. However, additional pathways may be important for physiological effects of BTX. For example, in the rat vas deferens, Sakamoto *et al*. (1985) found that BTX stimulated sodium channels on adrenergic nerve fibres, releasing norepinephrine from the nerve endings.

In addition, there appears to be a role for mast cells in the BTX-associated respiratory effects. Watanabe, Lockey and Krzanowski (1988) noted that BTX could combine with a separate site on the h gates of the sodium channel, causing the release of neurotransmitters from autonomic nerve endings. In particular, this can release acetylcholine, leading to smooth tracheal muscle contraction, as well as massive mast cell degranulation. The mast cell contribution to the adverse airway effects of BTX is supported by studies in a sheep model of asthma. In this model, aerosolized BTX causes bronchoconstriction (i.e. reversible pulmonary airway constriction) that can be blocked by the mast cell stabilizing agent cromolyn and the histamine H_1 antagonist chlorpheniramine (Singer *et al*., 1998). Thus, in addition to the direct neural component, BTX appears to induce the release of histamine from mast cells and the combination of these actions results in adverse airway effects. Furthermore, because BTX exposure by the respiratory route results in systemic distribution of BTX, the initial bronchoconstriction may only be part of the overall consequences associated with toxin inhalation, including direct effects on the CNS (Benson, Tischler and Baden, 1999; Apland, Adler and Sheridan, 1993).

Computer modelling suggests that BTX is a possible enzymatic binding inhibitor of cysteine cathepsins. Cathepsins are powerful lysosomal proteinases and epitope

presenting enzymes, found within cytosol or lysosomes of macrophages, lymphoid tissues and other cells (Bossart *et al*., 1998; Sudarsanam *et al*., 1992). Sudarsanam *et al*. (1992) demonstrated conclusively the competitive inhibition pattern of PbTx-2 on both papain (the parent for the superfamily to which cathepsins B, H, and L belong) proteolytic activity as well as with procathepsin L experiments. Bossart *et al*. (1998) postulated that the effects of aerosolized BTXs might be chronic, not just acute. These chronic effects would begin with the initial phagocytosis by macrophages, inhibition of cathepsins, and apoptosis of these cells, followed by the phagocytosis of the debris by new macrophages, ultimately resulting in chronic neuro-intoxication, haemolytic anaemia, and/or immunologic compromise.

BTXs undergo biotransformation in rodents and fish (Poli *et al*., 1990a, 1990b; Kennedy *et al*., 1992). In fish, the BTXs induce both cytochrome P4501A, and glutathione S transferase with a variety of pathways for metabolism (Washburn *et al*., 1996; Washburn *et al*., 1994). On the basis of evaluations of PbTx-3 on the sodium channels of rat sensory neurons, Jeglitsch *et al*. (1998) suggested that PbTx-3 metabolites might be more potent than PbTx-3 parent compound in affecting sodium channels. Work by Poli *et al*. (2000) evaluating metabolites in both the urine of three persons suffering from NSP and from the contaminated shellfish supported this conclusion; the authors suggested that these toxic metabolites from both the shellfish and the humans may be an additional cause of NSP and should be taken into account during regulatory testing. Unpublished work by Naar and Kubanek failed to show any oral potency using these metabolites (J. Naar, personal communication).

As discussed above, after its synthetic creation, a new ladder-frame polyether compound named "brevenal" containing five fused ether rings was isolated from cultures of the marine dinoflagellate *Karenia brevis*. This compound, together with the dimethyl acetal derivative isolated at the same time, displaces BTX from its binding site in rat brain synaptosomes. Significantly, they are also non-toxic to fish, and also antagonize the toxic effects of BTXs in fish and mammals, presumably by releasing BTX from its site of activity on excitable membranes (Purkerson-Parker *et al*., 2000; Purkerson, Baden and Fieber, 1999; Bourdelais *et al*., 2003; Bourdelais *et al*., 2004).

3.2 Toxicological studies

3.2.1 Acute toxicity

Fish, birds and mammals are all susceptible to the BTXs. In the mosquito fish (*Gambusia affinis*) bioassay, the LC_{50} (24 hour) is reported at 0.011 µg/litre (0.005–0.023), while with Japanese medaka (*Oryzias latipes*) the LC_{50} was reported to be 0.015–25 µg/ml (Bossart *et al*., 1998; Forrester *et al*., 1977; Geraci, 1989; O'Shea *et al*., 1991; Laverty, 1993; Trainer and Baden, 1999; Anderson, 1994; Sierra-Beltran *et al*., 1998; Cortes-Altamirano, Hernandez-Becerril and Luna-Soria, 1995; Ellis, 1985; ILO–UNEP–WHO, 1984; Poli, 1988). Fish kills associated with these red tides have been estimated up to 100 tonnes of fish per day during an active red tide. The fish are killed apparently through lack of muscle coordination and paralysis, convulsions and death by respiratory failure. Birds die acutely with neurologic and haematological effects.

Lu and Tomchik (2002) evaluated the effect of PbTx-3 on the hearing sensitivity of a teleost fish, the goldfish (*Carassius auratus*). The LD_{50} (24 hour) in goldfish that were intraperitoneally injected with PbTx-3 was 0.068 µg/g. Evoked auditory brainstem responses were recorded, and hearing threshold was determined using a correlation method. By comparing thresholds of fish before and after a sublethal-dose injection (0.064 µg/g) of the toxin, PbTx-3 significantly reduced auditory sensitivity up to 9 dB at low frequencies (100 Hz and 500 Hz), but not at a high frequency (2 000 Hz). Reduction of hearing sensitivity was recovered within 24 hours. Results of the study indicate that PbTx-3 could affect hearing capabilities of marine animals that survived exposure to red tides.

With respect to mammals (Table 1), the mouse LD_{50} (24 hour) is 0.170 mg/kg b.w. (0.15–0.27) intraperitoneally, 0.094 mg/kg b.w. intravenously and 0.520 mg/kg b.w. orally (Baden, 1983; Baden, Fleming and Bean, 1995; ILO–UNEP–WHO, 1984). Franz and LeClaire (1989) reported respiratory failure in less than 30 minutes in guinea pigs exposed intravenously to 0.016 mg/kg PbTx-3.

TABLE 1
Acute BTX toxicology

Brevetoxin (BTX)	Route	Observation time	24 hour LD50 value (µg/kg b.w.)	Vehicle	References
PbTx-2	oral (females)	24 hours	6 600	0.9% saline	Baden and Mende 1982, Baden *et al.* 1982
PbTx-3	oral (females)	24 hours	520	0.9% saline	Baden and Mende 1982, Baden *et al.* 1982
PbTx-3	intravenous (females)	24 hours	94	0.9% saline	Baden and Mende 1982, Baden *et al.* 1982
PbTx-2	intravenous (females)	24 hours	200	0.9% saline	Baden and Mende 1982, Baden *et al.* 1982
PbTx-1	intraperitoneal	24 hours	>100	0.9% saline + 0.1% Tween 60	Dechraoui *et al.*, 1999
PbTx-3	intraperitoneal (females)	24 hours	170	0.9% saline	Baden and Mende 1982, Baden *et al.* 1982
PbTx-2	intraperitoneal (females)	24 hours	200	0.9% saline	Baden and Mende 1982, Baden *et al.* 1982

Source: Adapted from FAO, 2004.

BTXs produce a variety of centrally and peripherally mediated effects *in vivo*; these include a rapid reduction in respiratory rate, cardiac conduction disturbances, and a reduction in core and peripheral body temperatures (cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004). In orally dosed mice, PbTx-3 caused tremors, followed by marked muscular contractions or fasciculations, Straub tail phenomenon (a stiff dorsiflexion of the tail resulting from cholinergic excitation), a period of laboured breathing and death. Mice injected with PbTx-3 exhibited the SLUD syndrome, i.e. salivation, lacrimation, urination and defecation. Hypersalivation was the most pronounced symptom, while copious urination and defecation were also common. Compulsive chewing motions and rhinorrhea were occasionally present at higher dosages. I.V. dosing to mice produced immediate effects, whereas i.p. and oral dosing caused latent (30 minutes and 5 hours, respectively) responses. The twofold more potency of PbTx-3 after i.v. dosing compared with i.p. dosing points to partial detoxification or excretion in the bile during the first passage to the liver as described above (Baden and Mende, 1982). In rats, gasping-like respiratory movements, head-bobbing, depression, ataxia, and, in some animals, the development of a head tilt were observed (cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004).

Groups of four male rats received, after surgical preparation and a 24-hour recovery, an i.v. infusion during 1 hour with vehicle only or with 12.5, 25, 50 or 100 µg PbTx-2/kg b.w. and were monitored for 6 hours or until death. All animals at the 100 µg/kg b.w. dose level died within 2 hours. One out of four animals at 50 µg/kg b.w. died during the 6-hour study; the remainder of the animals survived. Within 90 minutes, the respiratory rates at 12.5 µg/kg b.w. fell to near 60 percent of baseline value and at 25, 50 and 100 µg/kg b.w. to 20 percent of baseline value. Recovery to normal respiratory rates occurred 6 hours after exposure except in the 50 µg/kg b.w. group, which recovered to only 60 percent of baseline value. During the first 2 hours, dose-dependent decreases in core body temperature occurred in all treated groups, and a significant decrease in peripheral body temperature was seen in all but the 12.5 µg/kg b.w. group. An average decrease in peripheral body temperature of 0.5 °C was seen in the 12.5 µg/kg b.w.

group. Blood gas values remained normal, except terminally. Electrocardiography showed at doses ≥25 µg/kg b.w. heart block, premature ventricular contractions and idioventricular rhythms (cited from van Apeldoorn, van Egmond and Speijers, 2001).

Catheterized male Hartley guinea pigs received an i.v. infusion with PbTx-3 at a rate of 0.63 µg/kg/minute until death of the animal. The mean time until respiratory failure was 25 minutes. The mean dose of PbTx-3 at that time was 15.8 µg/kg. PbTx-3 caused lactic acidosis of unknown etiology that began early in the infusion period and was compensated for by increased minute volume. Airways resistance was not increased, nor was dynamic compliance decreased during intoxication, suggesting that neither central airways (upper airways, trachea and second-third generation airways) nor peripheral airways responded significantly (Franz and LeClaire, 1989; cited from van Apeldoorn *et al*., 2001; FAO, 2004).

The i.v. LD_{50} in mice of the haemagglutinative fraction separated from red tides of *Chattonella marina*, appeared to be 2–4 mg/kg b.w. The mice showed respiratory paralysis (cited from van Apeldoorn *et al*., 2001; FAO, 2004). I.P. injection of the haemagglutinative fraction, separated from red tides of *Chattonella marina*, in mice at a dose of 2.5 mg did not cause any abnormal sign (cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004).

Repeated inhalation of PbTx-3 (approximately 250 µg/m^3) resulted in neuronal damage in brains of mice exposed for just 2 days (6 hours total exposure; T. Murray, personal communication) and significant inhibition of antigen recognition in rats exposed 0.5 hour/day for 5 days (J. Benson, personal communication). Although the aerosol exposure concentrations are orders of magnitude higher than recently measured along Florida beaches during a moderate red tide (Pierce *et al*., 2003), to date, acute adverse effect levels for these toxic responses have not been identified in rodents through the aerosol route (J. Benson, personal communication).

3.2.2 Short-term toxicity

In animal studies with inhaled BTXs, suppressed splenic antibody production was observed among Sprague Dawley rats inhaling aerosols of crude *K. brevis* extract 4 hours/day for 1 and 4 weeks. No toxicity to the nervous, respiratory or haematopoietic systems was noted (Benson *et al*., 2004). The extract contained primarily PbTx-2 and -3, but also contained brevenal, a newly identified compound in *K. brevis* having pharmacological activity antagonistic to BTX-induced neurotoxicity and bronchoconstrictor activities (Bourdelais *et al*., 2003). BTX-induced suppression of splenic antibody production was confirmed in rats inhaling pure PbTx-3 at 500 µg/m^3 for 0.5 hour and 2 hours/day for 5 consecutive days (Benson *et al*., 2004). Antibody production was suppressed by > 70 percent in the low-exposure group (animals with exposure of 0.5 hours/day) and high BTX exposure groups (exposed for 2 hours/day). Small numbers of splenic and peribronchiolar lymphoid tissue macrophages stained positive for BTX. No biochemical or histological evidence of toxicity to the respiratory, nervous, or haematopoietic systems was found in the rats inhaling pure PbTx-3 for five days.

3.2.3 Long-term toxicity/carcinogenicity

Multiple die-offs of marine mammals have been reported in association with Florida red tide and BTXs (Geraci, 1989; O'Shea *et al*., 1991; Bossart *et al*., 1998). In 1996, a prolonged Florida red tide of several months in the Gulf of Mexico resulted in the documented deaths of 149 endangered Florida manatees (Bossart *et al*., 1998; Trainer and Baden, 1999). The BTX exposure of the manatees appears to have been prolonged inhalation of the red tide toxin aerosol and/or ingestion of contaminated seawater over several weeks to months. This manatee die-off investigation revealed severe catarrhal rhinitis, pulmonary haemorrhage and edema, and non-suppurative leptomeningitis,

as well as possible chronic haemolytic anaemia with multiorgan haemosiderosis and evidence of neurotoxicity (particularly cerebellar) in the dead manatees. Therefore, the respiratory tract, liver, kidneys and brains of the manatees were primary BTX targets, and the BTX exposures and effects were believed to be chronic rather than acute. PbTx-3 and its metabolites were identified by an immunohistochemical stain using a polyclonal primary antibody to BTX to be stored in the lung and other organs in alveolar macrophages and in the brain within lymphocytes and microglial cells. Immunohistochemical staining with interleukin-1-beta converting enzyme showed positive staining with a cellular trophism similar to the BTX antibody staining, suggesting that BTX may initiate apoptosis and/or release inflammatory mediators that culminate in fatal toxic shock. Additional studies demonstrated that BTX binds to isolated nerve preparations from manatee brain with a similar affinity as that reported for terrestrial mammals (Trainer and Baden, 1999).

3.2.4 Genotoxicity

The genotoxic potential of the BTXs has only recently been addressed (Kimm-Brinson and Ramsdell, 2001; J. Gibson, personal communication). Kimm-Brinson characterized adverse developmental effects of BTX-1 (PbTx-1) via microinjection of toxin reconstituted in a triolein oil droplet in Medaka fish (*Oryzias latipes*) embryos. Embryos microinjected with doses of 0.1-8.0 ng/egg parts per million (ppm) of BTX-1 exhibited pronounced muscular activity (hyperkinesis) after embryonic Day 4. Upon hatching, morphologic abnormalities were commonly found in embryos at the following lowest adverse effect levels: 1.0–3.0 ppm, lateral curvature of the spinal column; 3.1–3.4 ppm, herniation of brain meninges through defects in the skull; and 3.4–4.0 ppm, malpositioned eye. Hatching abnormalities were also commonly observed at BTX doses of 2.0 ppm and higher with head-first, as opposed to the normal tail-first, hatching, and doses > 4.1 ng/egg produced embryos that developed but failed to hatch. Developmental processes found in higher and lower vertebrates are similar and include membrane trafficking, genetic expression, and transcriptional processes; the teratogenic effects of BTXs likely occur among different phylogenetic classes.

Direct evidence for genomic effects has been demonstrated by J. Gibson (personal communication). Lymphocytes from a healthy human volunteer were extracted using the hypaque-ficoll method of lymphocyte isolation from blood. A single-cell gel electrophoresis assay, or comet assay, was conducted to determine and compare DNA damage (if any) following exposure to several agents. Human lymphocytes were exposed to the either Sigma or UNCW BTX-2, a positive control (hydrogen peroxide), a negative control (HBSS buffer), brevenal, brevenal and BTX-2 (Sigma and UNCW) or solvent (80 percent ethanol) for one hour. In addition, human lymphocytes were allowed to incubate in brevenal for one hour and then exposed to PbTx-2 or another known DNA damaging chemical (hydrogen peroxide) for a second hour. Similarly, human lymphocytes were allowed to incubate in PbTx-2 or hydrogen peroxide for one hour and exposed to brevenal for a second hour. This was done to determine if brevenal prevented or "repaired" DNA damage caused by PbTx-2 or hydrogen peroxide.

Following the treatment period, the lymphocytes were removed from chemical exposure, mixed with low-melting point agarose, and spread onto prefrosted slides. Once the agarose/lymphocyte layer was firmly affixed to the slide, the entire slide was immersed into a lysis solution containing salts and other detergents for a minimum of two hours to remove the cell membrane from the lymphocytes. The slides were then submersed into an alkalinizing solution for one hour to unwind the lymphocyte DNA. The slides were then put into an alkaline electrophoresis solution in a gel electrophoresis box where lymphocyte DNA undergoes separation. If DNA was damaged during the chemical treatment period, there will be many sections of fragmented DNA in the lymphocyte nucleus. If there was no damage to the DNA,

there will be no DNA fragments. In the gel box, an electric current was initiated. The charge on the DNA will force DNA separation. The more fragments of DNA present in the lymphocyte nucleus, the more the number of small fragments will be attracted to the negative electrode of the gel box because of the inherent charge on DNA. Following a 40-minute period in the gel box, the slides were removed. The slides were neutralized and allowed to dry. Once dry a fluorescent stain (SYBR green, Trevigen, Lot Number: 4250-050-05) was applied to the slide where the lymphocytes were located. The slide was then viewed under a fluorescence microscope and the comet size, head size and tail length were measured using an imaging system. The tail moment was calculated because the tail moment is generally preferred to the other comet parameters (Lee *et al*., 2004). PbTx-2 at concentrations as low as 10E-12M were shown to elicit single stranded and double stranded breaks in DNA, effects that were alleviated by 10E-8M brevenal. Brevenal had no effect on the ability of hydrogen peroxide to damage DNA.

Although the BTXs are known to exert their acute toxic effects through ion-channel mediated pathways in neural tissue, prior studies have also demonstrated that at least one form of the toxin (PbTx-6) is bound avidly by the aryl hydrocarbon receptor (AhR). Because AhR binding of a prototypical ligand, such as dioxin, is the first step in a cascade pathway producing major changes in gene expression, the investigators reasoned that PbTx-6 might produce similar genomic-wide changes in expression. Mice were injected i.p. with sublethal doses of PbTxs (either 1.5 or 3 µg/g b.w. of PbTx-6; or 0.15 µg/g b.w. of PbTx-2, a toxin not avidly bound by the AhR), and liver and brain tissues were sampled at 8, 24 and 72 hours and ribonucleic acid (RNA) was isolated. Changes in gene-specific RNA levels were assessed using commercially available mouse cDNA arrays (Incyte) containing > 9 600 array elements, including many elements from AhR-mediated genes. Histopathology of the two organs was also assessed. The investigators observed minor histopathologic effects and a total of only 29 significant (≥ 2.0-fold) changes in gene expression, most of which occurred in the liver, and most of which could be attributable to an acute phase inflammatory response. These results argue against the hypothesis that PbTx-6 acts via a classic AhR-mediated mechanism to evoke gene expression changes. However, given the avidity with which the AhR binds to PbTx-6, these findings have important implications for how PbTxs may act in concert with other toxicants that are sensed by the AhR (Walsh *et al*., 2003).

3.2.5 Reproductive toxicity

To date, no studies have addressed the placental transfer or developmental toxicity of BTXs in mammals although studies are now under way. Cell lysates of *K. brevis* caused mortality and developmental abnormalities in over 50 percent of sea urchin embryos (Moon and Morrill, 1976). *K. brevis* exposure has proved lethal to finfish larvae but not eggs (Riley *et al*., 1989). Recently, Colman and Ramsdell (2003) and Kimm-Brinson and Ramsdell (2001; see discussion above under Genotoxicity) exposed Medaka fish embryos to PbTx-1 by microinjection. Doses of from 0.1 to 8 ng/egg (ppm) BTX caused pronounced muscular activity in the eggs after embryonic Day 4. Upon hatching, morphologic abnormalities were commonly found. At oocyte doses less than 5 ppm, lateral curvature of the spinal column, herniation of brain meninges through defects in the skull and malpositioning of the eye were seen. These experimental conditions may be relevant to exposures encountered by marine life in red tide affected waters, but may be somewhat extreme compared with the human situation, where inhalation exposures may result in less than a microgram of BTX being deposited in a given day (ppb or ppt final concentrations in body tissues). However, the observed embryo toxicity and malformations raise the issue of the possible effects of repeated BTX exposure on foetuses of pregnant women eating contaminated shellfish and/or living along red-tide affected beaches and waterways. Results may also be of significance to

marine mammals and humans living in red tide affected waters (J. Benson, personal communication).

3.2.6 Special studies

Effects in isolated cells in vitro

The molecular target of BTXs is the voltage-gated sodium channel, a fundamental transmembrane protein involved in cellular excitability. The common binding site (the receptor-site 5) is located on the alpha subunit of this neuronal transmembrane protein. Electrophysiological studies of the mode of action of BTXs identify these toxins as specific sodium channel activators. Indeed, during the action of the BTXs, sodium channels remain permanently opened, at the resting membrane potential, which produces a continuous entry of sodium ions in most excitable cells. Such a sodium entry has various consequences on sodium-dependent physiological mechanisms, consisting in a membrane depolarization, which, in turn, causes spontaneous and/or repetitive action potential discharges and thereby increases membrane excitability. These neuronal discharges may be transient or continuous according to the preparation and the toxin tested. The increase in membrane excitability during the action of BTXs is responsible for the different effects exerted by these toxins on various chemical synapses and secretory cells. The BTXs also cause a marked increase in the volume of nodes of Ranvier of myelinated nerve fibres, motor nerve terminals innervating skeletal muscle and perisynaptic non-myelinating Schwann cell somata. This increase could be reversed by hyperosmotic external solutions and completely prevented by the blockade of voltage-gated sodium channels (Mattei *et al*., 1999).

The effects of PbTx-3 on various parameters of hepatic metabolism were evaluated in mouse liver slices. PbTx-3 inhibited oxygen consumption and increased Na^+ content and presumably intracellular Na^+ concentration of liver slices. PbTx-3 also activated a pathway that mediated K^+ efflux. No effect of PbTx-3 on the Na^+-K^+ pump activity was observed. The effect of PbTx-3 on liver slices Na^+ content was abolished by the sodium channel blocker tetrodotoxin; tetrodotoxin also antagonized the inhibition of oxygen consumption but the effect of PbTx-3 on K^+ movements was not affected by tetrodotoxin, suggesting that two distinct ion channels or pathways were activated by PbTx-3. The results of this study suggest that PbTx-3 can induce effects in the liver that appear to be similar to those observed in nerve and muscle membranes (Rodriguez, Escobales and Maldonado, 1994; Rodriguez-Rodriguez and Maldonado, 1996; Trainer and Baden, 1999; cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004).

The effects of PbTx-3 on hepatic cell structure were studied also in mouse liver slices. Light microscopy revealed hypertrophy and increased vacuolation of hepatocytes, and an increase in basophilia in the perivenous area of the lobules. Ultrastructurally, the vacuolation was related to swelling of the rough endoplasmic reticulum with water and/or protein retention without accumulation of fat droplets. Accumulation of proteins and/or degranulated ribosomes accounts for the increased basophilic reaction of the cells, especially in the perivenous area, an area where lipids are normally processed. Swelling in smooth endoplasmic reticulum, degranulation of rough endoplasmic reticulum, the deformities and lytic cristae in the mitochondria, and the presence of active lysosomes are evidence of the effects of PbTx-3 upon liver cells (cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004).

Positive inotropic and arrhythmogenic effects on isolated rat and guinea pig cardiac preparations were seen at concentrations between 1.25×10^{-8} and 1.87×10^{-7} M PbTx-2. The studies suggested that PbTx-2 is a potent cardiotoxin, exerting its effects by increasing sarcolemmal sodium permeability, and by releasing catecholamines from sympathetic nerve endings (Rogers *et al*., 1984; cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004).

Crude preparations of BTX produce airway contraction; however it was unknown if this mechanical response was coupled to changes in airway smooth muscle membrane potential, either to direct action on the airway smooth muscle cell membrane or indirectly via the release of endogenous acetylcholine at peripheral nerve terminals. Therefore, membrane potentials and contractility of *in vitro* canine trachealis smooth muscle preparations were measured before and during exposure to either the crude toxin (0.01–1.2 g/ml), or the purified fractions PbTx-2 and PbTx-3 (0.01–0.07 µg/ml). Membrane potentials in cultured airway smooth muscle cells were similarly studied (Asai *et al*., 1982).

The crude fraction of BTXs produced concentration-dependent depolarizations in airway smooth muscle preparations *in vitro*, as did the purified fractions PbTx-2 and PbTx-3; however, with an approximately tenfold higher potency than the crude BTXs. In all cases, depolarizations stabilized within four minutes. There was no significant difference in concentration-response relationship between PbTx-2 and PbTx-3. The effects of crude and purified toxins were fully reversed within 30 minutes of their washout from tissue bath. The results of this study suggested that BTXs did not produce direct depolarizing effects on airway smooth muscle cells, as BTXs were without any significant effect in *in vitro* preparations treated with tetrodotoxin, or in cultured cell preparations. BTX-induced bronchoconstriction is probably because of the depolarizing effect of endogenous acetylcholine, which is released from peripheral nerve terminals, on the airway smooth muscle cell (Asai *et al*., 1982; Kirkpatrick *et al*., 2004; cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004).

PbTx-3 was shown to be bound to isolated nerve preparations from manatee brain with similar affinity as that reported for a number of terrestrial animals. *In vitro* studies with ^{3}H-PbTx-3 showed binding to manatee brain synaptosomes with high affinity and specificity. The binding was saturable, there was competition of specific binding, and temperature dependence (decreased toxic-receptor affinity and lower measured percentages of specific binding as temperature increases from 0 to 37 °C); (cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004; Bossart *et al*., 1998).

3.3 Observations in domestic animals/veterinary toxicology

Red tides in Florida and elsewhere have always been associated with mass mortality in marine animals (fish, marine mammals and marine birds). These phenomena were recorded at least 24 times from 1844 to 1971, and the fact that they occurred before the development of agriculture, towns, industries and tourism indicate their natural rather than anthropogenic origin (Viviani, 1992). In addition to massive fish kills in the Gulf of Mexico and Asia, BTXs produced by a variety of dinoflagellates are believed to represent a serious threat to finfish aquaculture (Songhui and Hodgkiss, 2001; Sierra-Beltran *et al*., 1998).

BTX-associated mortality was postulated in bottlenose dolphins (*Tursiops truncatus*) in southwest Florida in 1946–47 (Bossart *et al*., 1998). This phenomenon was because of a bloom of *K. brevis*, which was identified in 1947 as the etiological agent and was considered the sole agent responsible for all the outbreaks described since 1844. BTX-associated mortality was postulated in bottlenose dolphins (*Tursiops truncatus*) along the mid-Atlantic coast of the United States in 1987–88 (Bossart *et al*., 1998) as well as more recently in the Florida Panhandle region in 2004 (J. Naar, personal communication).

Similar toxin-associated manatee mortality was speculated in southwest Florida in 1963 and 1982 (Bossart *et al*., 1998). In April 1996, at least 149 manatees (*Trichechus manatus latirostris*) died in an unprecedented epizootic along approximately 80 miles of the southwest coast of Florida (Charlotte Harbor area). At about the same time, a significant red tide dinoflagellate bloom, largely composed of *K. brevis*, producing BTX, was present in the same geographic area as the manatee epizootic.

Cell counts of *K. brevis* were approximately 23.3 × 10^6 cells/L. Autopsy results showed neurointoxication facilitated by oral and inhalation exposure. There are three potential routes of intoxication: 1) toxic aerosol inhalation, 2) toxic food ingestion, and/or 3) toxic seawater intake. In Florida, the poisoning of manatees by BTXs contained in epiphytes attached to sea grass was reported (Hallegraeff, Anderson and Cembella, 1995).

Mortality among the double-crested cormorant (*Phalacrocorax auritus*) was observed along the Florida gulf coast (Kreuder, Bossart and Ell, 1998; Kreuder *et al*., 2002; Kirkpartrick *et al*., 2004).

3.4 Observations in humans

The two known forms of red tide toxins-associated clinical entities in humans first characterized in Florida are an acute gastroenteritis with neurologic symptoms after ingestion of contaminated shellfish (i.e. NSP) and an apparently reversible upper respiratory syndrome after the inhalation of the aerosols of the dinoflagellate and their toxins (i.e. aerosolized red tide toxins respiratory irritation) (Asai *et al*., 1982; Baden, Fleming and Bean, 1995; Fleming and Stinn, 1999; Fleming *et al*., 1999; Fleming and Baden, 1998; Fleming and Easom, 1998; Morris *et al*., 1991; Music, Howell and Brumback, 1973; Fleming *et al*., 2001; Fleming, Backer and Rowan, 2002; Baden *et al*., 1982; Poli *et al*., 2000; Kirkpatrick *et al*., 2004; Backer *et al*., 2003).

3.4.1 Oral exposure

Neurotoxic shellfish poisoning has been reported from the southeast coast of the United States, the Gulf of Mexico and New Zealand (Ishida *et al*., 1996). Neurotoxic shellfish poisoning has been identified outside the typical geographic area where *K. brevis* is found. An unusual movement of ocean currents carried a red tide from the Gulf of Mexico to the coast of North Carolina in 1987 (Morris *et al*., 1991). People there were unaware of the risks associated with eating shellfish during a red tide, and an outbreak of NSP occurred that was associated with eating contaminated shellfish (Backer *et al*., 2004). However, in general, few cases of NSP occur in the United States of America because of comprehensive monitoring programmes that periodically sample shellfish and the waters where shellfish are harvested. In the endemic areas, if shellfish or seawater samples are contaminated with BTXs, harvesting is closed until the shellfish have naturally depurated the toxin and are again safe to eat (Backer *et al*., 2004).

Neurotoxic shellfish poisoning can be compared clinically with a milder form of PSP or ciguatera fish poisoning. In reported human cases of NSP, but based on very little data, the BTX concentrations present in contaminated clams have been reported to be 30–118 MU/100 g (i.e. 120–472 µg/mg) (ILO–UNEP–WHO, 1984). Poli *et al*. (2000) reported on the measurement of BTX in urine from three persons who suffered from severe NSP after eating contaminated shellfish from Florida; the urine BTX levels ranged from 42 to 117 ng/ml by radioimmunoassay (RIA) analysis on admission to the emergency department. As a comparison, in PSP, fatal paralysis can occur with as little as 1 mg of STX, while picogram levels of ciguatoxin in ciguatera fish poisoning have been reported to make adult humans severely ill. The shellfish reported to be associated with NSP when contaminated with BTX include oysters, clams, coquinas and other filter feeders (Keynes, 1979; Baden, Fleming and Bean, 1995; ILO–UNEP–WHO, 1984; Hughes and Merson, 1976; Poli *et al*., 2000; Cembella *et al*., 1995; Fleming, Bean and Baden, 1995; Fleming *et al*., 2001; Fleming, Backer and Rowan, 2002; Tibbets, 1998; Kirkpatrick *et al*., 2004).

Neurotoxic shellfish poisoning typically causes GI symptoms of nausea, diarrhoea, and abdominal pain, as well as the neurologic symptoms primarily consisting of paresthesiae similar to those seen with ciguatera fish poisoning (including reports of

circumoral paresthesia and hot/cold temperature reversal), beginning within minutes to hours after ingestion. Cerebellar symptoms such as vertigo and incoordination also reportedly occur. In severe cases, bradycardia, headache, dilated pupils, convulsions and the subsequent need for respiratory support have been reported. Death from NSP (rather than from PSP or ciguatera) has never been reported. Anecdotally, symptoms resolve within a few days after exposure; however, no studies have been reported evaluating possible chronic health effects after acute NSP (Morse, 1977; Sakamoto, Lockey and Krzanowski, 1987; Baden, Fleming and Bean, 1995; Fleming, Bean and Baden, 1995; Fleming *et al*., 2001; Fleming, Backer and Rowan, 2002; Morris *et al*., 1991; McFarren *et al*., 1965; Viviani, 1992; Hughes and Merson, 1976; Noble, 1990; Martin *et al*., 1996; Music, Howell and Brumback, 1973; Hopkins, Heber and Hammond, 1997; ILO–UNEP–WHO, 1984; Rheinstein, 1993; Dembert, Strosahl and Bumgarner, 1981; Kirkpatrick *et al*., 2004).

Morris *et al*. (1991) reported on an outbreak of NSP secondary to a red tide of *K. brevis* in October 1987 along the North Carolina coast. Ultimately, over 48 persons were diagnosed with NSP following consumption of cooked and raw oysters at 20 different meals. Acutely, 23 percent of the cases reported GI, and 39 percent reported neurologic symptoms. These symptoms were described as having a rapid onset (median incubation of 3 hours), mild, and of short duration (maximum malaise and vertigo up to 72 hours with median duration of 17 hours). Ultimately, 94 percent had multiple symptoms, and 71 percent had more than one neurologic symptom. Although no deaths or respiratory distress occurred, one woman was admitted to the intensive care unit because of severe neurologic symptoms. The illness attack rate increased significantly in association with the number of oysters eaten. Of note, 56 percent of the cases occurred before the first closure of affected shellfish waters to harvesting in early November; North Carolina had no red tide monitoring programme at that time.

On 16 June 1996, three patients were diagnosed with NSP by Sarasota County Health Department, the Bureau of Environmental Epidemiology, the Florida Department of Environmental Protection, and the United States Food and Drug Administration. All had eaten clams (*Chione cancellata*) and whelks (unidentified species) harvested from an area that had been closed to shellfish harvesting from 31 January 1996 to 8 June 1996 because of red tide of *K. brevis*, and then closed again on 11 June. The clams had been cooked until they opened; cooking time for the whelks was unknown (Hopkins, Heber and Hammond, 1997).

3.4.2 Dermal exposure

Because of the relative fragility of the *K. brevis* organism (*K. brevis* is a "naked" organism having no outer shell of polysaccharide plates as other dinoflagellates), it is easily broken open in the rough surf releasing the toxins. During swimming, direct contact with the toxic blooms may take place and skin irritation may occur; however, dermatologically confirmed rashes have not been reported (Cembella *et al*., 1995; Tibbets, 1998; Kirkpatrick *et al*., 2004). However, anecdotal accounts from numerous Florida red tide investigators indicate that exposure of mucus membranes to any measurable amount of BTX, salt spray from red tides, or BTX culture water results in severe discomfort; duration being 1–2 hours.

3.4.3 Inhalation of aerosolized BTX

Few reports have been published about human exposure and health effects associated with exposure to aerosolized red tide toxins in humans. The exposure usually occurs on or near beaches with an active red tide bloom. Onshore winds and breaking surf result in the release of the toxins into the water and into the onshore aerosols (Pierce, 1986; Pierce *et al*., 1989, 1990; Pierce and Kirkpatrick, 2001; Sakamoto, Lockey and Krzanowski, 1987; Music, Howell and Brumback, 1973; Backer *et al*., 2003, 2005;

Horstman *et al*., 1991; ILO–UNEP–WHO, 1984). After initial reports in Florida and Texas, Woodcock (1948) reported respiratory irritation during a severe red tide on the west coast of Florida in 1947. Pierce *et al*. (1990, 1989) simulated the red tide toxin aerosol in the laboratory by bubbling air through seawater cultures of lysed *K. brevis* cells; they recorded toxin enrichment in the aerosol of 5–50 times the concentration of original concentrations in the seawater. Collection of marine aerosols along the Gulf coast of Florida and the North Carolina Atlantic coast during natural red tide blooms showed that the aerosolized toxins were the same as those in the water and as those resulting from the *K. brevis* culture experiments (Pierce *et al*., 1989, 1990).

Inhalation of aerosolized red tide toxins reportedly results in conjunctival irritation, copious catarrhal exudates, rhinorrhea, non-productive cough and bronchoconstriction (Music, Howell and Brumback, 1973; Asai *et al*., 1982, 1984; Franz and LeClaire, 1989; Eastaugh and Shepard, 1989; Pierce, 1986; Temple, 1995; Sakamoto, Lockey and Krzanowski, 1987; Baden *et al*., 1982; Davis, 1994; Ahles, 1974; Hughes and Merson, 1976; Tommasi, 1983; Hopkins, Heber and Hammond, 1997; ILO–UNEP–WHO, 1984; Dembert, Strosahl and Bumgarner, 1981; Cummins, Jones and Stevens, 1971). Some people also report other symptoms such as dizziness, tunnel vision and skin rashes. In the normal population, the irritation and bronchoconstriction are usually rapidly reversed by leaving the beach area or entering an air-conditioned area (Steidinger and Baden, 1984; Baden, 1983).

However, people with asthma are apparently particularly susceptible; Asai *et al*. (1982) found that 80 percent of 15 asthmatic patients exposed to red tide aerosol at the beach complained of asthma attacks. The possibility of the susceptibility of asthmatics to the BTXs is corroborated by recent investigations with an asthmatic sheep model evaluating the exposure of aerosolized red tide toxins discussed above (Singer *et al*., 1998; Abraham *et al*., 2003; L. Fleming, personal communication). Furthermore, there are anecdotal reports of prolonged pulmonary symptoms even after exposure has ceased, especially in susceptible populations such as the elderly or people with chronic lung disease. Exposure to aerosolized red tide toxins can cause respiratory irritation, even in non-asthmatics and without obvious fish kills or high dinoflagellate cell counts in the seawater within a few feet of the seashore (Kirkpatrick *et al*., 2004; Backer *et al*., 2003, 2005). This may be because of the concentration of the BTXs in the aerosol of sea spray generated by waves hitting the shore during a red tide (Pierce *et al*., 1990, 1989; Music, Howell and Brumback, 1973; Cummins, Jones and Stevens, 1971). How far inshore this red tide toxins aerosol will travel, especially given strong offshore winds during a red tide bloom, is not known.

4. ANALYTICAL METHODS

4.1 Introduction

In the United States of America, the present system of testing and control appears to provide adequate protection from NSP to the consumer. In New Zealand, the United States methods for NSP testing and control, although informally adopted, have been questioned because of the different bivalve species and attendant toxin-metabolite profiles involved. Further, the co-occurrence interfering contaminants and algae species responsible for entirely different poisoning syndromes in humans (i.e. ASP, DSP, PSP) in New Zealand are known to obscure NSP test results. Notwithstanding the present status of the United States testing and control system for NSP and its validity under circumstances encountered by New Zealand, the continued use of live animal bioassays compromises ethical and scientific principles, and there is both public and scientific community consensus for discontinuation of this procedure. Recent studies suggest that *in vitro* and instrumental alternatives to live animal testing will serve well as quantitative measures of risk and provide levels of protection equivalent or superior to that afforded by the conventional MBA.

4.2 Shellfish extraction and test-sample clean-up

Neurotoxic shellfish poisoning (NSP) test samples intended for American Public Health Association (APHA) MBA are salted, acidified, boiled and then extracted in diethyl ether (see below). This procedure has been shown to exclude a significant portion of the toxic PbTx metabolites identified from contaminated shellfish by various groups. In consideration of the fact that the ether extraction and MBA procedure has been effective for many years in the United States of America, albeit less so in New Zealand, some proponents for replacing MBA with alternate method(s) suggest that the diethyl ether, or equivalent, extraction procedure be retained. Other parties to the debate suggest that thorough extraction of PbTx-related constituents followed by a clean-up step (e.g. solid phase extraction [SPE]) is most appropriate for an effective update of the NSP method and guidance level. The debate on selection of shellfish extraction and clean-up procedures is currently taking place under the auspice of the Association of Official Analytical Chemists (AOAC) International Task force on Marine and Freshwater Biotoxins, NSP subgroup.

4.3 NSP methods

4.3.1 Mouse bioassay (MBA)

NSP MBA: The APHA MBA protocol is the only officially recognized NSP method for assuring the safety of molluscan bivalves in the United States of America. It was also adopted for regulatory use in the management of NSP in New Zealand in 1993. The protocol for NSP MBA is specified in *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th ed. (The American Public Health Association, Inc., Washington, DC), pp. 61–66. The regulatory application of information derived from MBA is based upon studies conducted in the 1960s that compared the incidence of human illness with the incidence of death in mice following i.p. injection of crude residues extracted from shellfish in diethyl ether (McFarren *et al*., 1965). BTX structures and modes of action were not known in 1970 and the toxicity of the crude residues was expressed in terms of mouse units (MUs). One MU was defined as that amount of crude toxic residue that, on average, will kill 50 percent of the test animals (20 g mice) in 930 minutes. Any detectable level of toxin in shellfish tissue was considered potentially unsafe for human consumption. In practice, however, a residue toxicity > 20 MUs per 100 g shellfish tissue was adopted, and remains, the guidance level for prohibition of shellfish harvesting in the United States of America.

After the detection of NSP in New Zealand in 1993, a management strategy to monitor NSP toxins was developed by the regulatory authority. The sample preparation method used was based on acetone extraction of shellfish, followed by partitioning of lipophilic constituents into dichloromethane from aqueous suspension. This procedure required less time and was more efficient for extracting toxic constituents from shellfish than diethyl ether. However, the discovery of a novel bioactive compound (gymnodimine) produced by the dinoflagellate *Gymnodinium mikimotoi*, a commonly encountered species in New Zealand waters during their 1992–93 NSP event, compelled authorities to return to the diethyl ether extraction procedure of the APHA. Gymnodimine is not extractable by diethyl ether, but caused rapid mouse death when the acetone extraction procedure was used. Because gymnodimine is not considered a risk to human health, the monitoring programme now employs diethyl ether extraction as a means of discriminating gymnodimine activity from NSP toxicity (as cited in Fernandez and Cembella, 1995). As indicated above for the United States of America, any detectable level of BTXs in shellfish tissue was considered potentially unsafe for human consumption, but in practice, residue toxicity > 20 MU per 100 g shellfish tissue was adopted as the guidance level for prohibition shellfish harvesting in New Zealand.

Several studies comparing the APHA diethyl ether extraction with alternate solvents determined that the polar PbTx metabolites (amino acid conjugates) are not recovered

under these conditions. Nozawa, Tsuji and Ishida (2003) demonstrated using LC-MS of toxic cockle that diethyl ether is efficient for recovery of PbTx-3 but far less efficient than methanol or acetone for extracting BTX-B1 (i.e. PbTx-2 taurine conjugate). Dickey *et al*. (1999) found methanol and acetone extracts of toxic Eastern oysters 2.5- and 4-fold more toxic, respectively, than ether extracts, while recovery of PbTx-3 from spiked shellfish homogenate by all three solvents was comparable (90–108 percent). Naar *et al*. (2002) observed total recovery of a PbTx-2, -3, -9 mixture spiked into shellfish homogenate using acetone extraction, but only 25 percent recovery in diethyl ether. These studies support the contention that the APHA MBA, as performed, underestimates total toxin burden in PbTx-exposed shellfish. Further, with the evident expansion of NSP to geographic regions beyond the North America Gulf of Mexico, the APHA MBA has been questioned as a suitable regulatory method and reference point for scientific studies on NSP in other shellfish species (e.g. New Zealand *Perna canaliculus*, and *Austrovenus stutchburyi*).

The APHA MBA protocol has been protective in the United States of America as applied to NSP shellfish for more than 30 years; however, the continued use of live animal bioassays compromises both scientific and ethical principles, and there is both public and scientific community consensus for discontinuation of this procedure. Replacement by alternative methods (e.g. RBA and ELISA) will necessitate at least some comparison and derivation of a numerical value equivalent to MBA guidance level. Similarly, replacement by instrumental method will require establishment of a guidance level for an appropriate analytical marker compound(s), and availability of a suitable standard(s). This effort will be challenged somewhat by bivalve species differences in PbTx metabolite profiles, metabolite toxic potencies and rates of elimination, as illustrated above.

4.3.2 In vitro *assays*

Neuroblastoma cell assay: The toxins responsible for NSP exert their toxic effects by binding to voltage-gated sodium channels, inducing a decline in activation potential and inhibiting channel inactivation (Baden, 1989). This highly specific interaction with sodium channels in excitable cell membranes is the basis of the neuroblastoma cell assay. Any modification to the toxin molecule that interferes with its binding to the sodium channel and thus the shift in activation potential, would presumably also compromise its ability to elicit a toxic response *in vivo*. Detection is therefore based on functional activity rather than on recognition of a structural component, as is the case for an antibody-based assay or instrumental method. Moreover, the affinity of a toxin for its receptor is reported to be directly proportional to its toxic potency. Thus, for a mixture of toxins, a receptor-based assay will yield a response representative of the integrated potencies of those toxins present (reviewed in Cembella *et al*., 1995). The neuroblastoma cell assay can be used for detection of BTXs in contaminated shellfish tissue, but this assay cannot distinguish between individual BTXs and other sodium channel potentiating toxins (as cited in Hua *et al*., 1995).

A tissue culture technique using an established mouse neuroblastoma cell line (Neuro-2a: ATCC CCL 131) was developed for assay of sodium channel activating toxins (Manger *et al*., 1993, 1995). The assay measures PbTx-mediated ouabain-veratridine (O/V) dependent cell death using an end-point determination of mitochondrial dehydrogenase activity. The method incorporates a colorimetric detection element based upon the ability of metabolically active cells to reduce a tetrazolium compound, MTT (=3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium) to a blue formazan product. Assay responses to PbTx standards and test samples are determined by measuring the intensity of formazan colour development in viable +O/V cells following exposure to standards or test sample. Absorbance in treated wells is expressed as a percentage of formazan colour development measured in untreated +O/V control

wells (no sample or standard treatment). The responses of -O/V cells to standards and test samples are evaluated to ascertain the sodium channel specificity of cell response. Standard PbTx-3, PbTx-3 spiked extracts and toxic test samples have no effect on the viability of cells in the absence of ouabain and veratridine. This is consistent with the sodium channel specificity of the assay. Quantitative estimation of PbTx-3 equivalent response in toxic test samples is made by substitution of PbTx-3 standard dose causing 50 percent reduction in cell viability (standard ID_{50}) for the mean test sample dose causing 50 percent reduction in cell viability (test sample ID_{50}). The toxicity of test samples is quantified as PbTx-3 equivalent activity in units of µg/g tissue (ppm). Manger *et al.* (1995) reported a detection limit for PbTx-1 and -3 of 2.0 ng/ml. PbTx can be detected within 4–6 hours, but the detection limit can be improved with an incubation time of 22 hours. The linearity of assay response was tested by Dickey *et al.* (1999) using dilutions of PbTx-3 standard (range = 0.68–87.0 ng/ml). Assay responses were linear between 25 and 75 percent viable, which correspond to a PbTx-3 dosing range of 2.5–11.5 ng/ml (r = 0.9905, slope = 1.1).

A 13-laboratory comparative study (Dickey *et al.*, 2004) tested the performance of the neuroblastoma cell assay as an alternative to MBA for the determination of NSP toxins in the Eastern oyster. Other methods tested in this study included RBA, competitive ELISA and LC-MS. Three to five laboratories independently performed each method using centrally prepared test samples from non-toxic shellfish, PbTx-3 fortified shellfish at four levels: 0, 40, 80 and 160 µg/100 g and naturally incurred oyster samples at 29, 58 and 116 µg/100 g (7, 14 and 28 MU, respectively). The N2a cell assay showed the greatest inter-laboratory variability of the methods tested. The data rendered was unsuitable for statistical analysis. While this method has proved extremely valuable for detecting and tracking toxicity in seafood extracts in the research laboratory, and performs well within-laboratory in outbreak investigations, there appear to be technical aspects of the method that significantly influence reproducibility (i.e. between-laboratory). Further investigation will be needed to identify the source of this variation.

Similar cell assay methods have been devised to measure cell viability following toxin challenge, through various end-points and using colorimetric, fluorescent and luminescent detection elements. Yasumoto *et al.*, 1995, used XTT (a soluble formazan reagent) for colorimetric determination of PbTx toxicity via mitochondrial dehydrogenase activity in a manner similar to Manger *et al.* (1993). Louzao *et al.* (2004) utilized human neuroblastoma cell line BE(2)-M17 in the development of a fluorometric assay in which veratridine dependent sodium uptake is measured using the reagent Bis-oxonol. Quantitation of PbTx induced membrane depolarization was reported to be in the nanomolar range. Fairey, Edmunds and Ramsdell (1997) modified the cell assay of Manger *et al.* (1995) by expressing a stable c-fos-luciferase reporter gene in the N2a cells. The reporter gene assay utilizes luciferase-catalyzed light generation as detection element and a microplate luminometer for quantification. PbTx-1 caused a concentration-dependent and saturable increase in luciferase activity. Although additional characterization of these assays is still required to evaluate responses to PbTx metabolites, co-occurring contaminants and different shellfish matrices, the assays as reported met or exceeded the sensitivity of existing bioassays for sodium channel active algal toxins.

Trainer, Baden and Catterall (1995) reconstituted functional sodium channels into phospholipid vesicles. The reconstituted channel can be used for sodium channel binding competition in a manner similar to the RBA described below. Specific binding of PbTx-3 to purified rat brain sodium channels that were reconstituted into phospholipid vesicles, was demonstrated. This demonstration of specific binding of sodium channel toxins suggests that highly specific functional assays for the presence of these and other toxins in biological tissue may be engineered in the future.

Receptor binding assay (RBA): The toxins responsible for NSP exert their toxic effects by binding to voltage-gated sodium channels, and more specifically bind to site 5 of the sodium channel (Poli, Mende and Baden, 1986). On the assumption that differences in the chemical structure of congeners and molluscan metabolites of PbTx are reflected in their respective affinities for site 5, and that binding affinity is proportionate to toxicity *in vivo*, the receptor binding competition assay can be used to measure the composite toxic potency of PbTx in a sample, independent of which toxin congeners are present (Van Dolah *et al*., 1994). In the RBA, an extract of homogenized shellfish tissue is incubated, in the presence of [3H] PbTx-3, with rat brain synaptosomes containing sodium channels. PbTx-related toxins present in the sample compete with [3H] PbTx-3 for binding to the sodium channel. Incubation is carried out in a microplate format to minimize sample handling and to maximize sample throughput. The amount of BTX present (in PbTx-3 equivalents) in the sample is quantified by determining the amount of [3H] PbTx-3 bound to the receptor, relative to the amount that binds in the absence of competing toxin. Quantitation is based on a competition curve in which increasing concentrations of purified PbTx-3 standard compete for binding to the sodium channel in rat brain synaptosomes in the presence of [3H] PbTx-3. Measurements can be carried out either in a microplate scintillation counter or a traditional scintillation counter.

In a 1998 peer verification study conducted by Van Dolah *et al*. (personal communication), performance characteristics of the RBA method were assessed using extracts of the United States Eastern Oyster (*Crassostrea virginica*). The detection limit of the receptor assay was 1.6 + 0.4 nM PbTx-3, and the limit of determination in oysters was 3 µg PbTx-3 equivalents per 100 g oyster homogenate. In this study, each participating laboratory analysed oyster samples fortified with PbTx-3 at four levels: 0, 40, 80 and 120 µg/100 g and naturally incurred oyster samples at 40, 80 and 200 µg/100 g. Mean recovery of analytes from fortified samples was 103 percent. Repeatability and reproducibility (RSD_r and RSD_R, respectively) were 26.9 and 37.3 percent, respectively. Comparison of quantitation using the traditional versus microplate (i.e. 96 well) scintillation-counting methods yielded a correlation of 0.896. Quantitative comparisons between the RBA and MBA also showed linear correlation.

A 13-laboratory comparative study (Dickey *et al*., 2004) tested the performance of two variations of the RBA as alternatives to MBA for the determination of NSP toxins in the Eastern oyster. The variations were the traditional test-tube format (Poli *et al*., 1986) and the microplate format as compared by Van Dolah above. Other methods tested in this study included N2a neuroblastoma cell assay, competitive ELISA, and LC-MS. Three to five laboratories independently performed each method using centrally prepared test samples from non-toxic shellfish, PbTx-3 fortified shellfish at four levels: 0, 40, 80 and 160 µg/100 g and naturally incurred oyster samples at 29, 58 and 116 µg/100 g. Receptor binding homogeneity of variances for PbTx-3 fortified test samples passed Cochran's test at the 2.5 percent level of significance, and no statistical outlying laboratories were detected. For naturally incurred test samples, one outlying laboratory was identified for binding assay methods.

Recoveries of PbTx-3 from fortified test samples averaged 97 percent (test-tube format) and 136 percent (microplate format) (Table 2). The lower limit of detection was not tested for any of the methods, but in practice all methods easily measured fortified test samples one order of magnitude below the regulatory guidance level (2 MU/g or 0.8 ppm). After excluding data from outlying laboratories, within-laboratory variation (RSD_r) for all test samples averaged 27 percent (test-tube format) and 16 percent (microplate format). Between laboratory variation (RSD_R) for all test samples averaged 39 percent (test-tube format) and 23 percent (microplate format). HORRAT values averaged 2.40 (test-tube format), 1.40 (microplate format). This study of methods performance showed statistically acceptable correlation of MBA with the RBA

(microplate format) for the determination of NSP toxins in shellfish and concluded that this method was a suitable replacement for MBA. The microplate assay can be completed within three hours, and can analyse dozens of samples simultaneously. The assay is demonstrated to be useful for assessing algal toxicity, for purification of BTXs and for the detection of BTXs in seafood.

TABLE 2
RBA (96-well format) performance estimates

Parameter	Sample C	Sample A	Sample E	Sample B	Sample F	Sample D
N	9	9	9	6	6	6
Assay mean (ppm)	0.54	1.08	2.21	0.27	0.78	1.00
Spike level (ppm)	0.4	0.8	1.6	–	–	–
Incurred toxin (ppm)	–	–	–	0.29	0.58	1.16
Percent recovery	135	135	138	–	–	–
Percent of incurred value	–	–	–	92	134	86
Repeatability SD (S_r)	0.17	0.17	0.35	0.044	0.038	0.13
Repeatability RSD_r	0.31	0.16	0.16	0.17	0.049	0.13
Repeatability value, r ($2.8 \times S_r$)	0.47	0.48	0.98	0.12	0.11	0.38
Reproducibility SD (S_R)	0.17	0.17	0.79	0.065	0.13	0.13
Reproducibility RSD_R	0.31	0.16	0.36	0.24	0.17	0.13
Reproducibility value, R ($2.8 \times S_R$)	0.47	0.48	2.2	0.18	0.36	0.38
HORRAT ($RSD_R\%/PRSD_R\%$)	1.78	1.00	2.51	1.25	1.00	0.84
$PRSD_R\% = 2C^{-0.1505}$ where C = (estimated mean in ppm $\times 10^{-6}$)						

Statistical analysis: Data were analysed using the 1-way analysis of variance (ANOVA) for each test sample. In each analysis, the Cochran's test for homogeneity of variances was applied at the 2.5 percent level of significance. The repeatability and reproducibility values (r and R) indicate that the absolute difference of two test results from a single laboratory or from two laboratories (i.e., one result from each laboratory) is expected to be below r or R in 95 percent of the cases. Horwitz ratio (HORRAT) values less than 2 indicate that the method is acceptable according to the relative reproducibility standard deviation of historical data for AOAC International validated methods. It should be noted, however, that the number of laboratories performing each of the alternative methods in this study (3–5) is too small for AOAC International full collaborative validation status, but does meet the reduced format criteria for peer verification of methods.
Source: Plakas *et al.*, 2008.

Enzyme-linked immunosorbent assay (ELISA): At a time when only the structures of PbTx-2 and PbTx-3 were known, a competitive RIA to detect PbTx-2 and PbTx-3 with a detectability of 2 nM was developed. Detectability was improved later to approximately 1 nM (as cited in Trainer and Baden, 1991). Utilizing bovine serum albumine (BSA)-linked PbTx-3 as complete antigen, an antiserum was produced in goats. The RIA technique for PbTx is based on the competitive displacement of 3H-PbTx-3 from complexation with the antibody. Both PbTx-2 and PbTx-3 were detected in approximately equivalent manners. However, oxidized PbTx-2, which was not toxic in either the fish or MBA, also displaced PbTx-3 in RIA, an indication that potency was not reflected in competitive displacement assays using this antibody (as cited in Trainer and Baden, 1991).

Work has also advanced in the preparation of a reliable monoclonal antibody, ELISA. Trainer and Baden (1991) developed an ELISA method utilizing BTX coupled to either horseradish peroxidase or to urease with a goat antibody to purified BTX. A potential ELISA system for BTX detection from extracts of dinoflagellates or fish has been established with a limit of detection of 0.04 pM. The toxin can be linearly quantified from 0.04 to at least 0.4 pM BTX per well. In initial trials, BSA-linked PbTx-3 was used as the antigen and an antiserum was produced in a goat that was found to bind competitively to PbTx-2 and PbTx-3 (as cited in Cembella *et al.*, 1995). Because the assay is structural rather than functional, the antibody also binds to non-toxic PbTx derivatives with similar binding activity. When keyhole limpet haemocyanin was used instead of BSA, more efficient antibody production occurred (as cited in Baden *et al.*, 1988). Recent studies on epitopic recognition using naturally

occurring and synthetic BTX derivatives with two different anti-PbTx sera indicated that single antibody assays may not be adequate for detecting NSP toxin metabolites. Tests are being developed to utilize more than one antibody specifically for recognition of different regions of the polyether ladder (Baden *et al*., 1988; Levine and Shimizu, 1992; Poli, Rein and Baden, 1995; Trainer and Baden, 1991). In a later study (Baden *et al*., 1995) further modifications of the ELISA method are reported, which resulted in improved specificity and detectability. BTX in fish tissue could not be measured until 1995 by the ELISA because BTX is covalently conjugated via well-known cytochrome P450-monooxygenase detoxification pathways, and glutathione-S-transferase activities are induced as well. Normal tissue extraction will not release bound toxin in fish tissue. The ELISA was entirely satisfactory for detecting and quantifying BTXs in dinoflagellate cells, requiring as few as 10–50 cells. Shellfish tissue could be analysed with ELISA but at the expense of the detectability. The modifications and alternative techniques reported by Baden *et al*. (1995) made it possible to use the ELISA for BTX detection in dinoflagellate cells, in shellfish and fish seafood samples, in seawater and culture media, and in human serum samples. Naar *et al*. (1998) reported the improved development of antibody production to PbTx-2-type BTXs and developed a new RIA. The detection limit for PbTx-3 was 0.33 picomoles with a detectability range between 0.01 and 1100 picomoles. In a later study, Naar *et al*. (2001) described the production and characterization of mouse polyclonal and monoclonal antibodies (MAbs) specific for PbTx-2-type toxins using PbTx-3-carrier-conjugates prepared at the nanomolar level in a reversed micellar medium. The authors considered this first report on MAbs production to PbTxs most promising for the development of MAb-based assays to poorly available marine polyether-type potent neurotoxins. Naar *et al*. (2002) reported the development of a competitive ELISA for the detection of BTXs in seawater, shellfish extract or homogenate, and mammalian body fluid (urine and serum without pretreatment, dilution or purification) using goat antibrevetoxin antibodies obtained after immunization with keyhole limpet haemocyanin-brevetoxin conjugates, in combination with a three-step signal amplification process. The detection limit for BTXs in spiked oysters was 2.5 µg/100 g shellfish meat. Garthwaite *et al*. (2001) developed a group ELISA for ASP, NSP, PSP and DSP toxins including yessotoxin (YTX) as a screening system for contaminated shellfish samples. The system detects suspected shellfish samples. Thereafter, the suspected samples have to be analysed by methods approved by international regulatory authorities. Alcohol extraction gave good recovery of all toxin groups.

The competitive ELISA developed by Naar *et al*., (2002) was performance tested and compared with other methods in the 2002 study by Dickey *et al*., 2004. Other methods tested in this study included N2a neuroblastoma cell assay, RBA, and LC/MS. Three to five laboratories independently performed each method using centrally prepared test samples from non-toxic shellfish, PbTx-3-fortified shellfish at four levels: 0, 40, 80 and 160 µg/100 g, and naturally incurred oyster samples at 29, 58 and 116 µg/100 g. ELISA homogeneity of variances for fortified test samples passed Cochran's test at the 2.5 percent level of significance, and no statistical outlying laboratories were detected (Table 3). For the naturally incurred test samples one outlying laboratory was identified. Recoveries of PbTx-3 from fortified test samples averaged 87 percent. The lower limit of detection was not tested for any of the methods but in practice all methods easily measured spiked test samples one order of magnitude below the regulatory guidance level (2 MU/g or 0.8 ppm). After excluding data from outlying laboratories, within-laboratory variation (RSD_r) for all test samples averaged 10 percent. Between laboratory variation (RSD_R) for all test samples averaged 16 percent. HORRAT values averaged 0.99. AS the microplate format RBA discussed above, this study of methods performance showed statistically acceptable correlation

of MBA with the ELISA and concluded that this method was a suitable replacement for MBA.

TABLE 3
ELISA performance estimates

Parameter	Sample C	Sample A	Sample E	Sample B	Sample F	Sample D
N	9	9	9	9	9	9
Assay mean (ppm)	0.30	0.71	1.53	0.47	0.86	1.91
Spike level (ppm)	0.4	0.8	1.6	–	–	–
Incurred toxin (ppm)	–	–	–	0.29	0.58	1.16
Percent recovered	76	89	96	–	–	–
Percent of incurred value	–	–	–	162	148	165
Repeatability SD (S_r)	0.033	0.099	0.22	0.027	0.080	0.20
Repeatability RSD_r	0.1092	0.1393	0.1452	0.0577	0.0930	0.1035
Repeatability value, r ($2.8 \times S_r$)	0.093	0.28	0.62	0.076	.022	0.55
Reproducibility SD (S_R)	0.060	0.11	0.28	0.048	0.15	0.28
Reproducibility RSD_R	0.20	0.15	0.18	0.10	0.17	0.15
Reproducibililty value, R ($2.8 \times S_R$)	0.17	0.30	0.78	0.13	0.41	0.78
HORRAT ($RSD_R\%/PRSD_R\%$)	1.04	0.89	1.21	0.57	1.05	1.01
$PRSD_R\% = 2C^{-0.1505}$ where C = (estimated mean in ppm $\times 10^{-6}$)						

Statistical analysis: Data were analysed using the 1-way ANOVA for each test sample. In each analysis, the Cochran's test for homogeneity of variances was applied at the 2.5 percent level of significance. The repeatability and reproducibility values (r and R) indicate that the absolute difference of two test results from a single laboratory or from two laboratories (i.e. one result from each laboratory) is expected to be below r or R in 95 percent of the cases. HORRAT values less than 2 indicate that the method is acceptable according to the relative reproducibility standard deviation of historical data for AOAC International validated methods. It should be noted, however, that the number of laboratories performing each of the alternative methods in this study (3–5) is too small for AOAC International full collaborative validation status, but does meet the reduced format criteria for peer verification of methods.
Source: Plakas *et al.*, 2008.

4.3.3 Instrumental analysis

Liquid chromatography with mass spectrometry (LC-MS) techniques have been extensively employed for the identification and confirmation for marine biotoxins in general, and have been clearly demonstrated by a number of laboratories as instrumental in the characterization of PbTx and metabolites in molluscan bivalves. The high-sensitivity, high-specificity and multitoxin capabilities also make LC-MS attractive as a regulatory tool and Reference Method (Codex Type II). However, until recently, there had not been a strong effort put into establishing all the performance characteristics of LC-MS methods that would enable their use in quantitative analysis of PbTx and metabolites in shellfish. Many "discovery" reports demonstrate the potential of LC-MS, but expansion into the regulatory setting is impeded by the diversity of PbTx metabolites in different bivalve species, the lack of reference standards for key PbTx and metabolites, and the lack of information on their toxicological significance. Practical considerations also, such as requirements of technical expertise and the expense of instrument procurement and maintenance, hinder conceptual adoption of LC-MS for routine regulatory roles. Nevertheless, several studies suggest that selected PbTx and metabolites may serve well as quantitative "markers" of exposure and provide levels of protection equivalent or superior to that afforded by the conventional APHA MBA.

TABLE 4
LC-MS performance estimates

Parameter	Sample C	Sample A	Sample E	Sample B	Sample F	Sample D
N	15	15	15	15	15	15
Mean	0.295	0.615	1.316	1.163	2.167	4.047
Spike/toxin value (ppm)	0.4	0.8	1.6	0.29	0.58	1.16
Percent recovery	73.8	76.9	82.3	401.0	373.6	348.9
Repeatability SD (S_r)	0.057	0.082	0.112	0.158	0.366	0.442
Repeatability RSD_r	0.194	0.133	0.085	0.136	0.169	0.109
Repeatability value, r (2.8 × S_r)	0.160	0.230	0.314	0.443	1.024	1.239
Reproducibility SD (S_R)	0.065	0.146	0.241	0.988	1.247	2.397
Reproducibility RSD_R	0.221	0.238	0.183	0.850	0.576	0.592
Reproducibility value, R (w.8 × S_R)	0.183	0.410	0.674	2.767	3.493	6.713
HORRAT (RSD_R%/$PRSD_R$%)	1.15	1.38	1.19	5.43	4.04	4.57
$PRSD_R\% = 2C^{-0.1505}$ where C = (estimated mean in ppm × 10^{-6})						

Statistical analysis: Data were analysed using the 1-way ANOVA for each test sample. In each analysis, the Cochran's test for homogeneity of variances was applied at the 2.5 percent level of significance. The repeatability and reproducibility values (r and R) indicate that the absolute difference of two test results from a single laboratory or from two laboratories (i.e. one result from each laboratory) is expected to be below r or R in 95 percent of the cases. HORRAT values less than 2 indicate that the method is acceptable according to the relative reproducibility standard deviation of historical data for AOAC International validated methods. It should be noted, however, that the number of laboratories performing each of the alternative methods in this study (3–5) is too small for AOAC International full collaborative validation status, but does meet the reduced format criteria for peer verification of methods.
Source: Plakas *et al.*, 2008.

A 13-laboratory comparative study (Dickey *et al.*, 2004) tested the performance of LC/MS as an alternative to MBA for the determination of NSP toxins in the United States Eastern oyster (*Crassostrea virginica*). Other methods tested in this study included N2a neuroblastoma cell assay, RBA, and competitive ELISA. Five laboratories independently performed LC-MS analyses using centrally prepared test samples from non-toxic shellfish, PbTx-3-fortified shellfish at four levels: 0, 40, 80 and 160 µg/100 g and naturally incurred oyster samples at 29, 58, and 116 µg/100 g. MS data were acquired by SIM of the protonated molecules of PbTx-2 (MH+: m/z 895), PbTx-3 (m/z 897), and the more prominent conjugated cysteine metabolites of m/z 1018 and 1034, as described above. Authentic standards for the conjugated PbTx metabolites were not available. For LC-MS analyses, solutions of pure PbTx-3 were prepared for use as analytical standards to verify system response and retention times. For the conjugate ions m/z 1018 and 1034, partial separation of stereoisomers occurred and their areas were combined. LC-MS homogeneity of variances for PbTx-3-fortified test samples passed Cochran's test at the 2.5 percent level of significance, and one statistical outlying laboratory was detected. Recoveries of PbTx-3 from fortified test samples averaged 78 percent. The lower limit of detection was not tested for any of the methods but in practice all methods easily measured fortified test samples better than one order of magnitude below the regulatory guidance level (2 MU/g or 0.8 ppm PbTx-3). Excluding data from outlying laboratory, within-laboratory variation (RSD_r) for all test samples averaged 14 percent (Table 4). Between- laboratory variation (RSD_R) for all test samples averaged 44 percent. HORRAT values averaged 2.96 (where < 2 indicated acceptable performance).

The study showed LC-MS performed as well as RBA or ELISA on fortified test samples but was less consistent between laboratories for incurred test samples. Selection of appropriate PbTx and/or metabolite markers and production of accurate standards of those markers may resolve this discrepancy.

Additional LC-MS studies of PbTx in the Eastern oyster, *in situ* and in controlled laboratory conditions, supported the premise that the cysteine conjugates, particularly

those of B-type backbone structure, are useful LC-MS determinants of BTX exposure in the Eastern oyster, and potential marker(s) for composite toxin (Wang *et al*., 2004; Plakas *et al*., 2004). These studies acknowledged, however, that species specificity in metabolism of PbTx, differences in rate of elimination and relative toxicity of PbTx metabolites are considerations in the advancement of alternative methods to the MBA for prevention of NSP.

In a study of New Zealand shellfish from the 1992–93 NSP outbreak, Nozawa, Tsuji and Ishida (2003) developed procedures for quantitative determination of BTX-B1 together with PbTx-3 as marker candidates for subsequent monitoring. BTX-B1 was shown to contaminate cockle and Greenshell mussel, but not Pacific oyster, while PbTx-3 was found in all these shellfish species. Ishida *et al*. (2004c) considered BTX-B5, together with PbTx-3, good markers of shellfish neurotoxicity following *K. brevis* blooms, as these compounds were also isolated in Greenshell mussel (*Perna canaliculus*) and Pacific oyster (*Crassostrea gigas*) collected during the NSP outbreak in New Zealand.

McNabb and Holland (2003) reviewed research findings on NSP toxins in New Zealand shellfish by research teams at the Cawthron Institute, New Zealand, and the University of Shizuoka, Japan. The objective of the review and subsequent research was to identify reliable marker(s) for NSP in New Zealand shellfish. The review showed that PbTx-3 was a common contaminant in Greenshell mussel, Pacific oyster and cockle tissue as a result of metabolic reduction from PbTx-2; the main toxin produced by *Karenia* sp. However, PbTx-2 was not present in contaminated cockle samples and was difficult to measure quantitatively in the mussel and oyster. Consequently, PbTx-2 was not considered further as a potential marker for NSP toxins, and a quantitative LC-MS method for PbTx-3 was developed through the application of procedures similar to those developed at the University of Shizuoka. PbTx-3 was found to be a good marker for NSP and was detected in all samples tested by the Cawthron Institute from the 1993 NSP event and in feeding experiments using a toxic *K. brevis* culture. The profile of PbTX-2 and -3 was similar in freshly contaminated shellfish to samples from 1993 with similar APHA-MBA toxicity. The feeding studies showed that the LC-MS method is at least 25 times more sensitive to NSP contamination in Greenshell mussel than the APHA-MBA and can detect low levels of NSP contamination weeks after an event. The NSP LC-MS method proved to be significantly more reliable for New Zealand shellfish than the APHA-MBA, particularly for Greenshell mussel, which has yielded surprisingly variable results with APHA-MBA.

The following calculations indicate the base sensitivity required for an NSP LC-MS based on testing for PbTx-3 as a marker. The PbTx toxicity equivalence factor for the APHA MBA is that 1 MU corresponds to 4 micrograms of PbTx-3, i.e. the regulatory limit of 20 MU/100 g flesh corresponds to PbTx-3 only at 0.8 mg/kg shellfish. Depending on shellfish species, PbTx-3 contributes 4–26 percent of the total toxicity derived from PbTx extractable by methanol (PbTx-2, PbTx-3 and more polar metabolites; Nozawa, Tsuji and Ishida, 2003). For Greenshell mussels that contained total PbTx of 0.8 mg/kg, the PbTx-3 proportion of 4 percent would correspond to 0.03 mg/kg of PbTx-3 – a level readily detectable by LC-MS. This is the "worst case" because levels of total BTXs in shellfish must be much higher than 0.8 mg/kg to give an APHA-MBA result of 20 MU/100 g because of the poor extraction efficiency of ether for the predominant polar and toxic metabolites. The screen assay must have a very low false negative rate so that truly toxic samples are not missed. The screen assay should also have a low false positive rate, so that the number of unnecessary confirmation analyses required is minimized. An LC-MS assay for PbTx-3 as a "marker" NSP toxin can provide this type of test, because the method of detection is highly specific and the detection limit is low (in this case 0.01 mg/kg). The practicality of such a test

has been established based on recent research and method development. This method for quantitative determination of PbTx-3 in shellfish tissues has been validated in New Zealand.

McNabb and Holland concluded that, in New Zealand, the need to introduce the LC-MS NSP test is considered immediate because: 1) the APHA-ether test is ineffective for testing NSP-contaminated Greenshell mussel; 2) reliance on DSP/NSP "screen" testing is unfounded; and 3) the variability, low sensitivity and slow sample turnaround of the current mouse tests for NSP toxins may result in shellfish contamination events being missed. The arguments for adoption of NSP screening in New Zealand based on LC-MS testing included: 1) a very low false-negative rate because of the high sensitivity for PbTx-3–NSP contaminated samples that could be positive in the APHA-ether test will not be missed; 2) a low false-positive rate because of the high specificity of detection – confirmatory APHA-ether tests will only be required during true NSP contamination events; and 3) the LC-MS test is very sensitive and rapid so that low-level events can be investigated before shellfish toxicity develops. The new LC-MS test will not initially replace the use of the APHA-MBA but will act as an efficient first screen to detect low levels of NSP contamination that warrant further investigation by APHA-MBA or other appropriate tests.

4.3.4 Assessment of alternative test methods

In New Zealand, there has been no recurrence of NSP toxin producing blooms since the 1992–93 event. Consequently, there has been little opportunity to assess alternative methods under actual field conditions. In the United States Gulf of Mexico, *K. brevis* blooms occur annually and cell densities regularly reach millions of cells per litre. This circumstance permitted the assessment of alternative methods under actual field conditions.

In a study by Pierce *et al.* (2004), APHA-MBA and alternative methods (i.e. receptor binding, N2a neuroblastoma cell assay, ELISA and LC-MS) were used to monitor PbTx uptake and elimination during the time-course of a small-scale *K. brevis* bloom in Sarasota Bay, Florida, the United States of America. The comparison of results is given in Figure 5. MBA results showed oyster toxicity (> 20 MU/100 g) for about 4 weeks following the bloom, indicating a persistent public health risk. Although oysters were toxic by MBA, little or no parent PbTx was detected by LC-MS, suggesting that PbTx metabolites (e.g. fatty acid conjugates) contributed to APHA-MBA response. Results of the various assays exhibited good general agreement in the time-course of toxin uptake and elimination although numerical values differed substantially by method. The difference in numerical values is not unexpected considering the various detection end-points measured by the methods. The results of the comparison suggest that establishing equivalence to the existing NSP guidance level may be possible.

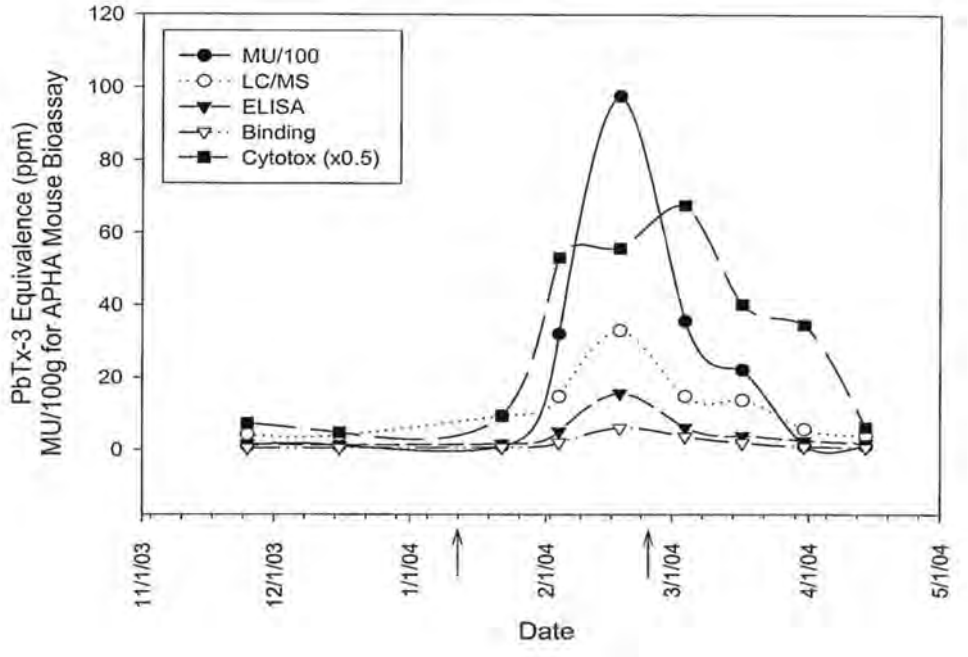
4.4 Conclusions

In the United States of America, PbTx and metabolites identified from naturally incurred toxic bivalves have been verified by several research laboratories, and confirmed in controlled exposures of bivalves to *K. brevis* cultures in the laboratory. Metabolites of PbTx-2 occur in greater relative abundance than corresponding metabolites of the other PbTx congeners. Cysteine conjugates of PbTx-2 and PbTx-1 (and their sulphoxides) represent the more prominent ions in LC-MS chromatograms, and particularly those of B-type backbone structure, are useful LC-MS determinants of BTX exposure in the Eastern oyster, and potential marker(s) for composite toxin. Similarly, the composite toxin methods, RBA and ELISA, compared well with the APHA-MBA response. Further work with naturally incurred toxic bivalves will enable refinement of toxin burden equivalence values as measured by *in vitro* assay and instrumental method, with those of the APHA-MBA. Such studies must consider

species specificity in metabolism of PbTx, differences in rate of elimination and the relative toxicity of PbTx metabolites in the advancement of alternative methods to the MBA for prevention of NSP.

In New Zealand, PbTx and metabolites identified from naturally incurred toxic bivalves have been verified by several research laboratories, and confirmed in controlled exposures of bivalves to *K. brevis* cultures in the laboratory. PbTx-3 and metabolites of PbTx-2 occur in greater relative abundance than corresponding metabolites of the other PbTx congeners. Oxidized taurine and cysteine conjugates of PbTx-2 represent the more prominent ions in LC-MS chromatograms. Because PbTx-3 was a common contaminant in all bivalve species tested from the 1992–93 NSP event, a quantitative LC-MS method for PbTx-3 was developed and is proposed as a first screen to detect low levels of NSP contamination until investigation by APHA-MBA is warranted. Inconsistency has been encountered, however, in the APHA-MBA of some bivalve species, particularly the commercially important Greenshell mussel, emphasizing the need for alternative method(s). Alternate LC-MS methods incorporating prominent PbTx metabolites (e.g. BTX-B1, -B2 and -B5) have been proposed and are under investigation, and preliminary results from an ELISA recently developed in New Zealand are promising. Further performance testing of these methods is anticipated. In the interim, PbTx-3 is the focus for an LC-MS method for use as a marker or indicator of NSP contamination, and potential toxicity, ancillary to APHA-MBA.

FIGURE 5
Comparison of assay methods for NSP-contaminated oysters



Notes: Before, during and after exposure to a *K. brevis* bloom in Sarasota Bay, Florida, the United States of America. Bloom initiation (arrow) 12 January 2004 to end (arrow) 27 February 2004. APHA-MBA data are plotted in MU/100 g. Bioassays results below 20 MU/100 g are depicted as 1 MU/10 g to indicate that the value is less than 20 but greater than 0 MU/100 g. Alternative results are expressed in PbTx-3 equivalence.
Source: Plakas *et al.,* 2008.

5. FOOD CONSUMPTION/DIETARY INTAKE ESTIMATES

No actual quantitative data exist that address the shellfish level of BTX that induces human illness. The regulatory limit is presently set at 20 MUs per 100 g of shellfish tissue (US FDA, 2001). According to Baden and Mende (1982), the i.p. LD_{50} for BTX is approximately 4 µg per mouse (or 80 µg per 100 g of shellfish tissue) (i.e. 20 MUs × 4 µg per MU = 80 µg). This is how the regulatory limit is defined. The highest levels of BTX measured in Florida shellfish approaches 4 000 µg/100 g of shellfish or 4 mg/100 g (i.e. 500-fold above the regulatory limit). Of note, although i.p administration is not the route by which humans are poisoned, the regulatory guidelines indicate that i.p is the route of choice for regulatory determinations. Therefore, assuming a 200 g meal size of shellfish, for human regulatory doses, the limit is 160 µg/person orally (or using an average 60 kg person, 2.7 µg/kg b.w.).

Noting that the highest levels measured in shellfish by Naar (J. Naar, personal communication) have been in excess of 4 000 µg/100 g of shellfish tissue, clearly shellfish in Florida regularly reach limits that could cause human illness. Consequently, analytical methods need to be able to measure toxin concentrations at or below the current 80 µg/100 g of shellfish. Specific data on the oral potency of any BTXs other than PbTx-2 and PbTx-3 are not available, nor are there any data to evaluate the effects of the brevenal antagonist in the mixture. Furthermore, in order to ascribe specific potency limits for humans, much more data need to be available on actual human intoxications. To the credit of the Florida public health and environmental monitoring officials, by using the cell counts in water rather than the toxin levels in shellfish to close shellfish beds, there has been a dearth of actual human NSP intoxications in Florida.

A recent dolphin die-off in April 1996 in the Florida Panhandle has raised the possibility that at least theoretically but under rare circumstances fish could be contaminated with BTXs in sufficient doses to cause poisonings. In this particular dolphin die-off, the dolphins died from brevetoxicosis and their stomach contents consisted of fish contaminated with BTX by ELISA (J. Naar, personal communication).

More data are needed on the other BTXs, their metabolites and antagonists levels in shellfish and fish.

6. PREVENTION AND CONTROL

Regulations for BTXs in shellfish are in force in a few countries (i.e. Italy, New Zealand and the United States of America) based on MBA. The action level is 20 MU/100 g shellfish flesh (~80 µg PbTx-2/100 g shellfish flesh) (US FDA, 2001). The regulatory application of information derived by using MBA is based upon studies conducted in the 1960s that compared the incidence of human illness with the incidence of death in mice injected with crude extracts from shellfish in diethylether (cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004).

Since the mid-1970s, the Florida Department of Environmental Protection has conducted a control programme with the closure of shellfish beds when *K. brevis* concentrations are greater than 5 000 cells/litre, until 2 weeks by testing for toxin with MBA testing. This should prevent cases of ingestion NSP related to contaminated shellfish consumption in most of the Florida human population, but not the respiratory irritation associated with exposure to aerosolized red tide toxins. There is monitoring of these red tides with their characteristic discoloration and massive fish kills by the Florida Department of Environmental Protection, as well as unsolicited reports to the Florida Department of Health of respiratory irritation. Although other states such as Texas have done otherwise, in Florida where the red tides are almost a yearly occurrence, beaches are not closed to recreational or occupational activities, even during very active near-shore blooms (Kirkpatrick *et al.*, 2004; Backer *et al.*, 2004).

Since the detection of NSP and BTX producing dinoflagellates in early 1993, New Zealand has rapidly evolved a management strategy. All commercial and non-commercial shellfish harvesting areas around the entire coastline are sampled on a weekly basis year round. Most major commercial growing areas have weekly phytoplankton sampling programmes and a "library" system of harvest sampling for the purpose of addressing the temporal and spatial spread of toxic events has been initiated. MBA (APHA method) is in force and 20 MU/100 g is employed as an acceptable level. This level corresponds to a survival time of six hours (Trusewich *et al*., 1996). Currently, shellfish testing involves MBA screen testing for NSP toxins with confirmatory testing (Busby and Seamer, 2001). A new biotoxin monitoring programme providing data that are highly accurate, in a shorter time and without the use of MBA is being developed. This new programme will implement test methods based on LC-MS providing chemical analytical data in place of bioassay screen test results. The development and implementation of new test methods are in discussion including funding, method validation, testing regulations, availability of analytical standards, comparison to existing tests, type of instrumentation and international cooperation (McNabb and Holland 2001; FAO, 2004).

In Denmark, a monitoring programme exists for several algal species, among others, *Karenia* spp. At 5.10^5 cells per litre (depending on species) fishery product harvesting areas are closed (Van Egmond, Speyers and Van den Top, 1992). In Italy, BTX-producing algae are monitored, and at simultaneous presence of algae in water and toxin in mussels, fishery product harvesting areas are closed. In Italy, provision of law is based on MBA and established "not detectable" in shellfish (Van Egmond, Speyers and Van den Top, 1992; Viviani, 1992). Argentina has a national monitoring programme of mussel toxicity in each coastal province throughout regional laboratories and one fixed station in Mar del Plata (Ferrari, 2001). Brazil had a pilot monitoring during one year, but not a national monitoring programme (Ferrari, 2001); Uruguay has a National Monitoring Programme on mussel toxicity and toxic phytoplankton (Ferrari, 2001; FAO, 2004). The primary prevention described above has been the most frequently recommended and the most obvious intervention for the human diseases associated with eating marine seafood contaminated with toxins. The populations most likely to be exposed to, and thus affected by, harmful algal bloom (HAB) toxins include those occupationally involved in seafood harvesting, shipping, and processing; seafood consumers (including those eating seafood they caught or seafood served in a restaurant); environmental workers (especially those collecting samples); persons who work and play on or near the water; and coastal communities, especially indigenous peoples who rely on seafood for a substantial proportion of their diet. Banning or severely limiting seafood harvesting also would significantly affect commercial and recreational fishing (Backer *et al*., 2004).

Epidemiologic and laboratory methods can help identify food and toxins associated with a particular disease outbreak. However, very little clinical research has been conducted to determine effective treatments for these diseases; and medical care is primarily supportive as described below (Backer *et al*., 2004; Blythe *et al*., 2001). Of note, Poli (1988) has recommended safety procedures for the safe laboratory handling of BTXs.

6.1 Depuration of shellfish

The loss rate of toxins from bivalves depends upon the site of accumulation, which may differ between phycotoxins. Scallops are the most intensively studied species and a two-phase detoxification was suggested: an initial rapid loss similar to the accumulation rate followed by a slower phase. During this process, the toxin profile may change between tissues, such as kidney and mantle, with toxic transfer between

tissue compartments or organs before excretion or secretion into the environment (Baden, Fleming and Bean, 1995; FAO, 2004).

The most common way of depurating bivalves is self-depuration, achieved by moving shellfish stock to clear water. Cooking and freezing is ineffective. One of the most promising treatments appeared to be ozone, which has been shown to assist in the depuration of mussel tissue of NSP (cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004). Oysters accumulate BTXs in less than 4 hours in the presence of 5 000 cells/ml, and depurate 60 percent of the accumulated toxin in 36 hours. Potency of depuration is species-specific and highly variable, even under controlled laboratory conditions. Commercial bivalves are generally safe to eat 1–2 months after the termination of any single bloom episode. Canning cannot be a way to decrease BTX concentration in bivalves (Baden, Fleming and Bean, 1995; Viviani, 1992; FAO, 2004).

In *Crassostrea virginica*, depuration of BTXs occurs 2–8 weeks after the bloom has dissipated. However, the metabolic fate of toxins in shellfish is species-specific.

Using a half-factorial experimental design, *K. brevis* cells were cultured and fed to Pacific oysters (*Crassostrea gigas*) at rates of between 10.45 and 24.5 million cells per oyster over 24-hour periods. Thereafter, the oysters were detoxified in various laboratory tanks over five-day periods. MBAs showed initial levels between 25 and 100 MU per 100 g of drained oyster meat, with larger oysters accumulating more toxin than the smaller ones. Experimental factors were temperature (15 and 20 °C), salinity (24 and 33-34 ‰), filtration (5 µm) versus no filtration, and treatment with ozone (to a redox potential of 350 mV in the shellfish tanks) versus passive UV light sterilization. Two experiments compared oysters that had been fed *K. brevis* over 5 days (5.0 or 3.5 million cells per oyster/day) with those fed for 24 hours (10.79 or 24.5 million cells per oyster). With the exception of one (4 tanks), all treatment combinations resulted in an initial decline of the BTX level reaching a minimum < 20 MU per 100 g by day 3 regardless of the initial toxin level or whether the toxin had accumulated over 1 or 5 days. The three-day period of decline was followed by a period of minimal reductions. None of the experimental factors had any statistically significant effect on the final toxin levels, suggesting that oysters will detoxify regardless of the conditions once they are placed in an environment free from toxic algae. The presence of final BTX levels just above 20 MU per 100 g (20.6 MU per 100 g) in some samples means that the process is not yet commercially viable (cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004).

6.2 Decrease of *K. brevis* cells and reduction of toxins

Cell cultures of *K. brevis* in artificial seawater were subjected to microwave irradiation at 2 450 MHz. Irradiation was for four 60 seconds intervals separated by 5 minutes intervals of cooling at 25 °C. A decrease in number of cells was seen. As a function of power (0–0.113 kJ/ml culture) the decrease in surviving cells was about 14 percent. A pronounced decrease or threshold effect was evident at energy levels above 0.08 kJ/ml of culture (cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004).

The effect of ozonated artificial seawater on *K. brevis* cells and toxins was studied. When artificial seawater, ozonated for 60 seconds, was added to *K. brevis* cultures the number of surviving cells decreased approximately 80 percent ($t_{1/2}$ = 10 seconds). When cultures of *K. brevis* in artificial seawater were directly ozonated for 60 seconds, non-intact cells were found ($t_{1/2}$ = 2.5 seconds). Experiments carried out in artificial seawater demonstrated that extracted *K. brevis* toxins (PbTx-1, -2, -3, -5, -7 and -9) reintroduced into artificial seawater, as well as toxins in whole cell cultures of *K. brevis* in artificial seawater at exposure to ozone for 0, 1, 5 or 10 minutes, displayed a marked reduction as ozone exposure increased. Total toxin concentration was reduced

99.9 percent after 10 minutes ozonization as determined by high-performance liquid chromatography (HPLC) analysis. Bioassays with the fish *Cyprinodon variegatus* confirmed the toxin reduction. As ozone exposure was increased to 5 minutes, total amounts of all toxins were reduced (Pierce, Henry and Rodrick, 2001; Baden, Fleming and Bean, 1995; cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004).

Doucette, McGovern and Babinchak (1999) studied the role of algicidal bacteria active against *K. brevis*. Two bacterial strains isolated from the Gulf of Mexico appeared to be lethal to *K. brevis*. The algicidal activity of one of these two strains was characterized. The strain was isolated from waters without *K. brevis* cells, suggesting that such bacteria are part of the ambient microbial community and are not restricted to areas of high *K. brevis* abundance. The bacterial strain examined, produced (a) dissolved algicidal compound(s) that was (were) released into the growth medium, and the algicide was effective against the four Gulf of Mexico *K. brevis* isolates tested, as well as against a closely related HAB species that also occurred in this region: *G. mikimotoi* (FAO, 2004).

Another control technique under examination in the laboratory setting is the addition of a blue-green alga, *Nannochloris*, into a *K. brevis* red tide culture (Martin and Taft, 1998; Perez, Sawyers and Martin, 1997). In laboratory studies, *Nannochloris* produces cytolytic agents called Apparent Oceanic Naturally Occurring Cytolin (APONINs), which react with the *K. brevis* cells and render them into a non-motile or resting form (Derby *et al*., 2003; Perez, Sawyers and Martin, 2001). Ongoing studies at Mote Marine Laboratory in Sarasota, Florida, the United States of America, indicate that adding *Nannochloris* cells to fish tanks containing *K. brevis* improves the health of the fish. Under the conditions used, the model showed that *N. oculata* would eradicate a *K. brevis* outbreak in little less than two days. The model demonstrated that *N. oculata* is a potential management method for *K. brevis* blooms, but further experiments need to be performed.

Interest in controlling the growth of HAB-forming marine organisms to protect food supplies has varied over the last 50 years. In 1958, copper sulphate was applied in west Florida waters to control a red tide bloom (U.S. Bureau of Commercial Fisheries, 1958). Although the number of viable organisms decreased immediately, the copper sulphate was not species-specific and did not last over time. The Asian aquaculture industry has used clay and clay compounds to coagulate the organisms where they sink to the bottom (Kim, 1988; Yu, Sun and Zhou, 2001). This technique is concurrently being examined in the United States of America under laboratory settings (Sengco, 2003).

Pierce *et al*. (2004) have also assessed the ability of phosphatic clay to remove the toxic dinoflagellate, *Karenia brevis*, and the potent neurotoxins (BTXs) produced by this species. Results showed that the addition of an aqueous slurry of 0.75 g (dry weight) clay to *K. brevis* culture containing 5×10^6 and 10×10^6 cells/litre, removed 97±4 percent of BTXs from the water column within 4 hours after the addition of clay. Clay flocculation of extra-cellular BTXs, released from cells ruptured (lyzed) by ultrasonication, removed 70±10 percent of the toxins. Addition of the chemical flocculant, polyaluminium chloride (PAC), removed all of the extra-cellular toxins. A 14-day study was undertaken to observe the fate of BTXs associated with clay flocculation of viable *K. brevis* cells. At 24 hours following the clay addition, 90±18 percent of the toxins were removed from the water column, along with 85±4 percent of the cells. The toxin content of clay diminished from 208±13 µg at Day 1, to 121±21 µg at Day 14, indicating that the phosphatic clay retained about 58 percent of the toxins throughout the 14-day period. These studies showed the utility of natural clay as a means of reducing adverse effects from HABs, including removal of dissolved toxins, in the water column, although considerable work clearly remains

before this approach can be used on natural blooms in open waters. A question remains as to whether the benthic deposition of toxin-containing cells is actually an advantage. Although not quantified, it can be postulated that the efficiency of the toxin and cell removal from water would be concentration dependent.

6.3 Degradation of BTXs

Brevetoxins are highly stable compounds, resistant to acids and heat (including normal cooking procedures) as well as being tasteless and odourless. BTX (associated with NSP) was not inactivated by exposure to 1 000 °F (537 °C) dry heat but was inactivated by exposure at 5 000 °F (2 760 °C) (Poli, 1988). These three compounds also are relatively stable in basic pH, and are resistant to autoclaving (Poli, 1988; Wannemacher, 2000).

Brevetoxins containing an aldehyde functional group on the terminal "tail" side chain, are easily converted to dimethylacetal structures in acidic solutions, while acid reaction to form the methyl ester at the head-side lactone ring proceeds slowly. Reactivity of BTXs to acid attack shows the following order: PbTx-1 > PbTx-2 > PbTx-9. Under basic conditions, head-side lactone ring opening initiated by hydroxide ion attack proceeds to completion in 120 and 50 minutes for PbTx-2 and PbTx-9, respectively, while that for PbTx-1 did not reach completion after 120 minutes. Base hydrolysis proceeds faster than acid hydrolysis under comparable acidic or basic conditions. However, these acid and base hydrolyses can be reversible reactions and they may not be reliable for degradation purposes. Brevetoxins are easily oxidized by potassium permanganate through addition across the double bond followed by cleavage. Brevetoxin oxidation treatment is an irreversible process and proceeds relatively fast, thus it could be a good means of BTX degradation (Hua and Cole, 1999; FAO, 2004).

6.4 Other preventive measures

Toxic blooms of *K. brevis* are generally detected by visual confirmation (water discoloration and fish kills), illness to shellfish consumers and/or human respiratory irritation with actual toxicity verified through time-consuming chemical analyses for BTXs within shellfish samples and MBAs. The exact environmental conditions leading to HABs are poorly understood. As a consequence, it is extremely difficult to predict the occurrence and magnitude of a bloom, thereby ensuring an "after-the-fact" management strategy dependent upon accurate water-quality evaluation. Monitoring programmes relying on microscopic identification and enumeration of harmful taxa in water samples generally suffice for preventing human intoxication. However, microscopic-based monitoring requires a high level of taxonomic skill, usually takes considerable time and can be highly variable among personnel. Therefore, an alternative and/or complementary evaluation system for predicting bloom occurrence and dynamics is highly desirable. Diagnostic pigment signatures and *in vivo* optical density spectra can effectively differentiate among most phylogenic groups of microalgae and macroalgae, and sometimes, taxa with a variety of habitats (Kirkpatrick *et al.*, 2004; FAO, 2004).

6.5 Treatment

The usual treatment for NSP has been supportive (Baden, Fleming and Bean, 1995; Blythe *et al.*, 2001). Of note, ciguatera fish poisoning is caused by the ingestion of large reef fish contaminated with ciguatoxin and other natural marine toxins (Lehane and Lewis, 2000); acute ciguatera can be treated (and the onset of chronic symptoms lasting weeks to months prevented) by the administration of i.v. mannitol within a few hours to days of exposure; the exact mechanism of action is unknown although may be related to diuresis of toxins and/or decrease of the swelling of the myelin sheath

on affected nerves (Mattei *et al*., 1999; Palafox *et al*., 1988). The BTXs are structurally very similar to the ciguatoxins, and bind to the same subunit of nerve cell sodium channels (Baden, Fleming and Bean, 1995). Therefore, it is possible that persons suffering from NSP may respond to i.v. mannitol (Mattei *et al*., 1999). Ramsdell *et al*. (2003) prefed cholestyramine to female CD 1 mice and then PbTx-3 via oral gavage at 230 and 300 µg/kg PbTx-3. These researchers stated that 5 percent cholestyramine in the diet attenuated the toxic effects of BTX in mice; however, at present the therapeutic effect of cholestyramine was not correlated with reduction of the blood levels of the toxin. Finally, in a sheep model of human asthma exposed to BTX aerosols, a range of commonly used asthma medications, as well as a newly identified natural antagonist (brevenal), have been shown to block the respiratory effects of aerosolized BTX exposure (Abraham *et al*., 2003; W. Abraham, personal communication).

7. DOSE RESPONSE ANALYSIS AND ESTIMATION OF CARCINOGENETIC RISK

Data are available, with a greater or lesser degree of detail, on the acute toxicity of BTXs in animals and humans through a variety of exposure routes. These materials are rapidly absorbed from the gut after oral administration and from the peritoneum after injection (as well as via inhalation of aerosols and possibly dermally). There are very little data available in either animals or humans concerning the health effects of chronic low dose exposure to BTXs.

The most obvious toxicity end-point for the BTXs is neurotoxicity, both central and peripheral. In addition, the *in vitro* data suggest that PbTx-3 can induce effects in the liver that appear to be similar to those observed in nerve and muscle membranes (Rodriguez-Rodriguez and Maldonado, 1996; Trainer and Baden, 1999; cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004). Additional *in vitro* data showed positive inotropic and arrhythmogenic effects on isolated rat and guinea pig cardiac preparations at concentrations between 1.25×10^{-8} and 1.87×10^{-7} M PbTx-2. The studies suggested that PbTx-2 is a potent cardiotoxin and exerted its effects by increasing sarcolemmal sodium permeability, and by releasing catecholamines from sympathetic nerve endings (cited from van Apeldoorn, van Egmond and Speijers, 2001; FAO, 2004). Finally, computer modelling suggests that BTX is a possible enzymatic binding inhibitor of cysteine cathepsins (Bossart *et al*., 1998; Sudarsanam *et al*., 1992). Therefore, Bossart *et al*. (1998) postulated that the effects of aerosolized BTXs may be chronic, not just acute. These chronic effects would begin with the initial phagocytosis by macrophages, inhibition of cathepsins and apoptosis of these cells, followed by the phagocytosis of the debris by new macrophages, ultimately resulting in chronic neuro-intoxication, haemolytic anaemia, and/or immunologic compromise. In addition, with the demonstrated differences in effect based on small alterations in toxin structure, it can be surmised that the profile of toxin presented will result in a variety of signs and symptoms.

Although classic animal carcinogenicity studies have not been performed, with regards to potential genotoxicity, only minor histopathologic effects and a total of only 29 significant (≥ 2.0-fold) changes in gene expression were observed in mice injected i.p. with BTXs. These results argue against the hypothesis that PbTx-6 acts via a classic AhR-mediated mechanism to evoke gene expression changes. However, given the avidity with which the AhR binds to PbTx-6, these findings have important implications for how PbTxs may act in concert with other toxicants that are sensed by the AhR (Walsh *et al*., 2003). Very preliminary experimental comet data indicate that PbTx-2 tests positive at nanomolar concentrations (Bourdelais *et al*., 2004; J. Gibson, personal communication).

The structure of the BTXs is of paramount importance for their toxicity. BTXs are characterized by a multiplicity of toxins that change in their potency profile with

stage and age of the red tide culture, and the presence and absence of antagonists and metabolites, as well as environmental conditions. PbTx-1 and PbTx-2 are the structural backbones of all other BTXs, their metabolites and antagonists; all other substances are derivative. Subtle change in the conformational preference induces a significant change in the gross shape of the molecule, which is believed to be responsible for the loss of binding affinity and toxicity (Rein *et al*., 1994). If more data become available, the toxicity of the different analogues could be expressed relative to BTX, similarly as it is done in the case of PSP, where the toxicity of STX analogues is expressed as STX.

Overall, it is safe to conclude that human illness (both acute and possibly chronic) because of the ingestion and inhalation of *K. brevis* products is complex, and is caused by at least ten toxins based on two structural backbones, with metabolites that have potential activity themselves, and with polyether antagonists that have the capability to modulate composite potency.

8. EVALUATION[1]

There are very limited quantitative human data (even from poisonings) available to derive the human oral ARfD or the tolerable daily intake (TDI) dose for BTX in shellfish. It is perhaps noteworthy that no human fatalities have been reported associated with consumption of shellfish contaminated with BTXs. Based on the fact that the mouse oral LD_{50} for PbTx-2 (the predominant toxin in blooms) is 6.6 mg/kg b.w., it can be postulated that the human oral ARfD is at or below 6.6 mg/kg b.w. (Loomis, 1968). With regard to the TDI, it is likely higher than the ARfD. This conclusion is based on data with animals where it was demonstrated that a lethal i.p. dose broken into several smaller doses over a period of several hours was not lethal (Baden and Mende, 1982).

No actual quantitative data exist that address the shellfish level of BTX that induces human illness. The regulatory limit is presently set at 20 MUs per 100 g of shellfish tissue (US FDA, 2001). According to Baden and Mende (1982), the i.p. LD_{50} for BTX is approximately 4 µg per mouse (or 80 µg per 100 g of shellfish tissue). This is how the regulatory limit is defined. The highest levels of BTX measured in Florida shellfish approach 4 000 µg/100 g of shellfish or 4 mg/100 g (i.e. 500-fold above the regulatory limit). It is important to note the following: although i.p. administration is not the route by which humans are poisoned, the regulatory guidelines indicate that i.p. is the route of choice for regulatory determinations. Therefore, assuming a 200 g meal size of shellfish, for human regulatory dose, the limit is 160 µg/person orally (or using an average 60 kg person, 2.7 µg/kg b.w.).

Specific data on the oral potency of any BTXs other than PbTx-2 and PbTx-3 are not available, nor are there any data to evaluate the effects of the brevenal antagonist in environmental mixtures. Furthermore, in order to ascribe specific potency limits for humans, much more data need to be available on actual human intoxications. The potency of tissue samples in ways other than by the oral route is of limited value in determining potential human intoxication.

The present reviewers conclude that there is limited information available on the mode of action of BTXs and its analogues, and the demonstration that BTXs and its analogues can cause defined alterations of cell functioning *in vitro*, with particular reference to low concentrations of BTXs. Therefore, there is a need for further investigations into the mechanisms of action of BTXs and its analogues, and an accurate assessment of long-term effects because of low-dose and/or repeated ingestion of BTXs and its analogues in animal experiments. In particular, studies of the possible

[1] The Expert Consultation arrived at conclusions in accordance with the draft chapter. The Evaluation Section of the present Background Document is similar to that of the draft chapter prepared for the Expert Consultation.

health effects of chronic low-level exposure are needed in humans and animals. With the demonstrated toxicological loci of neuronal, pulmonary, enzymatic and DNA damage, these studies need to be broad-based.

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Cyclic imines (gymnodimine, spirolides, pinnatoxins, pteriatoxins prorocentrolide and spiro-prorocentrimine)

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1. BACKGROUND INFORMATION[1]

The cyclic imines group includes gymnodimine, spirolides, pinnatoxins, prorocentrolide and spirocentrimine. The presence of this group of compounds in shellfish was discovered because of their very high acute toxicity in mice upon i.p. injections of lipophilic extracts. When present at elevated levels, they rapidly kill mice, and their presence may interfere with the MBA for OA, BTXs, and AZA groups. At sublethal doses, the mice recover rapidly. The toxic potential of the cyclic imines is much lower via the oral route. The regulatory significance of the cyclic imine toxins is still unclear. Although gymnodimine and spirolides are now known to commonly occur in microalgae and/or bivalve molluscs from several parts of the world (Canada, Denmark, New Zealand, Norway, Tunisia, the United Kingdom of Great Britain and Northern Ireland, and the United States of America), there have been no reports of adverse effects in humans.

2. BIOLOGICAL DATA IN MAMMALS

2.1 Biochemical aspects

2.1.1 Absorption, distribution, excretion and biotransformation

No information on the absorption, distribution and excretion of these materials has been found. The very rapid onset of toxic signs recorded with these substances after i.p. injection in acute toxicity studies (see below) suggests, however, that they are rapidly absorbed from the peritoneal cavity. Furthermore, the rapid recovery seen in animals following a sublethal dose of gymnodimine (Munday *et al.*, 2004a), desmethyl spirolide C (Richard *et al.*, 2001; Munday *et al.*, 2004b) or prorocentrolide (Hu *et al.*, 1996b) suggests that these compounds are rapidly detoxified or excreted in animals.

2.1.2 Effects on enzymes and other biochemical parameters

Spirolides do not affect kainate, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate or N-methyl-D-aspartate (NMDA) receptors *in vitro*. They do not inhibit protein phosphatases and have no effect on voltage-dependent sodium channels. They are weak activators of Type L calcium channels (Hu *et al.*, 1995).

[1] Corresponds to the "Background Information" section of the Expert Consultation Report.

2.2 Toxicological studies

2.2.1 Acute toxicity

Data on the acute toxicity of gymnodimine to mice are summarized in Table 1. Seki *et al.* (1995, 1996) and Stewart *et al.* (1997) reported MLDs of gymnodimine by i.p. injection of 450 and 700 µg/kg, respectively. A later study, using a fully characterized pure sample of the toxin, gave an MLD of 100 µg/kg and an LD_{50} of 96 µg/kg (Munday *et al.*, 2004a). In a study performed with extracts of naturally contaminated clams, Biré (2004) found that the LD_{50} of gymnodimine lay between 97 µg/kg, at which no deaths were recorded, and 110 µg/kg, at which all the test mice died within a few minutes. Gymnodimine was less toxic when administered orally by gavage, with an LD_{50} of 755 µg/kg. When given to fasted mice on a small piece of mousefood, it was even less toxic, and no effects were observed at a dose of 7 500 µg/kg. Furthermore, the b.w. gains of the mice fed gymnodimine over a subsequent three-week observation period were similar to those of control mice, and no lesions or changes in organ weight were recorded at necropsy (Munday *et al.*, 2004a). No deaths or symptoms of toxicity were recorded in mice given gymnodimine in extracts of naturally contaminated mussels at up to 1 225 µg/kg when administered by gavage (Biré, 2004).

TABLE 1
Acute toxicity of gymnodimine and gymnodamine to mice

Compound	Route of administration	Sex	Parameter	Acute toxicity (µg/kg body weight)	References
Gymnodimine	Intraperitoneal	?	MLD	450	Seki *et al.*, 1995, 1996
Gymnodimine	Intraperitoneal	?	MLD	700	Stewart *et al.*, 1997
Gymnodimine	Intraperitoneal	Female	LD_{50}	96 (79–118)**	Munday *et al.*, 2004a
Gymnodimine	Intraperitoneal	Female	LD_{50}	Between 97 and 110	Biré, 2004
Gymnodamine	Intraperitoneal	?	MLD	>4,040	Stewart *et al.*, 1997
Gymnodimine	Oral (gavage)	Female	LD_{50}	755 (600-945)**	Munday *et al.*, 2004a
Gymnodimine	Oral (gavage)	Female	MLD	>1 225	Biré, 2004
Gymnodimine	Oral (feeding)*	Female	LD_{50}	>7 500	Munday *et al.*, 2004a

* A solution of pure gymnodimine was added to a small piece of dry mousefood, and fed to a mouse that had been fasted overnight.
** Figures in brackets indicate 95 percent confidence limits.

The clinical signs of gymnodimine intoxication are highly characteristic. Immediately after i.p. injection of lethal doses of gymnodimine, mice became hyperactive. After one minute, however, movement became slower, and the animals walked with a rolling gait. Soon after, the hind legs became paralysed and extended. The mice subsequently became completely immobile and unresponsive to stimuli. Respiratory distress was apparent, with marked abdominal breathing. The respiratory rate progressively decreased, until respiration ceased altogether. Pronounced exophthalmia was observed shortly before death, which invariably occurred within 15 minutes of injection. No macroscopic abnormalities were recorded at necropsy. At toxic, but sublethal, dose-levels, prostration and respiratory distress were recorded, but the mice recovered within 30 minutes to an apparently normal state, and no adverse effects were observed during the subsequent 21-day observation period. The symptoms of intoxication observed after administration of gymnodimine to mice by gavage were the same as those seen after injection, although the time to death was extended (Munday *et al.*, 2004a).

Histological examination of the spleen, liver, kidneys, thymus and brain of mice receiving a sublethal dose of gymnodimine either orally (1 225 µg/kg) or intraperitoneally (97 µg/kg) revealed no changes attributable to the test substance (Biré, 2004).

Reduction of the imine function, yielding gymnodamine, greatly decreased the acute toxicity of gymnodimine. The reduced compound induced no observable toxic effects when injected into mice at 4 040 µg/kg (Stewart *et al*., 1997).

The short-acting cholinesterase inhibitors, neostigmine and physostigmine, protected mice against a lethal dose of gymnodimine (Munday *et al*., 2004a).

Data on the acute toxicity of the spirolides to mice are summarized in Table 2. By i.p. injection, spirolides B and D are of similar toxic potential (Hu *et al*., 1995). In contrast, spirolides E and F, in which the cyclic imine moiety is destroyed, are much less toxic (Hu *et al*., 1996a). The LD_{50} of a mixture of spirolides, containing predominantly 13-desmethyl spirolide C, was found to be 40 µg/kg after i.p. injection in mice (Richard *et al*., 2001). The toxicity of a pure sample of desmethyl spirolide C was much higher, however, with an LD_{50} of only 6.5 µg/kg (Munday *et al*., 2004b). Dihydrospirolide B, in which the imine moiety is reduced, showed no toxicity at 1 000 µg/kg (Hu *et al*., 1996a). The spirolides are less toxic by oral dosing than by injection. The above-mentioned spirolide mixture had an LD_{50} of 1 000 µg/kg after dosing by gavage (Richard *et al*., 2001), while pure desmethyl spirolide C gave an LD_{50} of 157 µg/kg when administered by this technique. The latter compound was less toxic when fed to mice. The animals were reluctant to eat dry mousefood containing desmethyl spirolide C, but enough mice consumed the food within a reasonable period of time (< 10 minutes) to establish an LD_{50}. This was 625 µg/kg (Munday *et al*., 2004b). Other feeding techniques were also employed in order to disguise the presence of the test substance and thereby ensure its rapid consumption by the mice. Desmethyl spirolide C was mixed into powdered mousefood (~ 150 mg) that was then made into a paste by addition of water. This was rolled into a pellet and fed to a mouse that had been fasted overnight. It was readily accepted, and gave a similar estimate of the LD_{50} as that obtained with dry mousefood. Spirolide was also fed to fasted mice mixed with a pellet (~ 300 mg) of cream cheese, again giving a similar estimate of the LD_{50}. Because of the avidity of the mice for cream cheese, spirolide could also be fed in this matrix to mice without the need for fasting. When given to fed mice, the LD_{50} of desmethyl spirolide C (1 005 µg/kg) was almost twice that recorded in the fasted animals (Munday *et al*., 2004b).

Gill *et al*. (2003) showed that mice given lethal doses of desmethyl spirolide C became hunched and lethargic, with piloerection. They were uncoordinated in their movements, and showed jerky locomotion. The hind limbs became splayed, and lachrimation, exophthalmia and abdominal breathing were observed. Arching the tip of the tail forward towards the head also occurred, with mouth breathing, followed by respiratory arrest. Similar effects were recorded in a subsequent study, although in these experiments tail arching was not a prominent feature of the intoxication (Munday *et al*., 2004b). The symptoms of spirolide poisoning were the same whether the material was given intraperitoneally or orally (Munday *et al*., 2004b). Mice receiving lethal doses of spirolide died between 3 and 20 minutes after dosing (Richard *et al*., 2001). If the animals survived for 20 minutes or longer, they recovered fully, and their subsequent appearance and behaviour were normal (Richard *et al*., 2001; Munday *et al*., 2004b).

No macroscopic changes were seen in mice after lethal doses of desmethyl spirolide C (Pulido *et al*., 2001; Gill *et al*., 2003; Munday *et al*., 2004b). No histological changes were recorded in the retina, skeletal muscle, peripheral nerves, heart, liver, kidney, spleen, lungs, adrenals or gastrointestinal (GI) tract of mice receiving lethal doses of desmethyl spirolide C. No histological changes were observed in the brains of rats dosed with spirolide, but widespread neuronal damage was seen in mouse brains, particularly in the brain stem and hippocampus (Pulido *et al*., 2001; Gill *et al*., 2003).

TABLE 2
Acute toxicity of spirolides to mice

Compound	Route of administration	Sex	State of alimentation	Parameter	Acute toxicity (µg/kg body weight)	References
Spirolide B	Intraperitoneal	?	?	LD_{100}	250	Hu *et al.*, 1995
Spirolide D	Intraperitoneal	?	?	LD_{100}	250	Hu *et al.*, 1995
Spirolide E	Intraperitoneal	?	?	MLD	>1 000	Hu *et al.*, 1996a
Spirolide F	Intraperitoneal	?	?	MLD	>1 000	Hu *et al.*, 1996a
Spirolide mixture*	Intraperitoneal	Female	?	LD_{50}	40	Richard *et al.*, 2001
Desmethyl spirolide C	Intraperitoneal	Female	Fed	LD_{50}	6.5 (5–8)**	Munday *et al.*, 2004b
Dihydrospirolide B	Intraperitoneal	?	?	MLD	>1 000	Hu *et al.*, 1996b
Spirolide mixture*	Oral (Gavage)	Female	?	LD_{50}	1 000	Richard *et al.*, 2001
Desmethyl spirolide C	Oral (Gavage)	Female	Fed	LD_{50}	157 (123–198)**	Munday *et al.*, 2004b
Desmethyl spirolide C	Oral (Feeding, method 1)***	Female	Fasted	LD_{50}	625 (547–829)**	Munday *et al.*, 2004b
Desmethyl spirolide C	Oral (Feeding, method 2)***	Female	Fasted	LD_{50}	591 (500–625)**	Munday *et al.*, 2004b
Desmethyl spirolide C	Oral (Feeding, method 3)***	Female	Fasted	LD_{50}	500 (381–707)**	Munday *et al.*, 2004b
Desmethyl spirolide C	Oral (Feeding, method 3)***	Female	Fed	LD_{50}	1 005 (861–1 290)**	Munday *et al.*, 2004b

1. A solution of pure desmethyl spirolide C was added to a small piece of dry mousefood, and fed to a mouse that had been fasted overnight.
2. A solution of pure desmethyl spirolide C was fed to mice mixed with a pellet of moist mousefood.
3. A solution of pure desmethyl spirolide C was fed to mice mixed with a pellet of cream cheese.
* Predominantly desmethyl spirolide C.
** Figures in brackets indicate 95 percent confidence limits.
*** Feeding methods (for details, see text).

Transcriptional analysis of animals dosed with desmethyl spirolide C showed major changes in rat brain, but not in mouse brain. In the brain stem and cerebellum of rats, there was an increase in the early-injury markers HSP-72 and c-jun, and certain subtypes of muscarinic (mAChR1, mAChR4 and mAChR5), and nicotinic (nAChRα2 and nAChRβ4) acetylcholine receptors were upregulated (Gill *et al.*, 2003). Other markers, such as acetylcholinesterase and the glutamate receptors NMDAR1 and kainate (KA2), were unchanged (Gill *et al.*, 2003).

The time to death of animals given a mixture of spirolides was increased after pretreatment with physostigmine (Richard *et al.*, 2001). Conversely, the time to death was decreased when atropine or other acetylcholine antagonists were given before administration of spirolide (Richard *et al.*, 2001).

Data on the acute toxicity of pinnatoxin derivatives to mice are summarized in Table 3. The LD_{99} of natural (+)-pinnatoxin A was reported as 180 µg/kg (Uemura *et al.*, 1995) and 135 µg/kg (McCauley *et al.*, 1998) after i.p. injection. In contrast, synthetic (-)-pinnatoxin A was without toxic effect at a dose of 5 000 µg/kg (McCauley *et al.*, 1998). Pinnatoxin D was less toxic than pinnatoxin A (Chou *et al.*, 1996), but a mixture of pinnatoxins B and C, which are stereoisomers, was much more toxic to mice, with an LD_{99} of only 22 µg/kg (Takada *et al.*, 2001a). No information on the symptoms of intoxication by the pinnatoxins or on the histology of animals dosed with pinnatoxin derivatives has been found.

TABLE 3
Acute toxicity of pinnatoxins to mice

Compound	Route of administration	Sex	Parameter	Acute toxicity (µg/kg body weight)	References
(+)-Pinnatoxin A	Intraperitoneal	?	LD_{99}	180	Uemura *et al.*, 1995
(+)-Pinnatoxin A	Intraperitoneal	?	LD_{99}	135	McCauley *et al.*, 1998
(-)-Pinnatoxin A	Intraperitoneal	?	MLD	>5 000	McCauley *et al.*, 1998
Pinnatoxins B & C*	Intraperitoneal	?	LD_{99}	22	Takada *et al.*, 2001b
Pinnatoxin D	Intraperitoneal	?	LD_{99}	400	Chou *et al.*, 1996a

* 1:1 mixture of B & C. These compounds are stereoisomers.

Data on the acute toxicity of pteriatoxin derivatives to mice are summarized in Table 4. The LD_{99} of pteriatoxin A was 100 µg/kg when administered by i.p. injection to mice. A 1:1 mixture of pteriatoxins B and C was much more toxic, with an LD_{99} of 8 µg/kg (Takada *et al.*, 2001b). No information on the symptoms of intoxication by the pteriatoxins or on the histology of animals dosed with pteriatoxin derivatives has been found.

TABLE 4
Acute toxicity of pteriatoxins to mice

Compound	Route of administration	Sex	Parameter	Acute toxicity (µg/kg body weight)	References
Pteriatoxin A	Intraperitoneal	?	LD_{99}	100	Takada *et al.*, 2001a
Pteriatoxins B & C*	Intraperitoneal	?	LD_{99}	8	Takada *et al.*, 2001a

* 1:1 mixture of B & C. These compounds are stereoisomers.

No details of the acute toxicity of prorocentrolide are available. Torigoe *et al.* (1988) reported that the "lethality" of this substance was 400 µg/kg in mice, although whether this figure relates to the MLD or to a particular proportion of deaths in treated animals was not stated. It is reported that prorocentrolide is a fast-acting toxin, with deaths occurring within minutes of i.p. injection. At sublethal doses, the animals recovered completely (Hu *et al.*, 1996b). No information on the clinical signs or macroscopic pathology associated with administration of this substance is available, and no information on the histology of mice dosed with prorocentrolide has been found.

Spiro-procentrimine appears to be much less toxic than other cyclic imines. Lu *et al.* (2001) reported an i.p. LD_{99} of 2 500 µg/kg in mice. No information on the symptoms of intoxication with spiro-procentrimine has been found, nor has any information on the histology of animals dosed with this substance.

2.2.2 Short-term toxicity

No data on the effects of short-term repeated dosing of gymnodimine, pinnatoxins, pteriatoxins, prorocentrolide or spiro-prorocentrimine to animals have been found.

A small pilot study has been conducted in mice in order to assess the effect of multiple sublethal injections of spirolide (Pulido *et al.*, 2004). 13-Desmethyl spirolide C was dosed intraperitoneally to mice at 12.5, 25 and 35 µg/kg at 0, 7.5 and 21.5 hours. A lethal dose (75 µg/kg) was then given to each mouse at 25 hours. No ill-effects were recorded following the initial, second or third dose of the test material, except for one mouse that died 14 minutes after receiving the second dose of 35 µg/kg. All the mice died within six minutes after the lethal dose. All animals were subjected to macroscopic examination and samples of brain and internal organs were processed for histology. No macroscopic or histological changes were observed in any of these mice when compared with untreated controls.

2.2.3 Long-term toxicity/carcinogenicity

No data on the possible long-term effects of any of the cyclic imines have been found.

2.2.4 Genotoxicity

No data on the possible genotoxicity of any of the cyclic imines have been found.

2.2.5 Reproductive toxicity.

No data on the possible reproductive effects of any of the cyclic imines have been found.

2.2.6 Special studies

Effects in isolated cells in vitro

Gymnodimine did not lyse mouse erythrocytes or cause toxicity to NB41 or P388 cells *in vitro* at a concentration of 5 µg/ml (Seki *et al*., 1996).

It was reported that prorocentrolide was toxic to LC-1210 cells *in vitro*, with an IC_{50} of 20 µg/ml but not to *Aspergillus niger*, *Candida rugosa* or *Staphylococcus aureus* at a dose of 80 µg/disk (Torigoe *et al*., 1988). No details of the conditions employed in these experiments are available.

No information on *in vitro* effects of pinnatoxin derivatives, pteriatoxin derivatives or spiro-procentrimine has been found.

2.3 Observations in humans

Anecdotal reports from New Zealand indicate that no adverse effects are seen in humans after consumption of shellfish contaminated with gymnodimine (McKenzie *et al*., 1996; Munday *et al*., 2004a). To the authors' knowledge, although contamination of clams in Tunisia has been reported (Biré *et al*., 2002), no case of poisoning has been shown to be associated with the presence of this substance (R. Biré, personal communication). At this time, therefore, no specific syndrome can be attributed to consumption of gymnodimine by humans.

Episodes of toxicity, involving rather non-specific symptoms such as gastric distress and tachycardia, have been recorded in individuals in Nova Scotia consuming shellfish during times when spirolides were known to be present. The spirolides have not, however, been definitively linked to human illness (Richard *et al*., 2001).

Pinnatoxins have been isolated from shellfish of the genus *Pinna*. Several outbreaks of poisoning occurred in Japan between 1975 and 1991 among individuals consuming *Pinna pectinata* (Chou, Kama and Uemura, 1996b) and toxicity from *Pinna attenuata* was recorded in China in 1980 and 1989 (Uemura *et al*., 1995). The association between toxicity of the shellfish and the presence of the pinnatoxins therein has not, however, been established. Indeed, the poisoning incidents described by Uemura *et al*. are listed in documents from the Government of Japan as being caused by *Vibrio* sp. (Otofuji *et al*., 1981). Solid evidence for human intoxication by pinnatoxin and its analogues is required before these substances are included in a list of contaminants to be monitored in the international trade.

No information on the possible involvement of pteriatoxins, prorocentrolide or spiro-procentrimine in human intoxication has been found.

3. ANALYTICAL METHODS

3.1 *In vivo* assays

Mouse bioassay (MBA)

The AOAC MBA for PSP toxins (AOAC, 1990) does not detect cyclic imine toxins, because they are not extracted with sufficient yields into acidic aqueous extracts. The toxins are also not readily extracted from shellfish by diethyl ether, thus the APHA (APHA, 1970) procedure for NSP toxins does not detect them either. However, cyclic

imine toxins are detected by the mouse assay commonly used for lipophilic toxins (Yasumoto, Oshima and Yamaguchi, 1978; Yasumoto *et al.*, 1984). This is based on an acetone or methanol extraction of shellfish HP, followed by a hexane wash and a partition into dichloromethane or chloroform, evaporation, redissolution in a small volume of 1 percent Tween 60, and then i.p. injection of the crude extract into mice.

The symptoms for spirolides are fairly representative of the group. These include rapid deaths preceded by neurological symptoms (piloerection, abdominal muscle spasms, hyperextensions of the back, and arching of the tail to the point of touching the nose). They are very different from those associated with other known shellfish toxins, such as those from the OA or STX groups. If a mouse survives past 20 minutes while demonstrating symptoms, it will recover fully and quickly.

Gymnodimine has a reported LD_{50} of 96 µg/kg (Munday *et al.*, 2004a). Based on this value and an i.p. injection of 25 g whole tissue equivalent (5 g HP) into a 20 g mouse, the MBA should therefore show an LD_{50} at 74 µg/kg whole tissue. For spirolides, early reports assigned an LD_{50} for i.p. in the mouse of approximately 40 µg/kg b.w. (Richard *et al.*, 2001), but this was prior to the availability of accurate, purified standards and was probably closer to an LD_{100}. Recent work on a pure sample of desmethyl spirolide C gave an LD_{50} of only 6.5 µg/kg (Munday *et al.*, 2004b). Therefore, MBA should show an LD_{50} at 5 µg/kg whole tissue and an LD_{100} at approximately 20–25 µg/kg whole tissue.

No validation studies of the MBA method for these toxins have been carried out.

3.2 *In vitro* assays

None to date.

3.3 Biochemical assays

None to date.

3.4 Chemical assays

Liquid chromatography-mass spectrometry (LC-MS)

Several publications have reported LC-MS methods for gymnodimine and spirolides (Cembella, Lewis and Quilliam, 1999; MacKenzie *et al.*, 2002; Quilliam *et al.*, 2001; Stirling, 2001). These methods are based on reversed-phase LC coupled with electrospray ionization MS used in either the selected ion or selected reaction monitoring modes (SIM, SRM).

Shellfish tissues can be extracted with good yield using 70–90 percent aqueous methanol. The crude extract can be cleaned with a hexane wash. Direct analysis of the aqueous methanol phase is possible, or it may be further extracted with a chloroform or dichloromethane partition. Alternatively, a SPE procedure can be used. Detection limits vary with sample preparation procedures and detection method (SIM or SRM), but can be as low as 0.1 µg/kg whole tissue.

An interlaboratory study of an LC-MS method for determination of various lipophilic toxins in shellfish was carried out (Holland and McNabb, 2003). Gymnodimine was one of the analytes included. The eight participating laboratories generally obtained consistent sets of data for the broad group of toxins down to low levels. The method could reliably detect gymnodimine (from 0.03 to 3 ng/ml, equivalent to 0.03 to 3 µg/kg).

The LC-MS methods need to be evaluated with a full collaborative study before they will be sufficient to meet Codex requirements for a quantitative method to enforce Codex standards. The LC-MS approach continues to evolve and improve for quantification of marine biotoxins and is increasingly being employed in developed countries for marine biotoxin analyses.

3.5 Reference materials

Calibration solution CRMs are available for gymnodimine and one spirolide, 13-desmethyl-spirolide C, from the NRC-CRMP (Halifax, Canada).

4. FOOD CONSUMPTION/DIETARY INTAKE ESTIMATES

The spirolide content of mussels has been estimated at ~ 2 mg/kg of edible tissue (M. Quilliam *et al.*, personal communication). A typical meal is considered to be ~ 200 g of edible tissue, so that the potential exposure dose is 0.4 mg/person, which equates to a dose of 8 µg/kg for a 50-kg individual.

5. DOSE RESPONSE ANALYSIS AND ESTIMATION OF CARCINOGENIC RISK

Data are available, with a greater or lesser degree of detail, on the acute toxicity of gymnodimine, spirolides, pinnatoxins, pteriatoxins, prorocentrolide and spiro-procentrimine in mice. No data are available on the symptoms of intoxication by the pinnatoxins or spiro-procentrimine, but all the other cyclic imines are fast-acting toxins, with death occurring in a matter of minutes after administration of lethal doses. These materials must therefore be rapidly absorbed from the peritoneum after injection and from the gut after oral administration. Equally, they must be rapidly excreted or detoxified, because although animals may show severe toxic effects when given sublethal doses of the toxins, they recover quickly and completely (Hu *et al.*, 1996b; Richard *et al.*, 2001; Munday *et al.*, 2004a). For those compounds for which data are available, the symptoms of intoxication are remarkably similar, with death following respiratory arrest (Pulido *et al.*, 2001; Gill *et al.*, 2003; Munday *et al.*, 2004a).

However, most of the acute toxicity studies on the cyclic imines have involved i.p. injection of the test materials. In risk assessment, the route of administration to experimental animals should be the same as that for the individuals at risk (Perera, Brennan and Fouts, 1989). Shellfish contaminated with cyclic imines are eaten by humans, and oral data from animal experiments are therefore much more valuable in assessing possible harmful effects to consumers. When administered by gavage, gymnodimine was ~ 8 times less toxic, and desmethyl spirolide C ~ 24 times less toxic than when injected (Munday *et al.*, 2004a, 2004b). Furthermore, there is evidence that gavage gives an artefactually high estimate of the acute toxicity of rapidly absorbed and fast-acting toxins. In experiments with gymnodimine, it was noted that animals died within a short time after gavage. This was rather surprising because the material is delivered into the stomach, and few materials are readily absorbed from the stomach of rodents. It was considered possible that, because of the semi-solid nature of mouse stomach contents, material given by gavage could flow around the mass of food and rapidly enter the duodenum, where rapid absorption is to be expected. This was confirmed by administering a dye by gavage. The dye did not readily penetrate the stomach contents and was visible in the duodenum within two minutes of dosing. It was shown, however, that dye fed to mice in a small piece of mousefood became evenly distributed within the whole mass of stomach contents. In this situation, material will pass from the stomach to the duodenum relatively slowly. The acute toxicities of gymnodimine and desmethyl spirolide after voluntary intake by feeding to mice were, respectively, > 10 and 3–6 times lower than that following gavage (Munday *et al.*, 2004a, 2004b). Furthermore, as shown with desmethyl spirolide C, the state of alimentation of the animals is of crucial importance, with fed mice being less susceptible than animals that had been fasted overnight (Munday *et al.*, 2004b). Because shellfish are generally consumed as part of a meal, it may be argued that experiments using fed mice are more relevant to the human situation.

The imine function of cyclic imines is of paramount importance for their toxicity. When the imine group is reduced, as in gymnodamine and dihydrospirolide B, or

destroyed by ring-opening, as in spirolides E and F, the toxicity is greatly decreased (Stewart *et al*., 1997; Hu *et al*., 1996a). Indeed, there are no data on the acute toxicity of the latter substances, with no effects being reported at the highest dose-levels employed (4 040 or 1 000 µg/kg). In the case of spirolides A and B, but not C and D, ring-opening of the imine ring is mediated by acid (Hu *et al*., 2001), and shellfish metabolize spirolides A and B to the ring-opened products (Hu *et al*., 1996a). The metabolism of spirolides within a living organism suggests that the ring-opening may be enzymatically mediated, and Hu *et al*. (2001) suggested that the lability of the imine ring to acid or to enzymatic hydrolysis could be important for the toxicity of spirolides in humans. This is an important point, and illustrates another problem with the use of the mouse for risk analysis of compounds of this type. The pH of the contents of the human stomach is maintained at 1–3 whereas the pH of mouse stomach contents is 3–5 (Ilett *et al*., 1990). It is possible, therefore, that certain spirolides could be destroyed in the human stomach but not in that of the mouse.

The data on pinnatoxins and pteriatoxins permit some comment on structure-activity relationships among these substances. Pinnatoxins A–C and pteriatoxins A–C differ only in the nature of the substituent at position 33. In both, compounds B and C are stereoisomers. Pinnatoxin A, which has a carboxyl group at C-33, is ~ 14 times less toxic than a mixture of pinnatoxins B and C, which have a glycine residue at this site. The reason for this marked effect of aminoacid substitution at C-33 is not known. The situation with the pteriatoxins is even more remarkable. These compounds may be considered as derivatives of 3-(2-hydroxyethylthio)-2-aminopropanoic acid, $HOCH_2CH_2SCH_2CH(NH_2)COOH$, in which the macrocycle is substituted in the hydroxyethylthio chain. The only difference between pteriatoxin A and pteriatoxins B and C is that in the former the macrocycle is attached at the 2-position of the hydroxyethylthio moiety and in the latter it is attached at the 1-position. Yet pteriatoxins B and C are 12.5 times more toxic than pteriatoxin A (Takada *et al*., 2001a). Again, no explanation can currently be offered for this difference.

No deaths were observed in mice given twice the LD_{50} of gymnodimine after pretreatment of the animals with physostigmine or neostigmine. Under these conditions, control animals died within a few minutes (Munday *et al*., 2004b). Similarly, physostigmine increased the time to death of animals given a lethal dose of spirolide (Richard *et al*., 2001). Physostigmine and neostigmine are short-acting acetylcholine inhibitors, which increase acetylcholine concentrations in the synaptic cleft of nicotinic receptors, thereby competitively inhibiting binding of foreign compounds to the receptor. The results of Gill *et al*. (2003), showing upregulation of both muscarinic and nicotinic acetylcholine receptors in mice given lethal doses of desmethyl spirolide C are also consistent with a mechanism of toxicity involving acetylcholinergic receptors. These receptors are widely distributed in tissues, and are involved in CNS, autonomic ganglia and neuromuscular transmission. It is feasible, therefore, that these compounds act at multiple sites. The work of Gill *et al*. (2003) suggests a central toxic effect, although other factors and mechanisms cannot be ruled out. Interestingly, the symptoms of intoxication by the cyclic imines are very similar to those of tubocurarine, a known neuromuscular-blocking agent, suggesting that the neuromuscular junction may be a site of action for this group of compounds (Munday *et al*., 2004a). At present, there is limited information on the specific sites and mechanisms of action of the cyclic imines, and further work in this area is required.

6. EVALUATION[2]

There is no conclusive evidence that the cyclic imines have adverse effects on human health. There is no doubt that some of these substances are exceptionally acutely toxic to mice by i.p. injection, but their oral toxicity is much lower.

By feeding, the LD_{50} of gymnodimine in mice was > 7 500 µg/kg. Applying a 100-fold safety factor, a dose of 75 µg/kg b.w. may well be without acute toxic effect in humans. For a 50-kg human, this would translate to a dose of 3.75 mg. If it is assumed that the intake of shellfish for an adult is 200 g, a level of 1.88 mg gymnodimine per 100 g edible material of shellfish is unlikely to cause harm.

For desmethyl spirolide C, the LD_{50} by feeding was between 500 and 1 005 µg/kg, depending on the state of alimentation of the animal. Taking the latter value as the most relevant to the human situation, and applying a 100-fold safety factor, a dose of 10 µg/kg b.w. would be unlikely to cause acute effects in humans. The latter would equate to a dose of 0.5 mg for a 50-kg human. This is above the estimated intake of spirolides from mussels (0.4 mg/person), and, again, if an intake of shellfish of 200 g is assumed, a level of spirolide of 0.25 mg/100 g edible matter is unlikely to cause harm.

In view of the possibility that cyclic imines may be degraded enzymatically or by acid, a study of their stability under the conditions pertaining to the human stomach would be of interest, together with examination of the possibility of their destruction by the gut flora or enzymes of the intestinal lumen. A study of the absorption, distribution and metabolism of these substances, taking into account the physiological differences between rodents and humans, would also be of great value in their risk assessment.

Very little information on the toxicity of cyclic imines after repeated exposure is presently available, and nothing is known of species, sex or age differences with regard to susceptibility to these compounds. No information is available on their possible carcinogenicity, reproductive toxicity or genotoxicity. Such data are required for an accurate analysis of the risk of cyclic imines to human health and to assess the possibility that certain subpopulations of humans could be particularly vulnerable to the toxic effects of these substances.

Very few methods are available for cyclic imines determination. The lipophilic MBA (semi-quantitative) and an LC-MS method (quantitative) are the only two that have been used and neither has been validated for routine analysis in regulatory environments. However, both these methods appear suitable for routine growing and harvest area monitoring.

7. REFERENCES

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[2] The Evaluation section of the present Background document is similar to that of the Cyclic Imines draft chapter. The Expert Consultation arrived at conclusions close to the draft chapter. Nevertheless, no provisional acute RfD or TDI were established for the cyclic imines in the Report because the database was considered insufficient.

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Domoic acid

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1. BACKGROUND INFORMATION[1]

Domoic acid (DA) was identified as the toxin responsible for an outbreak of illness in Canada in 1987, caused by eating blue mussels that had accumulated DA as a result of the presence of *Pseudo-nitzschia pungens*. Effects on both the gastrointestinal (GI) tract and the nervous system were observed. Because some of those affected experienced memory loss, the syndrome was named amnesic shellfish poisoning (ASP). As a result of the episode of human illness in Canada, a regulatory level of 20 mg DA/kg of shellfish meat was established, and no further incidences of ASP have been reported. The presence of DA in shellfish has been reported in various regions of the world. There have been numerous reports of toxicity in a variety of wildlife species indicating that DA moves up the food chain in marine ecosystems. Routine monitoring using LC-UV is well established in most monitoring programmes and has adequate detection limits to regulate DA at current limits. More rapid techniques such as ELISA would be useful. The recent finding of significant amounts of certain naturally occurring DA isomers requires an investigation of their toxicological significance for potential inclusion in monitoring.

2. ORIGINS AND CHEMICAL DATA OF SHELLFISH

Domoic acid is an excitatory amino acid that was identified as the toxin responsible for an outbreak of ASP that occurred in Canada in 1987 following consumption of contaminated blue mussels (*Mytilus edulis*).

Domoic acid is a tricarboxylic produced by certain marine organisms, the best characterized of which are the red alga *Chondria armata* and the planktonic diatom *Pseudo-nitzschia multiseries* (formerly known as *Nitzschia pungens f. multiseries*), although other species are also known to produce DA (for review see Bates, Garrison and Horner, 1998). Chemically, DA is (2S,3S,4S)-2-carboxy-4-1-methyl-5(R)-carboxyl-l(Z)-3(E)-hexadienyl pyrrolidine-3-acetic acid ($C_{15}H_{21}NO_6$) and has an anhydrous molecular weight of 311.14. Structurally, DA is very similar to another known toxin, kainic acid (KA), and both are analogues of the amino acids glutamate and proline.

Extracts of *C. armata* containing DA have been used traditionally as antihelminthic agents (Takemoto and Daigo, 1960) and the compound is a potent insecticide (Maeda *et al*., 1984). Domoic acid was shown in the 1970s to have potent excitatory properties in the mammalian CNS (Biscoe *et al*., 1975, 1976) and is generally regarded as a selective agonist at the kainate subclass of non-NMDA ionotropic glutamate receptors (Monaghan, Bridges and Cotman, 1989).

In addition to being the causative agent of ASP in Canada, there are also documented cases of DA intoxications in wild animals and outbreaks of costal water contamination

[1] Corresponds to the "Background Information" section of the Expert Consultation Report.

in many world regions (Mos, 2001; Vale and Sampayo, 2001; Hess *et al.*, 2001; Amzil *et al.*, 2001; Work *et al.*, 1993a, 1993b; Wekell *et al.*, 1994; Scholin *et al.*, 2000; Walz *et al.*, 1994; Lefebvre *et al.*, 1999, 2002; Sierra-Beltrán *et al.*, 1997). Hence, DA continues to pose a global risk to the health and safety of humans and wildlife.

3. BIOLOGICAL DATA

3.1 Biochemical aspects

3.1.1 Absorption, distribution and excretion

3.1.1.1 Oral absorption

Although consumption of contaminated food products is the primary route of DA intoxication, there have been very few objective studies on the absorption of this toxin following oral administration. Based on these limited observations, it appears that DA absorption from the GI tract is erratic and bioavailability is approximately 10 percent.

Two publications by Iverson *et al.* (1989, 1990) reported on the relative toxicity of DA following oral or i.p. administration in mice. These authors used both extracts of contaminated mussels and purified DA, obtaining similar results with each. Using scratching behaviour, seizures and death as the criteria for DA toxicity, they reported consistently reproducible toxicity following i.p. administration of doses from 2.0 to 9.5 mg/kg but variable toxicity following oral administration of doses ranging from 35 to 104 mg/kg. For example, scapular scratching was observed in several animals receiving 35 mg/kg p.o., but five of seven animals receiving 80 mg/kg p.o. showed no observable toxicity. The authors concluded that oral doses approximately tenfold those of parenteral routes of administration are required for toxicity but that other unidentified factors also contribute to oral bioavailability.

The same publications (Iverson *et al.*, 1989, 1990) also report on oral and i.p. administration of DA in rats. Toxicity was observed following i.p. administration at doses comparable with those reported for mice (2.0–8.0 mg/kg), but much higher doses (80–82 mg/kg) were required to produce seizures following oral administration.

Similar findings were reported by Truelove *et al.* (1996) following repetitive oral dosing with DA in rats. These authors reported no changes in behaviour, clinical chemistry or histopathology in rats dosed orally with 0.1 mg/kg per day (equivalent to a 50-kg human consuming 250 g of shellfish containing 20 ppm DA each day) and only minor histopathological changes in the brains of rats dosed with 5.0 mg/kg per day.

Iverson *et al.* (1990) also reported observing fine motor tremors in one cynomolgus monkey dosed 12 hours previously with 5.0 mg/kg DA p.o. but no effect in another monkey dosed at 0.5 mg/kg p.o.

Another early study by Tryphonas *et al.* (1990a) reported on observations of five cynomolgus monkeys receiving oral doses of toxic mussel extract containing DA in concentrations ranging from 5.21 to 6.62 mg/kg, which were then observed for 17–44 days. These authors reported GI disturbance but inconclusive CNS toxicity in these animals. In this study, one monkey was also given monosodium glutamate (MSG) orally and one received DMSO orally to see if either compound facilitated oral absorption, but both were without apparent effect. In a companion experiment reported in the same paper (Tryphonas *et al.*, 1990a) four monkey were dosed orally with purified DA at doses of 0.0, 0.5, 5.0 and 10.0 mg/kg. Nausea and vomiting were observed with 0.5–1 hour of dosing with 10.0 and 5.0 mg/kg respectively, but "histopathological changes in orally treated monkeys were minimal, equivocal or non-existent".

Finally, a 30-day oral toxicity study in monkeys (Truelove *et al.*, 1996) administered DA orally to three cynomolgus monkeys at a dosage of 0.5 mg/kg per day for 15 days and then at 0.75 mg/kg per day for 15 days. These authors reported that 4–7 percent of the orally administered dose was absorbed in these animals.

3.1.1.2 Distribution

Only three studies have systematically investigated the distribution kinetics of DA.

A study by Suzuki and Hierlihy (1993) administered doses of 0.5 ng/kg, 0.5 mg/kg or 2.0 mg/kg DA via i.v. catheter and collected blood and urine samples over the next 130 minutes. They reported that DA distribution was limited largely to the vascular compartment and interpolated an apparent steady-state volume of distribution (Vd_{ss}) ranging from 0.24 to 0.27 litres/kg.

Similar results were reported by Truelove and Iverson (1994) in which small groups of rats were given either 0.5 (n=2) or 1.0 (n=4) mg/kg DA i.v. These authors reported Vd_{ss} values ranging from 0.16 to 0.36 litres/kg with a mean of 0.23 litres/kg. In the same paper, a dose of 0.050 mg/kg i.v. was administered to each of four cynomolgus monkeys and a mean Vd_{ss} of 0.16 litres/kg was obtained (Truelove and Iverson, 1994).

Preston and Hynie (1991) used injection of radiolabelled DA in a group of ten adult rats to estimate BBB transfer constants relative to labelled sucrose, which is largely impermeable. They sampled in seven brain regions (frontal cortex, occipital cortex, striatum, hippocampus, diencephalons, cerebellum and pons-medulla) over 30 minutes. Results indicated poor BBB penetration, with mean transfer constants ranging from 1.60 to 1.82 ml/g/sec × 10^6 (sucrose constants ranged from 1.00 to 1.24). These data argue against the existence of a carrier protein and suggest that the highly charged state of DA at physiological pH results in poor CNS penetration kinetics. Interestingly, however, in a subgroup (N=4) of rats that underwent nephrectomy surgery prior to DA administration, regional brain concentrations were increased by 50–120 percent, largely because of elevated plasma concentrations and reduced clearance (Preston and Hynie, 1991). These data were accurately modelled using physiologically based pharmacokinetic calculations (Kim *et al.*, 1998) and are consistent with the epidemiology of ASP toxicity in humans (Perl *et al.*, 1990a, 1990b, 1990c).

3.1.1.3 Excretion

The excretion kinetics for DA have been investigated in rats (three studies) and monkeys (one study) with similar results.

Preston and Hynie (1991) reported that a single i.v. dose of radiolabelled DA was almost completely eliminated from the serum of intact rats within 30 minutes. However, nephrectomy resulted in a significantly reduced clearance, such that detectable serum concentrations were still present at 60 minutes postinjection (the last time point studied).

In a more robust study of renal pharmacokinetics, Suzuki and Hierlihy (1993) reported that serum clearance of DA was almost entirely because of renal excretion with similar kinetics over a wide range of dosages (0.5 ng/kg –2.0 mg/kg i.v.). Analysis of serum concentration over time curves produced elimination rate constants (k) of 0.025–0.035 min^{-1} (equivalent to an elimination half-life of approximately 13 minutes). These authors also reported total body clearance values of 7.75–10.82 ml/min per kilogram and renal clearance values ranging from 8.80 to 12.20 ml/min per kilogram. Renal excretion in this study appeared to be mainly by glomerular filtration because kinetics were not altered by the presence of probenecid.

Truelove and Iverson (1994) confirmed that elimination is largely by renal excretion and generated similar values in adult rats. Their data indicated a mean serum terminal half-life of 21 minutes and a total body clearance value of 7.8 ml/min per kilogram. These values translate to a mean residence time of about 30 minutes in rats. Interestingly, however, these authors made similar calculations based on serum data in four monkeys and found considerably longer elimination half-lives in this species (mean = 114.5 min) because of reduced clearance (mean = 1.25 ml/min per kilogram) at a dose of 0.050 mg/kg i.v.

3.1.2 Biotransformation

There are no published data indicating that DA undergoes biotransformation in any species studied to date.

3.1.3 Effects on enzymes and other biochemical parameters

The only literature on the effects of DA on biochemical markers derives from a few isolated studies.

A report by Bose *et al.* (1992) showed that single injections of 1.0, 1.5 or 2.0 mg/kg DA in male mice reduced indicators of brain lipid peroxidation activity, and both the highest acute dose (2.0 mg/kg) or three daily injections of 1.5 mg/kg resulted in increased activity of superoxide dismutase.

Similarly, Appel *et al.* (1997) reported that both subconvulsive and convulsive doses of DA resulted in decreased regional incorporation of long-chain fatty acids in the brains of rats when measured 6–8 days later. The authors concluded that DA causes chronic effects on brain function as manifested by altered fatty acid metabolism and gliosis.

An isolated report by Nijjar and Grimmelt (1994) provided evidence of altered adenylate cyclase activity in rat brain homogenates exposed to DA.

Effects on brain neurotransmitter systems and/or precursors were investigated in three Spanish studies. A 1995 report by Arias *et al.* using rats demonstrated that acute injections of either 0.5 or 1.0 mg/kg result in significant reductions of 5-hydroxytryptamine (5-HT) (serotonin) content in the hypothalamus but not in midbrain or brainstem regions, although the major 5-HT metabolite, 5-HIAA, was significantly reduced in both the hypothalamus and the brainstem. In another paper (Durán *et al.*, 1995), the same group reported DA-induced changes in brain amino acid content related to neurotransmission. Rats receiving 0.2 mg/kg i.p. DA had significantly increased content of gamma-aminobutyric acid (GABA) and aspartic acid in the hippocampus, amygdala, cortex and midbrain without accompanying changes in glutamate, glycine, taurine or alanine concentrations. Amino acid concentrations in hypothalamus and striatum were unaffected in this study. Most recently, this research group used *in vivo* microdialysis to demonstrate DA-induced dopamine release in the striatum of rats (Alfonso *et al.*, 2003).

The effect of acute doses of DA on endocrine function was investigated by Arufe *et al.* (1995) and Alfonso, Duran and Arufe (2000) in the rat. Injections of both 0.5 and 1.0 mg/kg DA (i.p.) resulted in a temporary increase and subsequent significant reduction in circulating serum T4 concentrations (Arufe *et al.*, 1995) and the higher dose (1.0 mg/kg) caused an eightfold increase in circulating thyroid-stimulating hormone (TSH) 60 minutes postinjection (Alfonso, Duran and Arufe, 2000).

3.2 Toxicological studies

3.2.1 Acute toxicity

Available data on the acute toxicity of DA in mice, rats and non-human primates are summarized in Table 1 and discussed below.

3.2.1.1 Acute toxicity in mice

The vast majority of the acute toxicity studies on DA/ASP have been conducted on mice. Consequently, a detailed account of each is beyond the scope of this section, but summary notes on each of these articles are provided in Table 1. There are, however, several general points that should be emphasized.

TABLE 1
Summary of acute toxicity data for DA in mice, rats and cynomolgus monkeys

Species strain Sex Age Length of study	Dose	Route (N/grp)	Observed effects	LOAEL	NOAEL	LD50	Ref.
Mice Swiss-Webster up to 4 h	5.3–60 ml/kg of WMX	i.p. (6–19)	sluggishness, scratching, clustered huddling, phalanx huddling, hind limb stretching, gait in-coordination, clonic convulsions, respiratory distress, & death				2
Mice Swiss-Webster 15 day old up to 4 h	5–30 ml/kg WMX (whole mussel extract) 0.64–2.55 mg/kg DA from MHX (mussel hepatopancreas extract)	i.p. (4–6)	* same as above * 3–4 fold more sensitive to WMX * kynurenic acid (KYN) gave protection after onset; time frame of KYN protection increased by probenecid or tryptophan & probenecid * phenytoin & ethosuximide were only mildly effective in delaying toxicity of WMX * different sources of DA did not effect response	75 mg/kg MHX (0.64 mg/kg DA) for 80% 6.9 ml/kg WMX (1.04 mg/kg DA) for 80%			2
Mice Swiss-Webster PND15	300 mg/kg MHX	i.p. (4)	gastric and duodenal ulcers				8, 9
Mice Swiss-Webster Male	0.5, 0.7, 0.8 or 1 ml of WMX (whole mussel extract)	i.p. (10–18)	* 1 ml caused gastric ulcers & peritoneal ascites * 0.7/0.8 ml caused duodenal ulcers * 300 mg/kg of kynurenic acid (60 or 75 min after extract) prevented ulcers & ascites			LD84 = 1 ml/mouse	8, 9
Mice Swiss-Webster Female Infant	0.50, 0.84, 1, 4 or 8 mg/kg	i.p. (4)	4 & 8 mg/kg caused higher incidence of gastrointestinal bleeding				9
Mice CD1 Female Up to 4 h	various doses from whole mussel extracts	i.p. (3)	scratching & death			3.6 mg/kg	10

TABLE 1
Summary of acute toxicity data for DA in mice, rats and cynomolgus monkeys (continued)

Species strain Sex Age Length of study	Dose	Route (N/grp)	Observed effects	LOAEL	NOAEL	LD50	Ref.
Mice CF1 Male/female	12–233µg DA in whole mussel extract 20–200µg of negative control (clean mussels injected with DA)	i.p. (3–6)	inactivity, scratching, uncontrolled rolling/ twisting, loss of righting reflex, seizures & death	46 ppm	0.59 mg/kg or 24 ppm	5 mg/kg	11
Mice Female	12–233 µg in toxic mussels 20–200 µg in neg. control 20–200 µg DA	i.p. (3–6)	scratching, rolling, tremors, seizures		12 µg or 24 ppm		12
Mice	0.3–2.5 g-equivalent or original wet mussel tissue	i.p.	* times of death ranged from 100 to 10 min. after injection * plankton samples produced similar symptoms to mussels				28
Mice Swiss-Webster Up to 72 h	2, 3 or 7 mg/kg	i.p.	damage confined to circumventricular organs & their environs				3
Mice CD-1 Male	0.15–20 mg/kg of contaminated mussels, spiked mussels, pure DA, or algal DA	i.p. (4)	* hypo-activity, sedation, rigidity, stereotypy, loss of posture, convulsions & death in a dose-dependent manner such that max. severity increased and latency decreased with increasing dose * TD50 of contaminated mussels, pure DA, mussel extract "spiked" with DA & agal source DA was: 2.9, 3.9, 4.9 & 4.2 mg/kg, respectively	20 µg			22
Mice CD-1 Female 4–168 h	4.0 mg/kg in WMX	i.p. (2–4)	* sedation, rigidity, sterotypy, balance loss & seizures * neuronal damage in CA3>CA4>CA1>DG cells * 82.1% of cells damaged in CA3				21

TABLE 1
Summary of acute toxicity data for DA in mice, rats and cynomolgus monkeys (continued)

Species strain Sex Age Length of study	Dose	Route (N/grp)	Observed effects	LOAEL	NOAEL	LD50	Ref.
Mice ICR Female 15–24 min	1–4 mg/kg DA in 100 ul PBS	i.p.	* c-fos mRNA within 15 min * c-fos product in 1 hr. degeneration in CA1-3, lateral and medial septum olfactory bulb				15
Mice ICR Female	0.25–4.9 mg/kg	i.p. (4–5)	* hyperactivity, scratching & seizures * fos expression in brain regions controlling memory and gastrointestinal functions	0.5 mg/kg		4.0 mg/kg	16
Mice CD-1 Male		i.p.	* scratching, seizures & death * lowest toxicity at pH 3.7 & highest at pH 7.4 * pH effect diminished as doses increased				14
Mice CF-1 Female	8.8–104 mg/kg in WMX	Oral (gavage)	inactivity, scratching, uncontrolled rolling/ twisting, loss of righting reflex, seizures & death				11
Mice	35–104 mg/kg of WMX 20–80 mg/kg DA	Oral (gavage) (1–2)	scratching & seizures oral dose is tenfold less potent than i.p.				12
Mice CD-1 Male	0.06–0.3 nmol	intracerebroventricular.v.	* wet dog shakes, hypermotility, circling, loss of righting reflex & seizures * CD50 = 0.09 nmol /mouse * ED50 = 0.07 nmol / mouse				4
Mice DBA Male	1.0 and 2.0 mg/ kg acutely or on alternating days for 1 week	i.p. (8–10)	* dose-related toxicity consistent with previous reports * mice receiving single injection had impaired performance on match-to-sample task on first day post-training (i.e. impaired recall) * no significant recall deficits in groups receiving repeat injections				5
Mice (14 day old) DBA Male / Female	2.0 mg/kg	i.p. (6)	swim in circles – longer latencies in Morris water maze				18

TABLE 1
Summary of acute toxicity data for DA in mice, rats and cynomolgus monkeys (continued)

Species strain Sex Age Length of study	Dose	Route (N/grp)	Observed effects	LOAEL	NOAEL	LD50	Ref.
Mice CD-1 Pregnant (E13)	0.6 mg/kg to dam	i.v. (5)	* reduced seizure thresholds * neuronal damage in CA3, CA4 & DG * reduced GABA * increased Glu * increased KA receptor binding in hippocampus * enhanced 45Ca influx into cortex & hippocampus				6
Mice ICR & DBA Female Acute & subchronic	0.5, 1.0 & 2.0 mg/kg acutely in ICR & DBA strains 0.5 & 2.0 mg/kg every 48 hours for 8 days	i.p. (4–8)	* increasing behavioural toxicity in acute dose groups with DBA strain appearing more sensitive than ICR * no difference in serum kinetics between acute versus repeat doses * no change in behavioural toxicity in acute versus repeat doses				17
Rats SD Female	0, 1, 2, 4 or 7.5 mg DA/kg b.w.	i.p.	* 75% of 2 mg/kg had equivocal transient behavioural signs * one 2 mg/kg rat & all 4 mg/kg had uniequivocal behavioural & neurological signs (partial seizures & status epilepticus) * encephalopathy & retinopathy in severally affected				26
Rats SD Female	0, 1, 2, 4 or 7.5 mg DA/kg b.w.	i.p. (4 except for 7.5 mg/kg where n=1)	* 3/4 rats at 2.0 mg/kg showed withdrawal followed by hyperexcitation & scapular scratching * rats in 4.0 & 7.5 mg/kg group showed above plus wet dog shakes, rearing with forelimb extension (“praying”), seizures & death * 1/4 rats at 2.0 mg/kg had lesions in the hippocampus (CA3>CA1>CA4), hypothalamus, cingulum, frontoparietal cortex, septum & olfactory nuclei * all rats at 4.0 mg/kg & 7.5 mg/kg had histopathology as described above	2.0 mg/kg			27

TABLE 1
Summary of acute toxicity data for DA in mice, rats and cynomolgus monkeys (continued)

Species strain Sex Age Length of study	Dose	Route (N/grp)	Observed effects	LOAEL	NOAEL	LD50	Ref.
Rats SD Male	0, 0.22, 0.65 or 1.32 mg/kg	i.p. (12)	* 1.32 mg/kg rats had degenerating neurons in the CA1/CA3 & gliosis * increased auditory startle & decreased wt. loss in 1.32 mg/kg rats * 0.93 mg/kg rats had hypomotility & decrease in b.w. * pretreatment of scopolamine (2 m/kg) reduced effects				20
Rats Fisher Male	2.25 mg/kg	i.p.	* stereotype behaviour & convulsions in 60% of rats * increased GFAP in cerebral cortex, hippocampus, septum, caudate putamen & thalamus * DA rats without convulsions decreased relative [14C] AA incorporation in claustrum & pyramidal cell layer of hippocampus, CA1 & CA2 * relative rCMR decreased in all rats				1
Rats Long-Evans	0.5–1.0 mg/kg	i.v.	* seizure discharge in hippocampus, tonic-clonic convulsions & death * convulsions & death prevented by diazepam * learning maze task was impaired after intraventricular injection * DA interfered with relearning maze				13
Rats Long-Evans	0.04 µg	i.c.v. (7–11)	* seizure discharge in hippocampus, tonic-clonic convulsions & death * convulsions & death prevented by diazepam * pretreatment of mug/kg i.p. of diazepam prevented death but pyramid neuronal loss in CA3, CA4 & parts of CA1				13
Rats CD-COBS Male	0.03, 0.1,0.3 nmol	i.c.v. (8–11)	* 67% incidence of wet dog shakes within 5–20 min at lowest dose * 0.1 & 0.3 nmol doses produced more characteristic complete behavioural toxicity profile within 1–5 min of injection at 0.3 nmol and within 30–60 min at 0.1 nmol * ED50 estimated at 0.07 nmol/rat, compared with 0.51 nmol for KA				4

TABLE 1
Summary of acute toxicity data for DA in mice, rats and cynomolgus monkeys (continued)

Species strain Sex Age Length of study	Dose	Route (N/grp)	Observed effects	LOAEL	NOAEL	LD50	Ref.
Rat pups (PND 2 & 10) Long-Evans	0–1.5 mg/kg	i.p. (3–5)	* 0.1 mg/kg DA induced c-fos expression in the CNS & was partly inhibited by 2-amino-5-phosphonovaleric acid * induced reproducible effects at doses as low as 0.05 mg/kg & induced seizures in doses as low as 0.2 mg/kg * serum levels corresponded to levels that induce similar effects in adult rats			0.25 mg/kg (PND2) 0.7 mg/kg (PND10)	29
Rat pups (PND 0, 5, 14, 22) SD Male / Female	0.05 –1.0 mg/kg	i.p. (6)	* no difference between males & females * ED50=0.12, 0.15, 0.30, 1.06 mg/kg at PND 0, 5, 14 & 22, respectively * PND 8 rats were twofold more sensitive than PND 14 rats * status epilepticus at both 0.15 & 0.20 mg/kg on PND 8 & 0.30 mg/kg on PND 14				7
Rats SD Male / Female	0, 0.1, 5.0 mg/kg/day	Oral (gavage)	0.1 mg/kg/day considered equivalent to 50-kg human consuming 250 g portion containing regulatory limit of 20 µg/g 5.0 mg/kg/day considered equivalent to estimated max. dose in 1987 human intoxication incident * no clinical abnormalities or changes in haematology or clinical chemistry * 24-hour urinary excretion estimated at 1.8% of dose (unchanged) * no changes in light microscopy histopathology * histopathology by EM found changes in neurons and astrocytes		0.1 mg/kg/day		23
Monkeys Cynomolgous	4.0 mg/kg	i.p. (1)	* persistent chewing with frothing, varying degrees of gagging & vomit * in high doses: abnormal head & body movement, rigidity of movement, loss of balance & tremors * excitotoxic lesions in area postrema, hypothalamus, hippocampus, & layers of retina				25

TABLE 1
Summary of acute toxicity data for DA in mice, rats and cynomolgus monkeys (continued)

Species strain Sex Age Length of study	Dose	Route (N/grp)	Observed effects	LOAEL	NOAEL	LD50	Ref.
Monkeys	0.025–0.5 mg/kg	i.v.	* same as above				23
Cynomolgous		(5)	* 0.5 mg/kg caused excitotoxic lesions in area postrema, hypothalamus, hippocampus, & layers of retina				
			* i.v. injected DA is neuroexcitatory & a powerful emetic at doses 0.025–0.2 mg/kg				

Sources: 1. Appel *et al.*, 1997; 2. Bose, Pinsky and Glavin, 1990; 3. Bruni *et al.*, 1991; 4. Chiamulera *et al.*, 1992; 5. Clayton *et al.*, 1999; 6. Dakshinamurti *et al.*, 1993; 7. Doucette *et al.*, 2000; 8. Glavin, Pinsky and Bose, 1989; 9. Glavin, Pinsky and Bose, 1990; 10. Grimmelt *et al.*, 1990; 11. Iverson *et al.*, 1989; 12. Iverson *et al.*, 1990; 13. Nakajima and Potvin, 1992; 14. Nijjar and Madhyastha, 1997; 15. Peng *et al.*, 1994; 16. Peng and Ramsdell, 1996; 17. Peng *et al.*, 1997; 18. Petrie *et al.*, 1991; 19. Scallet *et al.*, 1993; 20. Sobotka *et al.*, 1996; 21. Strain and Tasker, 1991; 22. Tasker, Connell and Strain, 1991; 23. Truelove *et al.*, 1996; 24. Truelove *et al.*, 1997; 25. Tryphonas, Truelove and Iverson, 1990; 26. Tryphonas *et al.*, 1990a; 27. Tryphonas *et al.*, 1990b; 28. Wright *et al.*, 1990; 29. Xi, Peng and Ramsdell, 1997.

The majority of the early toxicity studies in mice were conducted shortly following reports of human intoxication in 1987. As a result, most of these investigators did not have access to purified DA and/or the precise nature of the toxin(s) causing ASP was unknown. For these reasons toxicity data are derived largely from i.p. injections of crude homogenates of toxic mussels (Wright *et al*., 1990) or of toxin-containing methanol/water extracts of whole mussels (WMX) (Iverson *et al*., 1989, 1990; Grimmelt *et al*., 1990; Bose, Pinsky and Glavin, 1990; Glavin, Pinsky and Bose, 1990; Tasker, Connell and Strain, 1990, 1991; Strain and Tasker, 1991) or mussel hepatopancreas extract (MHX) (Bose, Pinsky and Glavin, 1990; Glavin, Pinsky and Bose, 1990). Variability in sample preparation probably accounts for some of the variability in dose-response data in these papers. In addition, Novelli *et al*. (1992) reported that DA toxicity in mussel extracts could be altered by the presence of other amino acids, implying that even identically prepared extracts could have small differences in toxicity if different batches of shellfish are used. This is consistent with detailed dose-response data compiled by Tasker, Connell and Strain (1991) in which these authors reported differences in the dose response curves (DRCs) generated using DA from extracts of toxic mussels (concentrations confirmed by HPLC) and extracts of "clean" mussels that were "spiked" with known concentrations of purified DA.

Another feature worth noting are variations in the sex and strain of mice. Acute toxicity studies to date have used either exclusively male, exclusively female, or both male and female mice. Comparisons of the data obtained indicate quite consistently that there is no appreciable sex difference in DA toxicity in mice. There are, however, differences of opinion on whether different mouse strains respond differently to DA. Comparisons between studies suggest that there are no major differences in toxicity between studies that used Swiss-Webster, CD–1 or CF–1 strains. However, the only report to specifically compare between mouse strains concluded that the DBA strain of mouse is significantly more sensitive to DA toxicity that are ICR mice (Peng *et al*., 1997).

3.2.1.2 Acute toxicity in rats

As reviewed by Jeffery *et al*. (2004), most acute toxicity studies have been conducted in young adult rats and have focused on the CNS effects (Iverson *et al*., 1989, 1990; Tryphonas *et al*., 1990a, 1990b, 1990c; Sobotka *et al*., 1996; Ananth *et al*., 2001, 2003; Ananth, Gopalakrishnakone and Kaur, 2003a, 2003b; Appel, Rapoport and O'Callaghan, 1997; Appel *et al*., 1997; Nakajima and Potvin, 1992; Chiamulera *et al*., 1992; Stewart *et al*., 1990). Despite the differences in experimental design, there is consistency in the features of acute DA toxicity (Table 1). The early rat studies provided an initial confirmation of the syndrome described in association with ingestion of mussels contaminated with DA (Table 1 and Section 3.2.6.1.). Single i.p., i.v., s.c. and oral studies in rats show that DA induces dose-dependent neurotoxicity (Tryphonas and Iverson, 1990; Tryphonas *et al*., 1990 a, 1990b, 1990c; Stewart *et al*., 1990). Data indicate that DA causes a characteristic syndrome with clinical neurobehavioural signs and brain histopathology (Section 3.2.6.1.1.) similar to those reported for KA (Schwob *et al*., 1980; Stewart *et al*., 1990). Several publications discuss and compare the features of toxicity associated with these compounds and describe it as consistent with excitotoxicity (Tryphonas *et al*., 1990b; Tryphonas and Iverson, 1990; Stewart *et al*., 1990; Pulido *et al*., 2000). DA neurotoxicity is reported to be approximately 5–8 times greater than KA (Stewart *et al*., 1990) in rats, which is consistent with results obtained in mice (Tasker, Connell and Strain, 1991) (Table 1).

In a study (Tryphonas *et al.*, 1990a) with 17 female Sprague-Dawley adult rats, weighing 180 g ± 15 g, the animals were dosed once i.p. with 0, 1, 2, 4 or 7.5 mg DA/kg of b.w. and observed for a maximum period of 24 hours. Clinically, control rats and rats in the 1 mg/kg group were unremarkable. Seventy-five percent of the animals

in the 2 mg/kg group had equivocal transient behavioural signs. One animal that was given 2 mg/kg and all rats given 4 mg/kg of b.w. or higher doses developed unequivocal behavioural and neurological signs culminating in partial seizures and status epilepticus. Severely affected rats developed selective encephalopathy (Section 3.2.6.1.1).

Adult (12–14 weeks old) Sprague-Dawley rats treated i.p. with DA at 0, 0.22, 0.65 or 1.32 mg/kg b.w. were tested for passive avoidance, auditory startle or conditioned avoidance behaviours. Animals were divided in groups (I–III) with 12, 11 and 16 rats in each group (Sobotka *et al*., 1996). Clinical signs were observed only with the higher dose, appearing within 24 hours of dosing. The rats exhibited transient decreases in b.w. and exaggerated auditory startle responding. The other end-points were not affected. Examination of brains revealed a subset of animals receiving 1.32 mg/kg DA with degenerating neurons in the hippocampal CA1/CA3 subregions and gliosis. The decreased b.w. and increased startle suggest a hyper-reactivity syndrome possibly related to neuronal degeneration in the hippocampus (Sobotka *et al*., 1996). In a separate experiment, DA at an i.p. dose of 0.93 mg/kg b.w. was found to produce hypomotility in addition to a decrease in b.w. (Sobotka *et al*., 1996). These authors also report that pretreatment with scopolamine (2 mg/kg) reduced the DA-induced effects, suggesting a possible cholinergic involvement.

Domoic acid, given i.p. at 2.25 mg/kg b.w., was given to male Fisher rats (Appel, Rapoport and O'Callaghan, 1997; Appel *et al*., 1997). This dose caused stereotyped behaviour and convulsions in approximately 60 percent of rats. Six to eight days after DA or vehicle administration, they measured the regional brain incorporation of the long-chain fatty acids [1-(14)C]arachidonic acid ([14C]AA) and [9,10-(3)H]palmitic acid ([3H]PA), and the regional cerebral glucose utilization (rCMRglc) using 2-[1-(14)C]deoxy-D-glucose, by quantitative autoradiography. In other rats, they measured brain glial fibrillary acidic protein (GFAP) by ELISA. Domoic acid increased glial fibrillary acidic protein (GFAP) in the anterior portion of cerebral cortex, the caudate putamen and thalamus compared with vehicle. However, in rats that convulsed after DA, GFAP was significantly increased throughout the cerebral cortex, as well as in the hippocampus, septum, caudate putamen and thalamus. In the absence of convulsions, DA decreased relative [14C]AA incorporation in the claustrum and pyramidal cell layer of the hippocampus compared with vehicle-injected controls. In the presence of convulsions, relative [14C]AA incorporation was decreased in hippocampal regions CA1 and CA2. Uptake of [3H]PA into brain was unaffected. Relative rCMRglc decreased in entorhinal cortex following DA administration with or without convulsions. These results suggest that acute DA exposure affects discrete brain circuits by inducing convulsions, and that DA-induced convulsions cause chronic effects on brain function that are reflected in altered fatty acid metabolism and gliosis (Appel, Rapoport and O'Callaghan, 1997; Appel *et al*., 1997).

Nakajima and Potvin (1992) examined the acute electroencephalographic and behavioural effects of DA in 38 Long Evans rats. Injection of DA (0.5–1.0 mg/kg b.w. intravenously, or 0.04–0.08 microgram intraventricularly) caused seizure discharges in the hippocampus, tonic-clonic convulsions and death within a few days. Convulsions and ensuing death were prevented by diazepam. Animals pretreated with diazepam (5 mg/kg, i.p.) tolerated an intraventricular dose of 0.4 micrograms DA, but showed a loss of pyramidal neurons mainly in the CA3, CA4 and a part of CA1 areas of the dorsal hippocampus. Learning of a radial arm maze task was severely impaired in naive rats after intraventricular injection of DA (and diazepam, i.p.). In the animals previously trained on the maze task, DA interfered with relearning of the same task. In parallel to the findings observed in humans (Section 3.4.2), rat toxicological studies describe a neurological clinical and electrophysiological profile consistent with a DA-induced epileptogenic effect (Section 3.2.6.1.3).

Adult Sprague-Dawley rats weighing 350–450 g received an s.c. injection of DA at one of the three doses (3.0 mg/kg n=3; 2.5 mg/kg n=3; 0.6 mg/kg n=1) (Stewart *et al*., 1990). In this study, the controls were the investigators' own laboratory historical controls. Following injection, rats were quiet and generally hypoactive for the first 30–60 minutes. Thereafter, animals treated with higher doses (3 and 2.5 mg/kg b.w.) began displaying repetitive head scratching, wet dog shakes, ataxia and clonic seizure activity up to status epilepticus (continuous seizure activity). The frequency and intensity of the head scratching progressively increased. The authors describe this behaviour as characteristic of DA toxicity and different from KA. The animal that received the low dose (0.6 mg/kg b.w.) remained relatively hypoactive for several hours postinjection, but did not show any of the neurobehavioural signs. Animals were allowed to remain in status epilepticus for 60–150 minutes, after which they were sacrificed and the brains prepared for light and electron microscopy. The severity of the histopathology correlated with the length of time in status epilepticus. The histomorphology is described as consistent with excitotoxicity (Section 3.2.6.1.1).

The acute oral toxicity of DA was tested in ten Sprague-Dawley rats weighing 180 ± 3.5 g (Tryphonas *et al*., 1990c). Rats were dosed once by gavage with the mussel extract dissolved in water at the dose (DA equivalent) of 0 (control, n=4), 60 (n=1), 70 (n=1) or 80 (n=4) mg/kg b.w. DA-treated rats showed withdrawal followed by hyperexcitation and death (in one case). Time of onset of signs varied from 14 to 45 minutes in the high dose (80 mg/kg b.w.) and 45 minutes in the lower dose (60 mg/kg b.w.). Clinical signs were apparent up to 5.5 hours after the treatment. Mild to moderate histopathology (Section 3.2.6.1.1) lesions were present in the brain of rats treated with 80 mg/kg b.w. The eyes and spinal cords were unaffected.

Sutherland, Hoesing and Whishaw (1990) report that DA (25 ng/0.5 µg physiological saline) microinjections in the hippocampal formation (bilaterally at three sites) of two male rats (Long Evans) produced a long-lasting anterograde amnesia for special information in the Moris water task and neural injury consistent to that described by others in association with DA toxicity (Section 3.2.6.1.1).

Other special studies discussed in this document have incorporated methodologies such as magnetic resonance imaging microscopy (MRM), electroencephalography and immunohistochemistry (see Section 3.2.6.1)

In summary, data from acute rat studies indicate that DA induces a dose-dependent response (depending on the sensitivity of the diagnostic procedures used) with a time-specific sequence in the appearance of clinical signs and histopathological changes. These acute toxic effects are followed by memory impairment and permanent structural brain damage, particularly affecting the hippocampus, because this region of the brain is particularly involved in memory and learning. The signs of acute toxicity are more evident and consistent after parenteral than after oral administration. Disturbances in learning and memory processing are long-term consequences of DA intoxication, and are seen after higher doses of DA.

Neonatal, as well as aged rats appear to be more sensitive to excitotoxins than young adult animals (Section 3.2.6.1.4).

3.2.1.3 Acute toxicity studies in non-human primates

Several studies have been conducted using non-human primate models to assess the toxicity of DA (Tryphonas, Truelove and Iverson, 1990; Tryphonas *et al*., 1990a, 1990c; Scallet *et al*., 1993; Schmued, Scallet and Slikker, 1995; Slikker, Scallet and Gaylor, 1998). As reviewed by Jeffery *et al*. (2004), most acute toxicity studies in non-human primates have been conducted in small groups of monkeys or compiled the observations of individual isolated animals receiving a single dose by i.p., i.v. or oral administration.

Eleven cynomolgus monkeys (*Macaca fascicularis*) were dosed orally with mussel extract (n=4), crude DA (n=1), purified DA (n=4) or physiological saline (n=2).

Single doses of extracts of mussels contaminated with DA (6.2, 6.47, 5.63, 5.89 or 0 mg/kg DA equivalent in mussel extract) or DA isolated from toxic mussels (0.5–10 mg/kg b.w.) were given by oral administration (Tryphonas *et al.*, 1990a). Monkeys given mussel extract developed anorexia, salivation, retching, vomiting, diarrhoea and postration (fatigue). Signs of toxicity appeared as early as 2 hours after dosing and lasted intermittently for as long as 70 hours. Eventually, all treated monkeys recovered clinically. With the exception of diarrhoea and postration, monkeys dosed with crude or purified DA developed clinical signs similar to those given the mussel extract. In addition, DA-treated monkeys developed licking and smacking of the lips and empty mastication. These symptoms were observed up to 96 hours after treatment. Mild to moderate CNS lesions were observed in treatment animals (Section 3.2.6.1.1). The various treatments induced lesions with similar histopathology characteristics. The incidence, distribution and severity of the lesions varied considerably between animals. The hippocampus and cerebral cortex were the most affected areas. The eyes, spinal cord and other brain regions appeared unaffected. Monkeys treated with crude or purified DA had similar but less severe lesions. Addition of MSG (at 0.25 percent of mussel extract bolus) or dimethylsulphoxide (at 1 g per bolus) had no significant effect on the incidence or severity of clinical signs or on the appearance and severity of CNS lesions. These compounds were coadministered to two (one each) of the DA-treated monkeys. The wide variations in the response of test animals to the oral administration of DA were attributed to the protective effect of vomiting, and to suspected incomplete or slow GI absorption of the toxic agent.

Six adult cynomolgus monkeys were used to assess the toxicity of single doses of DA obtained from cultured mussels contaminated with this neurotoxin (Tryphonas, Truelove and Iverson, 1990a). Domoic acid was administered intraperitoneally (n=1) at the dose of 4 mg/kg b.w. or intravenously (n=4; 0.025, 0.05, 0.2 or 0.5 mg/kg b.w.). The control monkey received no treatment. All treated monkeys showed clinical signs of DA toxicity. Clinical signs of neurotoxicity were preceded by a short presymptomatic period (2–3 minutes). Prodromal signs of toxicity appeared within the first 3–4 minutes. Lip licking and mastication were reported as characteristic of premonitory signs. The authors indicate that, in high-dose treated monkeys, the premonitory signs were not always present. The symptomatic period proper was characterized by persistent chewing with frothing, varying degrees of gagging, and vomiting. The authors also indicate that with increasing dose, there was a trend toward a longer symptomatic period, an increase in the number of signs, and a longer duration of clinical signs. Monkeys in the higher dose regimen exhibited additional signs including abnormal head and body positions, rigidity of movements and loss of balance, as well as tremors. Recovery was characterized by cessation of gagging and vomiting and then chewing. With low dose, recuperation was rapid. Excitotoxic brain lesions consisting of DA toxicity (Section 3.2.6.1.1) were detected in the area postrema, the hypothalamus, the hippocampus, and the inner layers of the retina in monkeys given DA at 0.5 mg/kg intravenously and 4 mg/kg intraperitoneally. Hence, DA, administered intravenously, was neuroexcitatory and a powerful emetic, at doses of 0.025 to 0.2 mg/kg. At higher doses (0.5 mg/kg intravenously and 4 mg/kg intraperitoneally) domoate was strongly excitotoxic.

Juvenile (n=9, less than 4 years old, weighing 2.5 ± 0.3 kg b.w.) and adult (n=15 weighing 5.3 ± 5.3 kg) cynomologus monkeys were dosed intravenously with DA at one of a range of doses from 0.25 to 4 mg/kg (Scallet *et al.*, 1993). There were 17 females and 7 males that were distributed evenly among dose groups. All animals that received DA but none of the saline controls (n=4) showed signs of nausea, i.e. gagging and retching, beginning within a minimum of 8 minutes to a maximum of about 75 minutes after dosing. Animals that received the lowest dose had longer latencies until the first signs of illness than did animals that received a higher dose

(0.25 mg/kg, 50–70 minutes; 1.0 mg/kg, 13–26 minutes). The authors also noted that the four adults that received doses greater than 1.0 mg/kg of DA showed symptoms within 8 minutes after the dose, whereas the three juvenile animals took longer (30–43 minutes) to retch or gag for the first time. Several animals that received the higher doses (1.0 mg/kg and above) scratched ipsilaterally at their neck and/or behind their ears. Four animals became moribund within 2–7 hours of treatment and died thereafter. The survival animals resumed eating and appeared normal in gait and behaviour until sacrificed one week after treatment. The histological evaluation reported focused in the hippocampus and is discussed in Section 3.2.6.1.1.

3.2.2 Subchronic/short-term toxicity

3.2.2.1 Subchronic toxicity in mice

Only one study has investigated the effects of repeated doses of DA in mice (Peng *et al*., 1997). Using a previously published rating scale for behavioural toxicity (Tasker, Connell and Strain, 1991), these authors first established dose response curves for acute toxicity in ICR mice following i.p. injection. They found that the effect of 0.5 mg/kg was not significantly different from control mice and that 2.0 mg/kg produced severe but non-lethal toxicity in this strain. They then administered these two doses every other day over 7 days (i.e. four injections) or as a single dose on Day 7 and analysed for both serum concentrations of DA and behavioural toxicity. Results indicated that acute versus repeated dosing did not result in any significant difference in serum concentrations measured at either 60 minutes or 120 minutes postinjection, implying that repeated administration does not alter the clearance of DA in mice. Similarly, they found no difference in behavioural toxicity scores at either dose when they compared the response of mice to a single injection versus the acute response in mice that had received three previous injections. This implies that an alternate-day pretreatment does not result in either kindling (a progressively increasing response) or tolerance (a progressively decreasing response) to DA in ICR mice. Finally, these authors repeated both the acute toxicity and repeated exposure studies using a different strain of mice (DBA) and found the same result (i.e. no kindling or tolerance) although they did observe that DBA mice appeared to be slightly less sensitive to acute DA toxicity than were ICR mice.

3.2.2.2 Subchronic toxicity in rats

There is only one published rat study aiming to determine the effects of repeated consumption of DA in rats (Truelove *et al*., 1996). Male and female Sprague-Dawley rats were dosed by gavage for 64 days with 0, 0.1 or 5 mg/kg/day DA. The low dose (0.1 mg/kg b.w.) was approximately equivalent to the dose that would result from a 50-kg person consuming one 250-g portion of mussel meat containing the present regulated limit of 20 µg/g. The high dose (5 mg/kg b.w.) was equivalent to the estimated maximum dose received during the Canadian ASP incident in 1987. This dose of 5 mg/kg was approximately seven times less than that required to cause overt clinical signs in rats (Iverson *et al*., 1989, 1990). Treated animals showed no clinical abnormalities. Terminal values in haematology and clinical chemistry did not reveal differences between treated and control groups. The 24-hour urinary excretion rate for DA determined at three time points was approximately 1.8 percent of the dose and remained unchanged during the study. Light microscopy histopathology was unremarkable. Histopathology evaluation included regions such as the hippocampus, the area postrema and the retina, which are considered sensitive targets for acute DA toxicity. GFAP and amino-methyl proprionic acid/kainate (AMPA/KA) glutamate receptors (GluRs) immunohistochemistry did not show visually detectable differences between treated and control animals (Truelove *et al*., 1996). Electron microscopy of the hippocampus from animals in this study has been partially reported

separately (Pulido, Mueller and Gill, 2001; Pulido *et al*., 2001). Rats treated with 5 mg/kg revealed changes in neurons and astrocytes. These changes included neuronal shrinkage, cytoplasmic vacuolation, dilatation of dendrites and astrocytes processes and electron-dense mitochondrial profiles (Pulido, Mueller and Gill, 2001; Pulido *et al*., 2001). These findings were scattered within otherwise well-preserved tissue. No changes were observed in animals treated with 0.1 mg/kg or in controls. The morphology of the electron microscopic findings in this subchronic study is consistent with those reported in acute studies in rats (Section 3.2.6.1.1). Results from this subchronic study provide no evidence for a cumulative effect or that repeated exposures to DA induces greater toxicity or alters clearance from the serum. To the contrary, recent *in vitro* studies using hippocampal slices suggest that pretreatment with small doses of DA induces tolerance in young, but not old, rats (Kerr, Razak and Crawford, 2002).

3.2.2.3 Subchronic toxicity in non-human primates

Five adult cynomolgus monkeys were used to assess the effect of repeated doses of DA (Truelove *et al*., 1997). Domoic acid was orally administered by gavage to three cynomolgus monkeys at doses of 0.5 mg/kg for 15 days and then at 0.75 mg/kg for another 15 days. After the 30-day dosing period, the treated monkeys were euthanized. Parameters monitored included b.w., food and water consumption, clinical observations, haematology and serum chemistry. Light microscopy histopathology analysis was conducted on all major organs. Neuropathology evaluation included histochemical stains and procedures, such as GFAP immunohistochemistry, that are commonly used to visualize neural injury. Systematic evaluation of the brain included the hippocampus and other structures of the limbic system, hypothalamus, brainstem, cerebellum and retina. These are regions identified as preferential targets to DA toxicity. Domoic acid in serum and 24-hour urine samples were measured at several time points. All parameters measured remained unremarkable. DA concentrations measured in the 24-hour urine samples indicated that GI absorption in the monkey was approximately 4–7 percent of the administered dose, which is at least twice that previously reported for the rat. This study does not provide evidence for greater neurotoxic response or increase in sensitivity after the oral administration of repeated low doses of DA in non-human primates.

3.2.3 Long-term studies of toxicity and carcinogenicity

To date there have been no published studies or reports on the long-term (> 1 year) toxicity of DA in any species, including humans.

3.2.4 Genotoxicity

The *in vitro* genotoxicity of DA was evaluated in a hepatocyte-mediated assay with V79 Chinese hamster lung cells. Crystalline DA (purity not stated) was dissolved in distilled water and added to lung cell growth medium at doses of 27.2 or 54.4 µg/ml medium. Three additional treatments used were a vehicle control with 0.2 percent dimethyl sulphoxide v/v, 155 µg ethyl methanesulphonate/ml medium (direct acting genotoxin) or 2.6 µg 7,12-dimethylbenz[a]anthracene (indirect acting genotoxin). Domoic acid did not cause an increase in the frequency of mutations to thioguanine resistance or to oubain resistance, either alone or in the presence of rat hepatocytes, nor did it increase the frequency of sister-chromatid exchange or micronucleus frequency. Significant increases in all these parameters were observed with the positive controls. The results suggested that, within the limits of the test system, DA is not genotoxic in V79 cells (Rogers and Boyes, 1989).

3.2.5 Reproductive and developmental toxicity

In a teratology study, DA at 0, 0.25, 0.5, 1.0, 1.25, 1.75 or 2.0 mg/kg b.w. was administered i.p. on Days 7–16 of gestation to groups of 9–15 female Sprague-Dawley rats, ranging in weight from 175 to 225 g. All of the rats in the 2.0 mg/kg group died within 3 days and 6 of 12 rats in the 1.75 mg/kg group aborted preterm. All remaining females were euthanized on day 22 of gestation. No signs of maternal toxicity were observed at doses of up to 1.25 mg/kg b.w. Slight, but significant ($P<0.05$), reductions in the number of live foetuses per litter were reported in the 0.5 and 1.0 mg/kg DA groups, but not in the 1.25 or remaining 1.75 mg/kg group. This reduction was not dose-related and did not correlate with the percentage of resorbed and dead foetuses of total implants. There was an increased incidence of retarded ossification of the sternebrae in the 1.25 mg/kg group (6 percent), but the increase was comparable with that in historical controls (4 percent). No other indications of maternal or fetal toxicity were reported, implying that 1.0 mg/kg/day may be considered a NOAEL in this study. The results indicate that DA is not teratogenic under the test conditions used (Khera *et al*., 1994).

The same group also reported on a homeostatic study in which groups of 3–4 rat dams received 0, 0.5, 1.0 or 1.5 mg/kg (i.p.) DA on gestational Day 11. Analysis of urine and blood samples from these rats revealed elevated plasma sodium (Na) in all treated groups at 1 and 3 hours as well as elevated serum potassium (K) at 9 hours in all treated groups. Urinary Na was also increased in all groups in a dose-related manner. In addition, pCO_2 values were increased at 3 hours in all domoate-treated groups and at 9 hours in the group receiving the highest dose (1.5 mg/kg). These changes failed to cause any long-term effect on fetal development as observed in the term foetuses (Khera *et al*., 1994).

The effects of low (subconvulsive) doses of DA on brain development in the rat have been described in four papers (Dakshinamurti *et al*., 1993; Doucette *et al*., 2003, 2004; Tasker *et al*., 2005) and one preliminary report (Doucette, Ryan and Tasker, 2002) to date.

Dakshinamurti *et al*. (1993) reported that 10–30 day-old mice exposed to DA *in utero* (0.6 mg/kg i.v. in the pregnant dams) demonstrated generalized electrocortical depression associated with diffuse spike and wave activity in basal electroencephalogram (EEG) recordings and had reduced seizure thresholds to exogenous domoate. These changes were seen even in the absence of motor convulsions, and were accompanied by severe neuronal damage in the hippocampal CA3 and dentate gyrus region. This latter finding is of particular interest, because most authors have found that excitotoxins do not produce permanent histopathological damage when administered to neonatal rats.

Another study by Doucette *et al*. (2003) reported physiologically relevant changes in brain development in the absence of convulsions when neonatal rats were injected daily (s.c.) with very low doses of DA (5 and 20 µg/kg), or pharmacologically equivalent doses of KA (25 and 100 µg/kg) from postnatal day (PND) 8–14. This study showed that while neither compound had identifiable effects on typical measures of toxicity such as weight gain, acoustic startle, ultrasonic vocalizations (USVs), or maternal retrieval, drug administration did result in significant differences in eye opening, conditioned place preference and spontaneous activity. These authors conclude that low doses of DA can produce changes in brain development if given during particular "windows" of brain development. In this case, that window is the second perinatal week in the rat, which is a particularly dynamic period for kainate receptor expression, and which corresponds roughly to the latter part of the third trimester in humans. This same research team recently reported on a follow-up study (Tasker *et al*., 2005) in which they describe induction of a conditioned odour preference in rats administered 0.020 mg/kg DA s.c. by daily injection between PND 8–14 (the same time frame as the previous study). No observable toxicity was reported at this dose, but drug-treated

rats spent significantly more time over a conditioning odour previously paired with drug administration. These data indicate a DA-induced conditioned odour preference. Moreover, this effect was antagonized by concomitant injection of the NMDA receptor antagonist CPP, implicating a role for NMDA receptors in this phenomenon.

Finally, a preliminary report by Doucette, Ryan and Tasker (2002) described a highly reproducible "seizure-like" syndrome in adult rats that had been treated postnatally with very low doses of DA. These are the first data to demonstrate permanent changes in brain function following perinatal administration of low dose DA; a finding that could have considerable implication for establishing regulatory limits on DA consumption. A manuscript describing these findings in greater detail has recently been published online (Doucette *et al*., 2004).

3.2.6 Special studies

3.2.6.1 Neurotoxicity

3.2.6.1.1 Brain histopathology

Neuropathology reports from humans that died during the 1987 intoxication (see Section 3.4.1), from rodent and non-human primate studies (Tryphonas, Truelove and Iverson, 1990; Tryphonas *et al*., 1990a, 1990b, 1990c; Stewart *et al*., 1990, Strain and Tasker, 1991; Sutherland, Hoesing and Whishaw, 1990; Appel, Rapoport and O'Callaghan, 1997; Appel *et al*., 1997; Scallet *et al*., 1993; Schmued, Scallet and Slikker, 1995; Slikker, Scallet and Gaylor, 1998) and from sea lions that died because of DA intoxications (Section 3.3.1.) provide a comprehensive description of the brain injury associated with acute DA intoxication (see Sections 3.2.1 and 3.2.2). The features described in the initial reports (Tryphonas, Truelove and Iverson, 1990; Tryphonas *et al*., 1990a, 1990b, 1990c; Stewart *et al*., 1990; Strain and Tasker, 1991; Sutherland, Hoesing and Whishaw, 1990) still hold valid as being characteristic of the acute neurotoxic effects of DA, and have been confirmed in further studies conducted in rodents and non-human primates (Scallet *et al*., 1993; Schmued, Scallet and Slikker, 1995; Slikker, Scallet and Gaylor, 1998) treated with DA at various doses, through different routes of administration (Table 1). Despite the differences in study protocol, there is an overall agreement regarding the histopathology of the acute brain lesions associated with DA toxicity. Pivotal neuropathology studies (Tryphonas, Truelove and Iverson, 1990; Tryphonas *et al*., 1990a, 1990b, 1990c; Stewart *et al*., 1990) provide detailed description of the histopathology of acute DA toxicity in rodents and non-human primates, including distribution of the lesions in brain and retina. Tryphonas and Iverson (1990) review and summarize the histopathology of acute DA toxicity. They report light microscopy acute brain damage as early as 1.5–3 hours after parenteral administration of DA. This is characterized by neurodegenerative changes, consisting of neuronal shrinkage, vacuolization of the cytoplasm, cell drop out, edema, microvacuolation of the neuropil, hydropic cytoplasmic swelling of resident astrocytes and nerve cells hyperchromasia. These changes have preferential distribution within structures of the limbic system (Strain and Tasker, 1991; Tryphonas *et al*., 1990a, 1990b; Tryphonas and Iverson, 1990; Stewart *et al*., 1990). The hippocampus, among other brain regions, appears to be a specific target site having high sensitivity to DA toxicity, particularly the pyramidal neurons in the CA3, CA4 or hilus of the dentate gyrus; the dentate gyrus and CA1 region (Strain and Tasker, 1991; Tryphonas, *et al*., 1990a, 1990b; Sutherland, Hoesing and Whishaw, 1990; Stewart *et al*., 1990). Iverson *et. al*. (1990) report that rats sacrificed after 1–1.5 hours of the onset of status epilepticus exhibit a consistent pattern of lesions affecting limbic and related brain regions. The olfactory bulb, the piriform and entorhinal cortices, the lateral septum, the hippocampus and subiculum, and the arcute, middle dorsal thalamus, and several amygdaloid nuclei are commonly affected (Tryphonas and Iverson, 1990; Tryphonas *et al*., 1990a, 1990b, 1990c). Structural brain lesions observed by histopathology were consistent with other end-points assessed

on both rodents and non-human primate studies indicative of a dose-dependent acute toxic dose response more evident in animals treated by i.p or i.v. injections and to a lesser degree after oral administration (see Section 3.1.1.1) and with differences in species susceptibility (Table 1). Circumventricular organs and in particular the area postrema has also been identified as a target site (see Section 3.2.6.1.5).

Light and electron microscopy observations in rodent studies show two types of neuronal degeneration associated with DA and KA toxicity. Swollen, vacuolated neurons are described as the predominant feature, intermingled with some shrunken and darkly stained electron dense neurons (Ananth *et al*., 2001; Pulido, Mueller and Gill, 2001; Strain and Tasker, 1991; Tryphonas *et al*., 1990a, 1990b, 1990c; Schwob *et al*., 1980; Stewart *et al*., 1990). These findings are consistent with the view that the neuronal degeneration induced by DA is mostly necrotic, at least in the short term (Section 3.2.6.3.3). Ananth *et al*. (2001) investigated the DA neuronal damage in the rat hippocampus. The authors report neuronal damage at 1–21 days following the administration of DA, the degeneration being most severe at 5 days.

Brain damage following administration of DA, was compared using different anatomic markers in adult rats (Appel, Rapoport and O'Callaghan, 1997). Seven days after administration of DA (2.25 mg/kg i.p.) or vehicle, brains were collected and sectioned and stained to visualize Nissl substance using thionin, argyrophilia using a cupric silver staining method, astroglia using immunohistochemistry to detect glial fibrillary acidic protein-like immunoreactivity (GFAP-ir), and activated microglia using lectin histochemistry to detect Griffonia simplicifolia I-B4 isolectin (GSI-B4) binding in adjacent sections. In approximately 60 percent of rats to which it was administered, DA caused stereotyped behaviour within 60 minutes, followed by convulsions within 2–3 hours. Brains of DA-administered rats that did not manifest stereotyped behaviour or convulsions did not differ from brains of vehicle-administered controls. In animals that had manifested stereotyped behaviour and convulsions, Nissl staining was mostly unremarkable in brain sections. In contrast, there was intense argyrophilia in anterior olfactory nucleus, CA1 hippocampus, lateral septum, parietal (layer IV), piriform, and entorhinal cortices, ventral posterolateral thalamus and amygdala. Adjacent sections displayed astrogliosis, evidenced by increased GFAP-ir, which was more diffuse than the argyrophilic reaction. Activated microglia were revealed using GSI-B4 histochemistry. These data suggest activation of discrete brain circuits in rats that convulse following DA administration and subsequent pathological alterations.

Ananth *et al*. (2001) investigate the neuronal degeneration and microglial reaction in the hippocampus of DA treated rats. The temporal profile of bcl-2, bax, and caspase-3 genes in cell death or survival was assessed following the administration of DA. Immunoreactivity of bcl-2 and bax was increased considerably at 16 hours and 24 hours in the neurons of the hippocampus following DA administration. No significant change was observed in the immunoreactivity of caspase-3 in the controls and DA-treated rats at any time interval. Microglial cells in the hippocampus showed intense immunoreaction with the antibodies OX-42 and OX-6 at 1–21 days after DA administration, indicating the upregulation of complement type-3 receptors and major histocompatibility complex type-II antigens for increased phagocytic activity and antigen presentation, respectively. Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling (TUNEL) showed occasional positive neurons in the CA1 and CA3 regions at 5 days after DA administration, with no positive cells in the controls. RT-PCR analysis revealed that bcl-2 and bax mRNA transcripts in the hippocampus were significantly increased at 16 hours and gradually decreased at 24 hours following the administration of DA. Although bax and bcl-2 RNA expression is rapidly induced at early stages, *in situ* hybridization analysis revealed complete loss of bcl-2, bax, and caspase-3 mRNA at 24 hours after DA administration in the region of neuronal

degeneration in the hippocampus. The authors report that the pattern of neuronal degeneration observed during DA-induced excitotoxic damage was mostly necrotic.

Roy and Sapolsky (2003) examined the neuroprotective mechanisms of the viral caspase inhibitors, p35 and crmA, following DA-induced excitotoxicity in hippocampal neurons. They show that although p35 and crmA rescued neurons from toxicity, they did so under conditions of negligible caspase activation and morphological apoptosis. They characterized the novel neuroprotective effects of p35 and crmA and found that they attenuated the drop in the mitochondrial potential and blunted the decline in adenosine triphosphate (ATP) levels. In demonstrating the modulation of these processes, these data underline the capacity for classically "anti-apoptotic" proteins to alter other branches of cell death processes.

Histopathology findings resemble those reported with KA and are considered characteristic of acute excitotoxicity (Iverson *et al*., 1990; Strain and Tasker, 1991; Pulido *et al*., 1995, 2001; Pulido, Mueller and Gill, 2001; Schwob *et al*., 1980). The distribution of the brain regions preferentially affected by DA toxicity correlate with areas showing high affinity for AMPA/KA GluRs immunolabelling (Section 3.2.6.3.1). This correlation is also observed in peripheral tissues such as heart (Sections 3.2.6.2 and 3.3.1).

At the cellular level the dendrite appear as preferential early targets sites for excitotoxicity (Iverson *et al*., 1990; Stewart *et al*., 1990; Pulido *et al*., 1995; Pulido, Mueller and Gill, 2001; Polischuk, Jarvis and Andrew, 1998). Electron microscopy of the CA3 hippocampal region of rats exposed to low doses of DA shows dilatation of the dendrites while the body of the neurons are still preserved or with minimal changes (Pulido *et al*., 1995; Pulido, Mueller and Gill, 2001). These findings are supported by immunohistochemical studies using the microtubule associated protein (MAP2) antibody, which preferentially binds to the microtubules of the dendrite tree (Pulido, Mueller and Gill, 2001; Pulido *et al*., 2001). Furthermore, *in vitro* studies on hippocampal slices demonstrated a distinct excitotoxic sequence of events leading to acute neuronal damage, highlighting the changes in the dendrites (Polischuk, Jarvis and Andrew, 1998).

In addition to neuronal degeneration, there is also evidence suggesting involvement and injury of glial cells, including astrocytes and microglia (Pulido, Mueller and Gill, 2001; Mayer *et al*., 2001; Gill *et al*., 2003; Ananth *et al*., 2001). Acute injury of astrocytes has been observed by light and electron microscopy in rodents exposed to DA (Pulido, Mueller and Gill, 2001; Pulido *et al*., 2001; Tryphonas and Iverson, 1990). The lesion includes vacuolation and cell necrosis. In experimental animals surviving the acute episode of intoxication, there are structural permanent lesions of the hippocampus represented by gliosis (Tryphonas, Truelove and Iverson, 1990; Tryphonas *et al*., 1990a, 1990b, 1990c), similar to what has been observed in patients that have died as a consequence of DA exposure (see Section 3.4.2).

In the study by Scallet *et al*. (1993), juvenile and adult cynomologus monkeys were dosed intravenously with DA at one of a range of doses from 0.25 to 4 mg/kg (see Section 3.2.1.3). Animals were perfused one week later, histochemical staining using a silver method to reveal degenerating axons and cell bodies showed two distinct types of hippocampal lesions. One lesion, termed "Type A", was a small focal area of silver grains restricted to CA2 stratum lucidum. Type A lesions occurred over a dose range of 0.5–2.0 mg/kg in juvenile animals and 0.5–1.0 mg/kg in adult animals. One juvenile animal that received 4.0 mg/kg sustained a second type of lesion, termed "Type B", characterized by widespread damage to pyramidal neurons and axon terminals of CA4, CA3, CA2, CA1, and subiculum subfields of the hippocampus. Doses of more than 1.0 mg/kg in the adult monkeys either proved lethal or resulted in Type B lesions. Induction of c-fos protein had occurred in the hippocampal dentate gyrus and CA1 regions of moribund animals perfused within hours of their initial dose.

As summarized in several publications (Tryphonas and Iverson, 1990; Chandrasekaran, Ponnambalam and Kaur, 2004; Jeffery *et al*, 2004), the morphological characteristics of the DA-induced lesions in brain, and their distribution, is similar in rodents, non-human primate, humans and wild animals (Sections 3.2.1; 3.2.2; 3.3; 3.4.1). The anatomical extent of brain lesions induced by DA has also been identified by magnetic resonance imaging microscopy MRM in both human (Teitelbaum *et al*., 1990) (see Section 3.4.2) and rat (Lester *et al*., 1999) and is consistent with histopathology data.

See Sections 3.2.6.1.2–3.2.6.1.6 and Sections 3.2.6.3 and 3.3.1 for additional comments relevant to mechanisms of neurotoxicity and to pathology in other areas of the nervous system and outside the CNS.

3.2.6.1.2 Motor and sensory abnormalities

The clinical observations consistent with anterior horn cell and dorsal root ganglion pathology, or a diffuse axionopathy, seen in humans (see Section 3.4.1) have been supported by experimental data. In a study involving the developing spinal cord (Wang *et al*., 2000), toxic effects of DA were examined in neonatal rats (postnatal Day 7) given s.c. doses of DA (0.10–0.50 mg/kg b.w.). Motor seizures were induced at all doses. At a dose of 0.33 mg/kg b.w., DA caused forelimb tremor, hind limb paralysis in 65 percent of the animals, and death in 47 percent of the animals within 2 hours. At this dose, electrocorticograms showed synchronized interrupted electrical activities in the brain, but no brain damage was detected in these rats. Spinal cord lesions characterized by focal haemorrhage, neuronal swelling, and neuronal vacuolization were found in 73 percent of the animals that had shown the paralysis/tremor in their extremities, when examined 1–2 hours after DA injection. These lesions were seen at all spinal cord levels. Neuronal degeneration was mainly found in the ventral and intermediate gray matter, whereas cells in the dorsal portion of the spinal cord were relatively spared. These data suggested that some of the clinical neurological abnormalities observed were because of spinal cord damage rather than seizures or brain lesions (Wang *et al*., 2000).

Huettner (1990) showed that primary afferent C fibres in rat dorsal roots ganglia (DRG) cells were depolarized by the excitatory amino acids kainate and domoate. DRG cells were desensitized with prolonged agonist exposure, and half-maximal activation was achieved with much lower concentrations of kainate and domoate in sensory neurons than in CNS neurons from cerebral cortex. These findings support the clinical observations in humans intoxicated with DA and suggest that DRG cells are targets for DA-induced toxicity. In support of this, Sato *et al*. (1993) reported the presence of kainate receptors in DRG neurons, and a report by Kerchner *et al*. (2002) demonstrated presynaptic effects mediated by both GluR5 and GluR6 receptor subunits (both have high affinity for DA) in spinal neurons.

3.2.6.1.3 Epileptogenic effects and electrophysiological profile

In parallel to the findings observed in humans (see Section 3.4.1), in DA-induced convulsions that affect limbic structures such as the hippocampus and entorhinal cortex, different anatomic markers can detect these neurotoxic effects to varying degrees. Studies using DA, 2.25 mg/kg i.p., caused stereotyped behaviour and convulsions in approximately 60 percent of rats (Appel, Rapoport and O'Callaghan, 1997; Appel *et al*., 1997). Six to eight days after DA or vehicle administration, the animals were processed to measure regional brain incorporation of the long-chain fatty acids [1-(14)C]arachidonic acid ([14C]AA) and [9,10-(3)H]palmitic acid ([3H] PA), or regional cerebral glucose utilization (rCMRglc) using 2-[1-(14)C]deoxy-D-glucose, by quantitative autoradiography. Other rats were processed to measure brain GFAP by ELISA. Domoic acid increased GFAP in the anterior portion of cerebral cortex, the caudate putamen and thalamus compared with phosphate buffered saline

(PBS). However, in rats that convulsed after DA, GFAP was significantly increased throughout the cerebral cortex, as well as in the hippocampus, septum, caudate putamen and thalamus. In the absence of convulsions, DA decreased relative [14C] AA incorporation in the claustrum and pyramidal cell layer of the hippocampus compared with PBS-injected controls. In the presence of convulsions, relative [14C] AA incorporation was decreased in hippocampus regions CA1 and CA2. Uptake of [3H]PA into brain was unaffected. Relative rCMRglc decreased in entorhinal cortex following DA administration regardless of whether the animal experienced convulsions. These results suggest that acute DA exposure affects discrete brain circuits by inducing convulsions, and that DA-induced convulsions cause chronic effects on brain function that are reflected in altered fatty acid metabolism and gliosis.

Current evidence also suggests that DA directly promotes neuronal hyperactivity in region CA1, presumably because of tonic AMPA and/or KA-receptor mediated depolarization, and that DA-induced hyperactivity in the recurrently networked, AMPA/KA-receptor-rich CA3 region does not contribute to the onset and spread of limbic seizures during relatively mild DA intoxication (Sari and Kerr, 2001). On the other hand, electrophysiological studies, using unitary extracellular recordings obtained from pyramidal neurons of the CA1 and of the CA3 regions of the rat dorsal hippocampus, were undertaken to study the effect of KA and DA applied by microiontophoresis (Debonnel, Weiss and de Montigny, 1990). The activation induced by DA and kainate was more than 20-fold more potent in the CA3 than in the CA1 region. In the CA1 as well as in the CA3 region, DA was about three times more potent than kainate. A selective lesion of the mossy fibre system originating from the dentate gyrus and projecting to the CA3 region of the dorsal hippocampus drastically reduced the excitatory effect of KA and DA in this later area. These studies suggest regional differential susceptibility and dose responses, as well as interaction among various regions.

Generalized bilateral electrical seizure discharges with accompanying histopathology were also reported by Dakshinamurti, Sharma and Sundaram (1991) in a small group (n=5) of male Wistar rats receiving either 100 or 200 pmol microinjections of DA in hippocampal area CA3. Subsequent neurochemical analysis of the brain tissue from these rats revealed significant reductions in the concentrations of GABA and glutamic acid decarboxylase (GAD), implicating domoate-induced reductions in GABA as the cause of the abnormal discharges.

Abnormal EEG recordings have also been reported following parenteral administration of DA to freely moving non-anaesthetized rats. Fujita *et al.* (1996) administered vehicle or one of three doses of DA (1, 5 or 10 mg/kg i.p.) to male Wistar rats implanted with bipolar electrodes in the dorsal hippocampus. In the 1 mg/kg group, recurrent focal hippocampal seizures were recorded beginning about 1 hour postinjection and lasted for about 24 hours. Rats in the two higher dose groups displayed hippocampal seizures within minutes of injection that progressed to full limbic seizures propagating to the sensorimotor cortex and culminating in fatal status epilepticus (Fujita *et al.*, 1996). Scallet *et al.* (2004) studied the electroencephalographic, behavioural and c-fos responses induced in rats by acute exposure to a single intraperitoneal dose of 2.2 mg/kg b.w. or 4.4 mg/kg b.w. of DA. High and low doses caused significant electroencephalographic changes. In the high dose rats, the changes were observed within 30 minutes of DA administration, whereas with the lower dose changes were observed later. A rearing praying (RP) seizure and ear scratching were monitored as biomarkers for DA-induced behaviour. These behaviours, which are considered characteristic of DA toxicity, became significantly elevated in the high dose rats 60 minutes after injection. Rats exposed to the low doses showed no significant alterations in behaviour at any time during the session. Post-mortem examination of brains using c-fos immunohistochemistry showed that c-fos was activated in the

anterior olfactory nucleus by both high and low doses of DA. However, only the high dose increased c-fos immunoreactivity in the hippocampus. These data indicate that electroencephalography and c-fos responses were induced at a dose of DA (2.2 mg/kg) that in this study failed to induce behavioural responses. It also suggests that electroencephalography and c-fos immunohistochemistry are more sensitive than behavioural end-points to monitor the responses to DA exposure. The observation of c-fos response in the anterior olfactory nucleus, but not in the hippocampus of the animals exposed to a low dose of DA, suggests regional differential susceptibility.

Also relevant to this discussion are several reports of altered EEG and/or seizure-like behaviours that result from perinatal exposure to DA. A study by Dakshinamurti *et al*. (1993) reported that 10–30-day-old mice exposed to DA *in utero* (0.6 mg/kg i.v. in the pregnant dams) demonstrated generalized electrocortical depression associated with diffuse spike and wave activity in basal EEG recordings and had reduced seizure thresholds to exogenous domoate. These changes were seen even in the absence of motor convulsions and were accompanied by severe neuronal damage in the hippocampal CA3 and dentate gyrus region. Similarly, a preliminary report by Doucette, Ryan and Tasker (2002) and a later published paper by the same group (Doucette *et al*., 2004) described a highly reproducible "seizure-like" syndrome in adult rats that had been treated postnatally with very low doses of DA.

3.2.6.1.4 Age-dependent tolerance and susceptibility

During the toxic mussel poisoning incident of 1987, the short-term epileptogenic effects and the lasting neurological deficits were seen primarily in older patients, suggesting a heightened susceptibility to DA toxicity in the elderly. It was not clear whether the heightened vulnerability arose from an age-related alteration in neuronal response to the toxin, from alterations in the pharmacokinetics of the toxin (e.g. impaired clearance because of age or pre-existing disease), or from a combination of these factors (see Section 3.4.1). Further studies to address this issue have been conducted in animal models.

In vitro studies support the view that there is an age-dependent susceptibility to excitatory amino acid (EAA) excitoxicity and provide evidence for induced tolerance in the young, but not in the old animals (Kerr, Razak and Crawford, 2002). Hippocampal slices from young (3 months) and aged (26–29 months) rats assessed by CA1 field potential analysis before and after preconditioning with DA were used to investigate this phenomenon (Kerr, Razak and Crawford, 2002). The authors show that in naive slices from young animals, DA produced initial hyperexcitability followed by significant dose-dependent reductions in population spike amplitude during prolonged application. Following toxin washout, only small changes in neuronal activity were evident during a second application of DA, suggesting that a resistance to the effects of DA occurs in hippocampal slices that have undergone prior exposure to DA. This inducible tolerance was not antagonized by NMDA receptor blockers, mGluR blockers or AMPA/KA blockers. Field potential analysis revealed significant age-related reductions in CA1 EPSP strength, population spike amplitude and paired-pulse inhibition, but aged slices did not differ in sensitivity to DA relative to young. However, aged CA1 failed to exhibit any tolerance to DA following preconditioning, suggesting that a loss of inducible neuroprotective mechanisms may account for increased sensitivity to excitotoxins during ageing (Kerr, Razak and Crawford, 2002). Further investigations conducted by Hesp *et al*. (2004) also used hippocampal slices from young and aged rats and showed preconditioning by exposure to low-dose KA or to the AMPA-receptor selective agonist (S)-5-fluorowillardiine (FW). Tolerance induction was assessed by administration of high concentrations of KA or FW (respectively). FW preconditioning failed to induce tolerance to subsequent FW challenges, while KA-preconditioned slices were significantly resistant to the effects

of high-dose KA. KA preconditioning failed to induce tolerance in aged CA1. The authors also showed that a number of ionotropic KA receptor agonists and antagonists significantly reduced constitutive GTPase activity in hippocampal membranes from young but not aged rats. Furthermore, in young CA1, low concentrations of the AMPA/KA blockers induced tolerance to high-dose KA. These findings suggest that tolerance is triggered by a selective reduction in constitutive KA-sensitive G-protein activity, and that this potential neuroprotective mechanism is lost with age. This age-related loss of tolerance to excitotoxins may serve to explain some aspects of the supersensitivity of aged animals and humans to brain damage associated with domoic and KA toxicity.

Alterations in the pharmacokinetics of DA as a function of age have not been systematically investigated to date. However, it is known that domoate is very hydrophilic and consequently exhibits relatively poor BBB permeability (Preston and Hynie, 1991). It has also been established by Suzuki and Hierlihy (1993) that DA is cleared systemically almost exclusively by renal clearance. Both BBB integrity and renal function are known to be impaired in very young and very old animals relative to normal adults.

There is also considerable evidence for a higher susceptibility to DA toxicity during early post-natal development in rats (Xi, Peng and Ramsdell, 1997; Doucette *et al*., 2000, 2003, 2004; Tasker *et al*., 2005). In a report by Xi, Peng and Ramsdell (1997), DA induced a time-dependent neuroexcitotoxic effect in neonatal rats characterized by hyperactivity, stereotypic scratching, convulsions, and death at exposures 40 times lower by b.w. in neonates than reported in adults. Low doses of DA (0.1 mg/kg) induced c-fos in the CNS, which was inhibited in part by 2-amino-5-phosphonovaleric acid, an NMDA receptor antagonist. Domoic acid caused no evidence of structural alteration in the brain of neonates as assessed by Nissel staining and cupric silver histochemistry. Domoic acid induced reproducible behavioural effects at doses as low as 0.05 mg/kg and induced seizures at doses as low as 0.2 mg/kg. Determination of serum DA levels after 60-minute exposure indicated that serum levels of DA in the neonates corresponded closely to the serum levels that induce similar symptoms in adult rats and mice. These results suggest that neonatal rats are highly sensitive to the neuroexcitatory and lethal effects of DA and that the increased sensitivity results from higher than expected serum levels of DA. A reduced serum clearance of DA was suggested as the predisposing factor to the enhanced DA toxicity.

Doucette *et al*. (2000) reported on comparative toxicity-response data for DA and KA in neonatal rats. These authors used a 5 point behavioural rating scale to construct dose response relationships for each toxin at each age and reported a dramatic reduction in domoate potency between PND 0 and PND 22. There were no differences between male and female rat pups at any age tested, and probit analysis of DRC data revealed DA ED50 values of 0.12 mg/kg at PND 0; 0.08–0.15 mg/kg at PND 5–8; 0.19–0.30 mg/kg at PND 14; and 1.06 mg/kg at PND 22. These authors also compared the potency of DA and KA on PND 8 and 14 and found that at both ages DA was 4–5 times more potent than KA. Behaviours exhibited by rat pups at these ages were similar but not identical to those usually reported for adult rats, with young rats often demonstrating caudally directed foot biting and licking prior to the characteristic hindlimb scratching behaviour induced by DA. Caudally directed foot biting is a behaviour more commonly seen following NMDA receptor activation, implying an involvement of NMDA receptors in DA toxicity at these early ages. This is consistent with other data described elsewhere (see Section 3.2.6.3.1).

Another study by Doucette *et al*. (2003) reported physiologically relevant but subconvulsive responses in neonatal rats administered daily s.c. injections of low doses of DA (5 and 20 µg/kg), or pharmacologically equivalent doses of KA (25 and 100 µg/kg) from PND 8 to 14. This study showed that while neither compound had

identifiable effects on typical measures of toxicity – such as weight gain, acoustic startle, ultrasonic vocalizations, or maternal retrieval – drug administration did result in significant differences in eye opening, conditioned place preference and spontaneous activity. These data further support the view that the period of perinatal development in the rat is one in which there is higher susceptibility to domoate and kainite toxicity.

Expanding on the findings described in the above paragraph, a report by Tasker *et al.* (2005) describes induction of a conditioned odour preference in rats administered 0.020 mg/kg DA s.c by daily injection between PND 8 and 14. No observable toxicity was reported at this dose, but drug-treated rats spent significantly more time over a conditioning odour paired with drug administration indicating a DA-induced learned association. Moreover, this effect was antagonized by concomitant injection of the NMDA receptor antagonist CPP, implicating a role for NMDA receptors in this phenomenon.

Finally, a paper appearing online in late 2004 (Doucette *et al.*, 2004) expands upon some extremely compelling evidence of permanent toxicity following perinatal administration of very low (subconvulsive) doses of DA (Doucette, Ryan and Tasker, 2002). These authors report that rats administered 0.020 mg/kg DA (s.c.) daily from PND 8 to 14 reliably manifest a "seizure-like" syndrome as adults when exposed to novel environments. In addition, these rats displayed permanent changes in hippocampal morphology, including altered synaptic connectivity and cell loss in selected regions.

3.2.6.1.5 The blood brain barrier (BBB) and the circumventricular organs

The transport of glutamate and DA at the BBB has been studied by both *in vitro* cell uptake assays and *in vivo* perfusion methods (Smith, 2000). In most regions of the brain, the uptake of these compounds from the circulation was limited by the BBB. At physiologic plasma concentrations, glutamate flux from plasma into brain appears to be mediated by a high-affinity transport system at the BBB. Efflux from brain back into plasma appears to be driven in large part by a sodium-dependent active transport system at the capillary membrane. The concentration of glutamate in brain interstitial fluid is only a fraction of that of plasma and is maintained fairly independently of small fluctuations in plasma concentration. This restricted brain passage was also observed for DA (Preston and Hynie, 1991).

The circumventricular organs lacking a BBB and their environs, including the organon vasculosum of the lamina terminalis, subfornical organ, mediobasal hypothalamus and area postrema, are identified as targets for DA effects (Bruni *et al.*, 1991). Domoic acid was administered intraperitoneally at doses of 2, 3 or 7 mg/kg to Swiss-Webster mice (Bruni *et al.*, 1991). Brains were examined at 0.5, 1, 24, 48 or 72 hours postinjection for evidence of damage. Significant pathologic changes occurred only after the largest dose of DA. Damage was confined to the circumventricular organs. In this study, the neural damage induced by DA was evident at as early as 30 minutes after injection and increased by 60 minutes postinjection. The loci of DA-induced neuropathological changes accounts for several central and peripheral effects and toxicities observed following systemic domoate treatment; these included gastroduodenal lesions, hypodipsia, analgesia and blood pressure fluctuations (Bruni *et al.*, 1991).

Experimental evidence in rodent and non-human primate indicates that the area postrema is a target for the effects of DA, suggesting neuroexcitatory effects on brainstem regions associated with visceral function (Tryphonas, Truelove and Iverson, 1990; Tryphonas *et al.*, 1990c; Peng *et al.*, 1994). The area postrema is one of the circumventricular organs that is outside the BBB. This structure is located at the base of the IVth ventricle and is implicated in the central control of the vomit reflex, acting as a defence mechanism. Vomiting was a prominent feature both in humans (see Section 3.4.1) and non-human primates intoxicated with DA (Tryphonas, Truelove

and Iverson, 1990). These emetic effects were observed after oral and parenteral exposure, suggesting that both gastric and central neural control mechanisms are involved. The observation of histopathologic changes in the area postrema of monkeys exposed to parenteral DA suggests that this structure is a target site for DA-induced toxicity and may explain the induced emetic response after parenteral administration. Because rodents cannot vomit, this emetic response is not observed in rodents, but changes have been reported in the area postrema (Peng *et al*., 1994). Other circumventricular organs were affected in mice treated with various doses of DA by i.p. injection (Bruni *et al*., 1991) (see Section 3.2.6.1.1)

3.2.6.1.6 Retina

Acute studies on rodents and non-human primates showed histopathological evidence of retinal injury associated with acute DA and KA toxicity (Stewart *et al*., 1990; Tryphonas *et al*., 1990a, 1990b, 1990c). The lesion was more often found on the inner nuclear layer, but other layers can also be affected. The characteristic of the cellular lesion is similar to that observed in the hippocampus and it is consistent with excitotoxicity. Furthermore, as in brain, glutamate receptors are ubiquitous in the retina (Truelove *et al*., 1996, 1997). These data suggest that these receptors are also involved in the induction of the retinal injury. This view is supported by some *in vitro* studies using either DA (Nduaka *et al*., 1999) or KA (Ferreira, Duarte and Carvalho, 1998). Using isolated embryonic chick retina, Nduaka *et al*. (1999) provided supporting evidence that release of glutamate contributes to the excitotoxic injury, exacerbating the neurotoxic action of DA. Their study suggests that an increase in membrane permeability secondary to osmotic swelling and lysis rather than a calcium-dependent vesicular exocytosis mechanism may be responsible for glutamate and lactate dehydrogenase (LDH) release. In their study, excitotoxic lesions were produced at all concentrations tested, with amacrine cells in the inner nuclear layer being the most severely affected.

3.2.6.2 Cardiotoxicity and toxicity of other internal organs and systems

Recent observations suggest that several subtypes of GluRs are widely distributed in peripheral tissues (Gill *et al*., 1998, 1999, 2000; Pulido *et al*., 2000; Gill and Pulido, 2001). The demonstration that several subtypes of ionotropic and metabotropic GluRs are present in heart and other tissues outside the CNS suggests that these tissues may be additional targets for excitatory compounds. Using immunochemical and molecular techniques, the presence and differential distribution of several subtypes of GluRs was demonstrated in the rat and monkey heart, with preferential distribution within the conducting system, nerve terminals and cardiac ganglia. These observations could explain some of the cardiovascular manifestations such as the arrhythmia described with DA intoxication in humans (see Section 3.4.1). In addition, severe cardiac lesions were observed in some sea lions that died of DA intoxication on the coast of California, the United States of America, in 1998 (see Section 3.3.1). These reports are the first to describe structural cardiac changes associated with DA poisoning. These data support the view that the heart may be a target site for the toxic effects of DA, and if so, individuals with pre-existing cardiac conditions may be at higher risk of the toxic effects of DA and other excitatory compounds in food. Preliminary data on histological sections from archived human heart tissue show that glutamate receptors are also present in human heart (Pulido *et al*., 2004).

Glavin, Pinsky and Bose (1990) examined the GI effects of an acidic extract of blue mussels contaminated with DA. The authors also tested pure DA and the putative antagonist kynurenic acid. Mussel extract produced gastric (antral) ulcers, duodenal ulcers, gastric, duodenal hyperemia, bleeding and ascites. Kynurenic acid protected significantly against extract-induced gastropathy, particularly when given 60 or

75 minutes after extract. Pure DA resulted in fatalities in all infant mice tested. These animals exhibited gastric bleeding and haemorrhage, especially at the higher doses employed. In otherwise untreated rats, kynurenic acid exerted significant anti-stress ulcer and anti-gastric secretory effects. The authors concluded that there may be both peripheral as well as central effects of kynurenic acid in modulating normal and pathological gastric function.

The actions of glutamate (L-Glu), and glutamate receptor agonists on serum thyroid hormones (T4 and T3) and TSH levels were studied in conscious and freely moving adult male rats (Arufe *et al*., 1995). Several EAAs, including KA and DA, were administered intraperitoneally. Thyroid hormone concentrations were measured by enzyme immunoassay, and thyrotropin (TSH) concentrations were determined by RIA. Data collected from this study showed that injection of the non-NMDA glutamatergic agonists KA (30 mg/kg) and DA (1 mg/kg) produced short-term (1 hour) increases in serum thyroid hormones and TSH levels, suggesting that compounds play a role in the regulation of hormone secretion from the pituitary-thyroid axis.

3.2.6.3 Mechanisms of toxicity

3.2.6.3.1 Glutamate receptors (GluRs) as mediators of DA effects

Domoic acid is an analogue of the endogenous EAA glutamate and is known to be a potent glutamate receptor agonist (Zaczek and Coyle, 1982). Domoic acid is similar in structure and function to KA, which is found in the red macroalga, *Digenea simplex*. The high affinity of DA and KA to several subtypes of GluRs (notably "kainate" receptors) and the rich distribution of these receptors within brain regions preferentially injured by DA provided the initial parameters suggestive of GluRs as mediators of DA neurotoxicity (Debonnel, Beauchesne and de Montigny, 1989; Debonnel, Weiss and de Montigny, 1989; Stewart *et al*., 1990). Further investigations showed strong correlation in the distribution of the neural injury and the immunohistochemical distribution of GluRs (Sobotka *et al*., 1996). Furthermore, the immunoreactivity of the KA and amino-methyl proprionic acid (AMPA) classes of receptors is significantly decreased in the hippocampus of rats treated with an acute single dose of KA. These findings can be interpreted as support for the view that regions rich in GluRs are more susceptible to the toxic effects of these compounds and therefore the most affected, although regional differences in sensitivity might also be attributed specific configurations of receptors rather than just receptor density.

Much evidence supports the view that DA and KA are excitotoxic and that their toxic effect is mediated through the activation of GluRs with the participation and co-activation of both non-NMDA (AMPA/kainate) and NMDA receptor subtypes (Novelli *et al*., 1992; Larm, Beart and Cheung, 1997; Berman and Murray 1997; Berman, LePage and Murray, 2002; Hampson and Manalo 1998; Clements *et al*., 1998; Tasker and Strain 1998; Tasker *et al*., 2005). Evidence suggests a preferential involvement of the non-NMDA receptor group including kainate and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor subtypes located on postsynaptic membranes (Jakobsen, Tasker and Zimmer, 2002; Jensen, Schousboe and Pickering, 1999). These receptors are ligand-gated ion channels that are activated by glutamic acid and its agonists mediating a fast excitatory synaptic transmission in the mammalian CNS. Briefly, persistent activation of KA receptors results in elevated levels of intracellular calcium (Ca^{2+}) through the cooperative interactions with NMDA and non-NMDA glutamate subtypes and voltage-dependent Ca^{2+} channels (Fernandez-Sanchez and Novelli, 1996; Xi and Ramsdell, 1996; Berman, LePage and Murray, 2002). NMDA receptors also appear to be activated secondarily as a consequence of AMPA and kainate receptor-mediated stimulation of EAA efflux (Novelli *et al*., 1992; Berman and Murray, 1997; Clements *et al*., 1998; Berman, LePage and Murray, 2002).

Neurotoxicity caused by DA and KA appears to be similar and results largely from excess accumulation of intracellular calcium. However, it is known that DA and KA have differing affinities for certain protein subunits (DA interacts preferentially with the GluK5 and GluK6 proteins whereas KA has highest affinity for the KA1 subunit) (Johansen *et al*., 1993; Verdoorn *et al*., 1994), which may account for both differences in both potency and behavioural toxicity (Tasker , Connell and Strain, 1991; Johansen *et al*., 1993; Tasker, Strain and Drejer, 1996).

3.2.6.3.2 Implications for GABA inhibitory neurotransmission in excitotoxicity

The administration of DA in a 0.2 mg/kg i.p. dose induces changes in the levels of GABA in different rat brain regions (hypothalamus, hippocampus, amygdala, striatum, cortex and midbrain). GABA is known to be the main inhibitory amino acid neurotransmitter in the mammalian brain. The distribution of this DA-induced inhibition of GABA correlates to rat brain regions that seem to be the main target of DA action belong e.g. the limbic system (hippocampus, amygdala). These suggest that inhibition of GABA may be implicated in DA-induced neurotoxicity (Duran *et al*., 1995). Furthermore, studies in rat whole hippocampal synaptosomes show modulation of (GABA) release by activation of kainate receptor (Cunha, Constantino and Ribeiro, 1997). In this preparation, kainate (10–300 microM) and DA (0.3–10 microM) inhibited [3H]GABA release in a concentration-dependent manner, whereas alpha-amino-3-hydroxy-5-methyl-4-oxazolepropionate (AMPA, 10–100 microM) was devoid of effect. These results indicate that GABA release can be modulated directly by presynaptic high-affinity kainate receptors. Moreover, it has been reported that there are GluK5 containing kainate receptors on GABA-ergic interneurons (Rodriguez-Moreno, Lopez-Garcia and Lerma, 2000).

3.2.6.3.3 Other mechanisms of neurotoxicity and neuroprotection

Several factors have been implicated in modulating or protecting the brain from the acute and long-term neurotoxic effect of DA and other EAAs. The role they play varies with age, dose and frequency of exposure. Most experimental data emphasize the effects of DA on neurons. However, there is evidence that astrocytes and microglia are also affected (Gill *et al*., 2000; Mayer *et al*., 2001; Ananth *et al*., 2001; Ananth, Gopalakrishnakone and Kaur, 2003a). Ross *et al*. (2000) reported that DA induced decreased glutamate uptake in rat astrocytes *in vitro*. These data suggest that disruption of astrocytic neuroprotective mechanisms and failure of astrocytes to remove excess glutamate at the synaptic site contributes to the excitotoxicity of DA. In addition, Mayer *et al*. (2001) showed experimental evidence that DA, at *in vitro* concentrations that are toxic to neuronal cells, can trigger a release of statistically significant amounts of tumour necrosis factor (TNF)-alpha and matrix metalloproteinase 9 (MMP-9) by brain microglia.

Nijjar and Nijjar (2000) reviewed evidence in support of a proposal that DA-induced toxicity is mediated by Ca^{2+} overload, inhibition of Ca^{2+} and CaM-stimulated adenylate cyclase activity, and/or by enhanced glutamate release. Their studies in rat brain slices demonstrated that DA induced an elevation in $[Ca^{2+}]_i$ that was enhanced by glucose deprivation and removal of Na+ from the Krebs-bicarbonate medium. This suggested a relationship between glucose metabolism (cell energy), Na+ and Ca^{2+} transfer across neuronal membrane and DA toxicity. In addition, diminished Ca^{2+}-ATPase activity because of lack of ATP, and variable amounts and expression of calcium binding proteins, appeared to contribute to an elevation in $[Ca^{2+}]_i$ in response to DA. Domoic acid also inhibited Ca^{2+} and calmodulin-stimulated adenylate cyclase activity in brain membranes, resulting in reduced level of cyclic AMP and diminishing the feedback control of cyclic AMP on Ca^{2+} influx via Ca^{2+} channels, thereby allowing continuing enhanced Ca^{2+} influx, resulting in Ca^{2+} overload, which adversely affects many

intracellular processes to induce toxicity. Calcium binding proteins bind Ca^{2+} reversibly and provide intracellular Ca^{2+} buffering, thereby protecting neuronal cell from damage by Ca^{2+} overload in response to DA. Hence, it appears to interfere with the cross-talk between Ca^{2+} and cyclic AMP, which is necessary for neuronal cell function.

Investigations by Erin and Billingsley (2004) demonstrated that during excitotoxicity induced by DA, calcineurin-Bcl-2 and calcineurin-1,4,5-inositol-trisphosphate receptor (IP3-R) interactions increase. They also show that calcineurin-IP3-R interactions are mediated by Bcl-2 in brain slices following short-term treatment with DA (10 microM). Bcl-2 is considered to be a survival protein that protects neurons from ischemia and excitotoxin-induced damage. On the other hand, DA induced late neuronal death and caspase-3-like activity in organotypic cortical and hippocampal cultures. These experiments suggest that interactions between calcineurin and its target proteins may influence cellular responses and further define the mechanisms by which neurons respond to excitotoxic insults.

Cell death associated with excitotoxicity has historically been regarded as necrotic. Recent findings, however, indicate that apoptosis may participate in excitotoxicity. Roy and Sapolsky (2003) showed novel neuroprotective effects of p35 and crmA following DA-induced excitotoxicity in hippocampal neurons, and Ananth *et al*. (2001) reported on induction of apoptosis-related gene expression by DA. These data underline the capacity for classically "anti-apoptotic" proteins to alter other branches of cell death processes and provide new insight on the mechanisms of DA-induced neuronal injury.

3.2.6.4 Protective agents and potentials for therapeutics

Benzodiazepines appear to selectively suppress the neuronal activation induced by kainate and suggest that a rapid treatment with high doses of benzodiazepines could possibly prevent irreversible hippocampal damage (Debonnel, Weiss and de Montigny, 1990). During the human intoxication of 1987, some patients had seizures that were relatively resistant to DilantinR medication, requiring high doses of i.v. benzodiazepine and phenobarbital for control (Teitelbaum *et al*., 1990). More recent studies indicate that the pineal hormone melatonin is neuroprotective and attenuates the excitotoxic effect of DA and KA (Ananth, Gopalakrishnakone and Kaur, 2003b; Chung and Han, 2003). Furthermore, there is evidence from the literature that benzodiazepines modulate melatonin. These data suggest that melatonin holds potential for the treatment of pathologies associated with DA and other excitotoxins. It also opens new perspectives on the potential role of melatonin on DA-induced seizures and on the increased susceptibility of elderly patients, because atrophy of the pineal gland and decline of melatonin secretion are known features of aging.

Other emerging pharmaceuticals have been shown to either attenuate DA toxicity *in vivo* or have the potential to do so. A study by Tasker, Strain and Drejer (1996) demonstrated significant *in vivo* protection against domoate toxicity in mice pretreated with the experimental agent NS-102 (NeuroSearch A/S). This compound is known to be a selective antagonist of the GluK6 receptor subunit and, consistent with current theory, was shown to selectively protect against DA toxicity relative to KA (Tasker, Strain and Drejer, 1996). Although not tested to date in DA models, the experimental drug NS-3763 has recently been reported to selectively antagonize the GluK5 receptor subunit (Christensen *et al*., 2004) and should, therefore, theoretically block some of the actions of DA.

Studies conducted by Dakshinamurti, Sharma and Geiger (2003) indicate that pyridoxine has anti-seizure and neuroprotective actions to the excitotoxic effects of DA. These protective effects appear to be mediated through mechanisms similar to those targeted by current therapeutic strategies. To this extent, the authors found that sodium valproate and pyridoxine significantly attenuated DA-induced increases in levels of glutamate, increases in calcium influx, decreases in levels of GABA and

increases in levels of the proto-oncogenes c-fos, jun-B and jun-D. These data suggest that pyridoxine either as a treatment or in the diet holds potential for the prevention and treatment of DA toxicity. It also suggests that premorbid nutritional status may be a factor in the individual susceptibility to the toxic effects of excitatory compounds in foods or therapeutic products.

There is also evidence that exercise prevents and protects the brain from damage of different etiologies, including excitotoxic brain damage associated with DA (Carro *et al*., 2001). This protection appears to be mediated through an increase uptake of circulating insulin-like growth factor I (IGF–I) by the brain. This data suggest that physical activity may be a factor that plays a role of individual differential susceptibility to neural injury. These findings also open new avenues on the potential use of IGF–I in therapeutics.

Aromatase, the enzyme that catalyzes the biosynthesis of estrogens from precursor androgens, may be involved in neuroprotection. Azcoitia *et al*. (2001) showed that a dose of DA that is not neurotoxic in intact male mice induced significant neuronal loss in the hilus of the hippocampal formation of mice with reduced levels of aromatase substrates as a result of gonadectomy. Furthermore, the aromatase substrate testosterone, as well as its metabolite estradiol, was able to protect hilar neurons from DA toxicity. In contrast, dihydrotestosterone, the 5 alpha-reduced metabolite of testosterone and a non-aromatizable androgen, was ineffective. These findings suggest that aromatization of testosterone to estradiol may be involved in the neuroprotective action of testosterone in this experimental model. The same authors also found that aromatase knock-out mice showed significant neuronal loss after injection of a low dose of DA, while control littermates did not, indicating that aromatase deficiency increases the vulnerability of hilar neurons to neurotoxic degeneration. The effect of aromatase on neuroprotection was also tested in male rats treated systemically with the specific aromatase inhibitor fadrozole and injected with KA. Fadrozole enhanced the neurodegenerative effect of KA in intact male rats and this effect was counterbalanced by the administration of estradiol. These findings suggest that brain aromatase may therefore represent a new target for therapeutic approaches to excitotoxicity.

Finally, a brief paper by Tasker, Connell and Strain (1992) reported that low doses of morphine (2.0 and 4.0 mg/kg i.p.) significantly attenuated both the behavioural and hippocampal histopathological toxicity induced by DA, but not KA, in adult mice.

3.3 Observations in animals and veterinary toxicology

3.3.1 Sea lions

Many California sea lions (*Zalophus californianus*) died and many others displayed signs of neurological dysfunction along the central California coast, the United States of America, during May and June 1998. A bloom of *Pseudo-nitzschia australis* (diatom) was observed in the Monterey Bay region during the same period. This bloom was associated with production of DA (Lefebvre *et al*., 2000). This toxin was also detected in planktivorous fish, including the northern anchovy (*Engraulis mordax*) (Lefebvre *et al*., 1999; Lefebvre, Dovel and Silver, 2001) and in sea lion body fluids. These and other concurrent observations suggested that DA was responsible for the marine mammal mortality. In contrast to fish, blue mussels (*Mytilus edulus*) collected during the outbreak contained no DA or only trace amounts. Such findings reveal that monitoring of mussel toxicity alone does not necessarily provide adequate warning of DA entering the food web at levels sufficient to harm marine wildlife and perhaps humans (Scholin *et al*., 2000).

Scholin *et al*. (2000) provide a detail account of the clinical and histopathology observations of animals affected during the 1998 outbreak. Clinical signs in affected sea lions were ataxia, head weaving, muscle tremor, titanic convulsions, rubbing and lethargy. Forty-eight (69 percent) of the initially affected sea lions died or

were euthanized. In animals that died, seizures became increasingly frequent, often progressing to status epilepticus for over an hour, and showed no improvement in clinical signs over subsequent days, or were comatose. Some were euthanized (n=22). Seizures durations lasted from a few minutes to 30 minutes with frequency recorded between 1 and 30 in a 24-hour period. The seizures were usually bilateral. They had limited response to treatment. In animals that survived, the frequency of seizures gradually decreased over a one-month period.

Other clinical signs reported included blood-tinged mucus, diarrhoea, moist cough and blepharospasm. No vomitus was observed and at post-mortem examination of animals that died, stomachs were empty. Pathology examination of animals that died or were euthanized during the event suggested that foetuses had died in utero some time prior to the female's death. At post mortem, the most common gross lesion observed that was considered a feature of the toxic event was pallor of the myocardium (16/48). These lesions were observed more frequently in animals that died within 48 hours of stranding.

Histopathology showed that the principal lesions that were considered unique to this stranding event were in the brain and in the heart. There was acute cerebral edema and necrosis most severe in the dentate gyrus and pyramidal layers of the hippocampus in the anterior ventral region, with other areas of the brain appearing less severely or consistently affected. However, it is feasible that the extent of the lesions in other brain areas was underestimated. Short-term survivors had evidence of neuronal loss and gliosis in the same regions as the acute cases with more evidence of extension to the pyriform lobe and the amygdala. The acute lesion was considered to be consistent with DA- induced excitotoxicity. Myocardial edema and necrosis were also seen in animals that died rapidly during this event. There was also evidence of repair of the acute lesions in the short-term survivors. This is the first report of overt myocardial lesion associated with DA intoxication.

Other changes were also reported including swelling of kidney (13/48), gastric erosion and ulcers (22/48), and pulmonary congestion (14/48). Two animals had uterine ruptures with intra-abdominal delivery, two had uterine torsion and one had uterine prolapse. These changes were not considered unique to this toxic episode. However, in view of the recent demonstration of GluRs in all those tissues (Gill and Pulido, 2001), it is feasible that there is a causal relationship with DA exposure.

In 2000, a further 184 sea lions stranded with similar clinical signs (Gulland *et al*., 2002), but the strandings occurred both during detectable algal blooms and after the blooms had subsided. The clinical signs in these sea lions included seizures, ataxia, and head weaving, decreased responsiveness to stimuli and scratching behaviour. Affected animals had high haematocrits, and eosinophil counts, and high activities of serum creatine kinase. They were treated supportively by using fluid therapy, diazepam, lorazepam and phenobarbitone. Fifty-five of the 81 sea lions (68 percent) affected in 1998 and 81 of the 184 (44 percent) affected in 2000 died despite the treatment. Three of the 23 sea lions that survived in 1998 were tracked with satellite and radio transmitters; they travelled as far south as San Miguel Island, California, and survived for at least three months. Eleven of the 129 animals that were released stranded within four months of being released (Gulland *et al*., 2002).

3.3.2 Whales

In addition to sea lions, DA has been implicated as a causative toxic agent in several other marine mammal species; notably whales and sea otters. A 2002 report by the Working Group on Marine Mammal Unusual Mortality Events (Ch'ng *et al*., 2002) describes investigations into the deaths of 350 gray whales, 7 sea otters, 90 sea lions and 2 humpback whales. DA was detected in body fluids from 2 of 11 gray whales (*Eschrichtius robustus*) sampled and in the urine of affected sea otters. While such

detection is not evidence of a causal relationship, it does demonstrate bioaccumulation of DA in the marine food chain. Similarly, a report by Lefebvre *et al*. (2002) describes DA concentrations from 75 to 444 µg/ml in the viscera of various fish species that had been consumed by 3 humpback whales (*Megaptera novaeanglia*) and concentrations from 10 to 207 µg/g in whale faeces collected from 1 humpback and 2 blue (*Balaenoptera musculus*) whales; the blue whales presumed to have been feeding on contaminated krill although this was not confirmed. By extrapolating these concentrations, Lefebvre *et al*. (2002) arrived at estimate toxin loads of 1.1 mg/kg DA for humpback whales feeding on fish and 0.62 mg/kg DA for blue whales feeding on krill.

3.3.3 Sea birds

There have been numerous anecdotal reports of DA intoxication in piscivorous sea birds; however, the best documented cases are those described by Work *et al*. (1993a, 1993b) and by Sierra-Beltrán *et al*. (1997). Work *et al*. (1993a, 1993b) described the epidemiology of a DA epidemic in Monterey Bay, California, in which 95 Brant's cormorants (*Phalacrorax penicillatus*) and 43 brown pelicans (*Pelecanus occidentalis*) died. They also noted the deaths of several other species, including double-breasted cormorants, pelagic cormorants and western gulls in the same incident. The source of DA in these cases appeared to be anchovies that were recovered from the stomachs of many of the dead birds and were shown by HPLC analysis to have high concentrations of DA. Analysis of blood samples from 12 intoxicated pelicans and 5 cormorants yielded detectable concentrations of DA (0.5–48 µg/g) in 11 of 12 pelicans and in 2 of the cormorants (1.4 and 6.3 µg/g). In terms of behavioural toxicity, intoxicated cormorants showed few overt signs of CNS toxicity but were generally lethargic or docile. In contrast, affected pelicans displayed many signs of toxicity that were reminiscent of ASP in other species. These included side-to-side head motions, fine motor tremors, scratching, vomiting, loss of awareness, loss of postural control, convulsions and death. One additional interesting feature of this case was that the planktonic source of DA appeared to be *P. australis*.

In a similar incident involving 150 dead sea birds in Cabo San Lucas, Mexico, Sierra-Beltrán *et al*. (1997) reported on the presence of DA in viscera from five dead pelicans that they analysed by both MBA and HPLC. The other birds were not tested. In this case, the source of DA appeared to be consumption of contaminated mackerel.

3.4 Observations in humans

3.4.1 Epidemiology

3.4.1.1 Canada

Late in 1987, an outbreak of a newly recognized acute illness caused by eating blue mussels and characterized by GI and unusual neurological symptoms occurred in Canada. More than 107 people (47 men and 60 women) were affected, most from Quebec. A case was defined as the presence of GI symptoms (vomiting, abdominal cramps, diarrhoea) within 24 hours and neurological symptoms within 48 hours (severe headache and memory loss). The etiologic agent was found to be DA, an excitatory neurotransmitter amino acid, produced by *Nitzschia pungens f. multiseries* (now called *Pseudo-nitzschia multiseries).* Both *N. multiseries* and DA were present in the digestive glands of the causative cultivated mussels, harvested from the eastern coast of Prince Edward Island, and shipped to other parts of Canada (Perl *et al*., 1990a,b; Teitelbaum, 1990; Teitelbaum *et al*., 1990).

Of the 107 persons that met the case definition, 19 were hospitalized from 4 to 101 days; 12 of these were admitted to intensive care and 3 died in hospital at 12–18 days after admission. Another patient died after 3 months. Ninety-nine of the patients answered a questionnaire in which they provided information on the symptoms and on

the amount of mussels consumed. Of these patients, 49 were between 40 and 59 years old and 38 patients were 60 years or older.

Symptoms of illness included nausea (77 percent), vomiting (76 percent), abdominal cramps (51 percent), diarrhoea (42 percent), headache (43 percent) and memory loss (25 percent). None of the younger patients (20–39 years) suffered memory loss, and their only symptoms were of a GI nature. For patients that suffered memory loss or that required hospitalization, the odds ratios (95 percent confidence limits) for age per 10-year increment were 1.6 (1.2, 2.0) and 2.3 (1.7, 4.0), respectively, indicating that increased age was a risk factor for the severity of the illness. Similarly, males were more susceptible as indicated by odds ratios for M/F of 4.4 (1.5, 13.0) and 16.9 (3.5, 80.4) with respect to memory loss and hospitalization, respectively. Hospital charts were available for 16 of the 19 hospitalized patients, indicating that all severely ill patients less than 65 years old had pre-existing illnesses, including diabetes (3), chronic renal disease (2) and hypertension with a history of transient ischemic attacks (1) considered to be predisposing factors. All patients admitted to intensive care had serious neurologic dysfunction, including coma (9), mutism (11), seizures (8). Seven patients had unstable blood pressure or cardiac arrhythmias.

For nine patients and one person who did not become ill, analytical information on the unconsumed portion of the mussels, and recall information on portion size were available, and this was used to estimate exposure. The concentration of DA in these mussels was determined by MBA (characteristic hindleg scratching), and ranged between 31 and 128 mg/100 g. Increasing exposure correlated with the clinical course of events (Table 2). All patients reported GI illness, but only 1 of 6 patients who consumed between 60 and 110 mg DA suffered memory loss and none required hospitalization. All three patients who had consumed 270–290 mg DA suffered neurological symptoms and were hospitalized. One person who consumed only 20 mg DA did not become ill. The cognitive impairment observed in this new disease, attributed to DA, appeared to be persistent and led to the term "amnesic shellfish poisoning" (ASP) (Perl *et al*., 1990a,b; Todd, 1990). Based on this dose-response relationship, and assuming a b.w. of 60 kg, the committee estimated that the LOAEL in humans was 1 mg/kg b.w., and no ill effects were observed in a person who consumed 0.33 mg/kg b.w.

In a companion paper (Gjedde and Evens, 1990), the neurologic manifestations of DA intoxication from the 1987 Canadian outbreak were studied over a period of several years in 14 of the patients, all from the Montreal area. This study included neuropsychological testing, an assessment of motor function and positron emission tomography (PET) to assess glucose metabolism in specific regions of the brain. These 14 patients were between 44 and 87 years old, and 10 were male; they were not truly representative of the total 99 DA patients described in the above study, as 3 had more severe pre-existing CNS disease (cerebral infarct, pituitary adenoma and Parkinson's disease). In addition, 3 patients had adult-onset diabetes mellitus, 2 had hypertension and 1 suffered from chronic renal failure. Three of the 14 patients were examined during the acute phase of the illness and all 14 were examined within 4 months of the exposure. In addition to these 14 patients, the neurologic and subsequent neuropathologic findings of 4 other patients, who died within 4 months of exposure, were assessed.

During the acute phase, 13 of the 14 patients developed acute GI illness and 7 patients became comatose and suffered severe complications, including haemodynamic instability with hypotension and arrhythmias unrelated to cardiac disease. A fever developed soon after exposure in 6 patients, and 6 patients had short-term increases in serum creatinine and BUN levels. There were no signs of non-neurological illness in any of the 14 patients, at 4 months postexposure.

TABLE 2
Estimated exposure and clinical course of patients who ingested DA during the 1987 outbreak of ASP in Canada[a]

Patient	Age	Estimated weight of mussels consumed[a,b] g/person	DA in sample mg/100 g	Estimated DA consumed mg/ person	Clinical symptoms and treatment			
					GI[b,c]	Memory loss	Hospitalized	ICU
Unaffected	60	35	52	20	–	–	–	–
1	72	120	52	60	+	–	–	–
2	62	150	45	70	+	+	–	–
3	70	15	52	80	+	–	–	–
4	61	300	31	90	+	–	–	–
5	67	160	68	110	+	–	–	–
6	71	360	31	110	+	–	–	–
7	74	400	68	270	+	+	+	–
8	68	225	128	290	+	+	+	+
9	84	375	76	290	+	+	+	+

[a] Adapted from Perl *et al.*, 1990.
[b] From patient's recall or estimated as 375 g when amount consumed was not known.
[c] GI refers to gastrointestinal symptoms.

All 14 patients were confused and disoriented 1.5–48 hours postexposure. Their behaviour ranged from agitation or somnolence to coma, with maximal deficits between 4 hours (least affected) and 72 hours (for comatose patients) postexposure. Most improved within 24 hours to 12 weeks. Patients characteristically had anterograde memory disorder with relative preservation of other cognitive functions. Those individuals with a moderate memory disturbance were generally able to encode information, but had difficulty with delayed recall. More severely affected individuals had some difficulty learning verbal and visuospatial material and their delayed recall was also very poor. In addition, some of these latter patients also had retrograde amnesia extending several years back. The ability to form concepts was generally adequate in the patients.

Three of the 14 patients had generalized seizures, 2 had psychomotor seizures and 1 had focal motor seizures; myoclonus (primarily facial) was seen in many who suffered seizures. The seizures became progressively less frequent and ceased within 4 months of DA exposure. EEGs in 7 patients obtained within 1 week postexposure revealed moderate to severe generalized slowing of background activity. At about 4 months postexposure, the EEGs of 11 patients showed mild to moderate generalized disturbance of background activity and the other 3 had normal readings.

During the acute phase of the illness, all patients were unsteady and showed generalized weakness. Two patients had symmetric transient hyperreflexia and Babinski signs, and three victims had fasciculations. Two patients showed signs of a novel syndrome, characterized by alternating hemiparesis and ophthalmoplegia. Spastic hemiparesis persisted for 24–36 hours postexposure. Complete external ophthalmoplegia also developed during the acute phase of the illness, but resolved within 10 days postexposure. Two other patients had ophthalmoparesis without hemiparesis. The diplopia disappeared in one subject within 24 hours, but it persisted in the other patient for 12 weeks. When examined between 4 and 6 months postexposure, 11/14 patients had distal atrophy and mild weakness of the extremities and 8 had hyporeflexia. In addition, 1 patient had a loss of distal sensitivity to pain and temperature changes. Electromyographic evaluation indicated marked spontaneous activity and neurogenic recruitment in 2/11 patients tested within 1 month of exposure. One of these patients, comatose for two weeks, had slow nerve conduction velocities in typical compression sites of the left ulnar, left median and left common peroneal nerves. Between 4 and 6 months postexposure, 9/10 patients who underwent

electromyography had findings consistent with various degrees of acute denervation. A total of 7 patients had diminished compound motor-unit potentials. By 11–14 months postexposure, 7 patients who had a follow-up examination showed partial recovery of the acute signs of denervation but the compound motor-unit potentials remained unchanged. Sensory nerve condition in 7 patients (including 3 with diabetes mellitus) was mildly abnormal, and sural-nerve potential amplitudes were diminished or absent. The authors suggested that DA induced acute non-progressive neuronopathy involving anterior horn cells or diffuse axonopathy predominantly affecting motor axons.

Between 4 and 6 months postexposure, the rate of glucose metabolism measured through PET in four patients revealed normal age-related decline in the cerebral cortex in all four and decreases in the amygdala and hippocampus in the two most severely affected patients. These observations correlated with composite memory scores, which were normal for two subjects and severely depressed for the other two.

Neuropathological examination of the four patients who died indicated neuronal necrosis and astrocytosis particularly in the hippocampus and the amygdaloid nucleus. All four victims also had lesions in the claustrum, secondary olfactory areas, the septal area and the nucleus accumbens septi. Two had prominent thalamic damage, especially in the dorsal medial nucleus. The subfrontal cortex was also damaged in three of the patients. The authors noted the pattern of damage in the hippocampus appeared to parallel the pattern seen in animals that suffered neurotoxic reactions after administration of KA. Cerebrovascular disease was evident in 2 patients: an acute haemorrhagic infarct in the left posterior perisylvian cerebral in 1 victim and in the other cerebellar infarcts likely related to a myocardial infarct 3 days prior to death. Neurofibrillary tangles and senile plaques, typical features of Alzheimer's disease, were not observed in these patients (Teitelbaum *et al*., 1990).

A further report detailed the initial findings and follow up of an 84-year-old man who suffered DA intoxication during the 1987 outbreak in Canada and who subsequently died, 3.25 years after exposure. The patient developed nausea and vomiting 1 hour postingestion of the contaminated mussels, and became progressively disoriented and somnolent. On Day 3, he became comatose and had complex partial status epilepticus, eventually involving the right hemibody. EEGs showed a diffuse slowing of background activity, periodic lateralized epileptiform discharges over the left hemisphere and subsequently bitemporal independent epileptic abnormalities. Computed tomographic scans of the patient's brain showed only a mild ventricular enlargement and cerebral atrophy consistent with his age. The seizures were treated with medication and 4.5 months following intoxication, the patient was discharged from hospital. Although he was seizure-free, he had severe impairment of anterograde memory. Approximately one year postintoxication, he experienced complex partial seizures consisting of staring, twitching of the left lower part of the face and then clonic movements of the left arm and leg developed. Magnetic resonance images revealed a hyper-intense signal and marked atrophy of both hippocampi. A positron emission tomogram showed bitemporal decrease in glucose metabolism, and a neuropsychological evaluation indicated severe memory impairment for both verbal and nonverbal material. The cause of death was pneumonia. Gross examination of the patient's brain revealed atrophy of the hippocampi and slight dilatation of the ventricular system and of the sylvian fissure. Histologically, the hippocampi showed complete neuronal loss in the H1 and H3 (equivalent to CA1 and CA3 in rodents) regions, almost total loss in H4 (equivalent to CA4) and moderate loss in H2 (equivalent to CA2). The amygdala showed patchy neuronal loss in medial and basal portions with neuronal loss and gliosis in the overlying cortex. Mild to moderate neuronal loss and gliosis were seen in the dorsal and ventral septal nuclei, the secondary olfactory areas and the nucleus accumbens. Reactive astrocytes were found in the sixth cortical layer and subjacent white matter in the orbital and lateral basal areas, the first and second

temporal gyri, the fusiform gyrus, parietal parasagittal cortex and the insula, while no abnormality was seen in the frontal parasagittal, the cingulate, the lateral parietal, or any part of the occipital cortex. The thalamus showed only a mild increase in astrocyte nuclei in central areas. It was suggested that temporal lobe epilepsy following DA exposure might develop after a "silent period" of one year (Cendes *et al*., 1995).

3.4.1.2 Other outbreaks

An outbreak of DA poisoning may have occurred in October/November 1991 in Washington State, the United States of America. Approximately two dozen people became ill after ingesting razor clams harvested along the Washington and Oregon coasts. Although GI or neurological symptoms were observed within 36 hours of ingestion, no other symptoms consistent with DA intoxication were reported. A total of 21 possible DA victims and 43 non-victims were interviewed. Based on the concentrations of DA measured in razor clams, the mean total DA consumption by the 21 victims would have been 17 mg (estimated to be 0.28 mg/kg b.w.) and 8 mg by the unaffected individuals (0.13 mg/kg b.w.). A total of 13 patients developed neurological symptoms, but all recovered. However, the incident was not well reported and was unsubstantiated (Todd, 1993; Jeffrey *et al*., 2004).

3.4.2 Biomarkers of exposure

Because DA was not found in the serum of patients affected by ASP (Perl *et al*., 1990b), and because animal studies have shown a rapid excretion of DA (Section 3.1.1.3), there is no reliable, readily available biomarker of exposure.

High-resolution (< 50 micron) magnetic resonance imaging microscopy (MRM) has been used to identify brain regions and to localize excitotoxin-induced lesions in fixed rat brains, subsequently confirmed using standard histology (Lester *et al*., 1999). The anatomical extent of lesions identified by MRM was identical to that seen in histological sections and various histopathological changes could be visualized. In contrast to the time involved in preparing and examining histological sections, lesions in intact brains could be rapidly identified and visualized in three dimensions by examining digitally generated sections in any plane. This study shows that MRM has tremendous potential as a prescreening tool for neurotoxicity and neuropathology in human and experimental models.

3.4.3 Biomarkers of effect

There are no objectively validated biomarkers of effect for DA. Scapular scratching is consistently observed in mice injected with DA (Tasker, Connell and Strain, 1991) but is not exclusive to this toxin. Patients intoxicated with DA frequently manifest deficits in short-term visuo-spatial recall in conjunction with other clinical signs (Teitelbaum *et al*., 1990) but this deficit is not produced exclusively by DA toxicity.

4. ANALYTICAL METHODS

4.1 General

Methods of analysis for DA have been reviewed and appear in an FAO publication (FAO, 2004). However, the great majority of these methods have had little or no validation. The methods described below are those that have been validated or show potential to be used for regulatory or international trade purposes.

4.2 Methods

4.2.1 In vivo assays

4.2.1.1 PSP mouse bioassay

The AOAC mouse bioassay (MBA) for PSP toxins (AOAC, 1990) can detect DA at concentrations of approximately 40 g/g tissue. It involves acidic aqueous extraction of the tissue followed by i.p. injection of 1 ml of the extract into mice. Although the AOAC extraction procedure can yield substantial recovery of DA, the limit of detection of the AOAC bioassay procedure is not low enough to be used with confidence for regulatory purposes to quantify this toxin. No interlaboratory validation of this method for DA has been carried out.

4.2.2 **In vitro** *assays*

4.2.2.1 Receptor binding assays

A competitive microplate receptor binding assay (RBA) for DA using frog (*Rana pipiens*) brain synaptosomes has been developed. The analysis of DA was based upon binding competition with radiolabelled-[^{3}H]-KA for the kainate/quisqualate glutamate receptor. In 1997, Van Dolah *et al*. (1997) reported the further development of the receptor assay by replacement of frog brain by a cloned rat GLUR6 glutamate receptor to eliminate animals from the testing procedure. The limit of detection and selectivity of the assay were optimized through inclusion of the glutamate decarboxylase pretreatment step to eliminate potential interference because of high concentrations of endogenous glutamate in shellfish. The RBA of Van Dolah *et al*. (1997) is suitable for analysis of DA in seawater extracts from algae and for analysis of DA in shellfish. No interlaboratory study of this method has been carried out. However, it is being considered for study by AOAC International.

4.2.3 Biochemical assays

Immunoassays

Garthwaite *et al.* (1998) used ovine antibodies raised against DA to develop an indirect competitive ELISA for shellfish and seawater. This ELISA method has been commercialized by Biosense®, into a kit format, intended to be used in routine monitoring of DA in cultured bivalve molluscs to check compliance with the regulatory limits. According to the producer, it is also applicable for quantification of DA in other matrices (see www.biosense.com "Direct cELISA ASP assay", 2003). Compared with the original procedure of 1998, the limit of quantitation (LOQ) of the kit has been reduced to 10 µg/kg shellfish. The method has been validated by interlaboratory study. Results indicated that 14 of 16 laboratories were able to establish the method with good calibration curves, and they reported valid concentration data for all study samples. The participating laboratories achieved good interlaboratory precision estimates for the eight Youden pairs of shellfish samples of RSD_r 15 percent and RSD_R 23 percent, and the ASP ELISA appeared robust to operator-controlled variables. The precision estimates for the ELISA data did not show a strong dependence on the DA concentration in the study samples, and the overall precision achieved was within the acceptable range of the Horwitz criteria (mean HORRAT 1.7) established by AOAC International for precision of analytical methods. The analysis of shellfish samples spiked with certified reference materials demonstrated very good method accuracy (recovery of 104 percent). This was supported by an excellent correlation slope of 1.015 (R2 0.992) for the determined vs the expected values of DA in the spiked samples. There was also good correlation of the ELISA results with those for the instrumental LC analyses of the same samples extracts. This method appears suitable for the routine quantitative determination and monitoring of DA in shellfish.

Another antibody-based technique employing lateral-flow strip test technology has been commercialized (Jellett Rapid Testing Ltd.) and evaluated (MacKintosh and

Smith, 2002) for DA. This approach has much potential for qualitative rapid screening of shellfish. (A similar test kit by the same manufacturer is already in use in several countries for STXs and has demonstrated very good correlation with the PSP MBA.) The test is sensitive enough for screening for DA well below the regulatory guideline of 20 mg/kg shellfish tissue. Comparison data (to LC-UV or LC-MS) for this test kit would be welcomed.

Traynor *et al.* (2002) have described the detection of DA in bivalve molluscs with an immunobiosensor. In this application, DA is bound to the sensor surface and use is made of polyclonal antibodies. The assay was found suitable for rapid analysis of cockles, mussels, oysters and scallops. A limit of detection was found at 0.8 µg/g, and an intra-assay C.V. of 8 percent was found at a level of 20 µg/g, the current legal limit for whole body. A large-scale comparison with LC is under way but no results have yet been reported.

4.2.4 Chemical assays

4.2.4.1 Thin layer chromatography (TLC)

Domoic acid can be determined by TLC as a weak UV-quenching spot that stains yellow after spraying with a 1 percent solution of ninhydrin (Quilliam, Thomas and Wright, 1998). The detection is about 0.5 µg by this method, which permits detection in shellfish tissues at about 10 µg/g. It is also possible to detect DA on the TLC plate using some other spray reagents. Quilliam further studied TLC as a separation technique to detect DA, after extraction with aqueous methanol followed by SAX-SPE cleanup. The method was successfully applied to scallop, razor clam and anchovy samples contaminated with DA. It was concluded the method should prove successful for the routine screening of shellfish tissues in those laboratories not equipped with an LC system. It should also be useful as a chemical confirmation method for DA in samples tested positive by assay methods such as immunoassay. This method would be useful to developing countries where other methods may be too costly. No in-depth quantitative studies have been reported for this method. However, it has potential as a relatively inexpensive screening technique.

4.2.4.2 Liquid chromatography-ultraviolet detection (LC-UV)

Liquid chromatography-ultraviolet detection (LC-UV) is currently the preferred analytical technique for the determination of DA in shellfish, and a method is available, formally validated for mussels in an AOAC collaborative study (Lawrence, Charbonneau and Ménard, 1991). The detection of DA is facilitated by its strong absorbance at 242 nm. The LC-UV detection limit for DA is about 10–80 ng/ml, depending on the sensitivity of the UV detector that is used. If crude extracts (either acidic or aqueous methanol) are analysed without cleanup, the practical limit for quantitation is about 1 µg/g. This method has been standardized by the working group on biotoxins of the European Committee for Standardization (CEN) and is approved as European Norm EN 14176 (CEN, 2003).

An improved LC-UV method was developed by Quilliam, Xie and Hardstaff (1995). In this procedure, an aqueous methanol extraction is applied in combination with strong anion exchange-SPE cleanup, leading to chromatograms free from interferences. Other advantages of the method are more stable extracts, higher recoveries and a lower limit of detection (20–30 ng/g). A European two-phase interlaboratory validation study of this method was conducted in 2002–03 by the EU Community Reference Laboratory for Marine Biotoxins for a variety of shellfish and fish samples. This study is now complete and preliminary results indicate that the method performed very well. If the study yields acceptable results, the method also will be approved by CEN.

4.2.4.3 Liquid chromatography-mass spectrometry (LC-MS)

Liquid chromatography-mass spectrometry (LC-MS) techniques continue to make important strides in the detection and quantification of marine toxins and this is no exception with DA. It is the only method at present that can detect all isomers of the DA group, and new isomers continue to be discovered by the approach (Holland *et al*., 2003a; Rhodes *et al*., 2003). An interlaboratory study of an LC-MS method for determination of DA (and DSP toxins) in shellfish was carried out (Holland and McNabb, 2003). The eight participating laboratories generally obtained consistent sets of data for the broad group of toxins down to low levels (< 5 ng/ml, equivalent to 0.05 mg/kg). A method specifically for DA was developed and successfully submitted to single laboratory validation (Holland *et al*., 2003b). The LC-MS approach continues to evolve and improve for quantification of marine biotoxins and is increasingly being employed in developed countries for marine biotoxin analyses.

4.2.5 Other assays

Capillary electrophoresis (CE) is a relatively new technique that has gained acceptance in a number of areas. Domoic acid has been determined by CE (Pineiro *et al*., 1999; Zhao, Thibault and Quilliam, 1997) but the approach has not been widely used.

The FAO report (FAO, 2004) lists many variations of the above-described methods that have been developed for DA, and some of them offer potential for regulatory purposes. However, none of these methods has been submitted to interlaboratory study and, in most cases, they have not even been submitted to acceptable single laboratory method validation.

5. LEVELS AND PATTERNS OF CONTAMINATION OF SHELLFISH

Although the most common vector for DA, including the source in the 1987 human intoxication incident in Canada, is the blue mussel (*Mytilus edulis*) (Perl *et al*., 1987; Lawrence, 1990; Johnson *et al*., 1990; Dizer *et al*., 2001), several authors have also reported on the presence of DA in other shellfish (Wekell *et al*., 1994; Blanco *et al*., 2002; Bargu *et al*., 2003) and crustaceans (Powell *et al*., 2002). Among these, the most comprehensive study is that of Vale and Sampayo (2001) in which they sampled a variety of Portuguese shellfish and fish for the presence of DA. These authors reported considerable accumulation of DA in a variety of shellfish including the common cockle (*Cerastoderma edule*), the peppery furrow shell (*Scrobicularia plana*), the carpet shell (*Venerupis pullastra*), oysters (*Ostrea edulis*), razor clams (*Ensis* spp.) and common clam (*Ruditapes decussate*).

6. COMMENTS

- Domoic acid is a naturally occurring toxin produced by the diatom Pseudo-nitzschia multiseries (formerly known as Nitzschia pungens f. multiseries).
- Domoic acid can potentially enter the food chain by contaminating shellfish and other types of seafood. Although depuration occurs with time, harvesting and consumption of the shellfish at the time of contamination can lead to human or animal intoxication.
- Domoic acid was identified as the responsible agent in the human intoxication that occurred in Canada in 1987. A constellation of clinical symptoms and signs, initially observed in the affected individuals, have thereafter been considered as characteristic of the clinical syndrome associated with DA poisoning. Among the most prominent features described was memory impairment leading to the term of amnesic shellfish poisoning (ASP).

- Monitoring programmes have been successful in preventing other human incidents. However, outbreaks of wildlife poisoning and of contamination of coastal water are still occurring. Hence, DA continues to be of concern to human and animal health and safety.
- The human poisoning episode of 1987 and subsequent toxicological studies in experimental animals provided the basis for the toxicology of DA and for the establishment of the regulated limits of 20 µg DA/g shellfish flesh currently in effect in Canada, the United States of America and the EU. Further experimental studies conducted have supported this safety limit. Although this regulatory decision has not been challenged by more recent work, this review indicates that there are gaps in current knowledge. These require attention because they may affect future regulations and therapeutic emergency preparedness.
- Of clinical importance and high relevance to risk/safety assessment are the premorbid health conditions of the individual, including renal clearance capacity, cardio-vascular status, GI absorption, nutrition, stomach content/emptiness and emetic response. Data from the human intoxication suggest that these were important risk factors, i.e. persons with renal insufficiency were at higher risk. Domoic acid appears to be cleared rapidly from the systemic circulation exclusively by renal excretion in all species studied to date. Moreover, DA poorly penetrates the intact BBB in rodents. This indicates that conditions of impaired renal function or compromised BBB integrity confer additional risk.
- Also of clinical importance and high relevance to risk/safety assessment is the effect of age. Data from human intoxication indicate that the elderly are more susceptible to DA toxicity. In addition, there is considerable evidence in the animal literature that newborn animals are subject to both immediate and permanent toxicity when exposed to doses 50–100 times below those considered toxic in adult animals. Pregnant women may also be at greater risk for foetal toxicity although data on transfer of DA across the placenta are lacking.
- Data from a few available oral subchronic studies in rodents and non-human primates suggest that repeated exposures to DA do not lead to cumulative effects or added toxicity, at least for young and healthy adult animals. However, the age-dependent responses and differential susceptibility to repeated exposure of DA or other excitatory amino acids have yet to be assessed. This is particularly important in view of observations from the human poisoning episode and from acute experimental studies indicating higher susceptibility in the neonate and the aged. Furthermore, in vitro studies provide evidence for an age-dependent tolerance in the young, but not in aged animals pre-exposed to small amounts of DA. This age-dependent differential response may be the result of an age-associated decline of brain protective mechanisms.
- The acute excitotoxic, epileptogenic and neurobehavioural effects of DA have been the most studied. There is agreement that DA induces a dose-dependent acute neurotoxicity characterized by a constellation of neurobehavioural signs, ranging from disorientation to seizure, coma and death. Histopatholic hallmarks of acute excitotoxicity have a specific anatomical distribution. The hippocampus and other structures within the limbic system appear to be preferential targets. The lesion involves neurons and glia cells. In the neurons, a preferential dendritic effect has been described. Humans and animals surviving the acute episode were left with memory and learning impairments. Hippocampal gliosis is the histopathologic counterpart for the long-term sequel.

- Data from animal studies using magnetic resonance imaging, electroencephalography and markers of neural injury support the findings of preferential regional distribution of DA toxicity. Data from some of these studies also indicate that DA can elicit electroencephalographic responses and changes in the olfactory bulb without having been associated with an overt clinical syndrome, suggesting that these procedures are more sensitive markers. However, the significance of these findings for health and risk assessment needs to be further evaluated.
- A great body of knowledge has been acquired on the mechanisms involved in excitatory neurotoxicity and neuroprotection. Several mechanisms have been implicated as mediators for the effects of DA. Of particular importance is the role played by GLuRs as mediators of excitatory neurotransmission, the modulation of GABA inhibitory neurotransmission, the concomitant excess release of glutamate at the synaptic site, the activation or deactivation of protective mechanisms, and the involvement of cells and structures such as astrocytes, the BBB and the circumventricular organs. These data reveal a complex interplay of cellular, molecular and electrophysiological mechanisms that are at least in part dependent on the anatomical region, dose and age. In addition to enhancing understanding of excitotoxicity, mechanistic-based studies continue to provide valuable information relevant to degenerative neurological diseases and to potential therapeutics.

7. EVALUATION[2]

The results of the first outbreak of ASP that occurred in 1987 in Canada provide the best basis for developing an ARfD (tolerable single day intake, acute TDI). In this outbreak, a dose-related increase in severity of signs and symptoms was observed in patients consuming between 1 mg/kg b.w. (the LOAEL) and 5 mg/kg b.w. Studies in rodents and cynomolgus monkeys have generally supported these findings.

To cover the full spectrum of intrahuman susceptibility, and account for the fact that 1 mg/kg b.w. was a LOAEL, this value was divided by a safety factor of 10, to derive a provisional ARfD of 0.1 mg/kg b.w. This value seems reasonable, as one person who consumed 0.33 mg/kg b.w. did not become ill. The provisional ARfD of 0.1 mg/kg b.w. provided the basis for the establishment of the maximum residue limit for DA by Canadian authorities, which on the basis of an intake of 250 g shellfish and a b.w. of 60 kg, was 24, rounded down to 20 µg DA/g shellfish. If instead of 250 g shellfish, a value of 300 g shellfish were to have been used, the maximum residue limit would be exactly 20 µg DA/g shellfish.

Very few animal studies have been conducted on the subchronic and chronic toxicity of DA, and these limited data suggest that cumulative effects of low doses of DA are unlikely. In this regard, it was found, based on subacute mouse studies, that there were no differences in behavioural toxicity scores occurring upon re-exposure to DA compared with a single dose (i.e. behavioural equivalent of kindling). The available data indicate that chronic sequelae, such as epilepsy and memory deficit, were only observed in those patients who had suffered severe acute neurological effects (examined up to 3.5 years post the event) after they had ingested a single high dose of DA. It is therefore unlikely that persons who would habitually consume small amounts of DA, with exposures below 0.1 mg DA/kg b.w., would experience any chronic effects, so that this acute reference may also be considered as a provisional chronic TDI.

[2] The Expert Consultation arrived at conclusions close to the draft chapter. The Evaluation Section of the present Background Document is similar to that of the Domoic Acid draft chapter prepared for the consultation.

The best analytical methods currently available for DA are methods employing LC-UV absorbance detection or LC-MS. Several of these have successfully passed single laboratory or interlaboratory validation. Rapid tests based on immunochemistry offer much potential for routine monitoring and are in use in some countries.

With proper monitoring in place, high exposure to DA should not be encountered again. Indeed, no further cases of ASP have been observed in Canada since the introduction of the maximum residue limit of 20 µg DA/g shellfish, and this limit is currently in effect in several jurisdictions.

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Okadaic acid

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1. BACKGROUND INFORMATION[1]

Toxins from the okadaic acid (OA) group have been known to cause human illness since the late 1970s. The syndrome was named diarrhoeic shellfish poisoning (DSP) because of the dominating symptom. The OA group has been detected in microalgae and/or bivalve molluscs globally. Analyses for this group have been a key part of many biotoxin monitoring programmes. However, contamination by the OA group has been generally accompanied by other lipophilic toxins that often cause positives in animal bioassays and require further confirmatory testing to evaluate actual risks. This is leading to development of multitoxin methods based on liquid chromatography with mass spectrometry detection (LC-MS) so that the OA group can be regulated more accurately and quickly. The importance of ester forms is now widely recognized and has implications for testing programmes. Hydrolysis is required for detection of ester forms in methods other than *in vivo* assays. A regulatory level of 0.16 mg OA-eq/kg shellfish is implemented in some countries.

2. ORIGINS AND CHEMICAL DATA[2]

2.1 Origins and distribution

2.1.1 Source organisms

Okadaic acid group toxins are produced by dinoflagellates that belong to the genera *Dinophysis* spp. and *Prorocentrum* spp. Under favourable environmental conditions, these algae may grow to large numbers and produce algal blooms.

The production of these toxins has been confirmed in seven *Dinophysis* species: *D. fortii* (in Japan), *D. acuminata* (in Europe), *D. acuta*, *D. norvegica* (in Scandinavia), *D. mitra*, *D. rotundata* and *D. tripos*, and in the benthic dinoflagellates *Prorocentrum lima*, *Prorocentrum concavum* (or *P. maculosum*) and *Prorocentrum redfieldi* (Viviani, 1992). Three other *Dinophysis* species, *D. caudata*, *D. hastata* and *D. sacculus*, are also suspected (cited from Hallegraeff, Anderson and Cembella, 1995). Giacobbe *et al*. (2000) showed that *D. sacculus* contained OA and dinophysistoxin-1 (DTX1) concentrations of 110–400 and 8–65 fg/cell, respectively. Maximum toxin concentrations (OA+DTX1 455 fg/cell) were found in early spring blooms. The authors suggested that the role of *D. sacculus* in harmful events in the Mediterranean area may be far from negligible despite their low toxicity.

Detection of OA toxins in the heterotrophic dinoflagellates *Protoperidinium oceanicum* and *P. pellucidum* may reflect their feeding on *Dinophysis*. Toxin productivity varies considerably among species and among regional and seasonal morphotypes in one species. For example, *D. fortii* in northern Japan during March and June contains

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[1] Corresponds to the "Background Information" section of the Expert Consultation Report.

[2] It must be pointed out that the Expert Consultation used contributions from the Okadaic Acid drafting group, but that the draft was never finished. To complete the OA chapter, specific material was extracted by Dr Jim Lawrence from the DSP section of *Marine biotoxins* (FAO, 2004).

high concentrations of toxins and is associated with significant accumulation of toxins in shellfish. However, the same species in southern Japan during May and July shows slight toxicity and shellfish is free from toxins (cited from Hallegraeff, Anderson and Cembella, 1995).

Pan, Cembella and Quilliam (1999) reported the production of OA, OA diol ester, DTX1 and DTX4 by *Prorocentrum lima*. Caroppo *et al.* (1999) demonstrated the potential of the non-photosynthetic species *Phalacroma rotundatum* in the southern Adriatic Sea to produce OA, DTX1 and DTX2. The benthic dinoflagellate *Prorocentrum arenarium* isolated from the reef ecosystem of Europa Island (Mozambic channel, France) (Ten Hage *et al.*, 2000) and also *Prorocentrum belizeanum* from the Belizean coral reef ecosystem (United States of America) were found to produce OA (Morton *et al.*, 1998).

2.1.2 Uptake and elimination in aquatic organisms

Okadaic acid toxins associated with *Dinophysis* spp. and *Prorocentrum* spp. are readily accumulated by shellfish and little is known of the retention time of these toxins (cited from Hallegraeff, Anderson and Cembella, 1995). A few studies have described OA toxin kinetics in bivalves under either natural or controlled laboratory conditions. In juvenile and adult bay scallops, dynamics of OA toxins were examined by feeding cells of the epibenthic dinoflagellate *Prorocentrum lima* to scallops in controlled laboratory microcosm. Analysis of the toxins in dinoflagellate cells and scallops tissues was performed by means of LC combined with ion-spray mass spectrometry (LC-MS). Juvenile and adult clearance rates were not inhibited by exposure to *P. lima* cells and no scallop mortalities were seen. Scallops could exceed regulatory toxin limits of 0.2 µg OA group toxin/g wet weight in less than 1 hour of exposure to high *P. lima* cell densities. Toxin saturation levels (2 µg DSP toxin/g wet weight) were attained within 2 days, however toxin retention was very low (< 5 percent). Although most of the total toxin body burden was associated with visceral tissue, weight-specific toxin levels were also high in gonads of adult scallops. Rapid toxin loss from gonads within the first two days of depuration indicated that the toxin was derived primarily from a labile (unbound) component within the intestinal loop section through the gonads. Detoxification of visceral tissue, however, followed a biphasic pattern of rapid toxin release within the first two days of depuration, followed by a more gradual toxin loss over a two-week period, suggesting that faecal deposition may be an important mechanism for rapid release of unassimilated toxin and intact dinoflagellate cells (Bauder and Grant, 1996).

Also Sedmak and Fanuko (1991) observed two phases of toxin release during a decontamination phase of mussels. There is first a rapid decrease in toxin content followed by a slow decrease with the toxicity remaining above the quarantine level of 0.5 MU/g HP. The patterns of contamination and decontamination are specific for shellfish species and do not seem to depend on the type of dinoflagellate toxin.

Toxic scallops (*Patinopecten yessoensis*) cultivated in tubs in which filtered and sterilized seawater was circulated, with or without supply of planktonic diatoms as feed, showed a gradual decrease of DSP during cultivation (microbial assay method). Diarrhoeic shellfish poisoning decreased to 30 percent of initial value within 2 weeks when dense cultures of *Chaetoceros septentrionelle* were supplied as the feed. Relatively high toxicity scores of DSP were detected in excrement of cultivated scallops. When other diatoms, such as *Skeletonema costatum*, *Asterionella japonica*, *Rhabdonema* spp. and *Thalassiosira* spp., were supplied as feed not only the toxicity but also the amounts of glycogen, free amino acids and free fatty acids decreased, causing a deterioration in quality (cited from Van Apeldoorn, 1998).

During decontamination of mussels (*Mytilus galloprovincialis*) from Galicia, Spain, for 70 days under different environmental conditions (salinity, temperature, fluorescence, light transmission), fluorescence and light transmission appeared to have

the most prominent effect on depuration. In most cases, there was an inverse relation between depuration and b.w. It could not be clearly concluded whether the DSP depuration evolved following 1- or 2-compartment kinetics (Blanco *et al*., 1999).

In a study on the feeding behaviour of the mussel *Mytilus galloprovincialis* on a mussel farm in the Gulf of Trieste, Italy, during a DSP outbreak, the mussels seemed to feed selectively on dinoflagellates rather than diatoms. Further selection was observed among different dinoflagellate genera and a preference for the genus *Dinophysis* was particularly evident. The mussels seemed to open the thecae of *Dinophysis* cells and digest them more easily than other dinoflagellates (Sidari *et al*., 1998).

2.1.3 Occurrence in shellfish

In Japan, the shellfish causing DSP were found to be the mussels *Mytilus edulis* and *M. coruscum*, the scallops *Patinopecten yessoensis* and *Chlamys nipponesis akazara*, and the short-necked clams *Tapes japonica* and *Gomphina melaegis*. In European Atlantic coasts, particularly *M. edulis* but also *Ostrea* sp. were contaminated with DSP toxins (Viviani, 1992).

In Japan and the Atlantic coast of Spain and France, the infestation ranges from April to September, and the highest toxicity of shellfish is observed from May to August, though it may vary locally. In Scandinavia, in contrast, oysters in February and mussels in October have caused DSP. Data from the first DSP episode in the Adriatic Sea in 1989 indicated that the infestation period in some coastal areas ranged from May to November (Viviani, 1992).

Comparative analysis in various shellfish from one area in Japan revealed that the highest toxicity was found in blue mussels (*Mytilus edulis*) with less toxicity in scallops, and very little in oysters. Differences in toxicity were also noted between mussels cultivated at different depths, with concentrations differing by factors of two to three (Viviani, 1992). The highest toxicity was obtained in mussels from the upper level (3–6 m), whereas toxicity was reduced to half that level at 6–8 m and 8–12 m (Botana *et al*., 1996). Okadaic acid levels of 0.63 and 4.2 µg/g HP in adjacent mussels were reported within the same mussel growing site and levels of 0.63 and 10 µg OA/g HP in mussels grown at different depths along the same rope (cited from Van Apeldoorn, 1998).

Spanish mussels from Galician Rias contained OA as the major toxin besides less polar DSP toxins. The levels of less polar DSP toxins never exceeded the OA levels. The highest low-polar DSP levels corresponded to the highest OA levels. The authors hypothesized the low polar DSP toxins found in the hexane layer, which is usually discarded, belong to the acyl-derivatives group (Fernández *et al*., 1996).

Data from DSP episodes in the Adriatic Sea showed that not all species of bivalve molluscs absorbed and concentrated the enterotoxin in their tissues to the same extent, although these species were living in the same habitat infested by microalgae. In particular, *Mytilus galloprovincialis*, *Chamelea gallina*, *Tapes decussata* and *Venus verrucosa* were monitored for DSP toxins by means of the MBA, and DSP was detected only in mussels, although they were drawn from the same habitat in the Adriatic Sea. This uneven distribution of DSP will have its impact on developments of sampling plans for shellfish, as part of monitoring schemes for control purposes (Viviani, 1992). In *M. galloprovincialis* from the northern Adriatic Sea OA and DTX1 as well as yessotoxin (YTX) were detected (Ciminiello *et al*., 1997). Cooking did not alter the toxicity of the contaminated shellfish, but intoxication could be avoided if the digestive glands were eliminated beforehand (Viviani, 1992).

Okadaic acid homologues in the alga *D. fortii*, the scallops *Patinopecten yessoensis* and the mussel *Mytilus galloprovincialis*, collected at the same site in Mutso Bay, Japan, were determined (by LC-FL detection). Prominent toxins in scallops and mussels were DTX3 and DTX1, respectively, whereas only DTX1 was detected in *D. fortii*.

Toxin contents in mussels were significantly higher than those in scallops indicating that mussels have a higher potential to accumulate OA homologues than scallops (Suzuki and Mitsuya, 2001).

Persistent low levels of DSP toxins were found in green mussels (*Perna viridis*) from the Johor Strait, Singapore. Six isomers of OA and five of DTX1 were detected, and, generally, the levels of the isomers were higher than that of OA and DTX1. The highest concentration found was 97 ng/g mussel digestive tissue (wet wt.) of an isomer of DTX1 (DTX1a). The maximum level of OA was 24 ng/g. These values were below the threshold limit for consumption (Holmes *et al*., 1999).

Okadaic acid toxins were also widely distributed in different shellfish species along the Chinese coast. Out of 89 samples, 26 contained DTX1 or OA, but only 6 samples contained levels above the regulatory limit for human consumption (20 µg/100 g soft tissue). The highest level of 84 µg/100 g was found in *Perna viridis* from Shenzhen (Zhou *et al*., 1999).

Comments

Not all bivalves absorb and concentrate OA group toxins in their tissues to the same extent, and cooking does not seem to alter the toxicity of contaminated shellfish. Okadaic acid group toxins accumulate in the digestive gland and HP is therefore often used to prepare test portions for analysis.

2.1.4 Other aquatic organisms containing OA toxins

Okadaic acid toxins accumulate into mussels by plankton filter-feeding. However, plankton filter-feeding is largely a non-selective process that is also used by certain fish and may thus lead to accumulation of OA toxins in fish. Also, predators can accumulate significant amounts of toxins in only one meal, given that many bivalve molluscs concentrate toxins in the digestive gland. Okadaic acid may appear in predatory fish as a consequence of their preying on mussels and fish containing OA.

Codfish in cages fed toxic mussels showed the highest concentrations of OA, particularly in the cod liver (0.7 g/g). Lower concentrations were noted in muscle and gonads. Whereas the mussels used for feeding showed the presence of higher concentrations of DTX1 than of OA, DTX1 was nearly absent from fish tissue. After giving non-toxic feed, the OA levels disappeared in 1–2 months' time, least rapid from testis. Analysis of wild fish (cod, sea-cat, shark, herring) caught in Scandinavian waters in January–February 1992, when OA and DTX1 content of mussels in the vicinity was low, showed no OA. No OA was found in refined cod liver oil (cited from Van Apeldoorn, 1998).

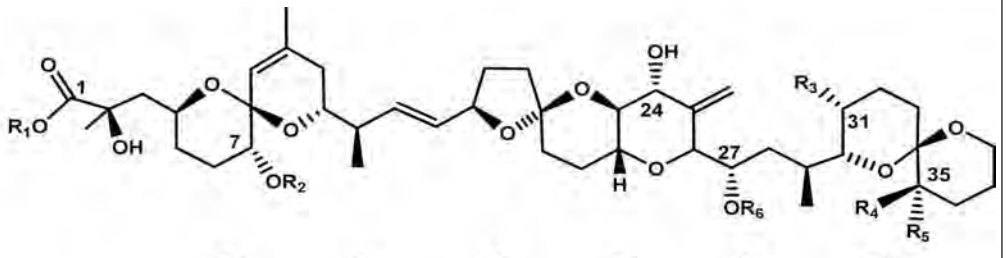
Traditionally, only filter-feeding molluscs are included in monitoring programmes. Shumway (1995) stressed the importance of including also higher-order consumers, such as carnivorous gastropods and crustaceans, in routine monitoring programmes, especially in regions where non-traditional species are being harvested. There are currently no records of OA toxins in gastropods or crustaceans, but this is, undoubtedly, only because no one has looked for them. Based on the data above, it cannot be excluded that OA toxins also accumulate in higher-order consumers.

2.2 Chemical structures and properties

Okadaic acid group toxins are all heat-stable polyether and lipophilic compounds isolated from various species of shellfish and dinoflagellates (Draisci *et al*., 1996) (Figure 1). Although diarrhoea is the most characteristic symptom of intoxication, several other effects may be observed, e.g. nausea, vomiting and abdominal pain (Van Egmond *et al*., 1993). Okadaic acid and its derivatives (DTX1, DTX2 and DTX3) are lipophilic and accumulate in the fatty tissue of shellfish. These compounds are potent phosphatase inhibitors and this property is linked to inflammation of the

intestinal tract and diarrhoea in man (cited from Van Apeldoorn, 1998; Hallegraeff, Anderson and Cembella, 1995). Okadaic acid and DTX1 are also tumour promoters in animal test systems (Draisci *et al*., 1996; Van Egmond *et al*., 1993). DTX1 was first detected in *Dinophysis fortii* in Japan; DTX2 was identified in shellfish in Ireland during a DSP episode (Van Egmond *et al*., 1993). DTX2 was isolated also from a marine phytoplankton biomass mainly consisting of *Dinophysis acuta* (James *et al*., 1997). A new isomer of DTX2, named DTX2B, was isolated and identified in Irish mussel extracts (James *et al*., 1997). DTX3 originally described a group of OA toxin derivatives in which saturated or unsaturated fatty acyl groups are attached to the 7-OH group. More recently, it has been shown that any of the parent toxins, OA, DTX1 and DTX2, can be acylated with a range of saturated and unsaturated fatty acids from C14 to C18 (cited from Hallegraeff, Anderson and Cembella, 1995; Wright, 1995). In a report of a European Union (EU) meeting, it was stated that chain length of the fatty acid can vary from C14 to C22 and that the number of unsaturation varies from 0 to 6. The most predominantly fatty acid in DTX3 was palmitoyl acid (cited from EU/SANCO, 2001). These acylated compounds also possess toxic activity. Because these compounds have only been detected in the digestive gland of contaminated shellfish, it has been suggested that they are probably metabolic products and not *de novo* products of toxin-producing microalgae (Wright, 1995). Suzuki, Ota and Yamasaki (1999) demonstrated the transformation of DTX1 to 7-O-acyl-DTX1 (DTX3) in the scallop *Patinopecten yessoensis*. The ester bond in the acylated compounds can be hydrolysed by heating in 0.5 N NaOH/90 percent methanol solution at 75 °C for 40 minutes. The ester bond in DTX3 was also easily hydrolysed by lipase and cholesterol esterase (cited from EU/SANCO, 2001).

FIGURE 1
Chemical structures of OA and DTXs



	R1	R2	R3	R4	R5	R6
OA	H	H	CH3	H	H	H
DTX1	H	H	CH3	CH3	H	H
DTX2	H	H	H	H	CH3	H
DTX3	H	acyl	as parent	as parent	as parent	H
Diol-ester	diol	H/acyl	as parent	as parent	as parent	H
DTX4	Diol-ester	H	as parent	as parent	as parent	H
DTX5	Diol-ester	H	as parent	as parent	as parent	H
27-O-acyl	H	H	CH3	H	H	acyl

Source: Adapted from Larsen *et al*., 2007.

Two naturally occurring ester derivatives, called diol esters, were isolated from some *Prorocentrum* species. These diol esters did not inhibit phosphatase *in vitro*. However,

it should be noted that these allylic diol esters may be somewhat labile and could be hydrolysed to yield the active parent OA toxin (cited from Hallegraeff, Anderson and Cembella, 1995). Draisci *et al*. (1998) reported the detection of another OA isomer and called it DTX2C. The structure of DTX2C is not yet elucidated. The compound was isolated from *D. acuta* collected in Irish waters.

3. BIOLOGICAL DATA IN MAMMALS

3.1 Biochemical aspects

3.1.1 Mechanism of toxicity

The discovery that OA caused long-lasting contraction of smooth muscle from human arteries was the first clue to elucidation of the mechanism of action of OA group toxins. Because smooth muscle contraction is activated by a subunit of myosin, it was supposed that the effect of OA was because of inhibition of myosin light chain phosphatase. Thereafter, OA was shown to be a potent inhibitor of the serine/threonine phosphatases protein phosphatae 1 and 2A (PP1 and PP2A); PP2A is about 200 times more strongly inhibited than PP1. Protein phosphatases are a critical group of enzymes linked closely with many crucial metabolic processes within a cell. Phosphorylation and dephosphorylation of proteins is one of the major regulatory processes in eukaryotic cells. Processes as diverse as metabolism, membrane transport and secretion, contractility, cell division and others are regulated by these versatile processes. It is indicated that phosphatases, which are sensitive to OA, such as PP1 and PP2A, are involved in entry into mitosis. It is suggested that diarrhoea in humans is caused by hyperphosphorylation of proteins that control sodium secretion by intestinal cells or by increased phosphorylation of cytoskeletal or junctional moieties that regulate solute permeability, resulting in passive loss of fluids (cited from Van Egmond *et al*., 1993 and Hallegraeff, Anderson and Cembella, 1995). Extensive structure-activity studies that measured the inhibition of protein phosphatase activity indicated that a free carboxyl group in the DSP molecule is essential for activity, because methyl and diol esters did not show phosphatase inhibition. However, the amide and reduced carboxyl (okadaol) derivatives are about half as active as OA, as are the naturally occurring DTX3 compounds (cited from Hallegraeff, Anderson and Cembella, 1995).

3.1.2 Pharmacokinetics

3.1.2.1 Studies in mice with OA

Adult Swiss mice received a single oral dose by gavage of 50 or 90 µg [^{3}H]OA/kg b.w. dissolved in 0.2 ml sterile water and methanol (50:50 [v/v]). Urine and faeces were collected during 24 hours and thereafter the animals were killed. At 50 µg/kg b.w., no clinical signs of toxicity were seen, whereas at 90 µg/kg b.w. diarrhoea was observed from 8 hours on. No mortality occurred. In brain, lung, spleen, heart, liver and gall bladder, kidney, stomach, intestine tissue, intestine content, skin, blood, muscle, urine and faeces, radioactivity was determined and OA was analysed with LC (fluorescence detection) after derivatization with 9-anthryldiazomethane (ADAM). Both methods gave similar results indicating that OA was not very much metabolized. Okadaic acid was absorbed from the gastrointestinal (GI) tract as it was found mostly in intestinal tissue and contents (49.2 percent of the dose) and urine (11.6 percent) after 24 hours. The high concentrations in intestinal tissue and contents after 24 hours demonstrated slow elimination of OA. Okadaic acid was found in all tissues. The total amount of OA in organs at 50 µg/kg b.w. was low compared with the amount excreted in urine and faeces (11.6 and 6.6 percent of the dose, respectively) and by far lower than the amount in intestinal tissue plus contents. As the dose increased from 50 to 90 µg/kg b.w., concentrations of OA in intestinal contents and faeces increased proportionally. The increase of OA in intestinal tissue at the higher dose correlated well with the diarrhoea observed. The fact that OA was present in liver and bile and all organs

including skin, and also fluids and the fact that concentrations in intestinal content were approximately 2–7-fold higher than in faeces after 24 hours, confirmed that enterohepatic circulation occurred (see study below). This study also demonstrated that in acute OA intoxication, the concentration in intestinal tissue reaches cytotoxic concentrations in accordance with the diarrhoea seen (Matias, Traore and Creppy, 1999). In recent studies in mice using anti-OA antibody, OA was detected in lung, liver, heart, kidney, and small and large intestines already 5 minutes after oral administration. Okadaic acid was detected in liver and blood vessels for 2 weeks after dosing and in the intestines for 4 weeks (cited from EU/SANCO, 2001).

Male and female adult Swiss mice received a single intramuscular injection with 25 µg [^{3}H]OA/kg b.w. dissolved in 0.1 ml sterile water and methanol (50:50 [v/v]). Okadaic acid was detected in bile and intestinal contents one hour after injection. Its elimination pattern showed biliary excretion and enterohepatic circulation. Administration of cholestyramine, which prevents enterohepatic circulation, changed the cyclic elimination profile of OA (Matias and Creppy, 1996a).

3.2 Acute toxicity in laboratory animals

3.2.1 Studies with mussel extracts

Toxicity of OA group toxins is usually measured by means of i.p. injection of extracts from contaminated mussels in mice. Although this is a crude comparison, it forms the basis of the most widely used screening and quality control methods.

When these toxins are given by the oral route, the lethal dose is 16 times higher than the i.p. dose, but the symptoms are the same (Yasumoto, Oshima and Yamaguchi, 1978).

Three to five mice (4–5 days old) receiving once orally by gavage 0, 0.05, 0.1, 0.2, 0.4 or 0.8 MU DSP toxins, as 0.1 ml of a crude extract from contaminated scallops containing a drop of 1 percent Evans Blue solution per ml animals, were kept for 4 hours at 25 ºC and sacrificed. Whole intestine was removed and fluid accumulation was determined as the ratio of intestinal weight to that of remaining b.w. (FA ratio). FA ratios in control, 0.05, 0.1, 0.2 and 0.4 MU groups, were 0.072, 0.073, 0.09, 0.108 and 0.112, respectively. At 0.8 MU, mortality occurred. The diarrhoeagenicity (as FA ratio) of the components of the crude mixture (OA, DTX1, DTX3, PTX1) in the suckling mice were as follows: OA and DTX1 had the same potency; diarrhoea was seen at doses ≥ 0.1 MU, with DTX3 diarrhoea seen at doses ≥ 0.05 MU, and PTX1 did not show diarrhoeagenicity at the doses tested (0.025–0.4 MU) (Hamano, Kinoshita and Yasomoto, 1986).

3.2.2 Oral studies in mice with OA

After oral administration of 75 µg OA/kg b.w. to adult mice, weights of small intestines were slightly increased within 1 hour (by fluid accumulation), but that of the liver slightly decreased. The lowest observable adverse effect level (LOAEL) in mice by acute oral administration was deduced to be 75 µg/kg b.w. (cited from EU/SANCO, 2001).

Within one hour after oral administration of OA to mice, severe mucosal injuries in the intestine were seen. The injuries could be divided into three consecutive stages (cited from Matias, Traore and Creppy, 1999): extravasion of serum into the *lamina propria* of villi; degeneration of absorptive epithelium of iliac villi; desquamation of the degenerated epithelium from the *lamina propria*.

Rat small intestine was stated to be the most sensitive and reproducible organ for studies of the diarrhoeic effects of marine toxins. When OA was injected in ligated loops from the middle duodenum of male rats (200 g) the following changes were seen within 15 minutes. Enterocytes at the top of the villi became swollen and subsequently detached from the basal membrane. Globet cells were not affected at the doses applied

(1–5 µg OA). After 60–90 minutes, most of the enterocytes of the villi were shed into the lumen and large parts of the flattened villi were covered by globet cells. The degree of the damage was dose-dependent: 3 µg OA affected only the top of the villi, while 5 µg led to collapse of the villous architecture. I.V. injection induced similar but less extensive changes (cited from Van Apeldoorn., 1998).

3.2.3 Oral studies in mice with DTXs

At oral doses of 100, 200, 300 or 400 µg DTX1 to mice, 1/5, 0/5, 2/4 and 3/4 animals, respectively, died (Ogino, Kumagai and Yasumoto, 1997).

3.2.4 Intraperitoneal (i.p.) studies

Thirty minutes to several hours after i.p. injection of OA toxins in mice, inactivation and general weakness were seen, and at sufficiently high concentrations mice died between 1.5 and 47 hours. Concerning the effects reported after oral administration, it is of interest to compare the i.p. toxicity of the different toxins in the OA group (see Table 1).

TABLE 1
Acute toxicity (lethal dose) of DSP toxins after i.p. injection in mice

Toxin	Toxicity (µg/kg b.w.)	Pathological effects
Okadaic acid (OA)	200	Diarrhoea
Dinophysistoxin-1 (DTX1)	160–200*	Diarrhoea
Dinophysistoxin-3 (DTX3)	500	Diarrhoea

* Ogino, Kumagai and Yasumoto, 1997.
Sources: Cited from Van Egmond *et al*., 1993, and Ritchie, 1993, except as indicated.

Mice receiving an i.p. injection with 160 µg DTX1/kg b.w. died within 24 hours while suffering from constant diarrhoea (cited from Van Egmond *et al*., 1993).

After i.p. injections of 50–500 µg DTX1/kg b.w. into suckling mice (7–10 g), duodenum and upper portion of small intestine became distended and contained mucoid, but not bloody, fluid. Villous and submucosal vessels were severely congested at the higher concentrations. No discernible changes in organs and tissues other than the intestines were seen. At ultrastructural level, three sequential stages of changes of intestinal villi were observed as was seen after oral administration (see above).

Marked dilation or destruction of Golgi apparatus suggests that DTX1 may directly attack this organelle (cited from Van Apeldoorn, 1998).

Okadaic acid and DTX1 induce also liver damage in mice and rats after oral as well as i.p. administration. The liver changes were expressed as degeneration of endothelial lining cells at the sinusoid. In addition, dissociation of ribosomes from the rough endoplasmic reticulum and autophagic vacuoles were seen in hepatocytes in midzone of hepatic lobuli. Haemorrhage in subcapsular region of the liver was observed. Furthermore, OA, DTX1 and DTX3 induced damage to the epithelium in the small intestine after both oral and i.p. dosing (cited from Van Apeldoorn, 1998).

3.2.5 Reproduction/teratogenicity studies

Studies in pregnant mice demonstrated the transplacental passage of [^{3}H]-OA by measuring the radiolabelled compound 24 hours after oral administration of 50 µg/kg b.w. (dissolved in sterile water and methanol 50:50) at Day 11 of gestation. Foetal tissue contained more OA than maternal liver or kidney: 5.60 percent of the administered label compared with 1.90 and 2.55 percent, respectively, as measured by scintillation counting and LC with fluorescent detection after derivatization with ADAM (Matias and Creppy, 1996b).

3.2.6 Mutagenic activity of OA

Okadaic acid did not induce mutations in *Salmonella typhimurium* TA 98 or TA 100 in the absence as well as the presence of a metabolic activation system, but it was strongly mutagenic in Chinese hamster lung cells without metabolic activation (mutagenic activity was comparable to that of 2-amino-N6-hydroxyadenine, one of the strongest known mutagens). Diphtheria toxin resistance (DTr) was used as marker of mutagenesis. Results indicated that OA increased the number of DTr cells by induction of a mutation from the DTr phenotype, and not by selection of spontaneously induced DTr cells. The authors suggested that induction of DTr mutation is not because of OA-DNA adduct formation, but probably operates via modification of the phosphorylation state of proteins involved in DNA replication or repair (cited from Aune and Yndestad, 1993).

Using the 32P-postlabelling method, DNA adduct formation was seen in two cell lines (BHK21 C13 fibroblasts and HESV keratinocytes) after treatment with OA for 24 hours (doses 0.01–5 nM). Low doses did not show adduct formation. Intermediate doses have given the most important number of adducts, and with higher doses, the number of adducts decreased dose dependently. Nineteen adducts were observed with BHK21 C13 cells and 15 with HESV cells. Ten adducts were similar in the two strains while 9 were specific of BHK21 C13 cell line and 5 of HESV keratinocytes (Fessard *et al*., 1996).

3.2.7 Tumour-promoting activity of OA and DTX1

Okadaic acid and DTX1 are tumour promoters in two-stage experiments on mouse skin. Okadaic acid and DTX1 do not activate protein kinase C (PKC) as do the phorbol esters, but inhibit the activity of protein phosphatase 1 and 2A, resulting in rapid accumulation of phosphorylated proteins. The effects of OA on protein phosphorylation in cellular systems emphasize the strong tumour-suppressing effect that PP1 and PP2A must have in normal cells. Okadaic acid and DTX1 distinguish themselves from phorbol ester promoters by the fact that they do not bind to the same receptors. Okadaic acid and DTX1 bind to a particulate fraction of the mouse skin. The binding sites of OA are also present in stomach, small intestine and colon, as well as in other tissues (Fujiki *et al*., 1988). Okadaic acid and DTX1 induce ornithine decarboxylase (ODC) in mouse skin (Fujiki *et al*., 1987). Furthermore, OA induced ODC in rat stomach and enhanced the development of neoplastic changes (adenomatous hyperplasia and adenocarcinomas) in the rat glandular stomach after initiation with N-methyl-N'-nitro-N-nitrosoguanidine (Suganuma *et al*., 1992).

Okadaic acid has been shown to promote morphological transformation of carcinogen (3-methyl-cholanthrene)-initiated BALB/3T3 cells. It was demonstrated that OA induced morphological transformation of BALB/3T3 cells also in the absence of an initiator (Sheu *et al*., 1995).

Induction of DNA adducts by OA was shown in baby hamster kidney (BHK) cells, human SV40 immortalised kiratinocytes (HESV) and human bronchial epithelial cells. Also, the induction of DNA adducts in zebra fish embryos was demonstrated. It was noted that the DNA adduct formation increased with the dose at lower and intermediate (non-cytotoxic) concentrations whereas higher concentrations caused toxic stress (Huynh *et al*., 1998).

3.2.8 Immunotoxicity of OA

The effect of OA on peripheral blood monocytes of man *in vitro* by means of effects on the interleukin-1 (IL-1) synthesis was studied. Okadaic acid induced a marked depression of IL-1 production in the monocytes at concentrations of 0.1–1.0 µg/ml. At higher concentrations, OA killed the cells. The suppressive effect of OA on IL-1 is

readily reversed by specific monoclonal anti-OA. The mode of action of this effect of OA is unknown (as cited in Aune and Yndestad, 1993).

3.2.9 In vitro *toxicity*

Okadaic acid and DTX1 were studied for their possible toxicity towards fresh rat hepatocytes by means of light and electron microscopy (cited from Van Apeldoorn, 1998).

Okadaic acid was the more toxic. At 1 µg/ml, blebs on the cell surface were seen. At increasing concentration, blebs increased in size and number. At high concentrations, the cells lost their circular appearance and became irregular. DTX1 showed at 2.5 µg/ml effects similar to those of OA, although to a lower degree. Neither of the purified toxins studied caused enzyme (lactate dehydrogenase) leakage from the cells.

Protein and DNA synthesis in Vero cells (from monkey kidney) were both inhibited by OA in a concentration-dependent manner (IC50 3.3×10^{-8} and 5.3×10^{-8} M, respectively). RNA synthesis was inhibited with an IC50 of 8.2×10^{-8} M. The time lag before DNA and RNA synthesis inhibition occurred was longer (8 hours) than the time lag before protein synthesis occurred (4 hours) indicating that protein synthesis is probably the main target and the first of OA's cytotoxic effect (Matias and Creppy, 1996c).

In a later study (Matias and Creppy, 1999b), the effect of OA on the production of oxygen reactive radicals as possible inducers of impairment of protein synthesis was studied in the presence and the absence of oxygen radical scavengers (SOD+catalase, vitamin E and/or vitamin C). Lipid peroxidation appeared to be a precocious marker of OA exposure. The radical scavengers (partially) prevented the lipid peroxidation, but the inhibition of protein synthesis induced by OA was not reduced to the same level. This indicates that a more specific mechanism might be responsible for inhibition of protein synthesis.

In the cell, free rabbit reticulocyte lysate specific mRNA is translated into globin. This was used to ensure that protein synthesis is a direct target of OA. Indeed, in this system protein synthesis was also inhibited by OA in a concentration-dependent manner (Matias and Creppy, 1996c).

Matias and Creppy (1998) studied the effect of OA on the five nucleosides (deoxycytosine, 5-methyldeoxycytosine, desoxythymidine, deoxyguanidine and deoxyadenine) in the DNA of Vero cells. At 7.5 ng OA/ml, no significant inhibition of DNA synthesis was seen, but hypermethylation of DNA was induced. The level of 5-methyl-deoxycytosine increased from 3.8 to 7.8 percent, indicating possible interference with DNA regulation, replication and expression. Higher levels of OA inhibited DNA synthesis, but failed to increase the rate of DNA methylation. Because OA is involved in tumour production, the most threatening effects are those possibly connected with DNA modification and/or regulation of gene expression, such as the rate of methylation. In other terms, the risks for humans and animals may be more related to repeated exposure to low OA concentrations in seafood that could assault the DNA several times within a life span.

The effect of OA on cultured human intestinal epithelial T84 cell monolayers was studied by measuring electrophysiological parameters, lactate dehydrogenase release, and 22Na+ and [3H]mannitol flux rates. Protein phosphorylation studies were carried out to identify potentially involved proteins. Okadaic acid did not directly stimulate Cl- secretion but increased the paracellular permeability of intestinal epithelia. This alteration may contribute to the diarrhoea of DSP poisoning (Tripuraneni *et al.*, 1997).

3.3 Observations in humans

Shellfish containing > 2 µg OA/g HP and/or > 1.8 µg DTX1/g of HP are considered unfit for human consumption (cited from Hallegraeff, Anderson and Cembella, 1995).

The predominant symptoms in humans are diarrhoea, nausea, vomiting and abdominal pain. The onset of symptoms, which were never lethal, ranged from 30 minutes to a few hours after ingestion of the toxic shellfish, with complete recovery within 3 days. The intensity of the symptoms in humans depends upon the amount of toxin ingested. Hospitalization is usually not needed. Among the DSP toxins, OA, DTX1 and DTX3 are the most important in causing diarrhoea in humans (cited from Aune and Yndestad, 1993). DTX2 was reported to be the predominant diarrhoeic DSP toxin in Ireland during a prolonged DSP episode (Carmody, James and Kelly, 1996). Epidemiological data from Japan (1976–77) indicated that as little as 12 MU was enough to induce a mild form of poisoning in humans (cited from EU/SANCO, 2001). MU was defined as the amount of toxin (later defined as DTX1 in the Japanese study) killing a mouse by i.p injection within 24 hours, and 12 MU corresponded to 43.2 µg, which can be considered as a LOAEL for DTX1 (EU/SANCO, 2001). However, Yasumoto, Murata and Oshima (1985) reported that the minimum dose of DTX1 for the induction of toxic symptoms in human adults was 32 µg. Fernandez and Cembella (1995) reported that 1 MU corresponded to approximately 3.2 µg DTX1 and 4 µg OA, which means that the minimum dose for toxic effects in humans is 38.4 and 48 µg for DTX1 and OA, respectively. The probable human health problems associated with tumour-promoting, mutagenic and immunosuppressive effects shown in animals and experimental systems cannot yet be quantified.

During a DSP episode in Norway in 1984, a few people were hospitalized with symptoms of severe exhaustion and cramps, in addition to the usual DSP symptoms. After i.v. injection of an electrolyte mixture, the patients recovered within a few days (cited from Aune and Yndestad, 1993). In a recent incident in Norway, about 70 people were served blue mussels during the opening ceremony of a new mussel farm. Among the guests, 54 percent were intoxicated with typical DSP symptoms. Diarrhoeic shellfish poisoning toxin levels in the leftovers were about 55–56 µg OA eq/100 g mussel meat (Aune, 2001).

4. ANALYTICAL METHODS

4.1 General

Okadaic acid and its derivatives, the dinophysistoxins (DTX1, DTX2 and DTX3) (Figure 1) may be produced by various *Dinophysis* and *Prorocentrum* species. They can occur in bivalves such as mussels, scallops, oysters or clams. The lipophilic toxins accumulate in the fatty tissue of the bivalves. Therefore, hepatopancreas (HP) is often used to prepare test portions for analysis. Several analytical methods have been published for the determination of these toxins in plankton and bivalves, but very few have been formally validated in collaborative studies according to the harmonized protocol of ISO/IUPAC/AOAC (Horwitz, 1995) so that the performance characteristics are not fully known. This means that only very incidentally can methods for OA group toxins be classified as "reference methods".

Mammalian bioassays for OA toxins are still applied widely, despite the growing resistance against the use of these assays for reasons of animal welfare and their inherent analytical variability. Chemical methods are applied primarily for confirmation of the results obtained in a bioassay, and the first standardized LC method for the determination of OA and DTX is available through the European Committee for Standardization (CEN, 2004). Further attempts to advance, develop and validate chemical methodology for OA and DTXs will be undertaken, e.g. in the STREP project "Biotox", within the European Commission's 6th Framework Programme for Research and Technological Developments (Biotox, 2004).

4.2 Classes of methods

4.2.1 Live animal tests

4.2.1.1 Mouse bioassay (MBA)

The most commonly used assay method is MBA developed by the Japanese Ministry of Health and Welfare (Yasumoto, Oshima and Yamaguchi, 1978; Japanese Ministry of Health and Welfare, 1981). Toxins are extracted from shellfish tissue using acetone and, after evaporation, the residue is dissolved in a small volume of 1 percent Tween 60. The extract is injected intraperitoneally into mice with a b.w. of approximately 20 g and the survival is checked from 24 to 48 hours. One MU (mouse unit) is defined as the minimum quantity of toxin needed to kill a mouse within 24 hours. The toxicity of the sample (MU/g whole tissue) is determined from the smallest dose at which two mice or more in a group of three die within 24 hours. In many countries, the regulatory level is set at 0.05 MU/g whole tissue. In this mouse assay, all OA toxins are likely to be detected. Also toxins that do not cause diarrhoea (pectenotoxins [PTXs] and YTXs) and have an unknown toxicity for humans are detected (see separate chapters). Other unknown toxin groups exhibiting ichthyotoxic and haemolytic properties may cause mortality of mice in this bioassay. Thus, major disadvantages of this assay are the lack of specificity (no differentiation between the various toxins that are detected), subjectivity of death time of the animals, and the maintaining and killing of laboratory animals. In addition, this assay is time-consuming and expensive, may give false positives because of interferences by other lipids (notably free fatty acids have shown to be very toxic to mice [Suzuki *et al*., 1996]) and shows variable results between whole body and HP extracts (as cited in Botana *et al*., 1996, and Van Egmond *et al*., 1993).

The problems observed with the original MBA of Yasumoto, Oshima and Yamaguchi (1978) have led to several modifications (Yasumoto *et al*., 1984; Lee *et al*., 1987; Marcaillou-Le Baut *et al*., 1990). This, in turn, has led to a situation where different countries use different variants of the mouse assay, which calls for harmonization. In an attempt to standardize the methodology of MBA, the European Union (EU) has included directions on how to perform this assay, in its new directive on toxins of the DSP complex and associated toxins (EC, 2002). The EU's Community Reference Laboratory on Marine Biotoxins has conducted an intercalibration exercise that showed reasonable agreement between EU National Reference Laboratories when they applied MBA on unknown shellfish extracts (CRL, 2001).

Fernández *et al*. (1996) warned that some bioassay procedures involve hexane washing steps, to avoid false positive results when free fatty acids are present. The hexane washing step should be reconsidered, taking into account the possible losses of the (lipophilic) DSP toxins, which may be solubilized in the hexane layer. This step must be avoided when analysing samples of unknown origin and with unknown toxin profiles.

4.2.1.2 Suckling mouse assay

In this procedure, an extract of shellfish tissue is administered intragastrically to mice that are 4–5 days old. The degree of fluid accumulation in the GI tract is determined after a four-hour period by measuring the ratio of intestine mass to that of the remaining body. Ratio values above 0.8–0.9 indicate a positive reaction. The assay time is shorter than with MBA, but quantification of the results is much more difficult. Diarrhoea causing substances (OA, DTXs) produce positive reactions. Detection limits for OA and DTX1 are 0.05 and 1 MU, respectively (as cited in Hallegraeff, Anderson and Cembella, 1995, and Van Egmond *et al*., 1993).

4.2.1.3 Rat bioassay

This assay is based on diarrhoea induction in rats. The (starved) animals are fed with suspect shellfish tissue (mixed into the diet) and observed during 16 hours for signs

of diarrhoea, consistency of the faeces and food refusal (Kat, 1983). The method is at best semi-quantitative (as cited in Hallegraeff, Anderson and Cembella, 1995, and Van Egmond *et al*., 1993). The test is still used routinely in the Netherlands and it is an officially allowed procedure in EU legislation (EC, 2002).

4.2.1.4 Daphnia magna assay

An assay in *Daphnia magna* was developed and used to analyse OA in mussel extracts. This method was reported to be inexpensive and sensitive. The method can be used in replacement of MBA for the screening of OA and some co-extracting toxins in mussels. The extraction method used allows OA and DTX1 to be determined. The *Daphnia* bioassay can measure OA levels ten times below the threshold of MBA method (Vernoux *et al*., 1994).

4.2.1.5 Intestinal loop assays

Fluid accumulation in the intestine of intact rabbits and mice has been used to detect OA group toxins. Suspensions of the toxins in 1 percent Tween 60 saline are injected into intestinal loops. A positive result is obtained when the ratio of the volume of accumulated fluid (ml) to the length of the loop (cm) is > 1.0 (Hungerford and Wekell, 1992).

The diarrhoeic activity of algal toxins in blue mussels was determined quantitatively in ligated intestinal loops of the rat by Edebo *et al*. (1988). HP from toxic mussels is disintegrated by freeze-pressing, and the homogenized tissue suspended in an equal amount (w/v) of buffer or in the liquid recovered after steaming. Rapid fluid secretion is seen after injection of the suspension into ligated loops of rat small intestine; maximum is reached within 2 hours (about 300 mg of weight increase per centimetre of intestine). Within a range of 50–200 mg/cm, dose-response relationship is close to linear. Average deviation from the mean is ±9 mg/cm (SD = ±4.9). Mussels yielding less than 100 mg/cm of weight increase per gram of HP were allowed for human consumption, a quantity agreeing with the allowed level of OA. The minimum quantity of OA that produces significant secretion in the rat intestinal ligated loop test is approximately 0.5 µg.

Comments

Although live animal tests for OA group toxicity are applied worldwide, there are large differences in performance of, for instance, MBA (toxicity criterion: animal death; no consensus on appropriate observation time) among different countries, resulting in differences in specificity and detectability. A major problem is the fact that MBA is unspecific and detects many toxins. The rat bioassay detects only OA and DTXs (and azaspiracids [AZAs]), because the criteria in this assay are soft stool, diarrhoea and feed refusal, effects known to be caused by OA and DTXs (and AZAs) only.

There is increasing pressure to replace mammalian bioassays, not only because they are considered less suitable for quantitative purposes, but also because of ethical reasons. In the EU, recently, a recommendation with supportive and convincing documentation has been issued by representatives of governmental institutions of Germany, the Netherlands and the United Kingdom of Great Britain and Northern Ireland and to the members of the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC) to stimulate the development of methodology that can replace the existing bioassays, not only for the OA group, but also for the saxitoxin (STX) group (Hess *et al*., 2006).

4.2.2 Functional assays

4.2.2.1 Cytotoxicity assays

An assay based on morphological changes in fresh rat hepatocytes when exposed to OA toxins has been developed by Aune, Yasumoto and Engeland (1991). With this method, a differentiation between the diarrhoeic lipophilic toxins OA and DTX1, and the non-diarrhoeic toxins PTX1 and YTX, is possible. Okadaic acid and DTX1 induce irregular-shaped cells with surface blebs; PTX1 induces dose-dependent vacuolization; and YTX does not cause changes in the shape of the cells but induced blebs on the surface. For OA and DTX1, the first signs appear at 0.5 µg/ml, for PTX at 5 µg/ml and for YTX at 10 µg/ml. This method is a valuable research tool in the separation between diarrhoeic and non-diarrhoeic lipophilic toxins. However, there are some disadvantages too; it is time-consuming, and confusing results may be obtained in the presence of mixtures of different algal toxins.

Okadaic acid has high toxicity for KB cells (a human cell line derived from epidermoid carcinoma) apparent already after three hours of contact. Amzil *et al*. (1992) developed a method to determine the minimal active concentration (MAC) based on direct microscopic study of toxin-induced changes in cell morphology. A high correlation is found between the MAC of tested extracts and corresponding OA concentrations in mussel HP as measured by LC. Tubaro *et al*. (1996a) developed a quantitative assay for OA using also KB cells. The method shows to be effective in detecting OA in mussel samples at a detection limit of 50 ng/g digestive gland tissue in a 24-hour end-point assay. The dose-dependent cytotoxicity assay is based upon the metabolic conversion of a tetrazolium dye (MTT) to yield a blue-coloured formazan product that can be read for absorbance with a microplate scanning spectrophotometer. Marcaillou-Le Baut *et al*. (1994) reported that results of the cytotoxicity assay with KB cells correlated well with results in the LC or the mouse test (by linear regression analysis).

Pouchus *et al*. (1997) compared the activity of contaminated mussel extracts on KB cells by direct interpretation of morphological changes and by a colorimetric method estimating the number of viable cells after staining. The latter technique reveals interferences, not detected by the former, with mussel cytotoxins. The results show that the technique, based on determination of the minimal active concentration of toxic extracts inducing morphological changes, is specific for OA and preferable to the determination of a 50 percent inhibition concentration (IC50) by a cell culture method.

Okadaic acid and related compounds in mussels possess a high toxicity to Buffalo green monkey (BGM) kidney cell cultures. A detection method for OA and related compounds based on the morphological changes in BGM cell cultures has been developed. A high correlation was found between MBA (Yasumoto *et al*., 1984) and this cytotoxicity test conducted on naturally contaminated samples of *Mytilus galloprovincialis* (Croci *et al*., 1997, 2001).

Other cytotoxicity assays for OA group toxins make use of fibroblasts (Diogene *et al*., 1995), as well as human cell lines (Oteri *et al*., 1998; Fairey *et al*., 2001; Flanagan *et al*., 2001). Further end-points used to assess the cytotoxicity of OA toxins include neutral red uptake (Draisci *et al*., 1998), vital staining (Flanagan *et al*., 2001) and inhibition of cell aggregation and apoptosis (Fladmark *et al*., 1998).

4.2.2.2 Acid phosphatase assays

An assay for DSP based on acid phosphatase activity in the protozoan *Tetrahymena pyriformis* has been developed. Toxins are extracted from shellfish using acetone/ether and cleaned up by silicic acid chromatography. *Tetrahymena* is cultured in the presence of the extract for 24 hours and the 50 percent acid phosphatase activity inhibitory concentration and the growth inhibitory concentration are determined and expressed

as MU equivalents (cited from Van Egmond *et al*., 1993, and Hallegraeff, Anderson and Cembella, 1995).

The specific inhibition of protein phosphatase Type 1 (PP1) and Type 2A (PP2A) by certain analogues (OA and DTX1) was used to develop a phosphatase radio assay using 32P-phosphorylase. The assay is used directly on shellfish extracts and on fractions collected after HPLC separation of the toxins from digestive gland extracts. Although the original technique, which is coupled with toxin fractionation by LC, is not wide in circulation as a regulatory tool, it has been used frequently in screening the phosphatase inhibition activity of putatively phycotoxic compounds and partially purified extracts of phytoplankton and shellfish. In its current format, this assay is based on the inhibition of PP1 by OA with a limit of detection as low as 10 fg OA/100 g tissue. A relatively rapid radioactive protein phosphatase (PP)-based assay has been developed and used (Honkanen, Mowdy and Dickey, 1996; Honkanen *et al*., 1996) to detect OA in oyster (*Crassostrea virginica*) extracts. In more than 320 assessments with spiked oyster samples, all samples containing ≥ 0.2 µg OA/g were positive. From the samples spiked with 0.1 µg OA/g, 16.7 percent were positive. Control samples and samples spiked with 0.02 µg OA/g were negative. A high correlation was seen between the results of this assay and LC.

Although the use of radiolabels in the PP assay leads to low limits of detection, colorimetric and fluorometric assays have been developed to allow a more widespread adoption of the PP assays (cited from Quilliam, 1998). A colorimetric phosphatase-inhibition bioassay has been developed for the quantitative measurement of OA by Simon and Vernoux (1994). The assay uses an artificial substrate, p-nitrophenylphosphate, and a semi-purified protein phosphatase PP2Ac containing extract prepared from rabbit muscle. The lowest detectable concentration of OA is 4 ng/ml in aqueous solutions and 40 ng/ml (i.e. 100 ng of OA per g of mussel tissue) in crude methanol mussels extracts. The rapidity, accuracy, reproducibility (within the laboratory), specificity and simplicity of the procedure provide a simple way to assay OA in buffered or complex solutions.

Tubaro *et al*. (1996b) developed a colorimetric PP assay using p-nitrophenylphosphate and a commercially available PP2A preparation to assess the presence of OA in mussels. The assay, which is employed in the microplate format, is accurate and reproducible (within the laboratory). Okadaic acid is detected in concentrations as low as 0.063 ng/ml in aqueous solutions and 2 ng/g in mussel digestive glands. Thirty naturally contaminated mussel samples were submitted to the PP2A inhibition assay as well as to an enzyme-linked immunosorbent assay (ELISA) and a MTT cytotoxicity assay, with similar results. The assay is sensitive, rapid and does not require expensive equipment according to the authors.

Lower limits of detection are possible with fluorometric PP assays. Vieytes *et al*. (1997) developed a fluorescent enzyme inhibition assay for OA using 4-methylumbelliferyl phosphate and fluorescein diphosphate as substrates for enzyme PP2A. The detection limit of OA is 12.8 ng/g HP in shellfish extracts. According to the authors, this assay can also be used for very dilute samples, such as phytoplankton samples.

Fluorometric protein phosphatase inhibition assays have not only been shown to perform better than colorimetric assays, but also to agree well with MBA and LC techniques (Quilliam, 1998; Vieytes *et al*., 1997; Mountfort *et al*., 1999). However, Mountfort, Suzuki and Truman (2001) have modified the fluorometric assay to overcome the lack of sensitivity towards the ester derivatives of OA and analogues and to reduce significantly the incidence of false negatives observed previously. At the time of writing, a European collaborative study of the fluorometric protein phosphatase inhibition method for the determination of OA and DTX1 was completed. However, the provisional results showed unacceptable performance because of both the study protocol and the poor quality of the PP2A enzyme. If these problems could be

overcome in the near future, the method may be standardized by the CEN because of the interest of this organization for this method, which could serve as an alternative for the currently applied rodent assays in the EU.

An attractive alternative for the protein phosphatase inhibition method could be the PP2A competitive displacement assay for OA and the DTXs. This assay was first described by Serres, Fladmark and Døskeland (2000), and is currently being redeveloped at Biosense® laboratories, Bergen, Norway (Kleivdal, 2004). The assay is based on the competitive binding between the toxin in the shellfish sample or the standard, and a predetermined amount of labelled tracer toxin, to the same inhibition site at PP2A. After the competitive binding step is completed, the amount of toxin in the sample is calculated from the remaining fraction of toxin bound to the PP2A. Because the competitive binding is entirely based on the direct binding of the toxin and the tracer to the inhibition site at the PP2A, and the assay does not rely on the dephosphorylation activity itself, this method is claimed to be less prone to misreporting. In addition, the method does not suffer from problems with the quality of the PP2A enzyme, a problem encountered with the PP2A enzyme inhibition assay (Kleivdal, 2004). The PP2A competitive displacement assay has not been subjected to comprehensive validation, but Biosense® laboratories have performed interlaboratory validation and compared current methodology through the a EC-sponsored project "Biotox", in which Biosense® is a partner (Biotox, 2004).

Comments

Functional assays, which are based on the actual effect of the toxin, are attractive because they mimic the mechanisms of a full-scale MBA. Cytotoxicity (hepatocytes, KB cells) assays and phosphatase assays seem to have potential to determine OA and DTX1. However, their value in practice is to be awaited from interlaboratory validation studies.

4.2.3 Immunoassays

There are several immunodiagnostic methods available for the detection of OA toxins, configured as either radioimmunoassay (RIA) or ELISA tests, all of which incorporate antibodies prepared against a single diarrhoeic agent OA (Hallegraeff, Anderson and Cembella, 1995).

An RIA for OA has been developed by Levine *et al*. (1988) (as cited in Hallegraeff, Anderson and Cembella, 1995). Antibodies to OA were prepared by immunizing rabbits with OA conjugated at the carboxy function to form an amide bond with an amino group of the immunogenic carrier, bovine albumin (using carbodiimide). Competitive binding of OA with 3H-OA in the test system and measurement by scintillation counting allows detection of 0.2 pmoles of toxin (about 0.2 pg/ml). Structurally related marine toxins (among others maitotoxin [MTX], palytoxin [PLTX] and brevetoxin [BTX]) do not inhibit binding of tritiated OA to the antibody.

ELISA test kits have been developed and are commercially available. The DSP-Check® ELISA test kit from UBE Industries, Tokyo, Japan, has been used throughout the world for screening OA and DTX1 at a claimed detection limit of 20 ng/g. Reports about its performance in practice vary. Inconsistencies including false positive responses when applied to either phytoplankton or shellfish samples have been reported many times. However, in a comparative experiment with LC (method of Lee *et al*., 1987), the DSP-Check® test kit was capable of detecting quantitatively DSP toxins in all tested contaminated samples containing only OA, provided that the parent toxins were within the range of detection and were not in the ester form (Vale and De M. Sampayo, 1999). The test was found to be more sensitive, specific and faster than LC.

The monoclonal antibody in the DSP-Check® test kit cross-reacts with DTX1 at a level comparable with OA but PTXs and YTXs are not reactive (cited from Hallegraeff, Anderson and Cembella, 1995). The Rougier Bio-Tech® ELISA test kit utilizes an anti-OA monoclonal antibody and an anti-idiotypic antibody that competes with OA for binding sites on the anti-OA antibody. The antibody in this test kit exhibits a much higher sensitivity (10–20-fold) for OA than either DTX1 or DTX2, and methyl-, diol- and alcohol derivatives of OA will also bind to the antibody, whereas DTX3 and BTX-1 do not cross-react at all. This test kit has undergone extensive comparison with alternative analytical methods for DSP toxins such as high-performance liquid chromatography (HPLC) and LC-MS and is found to be rather reliable for OA quantification in both mussel extracts and phytoplankton (cited from Hallegraeff, Anderson and Cembella, 1995).

Morton and Tindall (1996) compared the DSP Check® test and the Rougier Bio-Tech® test with LC (modification of method of Lee *et al*., 1987), and found both ELISA kits to provide accurate estimations of OA in extracts that were free of methylokadaic acid. However, the DSP Check® test underestimated quantities of total OA in extracts containing both analogues. Because outbreaks of DSP have been associated with OA, methyl OA, or a mixture of these and other related compounds, the ELISA kits may not accurately assess the total toxicity of shellfish samples.

Garthwaite *et al*. (2001) developed an integrated ELISA screening system for amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), paralytic shellfish poisoning (PSP) and DSP toxins; the system detects suspected shellfish samples. Thereafter, the suspected samples have to be analysed by methods approved by international regulatory authorities. Alcohol extraction gave good recovery of all toxin groups.

Immuno technology has also been applied in the development of biosensors for OA group toxins. Botrè and Mazzei (2000) defined a biosensor as "a self-consistent bioanalytical device incorporating a biologically active material, either connected to, or integrated within, an appropriate physico-chemical transducer, for the purpose of detecting –reversibly and selectively – the concentration or activity of chemical species in any type of sample". Marquette, Coulet and Blum (1999) described a semi-automated membrane-based chemiluminescent immunosensor for OA in mussels. The sensor is integrated in a flow injection analysis system. Anti-OA monoclonal antibodies were labelled with horseradish peroxidase for their use in a competitive assay, in which the free antigen of the sample competes with OA, immobilized on commercially available polyethersulphone membranes. The authors investigated the operational stability of the sensor over 38 OA determination cycles and found a stable response for the first 34 measurements. As well, the performance of five immunosensors (five different membranes) showed good repeatability for critically contaminated and blank mussel homogenates, with CVs of 12.6 and 7 percent, respectively. It may be expected that the development and application of biosensors for the determination of toxins of the DSP complex will advance in the coming years.

Another antibody-based technique is the application of immunoaffinity columns (IACs), to purify shellfish extracts prior to the determinative step in analysis procedures, usually LC. Puech *et al*. (1999) described the recent development and the characterization of IACs, which were elaborated using anti-OA monoclonal antibodies, for a specific retention of the OA group of toxins. The coupling yield and the stability of these columns were investigated as well as their capacity to remove interfering compounds. Cross-reactivity was observed between the antibodies and the DTX1 and the DTX2, allowing the detection of the different toxins in a single analysis. Different spiked or naturally contaminated matrices (mussel digestive gland and algae) were tested, and recoveries varied from 55 to 95 percent according to the matrices. The IAC purification was then included as a step of a global IAC/LC/spectrofluorimetric

detection method and the performance of the method was evaluated. Estimations of the linearity and the accuracy (percentages of the presumptive response for OA were in the range +101–114 percent) were satisfactory in accordance with the method validation criteria. Immunoaffinity columns have great potential as clean-up techniques in analytical methods, but their value in practice still has to be proved in interlaboratory validation studies.

Comments
Detection methods based on immunology (ELISA, RIA) are not yet fully developed and not formally validated for OA and the various DTXs. Nunez and Scoging (1997) reported that the ELISA assay detecting OA and/or DTX1, did not accurately detect low concentrations compared with the LC assay, the colorimetric phosphatase inhibition assay and MBA. Gucci *et al*. (1994) did not find either a clear quantitative agreement between four different test methods for DSP (MBA, rat bioassay, ELISA test and LC method). Also Draisci *et al*. (1994) reported that the ELISA method did not always give quantitatively reliable results compared with MBA and the LC method. Morton and Tindall (1996) compared the LC-fluorescence method with two commercially available ELISA test kits for the detection of OA and DTX1 in dinoflagellate cells (*Prorocentrum hoffmanium* and *P. lima*). Although false positive and false negative samples were not detected by the ELISA test kits, both test kits may underestimate total toxins present.

4.2.4 Physico-chemical methods

4.2.4.1 Thin layer chromatography (TLC)

Okadaic acid group toxins can be detected by TLC. After a clean-up (silica gel column chromatography or gel permeation) of the extracts, fractions are applied directly to a silica gel plate and eluted with a toluene-acetone-methanol mixture. The acidic DSP toxins themselves appear as a weak UV-quenching spot at Rf 0.4. Both the diol esters and the free acid toxins give a characteristic pinkish-red stain after spraying with a solution of vanillin in concentrated sulphuric acid-ethanol and allowing to stand at room temperature for several minutes. The free acids produce a bright pinkish-red colour whereas the colour is duller with the diol esters. When clean material is applied to a TLC plate, 1 µg of the toxin could be detected; with cruder fractions, 2–3 µg is required before detection was possible (cited from Hallegraeff, Anderson and Cembella, 1995). These rather high detection limits are a limiting factor for the use of TLC for determining these toxins.

4.2.4.2 Gas chromatography (GC)

Gas chromatography methods have been developed to detect and separate OA toxins. The toxins from diethyl ether extracts of dinoflagellate cultures are first isolated and purified using silicic acid, gel permeation chromatography and reversed-phase partition chromatography. Gas chromatography analysis of trimethylsilyl derivatives of intact toxin and methyl esters is carried out with hydrogen flame ionization detection (as cited in Hungerford and Wekell, 1992). In practice, this technique is rarely used.

4.2.4.3 Liquid chromatography (LC)

The method described below is one of the most commonly used analytical techniques for determination of OA and DTX1. The original method (Lee *et al*., 1987) involves sequential extraction of shellfish tissue with methanol, ether and chloroform; derivatization with 9-anthryldiazomethane (ADAM); silica Sep-pak cleanup; determination by HPLC with fluorescence detection. The ADAM method is very sensitive for DSP toxins being able to detect 10 pg of the OA derivative injected on the column. The minimum detectable concentration in shellfish tissue, however, is limited

not by detector sensitivity but by chemical background, which can vary considerably between samples. The practical quantitation limit is about 100 ng/g tissue. If digestive glands only are used in the analysis, this limit is equivalent to 10–20 ng/g for whole tissue of mussels.

Aase and Rogstad (1997) optimized the sample cleanup procedure for determination of OA and DTX1 with the ADAM derivatization method. The use of a solid-phase extraction silica column of 100 mg and of washing solvents composed of dichloromethane instead of chloroform were proposed to minimize the effect of stabilizing alcohol.

The unstable nature of ADAM and its limited availability have led several researchers to look for alternative derivatization reagents including 1-pyrenyldiazomethane and 1-bromoacetyl-pyrene, N-(9-acridinyl)-bromoacetamide, 4-bromomethyl-7-methoxycoumarin, 2,3-(anthra-cenedicarboximido)ethyltrifluoro-methanesulphonate. The polyaromatic hydrocarbon reagents ADAM, 1-pyrenyldiazomethane (PDAM) and 1-bromoacetylpyrene (BAP) have proved to be the most successful because they are less prone to interferences from reagent and reaction artefact compounds (James *et al.*, 1997). The ADAM-LC method has been collaboratively studied in an interlaboratory validation study conducted by the German Federal Laboratory for fish and fish products (Anonymous, 2001) for the determination of OA and DTX1 in mussel. This method was standardized by CEN as European Standard 14524 in 2004 (CEN, 2004).

DTX3 cannot be analysed directly by this method but must first be converted back to OA, DTX1 or DTX2 via alkaline hydrolysis. The diol esters of the DSP toxins cannot be analysed by the ADAM-LC method (cited from Hallegraeff, Anderson and Cembella, 1995, and Van Egmond *et al.*, 1993).

4.2.4.4 Micellar electrokinetic chromatography (MEKC)

Micellar electrokinetic chromatography with UV detection was applied to the determination of non-derivatized OA toxins. Okadaic acid was detected in mussels spiked with 10 ng/g whole tissue, and the presence of OA and DTX2 was observed in the crude extract of the dinoflagellate *Prorocentrum lima* (Bouaïcha, Hennion and Sandra, 1997).

4.2.4.5 Mass spectrometry (MS)

Hallegraeff, Anderson and Cembella (1995) reported the analysis of diol esters of OA, DTX1 as well as DTX3 toxins. Liquid chromatography combined with electrospray ionization mass spectrometry (LC-ESI-MS) appears to be a sensitive and rapid method of analysis for OA toxins. A detection limit can be achieved of 1 ng/g in whole edible shellfish tissue. Various analytical procedures continue to be developed for the determination of OA toxins, and recent reviews have described a comprehensive range of methods (cited from Quilliam, Hess and Dell'Aversano, 2001). Diarrhoeic shellfish poisoning profiling of bivalves (scallops and mussels) with LC-MS has been reported by Suzuki and Yasumoto (2000). They focused on OA, DTX1 (and PTX6). Negative electrospray ionization (ESI)-mode was found to be much more efficient than positive ESI-mode.

Matrix effects in the OA toxin analysis with LC-ESI-MS have been tackled in different ways. Suzuki and Yasumoto (2000) successfully used an alumina B column for sample clean-up. Hummert, Reichelt and Luckas (2000) applied size exclusion chromatography (SEC) for the clean-up of raw extracts from algae and mussel tissue containing either microcystins or OA group toxins. Although it is likely that improvements were obtained, the article fails in demonstrating that matrix effects could be removed completely (recovery data are missing, spiking was not applied).

Goto *et al*. (2001) paid more attention to the chemical properties of the different OA toxins by applying different extraction solvents and solvent partitioning.

Ito and Tsukada (2001) conducted an explicit study on matrix effects. They demonstrated a better performance by applying the standard addition method to each separate sample, which however requires two LC-MS runs per analysis. An alternative method, where the response factor was based on one model sample, was less satisfactory. The study demonstrates and emphasizes the matrix effect from shellfish extracts and demonstrates how that effect can be tackled for quantification purposes.

In the second half of 2002, an interlaboratory study took place of a new LC-MS method for determination of ASP and DSP toxins (including OA and DTX in shellfish [Holland and McNabb, 2003]). The eight participating laboratories generally obtained consistent sets of data for the broad group of analyte toxins down to low levels (< 5 ng/ml, equivalent to 0.05 mg/kg). In general: detectability is adequate to achieve the limits of detection (LODs) required. Most of the participating laboratories could detect the analyte toxins; and greater differences were observed for quantitation of some toxins, especially when no analytical standards were present. The participants used different MS detection modes. Some used single MS detection, others used tandem MS detection (MS/MS), and some used both. Although the use of the MS/MS mode is attractive in order to enhance specificity, it requires additional care for quantitation. Summarizing, the study was stimulating and encouraging for those who are interested in using an alternative method for MBAs.

Using minimal amounts of standards, supplied by internationally accepted authorities, and contaminated shellfish extracts, a sample extraction and multiple toxin LC-MS technique has been developed for the quantitative detection of OA, DTX1, DTX2 and various other lipophilic toxins in shellfish. A further previously described LC-MS technique (Mountfort *et al.*, 1999) with an additional sample hydrolysis step has also been assessed for the detection of diol esters and DTX3 compounds. The multiple toxin LC-MS method has been validated for OA and some of the other lipophilic toxins (Stobo *et al*., 2005).

Comments

Physico-chemical methods (mainly LC) are useful for identification and quantification of OA or DTXs and the first validated method for OA and DTX1 has been standardized by CEN. For the other DSP toxins, some LC methods exist, but they have not yet been validated. The rapid developments in LC-MS methodology are promising, but improvement and interlaboratory studies will be necessary before these techniques can become generally accepted tools in regulatory analysis. A serious problem is that pure analytical standards and reference materials are hardly or not readily available, which hampers the further development and validation of analytical methodology for the various OA toxins. As is the case for all analytical methods for these toxins, also with the chemical methods, it should be realized that the hexane layer, usually discarded, can be rather rich in these lipophilic toxins.

4.3 Reference methods

At the time of writing, only one quantitative method for OA (and DTX) has been formally validated through an interlaboratory validation exercise, carried out according to the harmonized ISO/IUPAC/AOAC protocol. The method is essentially a modified version of the procedure published by Lee *et al.* (1987). The method was collaboratively studied in Europe (Anonymous, 2001) and the study was evaluated by the CEN working group on biotoxins. The method has been standardized as method CEN EN 14524 (CEN, 2004). Some characteristics of the method are summarized below and in Table 2.

Scope

This European Union (EU) Standard specifies a method for the quantitative determination of the content of OA in mussels and mussel products. The content of OA is determined as free extractable acid of mussel hepatopancreas (HP). Okadaic acid, a fat-soluble toxin from dinophysis algae, is a main component of dinophysis toxins. The method has been validated in an interlaboratory study according to ISO general principles on assessing accuracy of measurement methods and results. The limit of determination of this method (signal/noise = 10) is 100 µg/kg for OA in mussel HP. The method has been validated for OA in cooked mussels at levels of 441–1 467 µg/kg. Laboratory experiences have shown that this method can also be used to determine other dinophysis toxins, e.g. DTX1, DTX2 and DTX3 (Lee *et al*., 1987; Stockemer and Gürke, 1993; Quilliam, Gago-Martínez and Rodríguez-Vázquez, 1998; Luckas and Meixner, 1998; Comesana-Losada *et al*., 1999; Fernandez *et al*., 1996).

Principle

Mussel HP is separated and homogenized. The toxins are extracted using methanol, derivatized with ADAM and the extract is cleaned up using a solid phase extraction (SPE) cartridge with silica gel. Chromatographic separation is performed on a gradient system with two HPLC pumps, followed by fluorescence measurement of the 9-anthryldiazomethyl ester of the toxin at 412 nm with excitation at 365 nm. Determination of OA is performed using the method of external standards. Variation of the clean-up step and/or the chromatographic conditions allows the detection of other DTXs.

TABLE 2
Performance characteristics as obtained in the collaborative study

Sample	A[a]	B[b]	C[c]	D[d]
Year of interlaboratory test	2000	2000	2000	2001
Number of laboratories	9	9	9	9
Number of laboratories retained after eliminating outliers	9	9	9	9
Number of outliers (laboratories)	0	0	–	0
Number of accepted results	45	45	–	52
Mean value $\bar{x}$, µg/kg	819	1 467	0	440.7
Repeatability standard deviation s_r, µg/kg	80	129	0	38.8
Repeatability relative standard deviation (RSD_r), %	9.76	8.79	0	8.89
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	226	365	0	109.7
Reproducibility standard deviation s_R, µg/kg	98	174	0	53.3
Reproducibility relative standard deviation (RSD_R), %	11.96	11.86	0	12.23
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	278	492	0	150.9
Recovery, %	102[e]	101[e]	–	97[e]

[a] OA contaminated mussel material from Wadden sea area harvested October 1995; stored at –20 °C at VUA Cuxhaven since Oct. 1995. Determination in 1995: 880 µg OA per kg material; result of homogeneity study (Dec. 1999): 800 µg/kg.
[b] Uncontaminated mussel material from Wadden sea area, harvested July 1999, stored at –20 °C at VUA Cuxhaven since July 1999; addition of 1 500 µg/kg OA per kg material; result of homogeneity study (Dec. 1999): 1 450 µg/kg.
[c] Uncontaminated mussel material from Wadden sea area, harvested July 1999, stored at –20 °C at VUA Cuxhaven since July 1999.
[d] Uncontaminated mussel material from Wadden sea area, harvested July 1999, stored at –20 °C at VUA Cuxhaven since July 1999; addition of 450 µg/kg OA per kg material; result of homogeneity study (Dec. 2000): 453 µg/kg.
[e] Based on homogeneity study result.
Source: CEN, 2004.

4.4 Need for standards and reference materials

As is the case with many of the marine biotoxins, sources for standards and reference materials for OA and the various DTX are rather scarce. Whereas calibrants for few of these materials are available from some commercial sources (Calbiochem, NRC Canada, Sigma, Wako Chemicals), (certified) reference materials (shellfish matrices with specified concentrations of toxins) are currently only available for OA in mussel material from NRC Canada.

5. LEVELS AND PATTERNS OF CONTAMINATION OF SHELLFISH

The number of DSP incidences, or at least the presence of DSP, appears to be increasing. This may be partly because of increasing knowledge about the disease and better surveillance programmes. However, it must be noted that toxin-producing algae and toxic molluscs are frequently reported from new areas (cited from Aune and Yndestad, 1993).

Diarrhoeic shellfish poisoning was first documented in 1976 from Japan, where it caused major problems for the scallop fishery. Between 1976 and 1982, some 1 300 DSP cases were reported in Japan; in 1981, more than 5 000 cases were reported in Spain; and in 1983, some 3 300 cases were reported in France. In 1984, DSP caused a shutdown of the mussel industry for almost a year in Sweden. The known global distribution of DSP includes Japan, Europe, Chile, Thailand, Canada (Nova Scotia) and possibly Tasmania (Australia) and New Zealand (cited from Hallegraeff, Anderson and Cembella, 1995).

In Japan, *Dinophysis fortii* has been incriminated as the organism producing DSP toxins (cited from Van Egmond *et al.*, 1993, and Viviani, 1992). However, in northern Japan, Sanriku coast, the OA-producing *Prorocentrum lima* occurred. The dinoflagellate was distributed on the surface of the algae, *Sargassum confusum* and *Carpopeltis flabellata.* This *P. lima* strain grew well in T1 medium at 15 °C, at which tropical strains do not grow, indicating that it is a local strain that adapted to cooler environments (Koike *et al.*, 1998).

On European Atlantic coasts, other dinoflagellate species are also involved: *D. acuminata* and *D. acuta* in Spain; *D. acuminata*, *D. sacculus*, *P. lima* in France; *D. acuminata*, *P. redfieldii* and *P. micans* in The Netherlands (cited from Van Egmond *et al.*, 1993, and Viviani, 1992); *D. acuta*, *D. sacculus*, *D. acuminata*, *D. caudata* and *P. lima* in Portugal (cited from Van Egmond *et al.*, 1993), *D. acuta*, *D. acuminata*, *P. lima* and *P. concavum* in Ireland; *D. acuta*, *D. acuminata*, *D. norvegica*, *P. micans*, *P. minimum*, *P. lima* in Scandinavia; and *D. sacculus*, *D. acuminata*, *D. tripos*, *D. caudata* and *D. fortii* in the Adriatic Sea (cited from Van Apeldoorn, 1998; Ciminiello *et al.*, 1997; Marasović *et al.*, 1998; Giacobbe *et al.*, 2000).

In the Gulf of Mexico, *D. caudata* was involved; in the Australian region *D. fortii*, *D. acuminata* and *P. lima;* and in eastern Canada, *D. norvegica* and *P. lima* (cited from Van Apeldoorn, 1998). In Johor Strait, Singapore, *D. caudata* was the most frequent and abundant species from March 1997 to February 1998. Other dinoflagellates observed were *Prorocentrum micans* and *Protoperidinium* spp. (Holmes *et al.*, 1999).

Phalacroma rotundatum, which has the potential to produce toxins of the OA group, was observed in Japanese waters, in northwest Spain (Ria Pontevedra) and along the southern Adriatic coast of Puglia (Italy) (Caroppo *et al.*, 1999).

Along the Chinese coasts in the South and East China Sea, DTX1 and OA were detected in shellfish species, implying that producers of OA and the DTXs also exist in this area. Frequency and shellfish toxin levels in southern parts of the coast were greater than those in northern areas (Zhou *et al.*, 1999).

D. acuminata and *Prorocentrum minimum* occurred in large numbers in the Peter the Great Bay (Sea of Japan, the Russian Federation) in summer 1995 and 1996 (Orlova, Selina and Stonik, 1998).

The benthic dinoflagellate *Prorocentrum arenarium* isolated from the reef ecosystem of Europa Island (Mozambic channel, France) (Ten Hage *et al.*, 2000) and also *Prorocentrum belizeanum* from the Belizean coral reef ecosystem (the United States of America) were found to produce OA (Morton *et al.*, 1998).

6. DOSE RESPONSE ANALYSIS AND ESTIMATION OF CARCINOGENIC RISK

In animal experiments, cancer-promoting and genotoxic effects of OA and DTXs are seen at relatively high doses and long exposure periods compared with the levels causing diarrhoea in humans shortly after consumption of contaminated shellfish. Consequently, it is unlikely that a substantial risk of cancer exists in consumers of shellfish because of these toxins. Therefore, human risk assessment is based on a N(L)OAEL from animal or human data with the use of an uncertainty factor. Human data are preferred when available.

Taking into account all human exposure figures, it can be concluded that the lowest levels causing diarrhoeic effects in humans vary from 32 to 55 µg OA and/or DTX1. These figures have been derived from Japanese and Norwegian human data. The effects seem to be restricted to diarrhoea, vomiting, headache and general discomfort. No serious and irreversible adverse health effects were seen at these levels (EU/SANCO (2001). Current EU Regulations allow maximum levels of OA, DTXs and PTXs together of 160 µg OA eq/kg edible tissue. If the consumption of shellfish is estimated to be between 100 and 300 g/meal, there is a margin of safety of about < 1–3.4 towards the diarrhoeic effects. These margins are quite small or there is no margin at all. EU/SANCO (2001) stated that, if the level of OA and DTXs in shellfish is not higher than 16 µg/100 g shellfish meat, there is no appreciable health risk at a consumption of 100 g mussel meat/day.

7. EVALUATION[3]

Okadaic acid and DTXs possess tumour promoting activity, and OA also shows genotoxic and immunotoxic activity. These effects raise questions as to the human health risks of (sub)chronic exposure to low levels of these compounds. A pressing problem is the lack of sufficient quantities of purified toxins to perform (sub)chronic animal toxicity studies. The 2004 Expert Consultation found that because of insufficient data on the chronic effects of OA, no TDI could be established.

The Expert Consultation established a provisional acute reference dose (ARfD) of 0.33 µg OA equ/kg b.w., based on the LOAEL of 1.0 µg OA/kg b.w., and a safety factor of 3 because of documentation of human cases including more than 40 persons and because DSP symptoms are readily reversible.

Gaps in the data

More studies on pharmacokinetics are needed. To establish a TDI, data on long-term/carcinogenicity and further studies on genotoxicity and reproductive toxicity are needed.

Although mammalian bioassays for OA group toxicity are applied worldwide, there are large differences in performance of, for instance, MBA (toxicity end-point is animal death; no consensus on appropriate observation time) among different countries, resulting in differences in specificity and detectability. A major problem is the fact that MBA detects all DSP components and probably also other toxins. However, it is not possible to distinguish between the various toxins whereas specific legal limits for the toxin groups have been established, e.g. in the EU. On the other hand, the rat bioassay detects only OA and DTXs (and possibly azaspiracids [AZAs]), because the

[3] Evaluation section of the present Background Document is taken from the Expert Consultation Report.

end-points in this assay are soft stool, diarrhoea and feed refusal, effects known to be caused by OA and DTXs only (and AZAs).

Chemical methods (LC) are useful for identification and quantification of selected diarrhoeic toxins (usually OA or DTXs). Chemical methods are applied as a regulatory tool primarily for confirmation of the results obtained in a bioassay.

None of the many approaches to determine OA group toxins in shellfish has been evaluated in a formal collaborative study according to ISO/IUPAC/AOAC, so that the performance characteristics are not fully known. The further development, evaluation and comparison of the various techniques would become significantly easier if reliable reference standards and reference materials (e.g. lyophilized mussel samples with certified contents of several OA toxins) could be developed and made available to the scientific community.

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Pectenotoxins

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1. BACKGROUND INFORMATION[1]

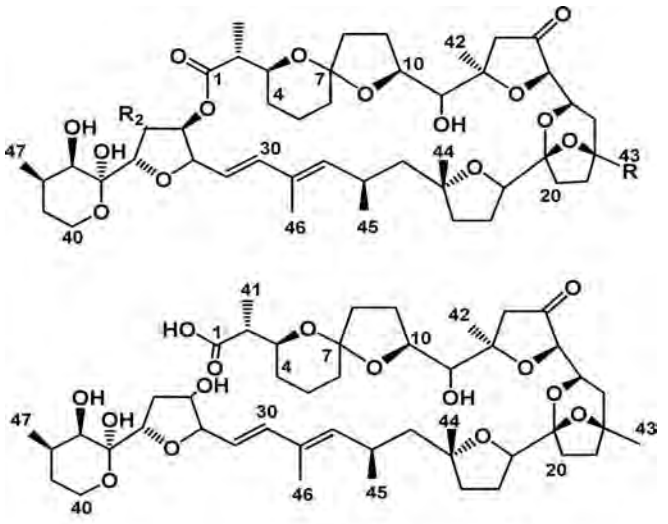
The presence of pectenotoxins (PTXs) in shellfish was discovered because of their high acute toxicity in mouse bioassay (MBA) after i.p. injections of lipophilic extracts. Pectenotoxins have been detected in microalgae and/or bivalve molluscs in Australia, Italy, Japan, New Zealand, Norway, Portugal and Spain. Animal studies indicate that they are much less potent via the oral route and that they do not induce diarrhoea. There are no data indicating adverse effects in humans associated with PTXs in shellfish. Pectenotoxins exclusively arise from *Dinophysis* spp. and are always accompanied by toxins from the okadaic acid (OA) group. Therefore, analytical methods must reliably distinguish these toxins, because they should be regulated separately. The provisional action level for PTX seco acids (20 mg/100 g shellfish) is implemented in some countries.

2. ORIGINS AND CHEMICAL DATA

Pectenotoxins are polyether macrolide toxins frequently associated with incidents of diarrhoeic shellfish poisoning (DSP) because of their co-occurrence with OA and dinophysistoxins (DTXs) (Figure 1). Pectenotoxin-2 (PTX-2) is also produced by the toxic dinoflagellate, *Dinophysis* spp. (Draisci *et al*., 1996; Draisci *et al*., 1999; James *et al*., 1999; Lee *et al*., 1989; MacKenzie *et al*., 2002; Pavela-Vrancic *et al*., 2001; Sasaki *et al*., 1999; Suzuki *et al*., 1998, 2003; Vale and Sampayo, 2002). It has been shown that pectenotoxin-1 (PTX-1), pectenotoxin-3 (PTX-3) and pectenotoxin-6 (PTX-6) are metabolites formed by oxidative conversion of PTX-2 in Japanese scallops *Patinopecten yessoensis* (Suzuki *et al*., 1998). PTX-2 seco-acid and its epimer 7-*epi*-PTX-2 seco acid (Daiguji *et al*., 1998) are metabolites of PTX-2 in mussels, *Perna canaliculus*, *Mytilus galloprovincialis*, and New Zealand scallops, *Pecten novaezelandiae* (Suzuki *et al*., 2001). Because PTX-2 seco acid is not toxic to mice after either oral administration or i.p. injection (Miles *et al*., 2004), the conversion from PTX-2 to PTX-2 seco acid in bivalves is a positive attribute in terms of detoxification. It has been shown that PTXs can undergo acid-catalyzed isomerization at the spiroketal carbon of the A/B ring system by ring opening and re-closure of the system (Suzuki *et al*., 2003; Sasaki, Wright and Yasumoto, 1998). Pectenotoxins are easily destroyed under strong basic conditions such as those used for hydrolysis of acyl esters of the OA groups.

[1] Corresponds to the "Background Information" section of the Expert Consultation Report.

FIGURE 1
Pectenotoxins (PTXs)



Note: The complete macrocyclic ester of the PTX-2 structure (and derivatives) is shown in the top figure while the bottom figure shows the structure of the hydrolysed compound (PTX-2SA), where the macrocylce is opened.

3. BIOLOGICAL DATA IN MAMMALS

3.1 Biochemical aspects

3.1.1 Absorption, distribution and excretion

A preliminary study of the absorption, distribution and excretion of PTX-2 and PTX-2 seco acid has been conducted (Burgess, 2003). After oral administration, significant amounts of the test compounds were found in the gastrointestinal (GI) contents and faeces, with only traces in tissue and urine. Analytical methods for only the administered compounds were available, however, and the total recovery was low. After i.p. injection, PTX-2 and PTX-2 seco acid were detected in the blood and internal organs, as well as in the GI tract and faeces, but again the total recovery was low.

3.1.2 Biotransformation

No information on pathways of PTX metabolism in mammals has been found.

3.1.3 Effects on enzymes and other biochemical parameters

PTX-2 was shown to form a 1:4 complex with G-actin (Hori *et al.*, 1999). The effects of the PTXs on actins and cytoskeletal structure within cells are described in Section 3.2.6.

3.2 Toxicological studies

3.2.1 Acute toxicity

The acute toxicities of PTX derivatives to mice are summarized in Table 1. PTX-1, PTX-2 and PTX-11 are of similar toxicity to mice by i.p. injection (Yasumoto *et al.*, 1985; Yoon and Kim, 1997; Miles *et al.*, 2004; Suzuki *et al.*, 2006), while PTX-3, 4 and

6 are less toxic (Yasumoto *et al*., 1989). PTX-7, 8 and 9, and PTX-2-seco acid are very much less toxic than the other derivatives (Sasaki *et al*., 1998; Miles *et al*., 2004). No deaths were recorded with the seco acid at 5 000 µg/kg, the highest dose-level that was employed (Miles *et al*., 2004). Fewer data are available on the oral toxicity of the PTXs. On the basis of the study by Ogino, Kumagai and Yasumoto (1997), it could be argued that the acute toxicity of PTX-2 administered by gavage is similar to that following i.p. injection. The data of this study are difficult to interpret, however, because the incidence of death was not dose-dependent. The mortality recorded at a dose of 25 µg/kg (25 percent) was higher than that seen in mice given 100 µg/kg (0 percent) or 200 µg (20 percent), while that recorded at a dose of 400 µg/kg (25 percent) was lower than that seen at 300 µg/kg (40 percent). A later study did not confirm the apparently high oral toxicity of PTX-2. No deaths were observed at an oral dose of 5 000 µg/kg, and the subsequent appearance and behaviour of the dosed mice were entirely normal (Miles *et al*., 2004). No signs of toxicity were recorded in mice given PTX-2 seco acid by gavage at 5 000 µg/kg (Miles *et al*., 2004).

TABLE 1
Acute toxicity of PTX derivatives in mice

Compound	Route of administration	Sex	Parameter	Acute toxicity (µg/kg b.w.	Reference
PTX-1	Intraperitoneal	?	MLD	250	Yasumoto *et al*., 1985
PTX-2	Intraperitoneal	?	MLD	260	Yasumoto *et al*., 1985
PTX-2	Intraperitoneal	?	LD_{50}	411	Yoon & Kim, 1997
PTX-2	Intraperitoneal	Female	LD_{50}	219 (183–257)*	Miles *et al*., 2004
PTX-3	Intraperitoneal	?	MLD	350	Yasumoto *et al*., 1989
PTX-4	Intraperitoneal	?	MLD	770	Yasumoto *et al*., 1989
PTX-6	Intraperitoneal	?	MLD	500	Yasumoto *et al*., 1989
PTX-7	Intraperitoneal	?	MLD	>5 000	Sasaki *et al*., 1998
PTX-8	Intraperitoneal	?	MLD	>5 000	Sasaki *et al*., 1998
PTX-9	Intraperitoneal	?	MLD	>5 000	Sasaki *et al*., 1998
PTX-11	Intraperitoneal	Female	LD_{50}	244 (214–277)*	Suzuki *et al*., 2006
PTX-2 seco acid	Intraperitoneal	Female	MLD	>5 000	Miles *et al*., 2004
PTX-2	Oral (gavage)	Male	LD_{50}	~200**	Ogino *et al*., 1997
PTX-2	Oral (gavage)	Female	MLD	>5 000	Miles *et al*., 2004
PTX-2 seco acid	Oral (gavage)	Female	MLD	>5 000	Miles *et al*., 2004

* Figures in brackets indicate 95% confidence limits.
** This estimate is questionable because of the absence of a dose-response in this study (see text).

Symptoms of intoxication were observed within minutes of injection of toxic doses of PTX-2. The animals became hunched and lethargic, and showed ataxia (Yoon and Kim, 1997; Miles *et al*., 2004). Subsequently, respiration became laboured, with abdominal breathing, and the respiration rate progressively decreased (Miles *et al*., 2004). Cyanosis and a decrease in body temperature were also recorded (Yoon and Kim, 1997). Deaths generally occurred between 4 and 10 hours after dosing, although one animal, receiving a dose close to the median lethal dose (LD_{50}), survived for 22 hours. At this time, it was in very poor condition, and was humanely killed (Miles *et al*., 2004).

The ability of PTX derivatives to cause diarrhoea has been a matter of dispute. PTX-1 did not cause diarrhoea when injected intraperitoneally into suckling mice (Terao *et al*., 1986), or when given by gavage (Hamano, Kinoshita and Yasumoto, 1986). Furthermore, the latter authors showed that PTX-1, unlike OA or the DTXs, caused no fluid accumulation in rabbit or mouse intestinal loops. Similarly, mature mice dosed either orally or intraperitoneally with PTX-2 showed no diarrhoea. No macroscopic changes were recorded in their intestines, and the consistency of the

intestinal contents of these animals was entirely normal (Munday, Munday and Miles, 2004). In contrast, Ishige, Satoh and Yasumoto (1988) reported viscous or watery diarrhoea after oral administration of PTX-2. The diarrhoea was associated with dilatation of the intestines, which were full of watery material. A mixture of 35 percent PTX-2 seco acid and 65 percent 7-*epi*-PTX-2 seco acid was also reported to cause diarrhoea in mice after oral intubation (Burgess *et al*., 2002). However, later work by these authors indicated that the previously observed diarrhoea was most likely because of contamination of the PTX-2 seco acids with OA esters (Burgess and Shaw, 2003), and no diarrhoea or any other toxic effects were seen in mice dosed with a different batch of PTX-2 seco acid (Burgess, 2003).

Yoon and Kim (1997) reported a decrease in liver weight in mice dosed with PTX-2, although this was not confirmed in a later experiment (Munday, Munday and Miles, 2004). In the latter study, i.p. injection of PTX-2, at doses close to the LD_{50}, caused no change in the relative hepatic, cardiac, splenic or pulmonary weights of the animals. A significant increase in renal weight was observed, however, in animals receiving PTX-2.

Terao *et al*. (1986) reported that by 60 minutes after i.p. injection of 1 000 µg/kg of PTX-1, the surface of the liver of suckling mice was finely granulated. Ascites and severe pleural effusion were recorded in mature mice injected with PTX-2 at 250 µg/kg (Munday, Munday and Miles, 2004). No other macroscopic changes were recorded in mice dosed with PTX-1 or PTX-2 (Terao *et al*., 1986; Munday, Munday and Miles, 2004).

Serum activities of alanine aminotransferase, aspartate aminotransferase and sorbitol dehydrogenase were significantly elevated in mice injected with PTX-2, but glucose-6-phosphatase activities were unchanged (Yoon and Kim, 1997).

Malondialdehyde levels were increased in the urine of some mice receiving PTX-2 and PTX-2 seco acid (Burgess, 2003). There was no dose-response relationship, however, and at the end of the experiment, the animals employed in this study were found to be suffering from a urinary tract infection.

Some data on the histological changes in animals dosed with PTX derivatives are available. Terao *et al*. (1986) observed multiple vacuoles in the periportal region of the livers of suckling mice 60 minutes after i.p. injection of PTX-1 at 1 000 µg/kg. The vacuoles were up to ~15 µm in diameter and did not contain fat. Similar changes were recorded in the livers of mice given 500 or 700 µg/kg of PTX-1 2 hours after dosing, and eosinophilic granules were also seen in the vacuole-containing hepatocytes. Only minor changes were seen in the livers of mice receiving the test compound at 150 or 200 µg/kg. After 24 hours, almost all hepatocytes containing numerous vacuoles and granules had become necrotic. Electron microscopy showed that the vacuoles resulted from invagination of the hepatocytic plasma membrane. No histological changes were observed in the small and large intestines of the mice, or in their kidneys or hearts (Terao *et al*., 1986). In contrast, it was reported (Ishige, Satoh and Yasumoto, 1988) that mice dosed orally with PTX-2 at 250 µg/kg or more showed damage to the mucous membranes of the GI tract, with vacuolation and necrosis of villi. Hyaline droplets and granular degeneration of hepatocytes was also noted in the peripheral areas of the liver lobule, with congestion and vacuolar degeneration. At an oral dose-level of 875 µg/kg, a mixture of 35 percent PTX-2 seco acid and 65 percent 7-*epi*-PTX-2 seco acid caused necrosis, oedema and haemorrhage of the glandular stomach of mice. Necrosis of the microvilli of the duodenum was also observed, with extensive mucosal and submucosal haemorrhage. Some muscle damage also occurred in the duodenum, as reflected by oedema of the muscle and submuscular haemorrhage (Burgess *et al*., 2002). In view of the likely contamination of the test material by OA derivatives (see above), this result must be interpreted with caution. In a later experiment, mice were dosed with pure PTX-2 intraperitoneally at 250 µg/kg. The animals died or were killed between 4.8 and 8.2 hours later. The major histological change observed in these animals was splenic,

renal and hepatic congestion. Centrilobular vacuolar degeneration of the liver was also recorded, but no changes in the small or large intestine were observed (Munday, Munday and Miles, 2004).

3.2.2 Short-term toxicity

No data on the effects of short-term repeated dosing PTX derivatives to animals have been found.

3.2.3 Long-term toxicity/carcinogenicity

No data on the possible long-term effects of the PTXs have been found.

3.2.4 Genotoxicity

No data on the possible genotoxicity of the PTXs have been found.

3.2.5 Reproductive toxicity

No data on the possible reproductive effects of the PTXs have been found.

3.2.6 Special studies

Effects in isolated cells in vitro

PTX-1 caused vacuolation in isolated rat hepatocytes *in vitro*. Unlike OA and DTX-1, PTX-1 did not cause blebbing of the hepatocyte membrane, and it caused no membranal disruption, because no lactate dehydrogenase (LDH) leakage or Trypan Blue uptake occurred (Aune, 1988, Aune, Yasumoto and Engeland, 1991). Incubation of primary cultures of chick hepatocytes with PTX-1 led to cell shrinkage. This was associated with loss of microtubules in the perinuclear regions, and remaining microtubules lost their radial arrangement and became twisted. Stress fibres disappeared from the cytoplasm, but cytoplasmic vacuolation was not observed. At low concentrations of PTX-1, the changes were reversible (Zhou *et al*., 1994). PTX-1 caused apoptosis in salmon and rat hepatocytes, associated with nuclear and cellular shrinkage. Again, no membrane disruption occurred (Fladmark *et al*., 1998). PTX-1 was neither fungitoxic nor bactericidal (Nagai, Satake and Yasumoto, 1990). PTX-2 caused disruption of actin stress fibres in the centre, but not the periphery, of A10 cells *in vitro* (Hori *et al*., 1999). PTX-2 caused loss of F–actin bundles in liver sinusoidal endothelial cells, and produced fenestrations within the cytoplasm. It sequestered monomeric actin *in vitro* (Spector *et al*., 1999). PTX-2 was toxic to KB cells *in vitro* at a concentration of 0.05 µg/ml. In contrast, no evidence of a toxic effect to these cells was seen with PTX-2 seco acid or 7-*epi*-PTX-2 seco acid at a concentration of 1.8 µg/ml (Daiguji *et al*., 1998). PTX-2 has been screened for selective cytotoxic activity against 60 human tumour cell lines by the United States National Cancer Institute. It was selectively toxic to several cell lines of ovarian, renal, pulmonary, colonic, cerebral and mammary tumours, but not against leukaemia or prostate cancer cell lines (Jung, Sim and Lee, 1995). PTX-6 caused depolymerization of F–actin in neuroblastoma cells. The observed cytoskeletal disruption was not associated with changes in mitochondrial membrane potential, total DNA content or cell attachment. Apoptosis was not observed, and cell viability was not compromised (Leira *et al*., 2002).

3.3 Observations in humans

It was suggested (Burgess and Shaw, 2001) that PTXs could have been involved in outbreaks of human illness in Australia in 1997 and 2000. The symptoms of intoxication were nausea, vomiting and diarrhoea, and shellfish responsible for the toxic effects were found to contain PTX-2 seco acid. However, these symptoms are characteristic of diarrhoetic shellfish poisons, and later work showed that the shellfish also contained

OA esters at concentrations sufficient to cause human intoxication (Burgess, 2003). It is therefore likely that the symptoms observed in these outbreaks were caused not by the PTX derivatives but by derivatives of OA.

4. ANALYTICAL METHODS

4.1 General

Pectenotoxins can be detected either by MBA or by chemical analysis although there are no officially validated methods at the moment. Despite the worldwide application of mammalian bioassays for DSP toxicity, there are large differences in the procedures and in their performance in different countries. Several chemical analytical methods for the determination of PTXs have been published. These methods are based on liquid chromatography (LC) with detection by ultraviolet absorption, fluorescence or mass spectrometry (MS).

4.2 Distribution of PTXs in bivalve compartments

No reports concerning the distribution of PTXs in bivalve compartments have been published. Investigation about this should be carried out. Mixtures of OA, DTX1, PTX-6 and yessotoxin (YTX) were administered via syringe to scallops, *P. yessoensis*, and their distribution in the hepatopancreas (HP), adductor muscle, and combined other tissues (mantle, gill, gonad) was determined by LC-MS. Toxins exclusively remained in the HP irrespective of the injection site, including the adductor muscle or HP (Suzuki *et al.*, 2005).

4.3 Screening tests

There are no validated rapid screening tests reported for PTXs. Enzyme-linked immunosorbent assay (ELISA) methods for PTXs are under development in several countries, and a preliminary method without performance data has been reported (Garthwaite, 2000). Silica gel thin layer chromatography (TLC) with chloroform/methanol/distilled water (100:15:1, v/v/v) as a developing solvent is useful to identify PTXs in semi-purified samples; however, it is difficult to use TLC for screening tests of toxins in crude bivalve extracts because of the poor selectivity.

4.4 Bioassays

Intraperitoneal injection (i.p.) MBAs (Yasumoto, Oshima and Yamaguchi, 1978), a suckling MBA (Hamano, Kinoshita and Yasumoto, 1986) and a *Daphnia magna* bioassay (Vernoux *et al.*, 1993) have been used for detection of DSP toxins. However, recent studies have shown that PTXs do not cause diarrhoea (Miles *et al.*, 2004). This indicates that assays based on oral exposure, such as the suckling MBA, are not able to detect PTXs. It has already been shown that PTXs are not detected by the oral rat bioassay (Van Egmond *et al.*, 1993). The *Daphnia magna* bioassay has been developed for detection of toxin of the OA group, but the applicability to PTXs is unknown. In the i.p. MBA, toxins are extracted with acetone, or combinations of acetone and methanol. The test protocols can differ in the observation times for the survival of mice from 5 to 24 hours. The bioassay may include a clean-up step to remove some hydrophilic interferences.

4.5 Chemical methods

4.5.1 Extraction and clean-up for chemical methods

Pectenotoxins are extracted from bivalve samples with 4–9 times volume of 80 or 90 percent methanol (Yasumoto *et al.*, 1995; Goto *et al.*, 2001). These solvent systems have been shown to be equally efficient for PTXs, although 90 percent methanol is more efficient for DSP toxins and their esters (Holland and McNabb, 2003; McNabb, Selwood and Holland, 2005). Clean-up has been accomplished using

liquid/liquid partitioning between methanolic solution and chloroform. Pectenotoxins are partitioned into the chloroform layer and toxins are analysed directly by LC-MS after solvent evaporation and dissolution in methanol (Suzuki and Yasumoto, 2000). The method of McNabb, Selwood and Holland (2005) uses a hexane wash of the crude methanolic extract to remove non-polar lipids before direct LC-MS analysis of the relatively dilute extract (0.1 g equiv/ml). Solid phase extraction (SPE) clean-up is useful in situations where the quantification of the toxins is interfered with by coextractives from biological matrices (Goto *et al*., 2001). Solid phase extraction is also useful to extract PTXs from frozen plankton net samples (Suzuki *et al*., 1998).

4.5.2 Calibration standards

Calibration standard of PTX-2 and PTX-2 seco acid is available from the National Research Institute Canada (Thomas *et al*., 2003). There is a standard toxin distribution project organized by the Japanese Government for domestic use (Goto *et al*., 2001). PTX-1, PTX-2 and PTX-6 are available in Japan from the Japan Fisheries Resource Conservation Association.

4.5.3 Methods

4.5.3.1 Liquid chromatography (LC)-ultraviolet (UV) detection

Quantification of PTX-2 by isocratic LC-UV detection at 235 nm has been reported (Lee *et al*., 1989; Draisci *et al*., 1996; Suzuki *et al*., 1998). Because the other PTX analogues have a conjugated diene in a macrolide skeleton, the LC-UV detection between 235 and 239 nm is applicable to the other PTX analogues. The LC-UV detection is not sensitive and specific for PTXs in bivalve extracts because of biological matrices, although this approach is useful for plankton net extracts. The detection limit of PTX-2 by LC-UV detection is approximately 2.0 µg/g HP (Suzuki *et al*., 1998).

4.5.3.2 Liquid chromatography (LC)-fluorescence (FL) detection

Precolumn fluorimetric analysis of PTX-2 by isocratic LC-FL detection has been reported (Sasaki *et al*., 1999). PTX-2 is derivatized with a dienophile fluorophore, DMEQ-TAD, 4-[2-(6,7-dimethoxy-r-methyl-e-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione. The fluorescence detector was set for 370 nm excitation and 440 nm emission, respectively. The fluorescence derivative of PTX-2 was quantitatively detected in the range 1–200 ng. Application of this method to bivalve samples has not been reported although it has been used to confirm the occurrence of PTX-2 in plankton net samples.

4.5.3.3 Liquid chromatography (LC)-mass spectrometric (MS) detection

Several analytical methods for the determination of PTXs by LC-MS are reported (Draisci *et al*., 1996; Suzuki *et al*., 1998; James *et al*., 1999; Draisci *et al*., 1999; Pavela-Vrancic *et al*., 2001; Vale and Sampayo, 2002; MacKenzie *et al*., 2002; Suzuki *et al*., 2001, 2003; Yasumoto *et al*., 1995; Goto *et al*., 2001; Suzuki and Yasumoto, 2000; Quilliam, Hess and Dell'Aversano, 2001; Holland and McNabb, 2003; Holland *et al*., 2004). Selected ion monitoring (SIM) for $[M-H]^-$ or $[M+MH_4]^+$ of PTXs or multiple reaction monitoring using loss of water molecules from $[M+MH_4]^+$ are applicable to the quantification. Determination of PTXs by LC-MS is usually carried out using reversed phase chromatography and isocratic or gradient elution with distilled water/acetonitrile mobile phases containing acidic modifiers such as acetic acid or formic acid/ammonium formate. LC-MS/MS spectra for $[M+MH_4]^+$ of PTXs are useful for confirmation of identity (Suzuki *et al*., 2003; Morris *et al*., 2004). The detection limit of PTXs is 5–80 ng/g HP (Goto *et al*., 2001; Suzuki and Yasumoto, 2000) or 0.01 mg/kg whole flesh (Holland and McNabb, 2003; McNabb, Selwood and Holland, 2005). An LC-MS method for a wide range of toxins has been subjected to a

full within-laboratory validation (Holland and McNabb, 2003; McNabb, Selwood and Holland, 2005). Tissues from four different shellfish species were studied. Recoveries for fortifications were 100.5 ±16.2 percent at 0.05–0.1 mg/kg and 98.3 ±9.7 percent at 0.5–1.0 mg/kg (mean percent ±SD; reproducibility conditions). PTX-2 seco acid and 7-epi-PTX-2 seco acid were also detected with good reproducibility but showed evidence of signal enhancement (McNabb, Selwood and Holland, 2005). A preliminary interlaboratory study has also been completed (Holland and McNabb, 2003; McNabb, Selwood and Holland, 2005). Three contaminated shellfish extracts containing PTX-2 at equivalent of 0.04–0.11 mg/kg were tested. Detection of the analytes was consistent although the reproducibility relative standard deviations (RSDs) were high (44–57 percent; HORRAT 1.9–2.1). The results were considered encouraging taking into account the low levels of toxin and the relative inexperience of the participating laboratories with LC-MS of this toxin group. There was no significant difference between the results for single MS or MS/MS detection, showing less expensive instruments should be applicable to screening.

5. FOOD CONSUMPTION AND DIETARY INTAKE ESTIMATES

The Canadian Food Inspection Agency has reported levels of PTX up to 91 µg/100 g shellfish digestive tissue in Atlantic Canada. This is equivalent to levels of up to 18.2 µg/100 g whole shellfish flesh if it is assumed that the distribution of PTX in mussels is the same as that found with DTX1 (5:1 ratio of toxin concentration in digestive tissue: whole shellfish flesh) (Health Hazard Assessment of OA and DTX in Mussels, memo from Chemical Evaluation Division, Health Canada, 16 August 1991).

In a later study (Aune *et al*., 2002), a peak level of 48.6 µg PTX/100 g shellfish meat was recorded in Norwegian mussels.

To assess acute toxicity, a probable acute single intake of 0.61 µg PTX /kg b.w. was estimated based on a shellfish intake (whole flesh) of 200 g/day (Nutrition Canada Survey 1972; 90th percentile, adult, "one-day", "eaters only"), a b.w. estimate of 60 kg, and a PTX concentration of 18.2 µg/100 g whole shellfish flesh (Gully and Kuiper-Goodman, 2004). Using the same parameters, a PTX concentration of 48.6 µg/100 g edible tissue would give a probable acute single intake of 1.63 µg/kg b.w.

For chronic toxicity, using a shellfish intake (whole flesh) of 22 g/day (every day for "average" consumers), a b.w. estimate of 60 kg, and a PTX concentration of 18.2 µg/100 g whole shellfish flesh, the probable daily intake of PTX would be 0.07 µg/kg b.w. (Gully and Kuiper-Goodman, 2004). For the level established in Norwegian shellfish, the probable daily intake would be 0.187 µg/kg b.w.

6. DOSE RESPONSE ANALYSIS AND ESTIMATION OF CARCINOGENIC RISK

Certain PTX derivatives are acutely toxic to mice after i.p. injection. The degree of toxicity is, however, strongly dependent upon structure. PTX-1, 2, 3 and 6 differ only with regard to the substituent at C-18. In PTX-2, the substituent is CH_3, and this substance is one of the more toxic derivatives. Oxidation of the methyl group to CH_2OH, forming PTX-1, has little impact upon toxicity, but further oxidation to the aldehyde (PTX-3) or the carboxylic acid (PTX-6) decreases toxicity. Epimerization at C-7 also greatly decreases toxicity. PTX-4 (7-*epi*-PTX-1) is less toxic than PTX-1, and PTX-7 (7-*epi*-PTX-6) is less toxic than PTX-6. In contrast, the toxicity of PTX-11 (34-β-hydroxy PTX-2) was not significantly different from that of PTX-2, indicating that such substitution at the 34 position is without major effect on toxicity. Opening of the lactone ring of PTX-2, to yield PTX-2 seco acid, caused a pronounced decrease in toxicity. No data on the acute toxicity of 7-*epi*-PTX-2 seco acid are available at present, but in view of the low toxicity of PTX-2 seco acid and the decrease in toxicity seen in other PTX derivatives following epimerization at the 7 position, it would be expected that this compound would be relatively harmless.

Although Ogino, Kumagai and Yasumoto (1997) reported that PTX-2 was toxic after oral administration to mice at low dose-levels, the data are hard to interpret because of the lack of a dose-response. In another study, no deaths or other changes were recorded with PTX-2 at a dose of 5 000 µg/kg, indicating that, like many other algal toxins, PTX-2 is much less toxic orally than by i.p. injection. This may reflect poor absorption from the GI tract or conversion to a less toxic material, such as PTX-2 seco acid, in the gut (Miles *et al*., 2004). This is an important point. PTX-2 is converted to the seco acid by incubation with homogenates of shellfish tissue (Suzuki *et al*., 2001; Miles *et al*., 2004). Mammalian tissues similarly contain lactonases (Fishbein and Bessman, 1966), thus a similar conversion in the mouse is feasible. Further work in this area is required, and studies on the acute toxicity of PTX derivatives by feeding to mice (which may give a more accurate estimate of the potential risk to humans [Munday *et al*., 2004]) also would be of interest.

Although the PTXs have been classified within the diarrhoetic shellfish poison group, most available information now indicates that these compounds do not cause diarrhoea in experimental animals. The observation of Burgess and Shaw (2003) that the diarrhoea observed after administration of PTX-2 seco acid was because of contamination of the test material with OA esters is of particular interest. It was later shown (Miles *et al*., 2004) that the C_8-diol ester of OA is co-extracted with PTX-2 and PTX-2 seco acid, so that without careful purification, contamination of the PTXs with diarrhoetic substances is likely. Such contamination could likewise account for the diarrhoea observed in mice given PTX-2 (Ishige, Satoh and Yasumoto, 1988).

The liver appears to be a major target organ of the PTXs. Hepatic congestion and vacuolar degeneration of hepatocytes was recorded with both PTX-1 and PTX-2 after i.p. injection (Terao *et al*., 1986; Munday, Munday and Miles, 2004). The observation of elevated serum activities of alanine aminotransferase, aspartate aminotransferase and sorbitol dehydrogenase (Yoon and Kim, 1997) is also consistent with hepatic damage. In accord with the *in vivo* studies, Aune, Yasumoto and Engeland (1991) observed vacuolation in hepatocytes *in vitro.* Several reports (Zhou *et al*., 1994; Fladmark *et al*., 1998; Hori *et al*., 1999) have described effects of the PTXs on actin and microtubules. Damage to the latter structures could account for the toxic change observed in hepatocytes. The observation of severe pulmonary effusion and ascites in animals given toxic amounts of PTX-2, together with tissue congestion (Munday, Munday and Miles, 2004), suggests an impairment of circulation, either via the heart or the blood vessels. A study of the possible involvement of circulatory insufficiency in the lethal effects of the PTXs would be of interest.

Histological changes in the intestine were recorded in those experiments with PTX-2 and PTX-2 seco acid in which diarrhoea was reported (Ishige, Satoh and Yasumoto, 1988; Burgess *et al*., 2002). As discussed above, the diarrhoea may reflect contamination with OA derivatives, and this may also account for the changes recorded in the intestine. The histological changes reported in these studies resemble those described in animals receiving OA (Terao *et al*., 1986).

The preliminary study on the absorption and excretion of PTX-2 and PTX-2 seco acid (Burgess, 2003) suggests that these materials are very poorly absorbed from the GI tract. However, the low recovery of the test materials in this study suggests that metabolism to other, unidentified, compounds may occur. Further studies on the pharmacokinetics of the PTXs are required.

The increase in urinary malondialdehyde concentrations in mice dosed with PTX-2 or PTX-2 seco acid (Burgess, 2003) could indicate an ability of these substances to cause oxidative damage *in vivo*. The results are difficult to interpret, however, because of the absence of a dose-response and because of the unsatisfactory health status of the animals employed.

7. EVALUATION[2]

There appears to be no conclusive evidence that PTX derivatives *per se* have caused toxicity problems in humans. While it has been suggested that such compounds could be involved in cases of human poisoning in Australia (Burgess and Shaw, 2001), the symptoms (nausea, vomiting and diarrhoea) are consistent with the involvement of OA esters, rather than PTXs, in the effects on humans.

In order to provide some estimate of the acute risk to human health of the PTXs (and, indeed, other shellfish contaminants), the most relevant parameter is the acutely toxic dose by oral administration. In the case of PTX-2, the latest results indicate that this is greater than 5 000 µg/kg in the mouse. Although no data on the oral toxicity of other PTX derivatives, apart from PTX-2 seco acid, are available, there is no reason to suppose that the oral toxicity of these substances would be higher than that of PTX-2. In the case of the seco acid, the acute toxicity by both oral and i.p. administration was > 5 000 µg/kg. The latter dose level was the highest employed, thus, no estimate of the LD_{50} is available. Because no effects were recorded at 5 000 µg/kg, however, the LD_{50} may well be considerably higher than this.

The calculated acute human intake of the PTXs in Canada is 0.61 µg/kg b.w. (Gully and Kuiper-Goodman, 2004) while the Norwegian data indicate an intake of 1.63 µg/kg b.w. The acutely toxic dose in the mouse is thus between approximately 3 000 and 8 000 times the estimated acute human intake.

No comment on the potential long-term effects of the PTXs in humans can be made at present, because no data appear to be available on the chronic toxicity of these substances in animals. In order to assess the possibility of longer-term risks of these substances to humans, chronic feeding studies in animals are required. Data on the absorption, metabolism and excretion of these substances would also be valuable. In view of the confounding effect of other shellfish-derived contaminants in PTX samples used in previous toxicological studies, it is imperative that future work is conducted with pure, fully-characterized samples of the PTX derivatives.

Pectenotoxins are currently regulated against, and the European Union (EU) allows a maximum total of 16 µg of OA, DTXs and PTX per 100 g of shellfish (Commission Decision 2002/225/EC). However, because the PTXs do not belong in the diarrhoetic shellfish poison group, a re-evaluation of this regulation would be appropriate, and regulatory limits for PTXs alone should be set.

There are no fully validated analytical methods to detect and quantify PTXs. Liquid chromatography with mass spectrometry detection has been shown to have potential for quantitative testing of PTXs in bivalve samples. Further interlaboratory study of LC-MS methods is required to establish them as Codex reference methods. Liquid chromatography with ultraviolet or fluorescence detection is applicable to quantify toxins in plankton net samples. Because the i.p. MBA for DSP toxicity is considered to be semi-quantitative, the method is probably semi-quantitative for PTXs in isolation. However, these toxins invariably co-occur with OA analogues, and the performance of MBA on mixtures is not well established. While the accuracy of the i.p. MBA for PTXs is unknown, the method may still be applicable to monitoring of PTXs. Further detailed studies about the i.p. MBA for PTXs are recommended, particularly in mixtures with OA analogues. Investigations into the distribution of PTXs in bivalve compartments should be carried out. Development of rapid screening test for PTXs will be useful for efficient monitoring of toxins.

[2] It must be pointed out that, as a result of the Expert Consultation, the evaluation section of the Report differed from that of the Pectenotoxins draft chapter. The Evaluation Section of the present Background Document has been adapted by the authors to fit the outcome of the Expert Consultation Report.

8. REFERENCES

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Saxitoxins

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1. BACKGROUND INFORMATION[1]

Paralytic shellfish poisoning (PSP), associated with intake of toxins from the saxitoxin (STX) group, has been known for a long time, and has caused many fatalities. On the basis of case reports, the intake of toxins necessary to induce various PSP symptoms varies greatly. This may be because of differences in susceptibility among individuals, as well as a lack of precision in exposure assessments because of problems with sampling and analysis of contaminated shellfish at the time of intoxication. Saxitoxins have been found worldwide. A regulatory level of 0.8 mg/kg shellfish meat as STX equivalents has existed in North America for about 50 years, and the probability of suffering PSP from commercially harvested shellfish is extremely low. The same regulatory limit is currently used in many other countries. The mouse bioassay (MBA) has been widely used in monitoring programmes.

2. ORIGINS AND CHEMICAL DATA IN SHELLFISH

Saxitoxins are a group of low molecular weight (STX=299 dalton) non-protein toxins, with about 20 naturally occurring analogues (Figure 1). Saxitoxins have been found to occur worldwide. They are produced by *Alexandrium* spp. and other species and affect a wide variety of shellfish.

3. BIOLOGICAL DATA

3.1 Biochemical aspects

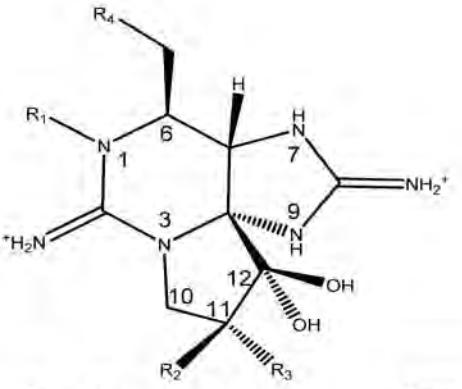
3.1.1 Absorption, distribution and excretion

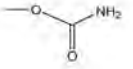
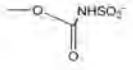
3.1.1.1 Absorption

Paresthesia and numbness around the lips, tongue and mouth, which appeared within minutes after eating toxic food, indicated local absorption of the toxin through the buccal mucous membranes (Kao, 1993). From the short onset time reported in many of the intoxication cases, it is evident that the toxins are quickly absorbed from the gastrointestinal (GI) tract and transported to the other organs by blood stream, although no human data are available. When the pyloric stomach was closed surgically in cats, 80 percent of orally administered GTX2/GTX3 was retained in the stomach after 5 hours, and the toxins were not detected in plasma and urine, indicating that absorption of the toxins occurred only at intestinal level (Andrinolo *et al*, 2002a). Andrinolo *et al*., (2002b) determined the mechanisms involved in toxin absorption, using layers of intestinal epithelial cell lines derived from human and rat, and concluded that GTX2 and GTX3 were transported across the epithelium by the paracellular route.

[1] Corresponds to the "Background Information" section of the Expert Consultation Report.

FIGURE 1
Saxitoxin (STX) and analogues



R_1	R_2	R_3	R_4	Toxin
H	H	H		STX
H	H	OSO_3^-		GTX2
H	OSO_3^-	H		GTX3
OH	H	H		NEO
OH	H	OSO_3^-		GTX1
OH	OSO_3^-	H		GTX4
H	H	H		GTX5 (B1)
H	H	OSO_3^-		C1
H	OSO_3^-	H		C2
OH	H	H		GTX6 (B2)
OH	H	OSO_3^-		C3
OH	OSO_3^-	H		C4
H	H	H		dcSTX
H	H	OSO_3^-		dcGTX2
H	OSO_3^-	H		dcGTX3
OH	H	H		dcNEO
OH	H	OSO_3^-		dcGTX1
OH	OSO_3^-	H		dcGTX4

Source: FAO, 2004.

3.1.1.2 Distribution

There are few data on the distribution of toxins in the body of human patients because of the difficulty of analysis as well as obtaining samples. In a post-mortem examination of the samples from a victim of toxic coral crab, only trace amounts of toxin were detected in the liver by radioreceptor binding assays, while fairly large amounts were found in the gut contents, blood and urine (Llewellyn *et al*, 2002). In experiments with cats, STX injected as a single bolus into blood disappeared quickly with a serum half-life of 22 minutes. Saxitoxin was detected by liquid chromatography (LC) analysis in the blood and urine as well as spleen, liver, medulla oblongata and brain (in the order of concentration) (Andrinolo *et al*., 1999). Another experiment that used tritiated saxitoxinol ([^{3}H]STXOL), a radioactively stable derivative of STX, showing very low affinity to Na channels, also showed quick disappearance of toxin from blood (29 min serum half-life). One hour after i.v. administration, 5.0, 2.2, 2.2, 1.3, 0.8, 0.2, 0.1 and 0.04 percent of the total dose of radioactivity was detected in the muscle, liver, kidney, small intestine, large intestine, lung, heart and spleen, respectively (Naseem, 1996).

3.1.1.3 Excretion

In several patients from Alaska, the United States of America, using four different STX binding assays and LC analysis, toxin levels of 2.8–47 nM were observed in

serum during acute illness, compared with 65–372 nM in urine after acute symptom resolution (Gessner *et al*., 1997). The detection of high concentration of toxins in the urine of patients indicates that urinary excretion is a primary route of human toxin excretion. This was supported by animal experiments in which toxins were administered intravenously. An early study by Prinzmetal, Sommer and Leake (1932) reported that 40 percent of the toxins were detected in the urine of a dog, by MBA, 2 hours after i.v. injection of crude toxin. From these data, Kao (1993) estimated the half-life of toxin elimination from the body as being in the order of 90 minutes. More recent studies in rats and cats by more sophisticated methods of toxin analysis showed a slower rate of elimination from the body. In rats, intravenously administered STX, at a dose of 2 µg/kg b.w., was estimated to have a half-life of 17.8 hours (Stafford and Hines, 1995). Similar results were observed in cats, using LC analysis, in which 25 and 10 percent of the administered toxin was excreted in urine within 4 hours after STX had been injected at doses of 2.7 µg/kg b.w. and 10 µg/kg b.w., respectively (Andrinolo *et al*., 1999). In the latter report, the authors concluded, based on the similarity of STX clearance to inulin clearance, that glomerular filtration plays a major role. In experiments with rats with [^{3}H]STXOL, the half-life of toxin elimination from the body was estimated to be 12.3 hours. Small quantities of non-metabolized STXOL were detected in rat urine up to 144 hours after i.v. administration (Hines, Naseem and Wannemacher, 1993, Naseem, 1996). Faecal elimination is unlikely, because STX was not detected in the bile of cats (Andrinolo *et al*., 1999) and no radioactivity was recovered in the faeces of rats injected with [3H]STXOL (Stafford and Hines, 1995).

3.1.2 Biotransformation

There are two reports on the suspected biotransformation of the toxins in the human body based on the different toxin profiles observed between the causative foods and human biological specimens by LC analysis. Gessner *et al*. (1997) found higher proportions of C1, C2 and less GTX2 in the serum and urine compared with the cooked mussels as the leftover of implicated meal. The gut contents and urine sample of the crab poisoning victim mentioned before showed higher proportion of STX and less GTX2, 3 compared with uneaten crab. Also, GTX1, GTX4 and dcSTX, which were not detected in the crab, were found in the urine (Llewellyn *et al*., 2002). The authors speculate whether reductive cleavage of 11-hydroxy sulphate (often observed in shellfish during toxin accumulation), oxidation of N-1 (very unusual) and decarbamoylation (found in certain clams) took place in the gut as well as in the body. However, toxin identification was rather ambiguous because of many interfering peaks in the chromatograms shown in the paper. More conclusive data are needed for the biotransformation of toxins in the human body. No apparent change was observed in GTX2 and GTX3 incubated with cat liver homogenate (Andrinolo *et al*., 2002a).

Toxins having a N-sulphocarbamoyl moiety (such as B1, B2, C1–C4) as a side chain showed low mouse toxicity. When heated at low pH, they were easily converted to the corresponding carbamate toxins through hydrolysis. Because the reaction resulted in a several-fold increase in toxicity, a potential danger of these toxins was suggested (Hall and Reichaedt, 1984). To examine this phenomenon experimentally, B1 (GTX5) was incubated at modelled conditions for the human stomach and analysed by MBA. After 5 hours' incubation at 37 °C, twice increase of toxicity corresponding to 9 percent conversion of toxin was observed in the artificial gastric juice at pH 1.1 and no apparent increase of toxicity in rat gastric juice at pH 2.2 (Harada, Oshima and Yasumoto, 1984). Similar experiments carried out on C1, C2 with LC analysis showed that 5.5 percent of the toxins were converted to GTX2, 3 at pH 1.6 and 1.5 percent at pH 2.2 after 4 hours' incubation (unpublished data by Oshima). These data indicated that the increase in toxicity in the human body because of the hydrolysis of N-sulphocarbamoyl toxins may not be significant.

In addition to the above toxin conversion, the following chemical transformations were reported and often observed during toxin accumulation by shellfish, but they have not been reported in warm-blooded animals (Oshima, 1995a): epimerization of 11-hydroxysulphate at neutral pH; reduction of N1-OH; reductive elimination of 11-hydroxysulphate (recently, stable thioether intermediates of this reaction were reported by Sato, Sakai and Kodama, 2000); hydrolysis of N-sulphocarbamoyl at neutral pH.

Hydrolases catalyzing the hydrolysis of the N-sulphocarbamoyl and/or carbamoyl moiety were reported in three species of clams, *Protothaca staminea* (Sullivan *et al*., 1983a), *Mactra chinensis* and *Peronidia venulosa* (Oshima, 1995a; Lin *et al*., 2004). N-sulphotransferase involved in the biosynthesis of toxins was also observed in the dinoflagellates (Oshima, 1995a; Sako *et al*., 2001).

In the toxicokinetic study with [^{3}H]STXOL, unidentified radioactive peaks were observed by LC in the extracts of rat organs (Naseem, 1996). However, no data are available on the metabolism of the actual toxins in the human body.

3.1.3 Effects on enzymes and other biochemical parameters

3.1.3.1 Enzymes

No data are available about the effects of paralytic shellfish toxins on enzymes.

3.1.3.2 Sodium channels and receptor binding

The systemic manifestations of poisoning are the result of selective blockade of the voltage-gated sodium channels on excitable membrane (Kao, 1993). Sodium channels conduct the electrical impulse in excitable tissues through highly selective permeation of Na+ and voltage-dependent gating. The principal functional unit of the Na+ channel is the α subunit, approximately 260 kDa in size, which consists of four internally homologous domains, each containing six trans-membrane segments. These four homologous domains are pseudo-symmetrically arranged around a central pore whose structural constituents determine the selectivity and conductance properties of the channel (Catterall, Doldin and Waxman, 2003).

Saxitoxin selectively binds to the neurotoxin receptor site 1 on the α subunit. Tetrodotoxin of puffer fish and μ-conotoxin of the Conus snail also bind to this site. The receptor sites for these toxins are formed by amino acid ligands in the pore loops and immediately on the extracellular side of the pore loops at the outer end of the pore (Catterall, Doldin and Waxman, 2003).

All the analogues of STX occupy the same receptor, although affinities differ greatly. From the structure-activity studies of STX and its analogues, 7,8,9-guanidine and geminal diol at C12 were most important for the binding of toxins to the receptor. The carbamoyl side chain also contributes to binding at some extent, and introduction of N-sulphonate causes low affinity to the receptor probably because of ionic interaction with the channel protein. N1-hydroxy and 11-hydroxysulphate moieties of both stereochemistries showed little effect on the affinity to the receptor site (Kao, 1993).

As shown in the section of bioconversions, when 11-hydroxysulphate toxins such as GTX1–GTX4 were reduced by thiol compounds, thioether-linked intermediates were observed (Sato, Sakai and Kodama, 2000). The reaction occurred with glutathione and cysteine in shellfish. Only trace amount of toxins bound to proteins were detected in the highly contaminated scallops (Kodama, personal communication). It is not known whether the reaction takes place in the human body and causes adverse effects.

3.2 Toxicological studies

3.2.1 Acute toxicity

The main adverse effect of STXs in animal species and humans is neurotoxicity. (See also Section 3.4, Observations in humans.)

Saxitoxin, when injected intraperitoneally, typically kills mice within 5–7 minutes because of respiratory arrest. Characteristic dose-death time relationship were observed and used for the quantification of toxin as shown in Section 4, Analytical methods. The intraperitoneal injection (i.p.) median lethal dose (LD_{50}) of STX is in the order of 10 µg/kg b.w., which for a 20 g mouse equates to 50 MU. Thus, 1 MU corresponds to 0.2 µg STX equivalents.

The acute i.p. toxicity of other analogues has also been measured in mice. Usually, reports on the structural elucidation of these analogues showed the toxicity on a weight basis. However, often these values were not accurate because overdrying of the toxins often caused degradation or transformation to other toxins (Genenah and Shimizu, 1981). For a comparison among the large number of analogues known to date, a short description of specific activities (i.p. route) of major 14 analogues is shown in Table 1 (Oshima, 1995b), measured during the preparation of LC standards. The toxicity of each pure toxin solution was determined using Sommer's table for MBA, while toxin concentrations on a molar basis were based on the nitrogen content of the solution through combustion analysis, so that the values were given in MU/µmole. The data are close to the graphically presented data of Hall *et al.* (1990). It is noteworthy that i.p. toxicity to mice and binding affinity were almost parallel for most toxins (Hall *et al.*, 1990).

TABLE 1
Specific i.p. toxicities of saxitoxin (STX) analogues

Toxin	Specific toxicity (MU/µmole)	Relative toxicity
STX	2 483	1
neoSTX	2 295	0.92
GTX1	2 468	0.99
GTX2	892	0.36
GTX3	1 584	0.64
GTX4	1 803	0.73
dcSTX	1 274	0.51
dcGTX2	382*	0.15
dcGTX3	935*	0.38
B1 (GTX5)	160	0.064
C1	15	0.006
C2	239	0.096
C3**	33	0.013
C4**	143	0.058

* After re-examination.
** Estimated by the measurement of GTX1, GTX4 formed by acid hydrolysis.
Source: Oshima, 1995b.

The LD_{50} values of purified STX dihydrochloride to mice by the different routes of administration were determined at Health Canada by Wiberg and Stephenson (1960). As shown in Table 2, the intraperitoneal toxicity was 1/25th of the oral toxicity.

TABLE 2
Acute toxicity of STX in mice by different routes of administration

Route	LD_{50} in µg/kg b.w.
Oral	260–263
Intravenous	2.4–3.4
Intraperitoneal	9.0–11.6

Source: Wiberg and Stephenson, 1960.

For a comparison of susceptibility among different animals, only one report is available. The oral LD_{50} values for several species of warm-blooded animals were examined by McFarren *et al*. (1960) as shown in Table 3. Not much difference was observed among the mammals tested.

TABLE 3
Oral toxicities (LD_{50} in µg/kg b.w.) of STX in various species

Animal	Mons, Van Egmond and Speijers (1998); McFarren *et al*. (1960)	
Mouse	420	
Rat	192–212	212
Monkey	277–800	400–800
Cat	254–280	280
Rabbit	181–200	200
Dog	180–200	200
Guinea pig	128–135	128
Pigeon	91–100	100

Prior exposure to non-lethal doses of PSP toxin seems to lower the susceptibility of rats to lethal doses of PSP toxin. In a study using Sprague-Dawley rats (sex not indicated), the oral LD_{50} value for the purified PSP extract was determined (McFarren *et al*., 1960). One group of rats was given a non-lethal dose of PSP (about one-third of the LD_{50}) 14 days before the test. The LD_{50} for the pretreated rats was about 50 percent higher than that for untreated rats. This finding corroborates the fact noted by Prakash, Medcof and Tennaut (1971) that fishers who habitually eat shellfish containing low levels of PSP toxins may be less susceptible to developing PSP.

Besides the acute lethal toxicity and toxicokinetics described above, other acute toxicological effects on experimental animals were reviewed by Mons, Van Egmond and Speijers, 1998. These authors described the effects of STX on the respiratory system, myocardium, muscle and nervous tissue (both peripheral and central) in various animal species. (See Section 3.2.3., Special studies, below.)

3.2.2 Other toxicity data

No data available on: short-term or long-term toxicity; carcinogenicity; genotoxicity; reproductive toxicity; or developmental toxicity.

3.2.3 Special studies

3.2.3.1 Effects on the respiratory system

When PSP intoxication occurs, the effects on the respiratory system are responsible for the fatal outcome. The cause of death is asphyxiation because of progressive respiratory muscle paralysis. In animals (cat, rabbit), doses of 1–2 µg STX/kg b.w., administered intravenously, caused decreased respiratory activity reflected in both a decline in amplitude and velocity. When the dose was raised to 4–5 µg STX/kg b.w., a strong depression of respiration was observed, which resulted in death unless artificial respiration was provided. At lower doses, respiration may return spontaneously. In animal experiments, only peripheral paralysis has been noted, because of a direct effect on the muscles of the respiratory system, and the respiratory centre of the nervous system is not inhibited. Action potentials are sent off to the midriff and the middle rib muscles. Other investigators, however, suggest a possible central role on the respiratory neurons. For comparison, the occurrence of paresthesia and feeling of lightness in humans are often connected with a central effect, but the peripheral effects on the nervous system may be the cause of these symptoms.

3.2.3.2 Cardiovascular effects

In anaesthetized animals, doses above 1 µg STX/kg b.w. (i.v.) can provoke hypotension, with paralysis of muscles already observed at lower dose levels. This cardiovascular effect is seldom observed in human cases of intoxications and is more likely the reflection of peripheral effects, although the central nervous system (CNS) might be involved to a certain extent. There are uncertainties about this peripheral action. Apart from a direct effect on the muscle tissue the possibility of an axonal blockade of the sympathetic nervous system cannot be excluded. Most investigators agree on the fact that no or hardly any direct cardiac effects occur.

3.2.3.3 Neuromuscular effects

An i.v. dose of 1-2 µg STX causes a fast weakening of muscle contractions; both contractions by direct stimulation as well as contractions by indirect motoneuron stimulation are affected. The effects include all skeletal muscle tissues. This dose level induces also a decrease of the action potential-amplitude and a longer latency time in the peripheral nervous tissue. Both motor and sensory neurons are affected, but the sensory neurons are already inhibited at lower dose levels. Through this effect on the sensory system, the numbness and the proprioceptive loss may be explained, but not the paresthesia. About the possible mechanism of the toxicity, no clarity has been achieved, and many scientific debates reflect this.

3.2.3.4 Effects on the CNS

There are uncertainties about the existence of an effect of the toxins on the CNS. Most symptoms can be attributed to peripheral effects. However, central effects cannot be excluded.

3.2.3.5 Immunotoxicity

No data available.

3.3 Observations in domestic animals/veterinary toxicology

Several authors have indicated that domestic animals (cats, hens, ducks, sea birds) have become ill or died after they had eaten discarded parts from shellfish being prepared for human consumption during a PSP episode or scallop rims shucked overboard by fishers (Prakash, Medcof and Tennaut, 1971).

3.4 Observations in humans

3.4.1 Symptomatology and clinical observations of human cases

Paralytic shellfish poisoning is an acute illness that may affect persons who have consumed shellfish containing PSP toxins. The signs and symptoms of PSP in humans may range from a slight tingling and numbness about the lips to complete paralysis and death from respiratory failure (Medcof *et al.*, 1947; Meyer, 1953; McFarren *et al.*, 1960). Typically, the tingling sensation around the lips, gums and tongue develops within 5–30 minutes of consumption. In moderately and extremely severe cases, this is followed by a feeling of numbness in the fingertips and toes, and within 4–6 hours the same sensation may progress to the arms, legs and neck, so that voluntary movements can be made only with great difficulty. In fatal cases, death is usually caused by respiratory paralysis within 2–12 hours of consumption of the PSP-containing food. Case severity has been defined as mild, moderately severe or extremely severe (Prakash, Medcof and Tennaut, 1971). The following are descriptions of typical symptoms in each category:

- Mild. Tingling sensation or numbness around lips, gradually spreading to face and neck; prickly sensation in fingertips and toes; headache, dizziness, nausea, vomiting.

- Moderately severe. Incoherent speech; progression of prickly sensation to arms and legs; stiffness and incoordination of limbs; general weakness and feeling of lightness and floating (light headedness); slight respiratory difficulty; rapid pulse; backache as a late symptom.
- Extremely severe. Muscular paralysis; pronounced respiratory difficulty; choking sensation; high probability of death in absence of artificial respiration.

Clinicians have observed that, if patients survive for 24 hours either with or without mechanical ventilation, chances for a rapid and full recovery are excellent. In rare cases, severe hypertension was observed in patients, although only nanomolar serum levels were detected (Gessner *et al*., 1997).

3.4.2 Biomarkers of exposure

If samples of serum or urine are collected soon after exposure, the presence of STX or related congeners can be found to confirm exposure to PSP toxins (Gessner *et al*., 1997).

3.4.3 Epidemiological studies[2]

There are several case reports or reports that summarize case series on PSP. Most of these reports, indicated below, showed occurrence data of PSP toxins in the implicated food, and some included an estimation of the amount of toxins consumed by the patients, either as a total dose per person or on a b.w. basis. Toxin levels were determined by MBA, using the leftover food or, more often, those of shellfish samples from the same origin. Some authors applied factors for the degradation of toxins by cooking. To make comparison easier, the values given in MU originally were converted to µg STX equivalents, assuming a conversion factor 0.18 to 0.2 µg STX eq. per MU (see Sections 3.2.1 and 4.2).

Prakash, Medcof and Tennaut (1971) provided data on cases of PSP that occurred in New Brunswick, Canada, from 1945 to 1957. In their studies on the effect of various cooking methods on the presence of PSP toxins, they noted that as much as 90 percent of the toxins can disappear as a result of cooking. They therefore applied an average conversion factor of 0.3 to the concentration of PSP toxins in raw shellfish if no samples of cooked leftover shellfish meal were available, and thus the estimated oral intake was corrected for the effect of cooking on toxin levels in shellfish. They categorized 49 cases, two of which were children of 2 and 8 years old, as having mild, severe and extreme symptoms, and reported an associated estimated toxin exposure within the range of 85–4 128, 90–9 000, and 390–7 000 µg STX eq. per person, respectively. In the same report, they also showed 82 persons, including 4 children, who did not show any symptoms with a range of 50–2 800 µg STX eq. per person.

More recent Canadian data on PSPs, from 1970 to 1990, were analysed and compared with selected other outbreaks and case series in an unpublished Health Canada report by Kuiper-Goodman and Todd. These authors assessed the clinical information based on the original case histories of persons for which occurrence data of PSP toxins in shellfish were also available (raw or cooked shellfish), and they determined the corresponding exposure, adjusted for the effect of cooking. Similar to Prakash, Medcof and Tennaut (1971), cases were rated as mild, moderately severe and extremely severe. Although there was an overlap in the doses that were associated with the three categories of severity, a clear and steep dose response was apparent. The more severe cases generally involved an exposure of from > 10 to 300 µg per kg b.w. It is of interest that the upper range of these values was similar to oral LD_{50} values observed in

[2] The draft STX chapter presented at the Expert Consultation in Oslo, September 2004, was not complete. During the meeting, some information on effects of cooking on STX level was presented. In the present revised Background Document, this aspect has been increased. Nevertheless, in order to fit the outcome of the Expert Consultation, this aspect has not been considered in the Evaluation Section.

experimental animals (see Section 3.2.1, Acute toxicity). With one exception, mild cases generally had consumed between 3 and 30 µg/kg b.w., and for the moderately severe category, exposure was somewhere in between. As noted by others, there was great variability in individual sensitivities, as there were persons who had consumed between 5 and 50 µg per kg b.w., without becoming ill.

In an incident off the Nantucket coast (in the northeast of the United States of America) in 1990, six fishermen contracted PSP after consuming blue mussels that had been harvested in deep water and cooked for 90 minutes. The fishermen had consumed from 3 to 48 mussels, which contained 24 400 and 4 280 µg STX eq/100 g in raw and cooked shellfish, respectively. All the fishermen became ill, with onset of illness and severity of symptoms related to number of mussels they had consumed (Sharifzadeh *et al*., 1991).

Gessner and Middaugh (1995) reviewed 54 incidents of PSP in Alaska, the United States of America, from 1973 to 1992, involving 117 patients (including one fatal case, and four cases requiring mechanical ventilation). The estimated dose of toxin appears not to have been corrected for the effect of cooking and, for 33 ill persons, ranged from 13 to 123 457 µg STX eq. (mean 19 521 µg STX eq.). For ten non-ill people, the estimated dose was 17–36 580 µg STX eq. (mean 12 959 µg STX eq.). The authors indicated that they might have miscalculated the dose by assuming that toxin levels from tested shellfish were identical to levels of ingested shellfish, and the reviewers noted that, if the effects of cooking had been considered, the suggested doses would probably have been much lower. Gessner *et al* (1997) provided the estimated dose per person and on a b.w. basis from four outbreaks in Alaska. For most cases this was adjusted for the effect of cooking, but full details were not provided. Levels of PSP toxins in raw shellfish ranged from 1 778 to 19 418 µg STX eq/100 g. The lowest dose that caused illness was estimated to be 21 µg STX eq/kg b.w. Among four persons with respiratory arrest, who may be considered to have consumed a lethal dose, the dose ranged from 230 to 411 µg STX eq/kg b.w.

A large epidemic of PSP occurred in Guatemala in 1987, affecting 187 patients. The patients, 26 of whom died, had eaten clams (*Amphichaema kindermani*) that contained STXs through uptake from the dinoflagellate *Pyridinium. bahamense*. The estimated intake from clam soup for one fatal case was 630 µg STX eq. per person and 25 µg STX eq/kg b.w. In this episode, the case fatality rate was 50 percent in children less than 6 years of age, compared with 7 percent in adults. Although neurological symptoms subsided in most patients within 24–72 hours, persistent headaches, memory loss and fatigue were reported for weeks in some instances (Rodrigue *et al*., 1990). Of the control cases, 5 of 41 had consumed clams, but no information on their dose was provided.

Other estimated intakes, given as µg STX eq. per person, from a single or small number of incidents were 3 060–7 560 (three cases in the United States of America [Meyer, 1953]); 77–5 634 (four cases in Canada [Tennant, Naubert and Corbeil, 1955); 117–1 044 (seven cases in Canada [Tennant *et al*., 1955]); 2 700 (two cases in California, the United States of America [Seven, 1958]), 352–2 720 (two cases in Canada [Quayle, 1969]); 4 050–5 166 (four cases in England, the United Kingdom of Great Britain and Northern Ireland [McCollum *et al*., 1968]); and 90–10 530 (16 cases in South Africa [Popkiss, Horstman and Harpur, 1979]). It was suggested that a range of 120–304 µg STX eq. per person is associated with mild symptoms in humans (FAO, 2004).

As observed in the incident in Guatemala, high susceptibility of children to the toxins was mentioned in several other papers (Meyer, 1953; Prakash, Medcof and Tennaut, 1971; Quayle, 1969). In the Philippines, among 44 fatal cases whose ages were recorded, 27 were from 3 to 11 years old (Furio and Gonzales, 2002). The reviewers noted that the higher susceptibility in children was undoubtedly because, in part, of their lower b.w.

Some publications noted the median or mean exposure to STXs for persons who became ill or not ill (Prakash, Medcof and Tennaut, 1971; Gessner and Middaugh, 1995, Gessner *et al.*, 1997). Prakash, Medcof and Tennaut (1971) pointed out, however, that because of the large person-to-person variation in susceptibility, average doses are not very informative for the purpose of deriving a safe-dose estimate. It is more important to know the limits of the range in sensitivity and particularly the minimum dose that may induce illness.

The case fatality rate of PSP varies considerably. In recent outbreaks in North America and Western Europe involving more than 200 people, there were few deaths; but in similar outbreaks in southeast Asia and Latin America, case fatality rates of 2–14 percent have been recorded. Part of the difference may be related to how readily victims have access to hospital care. In addition, when the disease occurs in new geographic areas, the local population and health professionals may have never before encountered such poisonings.

4. ANALYTICAL METHODS

4.1 Introduction

Because of the potential hazard to humans and animals, a quick, sensitive and specific method is needed to determine the presence of the toxins in shellfish. Traditionally, the presence of toxins has been determined using MBA. However, the controversial issue of using mammals for testing, in addition to the inherent problems and limitations of mammalian bioassays, encourages the development of alternative assays such as pharmacological assays, immunoassays, chemical assays and alternative bioassays to detect marine toxins in seafood.

The development of analytical methods for the STX group is made challenging for several reasons, including: the presence of a large number of STX analogues with closely related structures; a great variation in toxicity (lethality to mice) among analogues; and a wide variation in toxin composition in different shellfish species and in different geographic locations.

4.2 Bioassays

4.2.1 In vivo *assays*

4.2.1.1 Mouse bioassay (MBA)

Mouse bioassay was first applied to toxin-contaminated shellfish by Sommer and Meyer (1937). Later, the bioassay procedures were standardized (Medcof *et al.*, 1947) and subjected to an Association of Official Analytical Chemists (AOAC) collaborative study (McFarren, 1959). Since then, MBA has been used exclusively in most of the toxin monitoring worldwide.

The method involves a simple aqueous heat extraction of shellfish homogenate with 0.1 N HCl (1:1), followed by i.p. injection of 1 ml of the extract into mice, and measurement of time of death after injection. Toxicity scores in MU are obtained by applying the median death time of mice to the table that shows dose-death time relationship for STX originally reported by Sommer and Meyer (1937). MU is defined as an amount of toxin to kill a 20 g mouse in 15 minutes after i.p. injection. Although the dose-death time relationship is given as a table in the protocol, it can be expressed by the equation: $\log (MU) = 145/T + 0.2$ (where T is the median death time in seconds). The assay is quantitative only between death times of 5 and 7 minutes, corresponding to the toxicity between 1.92 and 1.38 MU/ml. Dilution of the extract is required until the median death time drops within the range. The assay is standardized by parallel testing with the standard solution of STX dihydrochloride distributed by the United States Food and Drug Administration (USFDA), to minimize the effect of different sensitivity among mouse colonies (strain, sex or physical conditions). This standardizing procedure gives a factor converting MU to µg STX (ug STX/MU). By

multiplying the toxic scores by the conversion factor examined for the STX standard, the results are given in the unit of µg/100 g shellfish tissue. (Because the value means equivalent toxicity, it will be better to express it in µg STX eq/g, and this unit will be used in this chapter hereafter.) In some countries where the USFDA STX standard solution is not available, toxicity values are just expressed in MU/g. In Japan, sex (male) and strain (ddY) are specified for the assay to minimize fluctuations of the data (Yasumoto, 1981). The detection limit of the method varies depending on the sensitivity of mice colony, and around 0.50 µg STX eq/g, if a common conversion factor of 0.18 µg STX/MU is applied to the quantitative detection limit of 2.8 MU/g. The value 2 MU/g or 0.4 µg STX eq/g is often referred in the literatures but this is the detection limit in qualitative mean based on 1 MU/ml. The precision of the method is often given as ±20 percent.

Interferences

The most important interfering factor in the assay is the extractable materials co-existing with the toxins. Crude extracts of shellfish added to pure toxin elongate the death time of mice and cause underestimation of the toxin quantity (Wiberg and Stephenson, 1960, Park *et al*., 1986a, Oshima, 1995a, LeDoux and Hall, 2000). The phenomenon was recognized even earlier when the method was officially adopted by United States of America and Canada (Schantz *et al.*, 1958), and so far NaCl is the only identified substance to cause this interference (Wiberg and Stephenson, 1960). This phenomenon, the so-called "salt effect", is apparent for the low toxic samples including those close to the regulation level of 0.8 µg STX eq/g, and may be the cause of discrepancy to the results obtained by other analytical methods.

It has not been recognized in shellfish but some marine organisms (crab and pufferfish) contain both tetrodotoxins and STXs, which share the same binding site on the Na channel. MBA cannot distinguish these by symptoms in mice, although tetrodotoxin shows a slightly different dose-death time relation (Kawabata, 1978). High zinc chloride in the oyster extracts sometimes causes acute death in mice, but can be distinguished by the symptoms in mice (McCulloch *et al.*, 1989; Aune *et al*., 1998).

Applicability to other analogues

The MBA method was originally developed only for STX, before the presence of other analogues was recognized. Now it is common knowledge that toxic shellfish occurring worldwide mainly contain analogues other than STX. (Alaskan butter clam [*Saxidomus giganteus*] originally used for the purification of STX standard is rather exceptional.) This questions whether the method is applicable to other analogues with a wide variation in potency. Experimentally, only two analogues were shown to have the same dose-death time relation in mice as STX. The regression lines between log (MU) and the inverse of time to death for B1 (Harada, Oshima and Yasumoto, 1984) and dcSTX (Oshima *et al*., 1989) were shown to be parallel to that of STX, especially at the death times ranging between 5 and 7 minutes. Good correlation between MBA results and the estimated toxicity by LC analysis might be further evidence for the applicability of MBA to a wide range of analogue mixtures, because in the latter case total toxicity was estimated by the summation of each analogue. Especially good agreement was observed for the samples with high toxicity that escaped from the salt effect in MBA.

Another matter often suggested as a possible cause of variation in MBA is the hydrolysis of N-sulphonate at the side chain of some low toxic analogues, such as C1-C4, B1 and B2. However, as described in the section on toxicity, these toxins are most stable at a pH range between 3 and 4, the preferred pH in the AOAC protocol for the extraction. Thus, at the moment there are no significant experimental data that would necessitate a change in the AOAC protocol.

In France, a proficiency study was conducted in which eight laboratories applied the mouse assay for the analysis of oyster samples contaminated with PSP toxins at levels from non-detectable to levels of 1.53 and 3.35 µg STX eq/g meat. The authors concluded that, on the basis of overall performance, all eight participating laboratories were proficient in their use of the AOAC mouse assay. Within-laboratory variations and between-laboratory variations ranged from 5 to 10 and from 8 to 40 percent, respectively (LeDoux and Hall, 2000).

4.2.1.2 Other in vivo *assays*

Other live animal bioassays reported for STXs are: killifish (McLaughlin and Downs, 1969); house fly (Siger, Abott and Ross, 1984); desert locust (McElhiney *et al.*, 1998); chicken embryo, brine shrimp and bacterial bioassays (Park *et al.*, 1986b). Little work has been done on these to evaluate them for application to regulatory monitoring and control.

4.2.2 In vitro *assays*

4.2.2.1 Receptor binding assays (RBAs)

4.2.2.1.1 Na channels

Saxitoxins specifically bind on receptor site 1 of Na channels in a reversible manner and are used as a tool to investigate the mechanism of neurotransmission in neurophysiology. Davio and Fontelo (1984) were the first to use this binding activity for the detection of toxin. The binding of toxins on rat brain preparation was detected by displacement of radiolabelled STX (11-[^{3}H]STX). The method was later improved using microplate scintillation to give a high throughput operation and tested for toxin measurement in shellfish as well as dinoflagellates by many authors (Vieytes *et al.*, 1993; Doucette *et al.*, 1997; Velez *et al.*, 2001). Because the i.p. mouse toxicities of STX analogues corresponded well to binding activities to Na channels (Hall *et al.*, 1990), good correlation was reported in most of the papers. Vieytes *et al.* (1993) tested three samples of purified toxins with different toxin composition and found good correlation with the MBA. Doucette *et al.* (1997) tested 20 shellfish extracts with r=0.94 and found higher toxic scores by the binding assay than MBA. Velez *et al.* (2001), using chick cerebellum instead of rat brain synaptosome, reported r=0.97 for 40 shellfish samples. Llewellyn *et al.* (2001) also found good correlation (r=0.88) on the analysis of 35 shellfish extracts from Alaska. Ruberu *et al.* (2003) reported the optimization of procedures for the receptor assay and the results of an interlaboratory comparison with high throughput using microplate scintillation counting. Shellfish samples tested (n=75) ranged from non-detectable by MBA (< 0.4 µg STX eq/g) to 1.37 µg STX eq/g. The detection limit of the optimized assay was 0.002 µg STX eq/g with a between-assay relative standard deviation of 10 percent. The ratio of toxin concentrations reported by the two laboratories averaged 0.9. The results were somewhat higher than those by MBA for samples < 0.8 µg STX eq/g, as has been previously observed. It is likely that the shellfish matrix provides a "protective" effect for the mouse, as it is injected undiluted at these low levels.

The receptor binding assay (RBA) is sensitive and rather specific, but requires [^{3}H]STX. As for many other assays for the STX group, a major impediment to progress in validation trials of receptor assays is the classification of STX as a class 1 chemical weapon by the Chemical Weapons Convention, which has severely hampered the availability of [^{3}H]STX. However, the availability of [^{3}H]STX as a diacetate salt (Amersham), recognized as a different chemical form, has recently eased restrictions. American Radiolabeled Chemicals, in collaboration with the USFDA and the International Atomic Energy Agency (IAEA), produced an exchange-labelled tritiated STX as an alternative and equivalent to the product previously produced by Amersham. This product has proved to be stable for six months in an ongoing storage

stability trial. This material is being distributed internationally by the IAEA through a Technical Cooperation Project. The intercalibration exercises have been successful (Van Dolah, 2005). Tritiated tetrodotoxin, which was shown to work as well as [^{3}H] STX for the RBA by Doucette *et al.* (2000), might be another option for this problem.

4.2.2.1.2 Saxiphilin

Besides the Na channel preparation, soluble proteins that specifically bind STX were found in the circulatory fluid of several different animal species. These proteins, the saxiphilins, have been used in a competitive binding assay with [^{3}H]STX by Llewellyn's group (Llewellyn *et al.*, 1998; Negri and Llewellyn, 1998; Llewellyn and Doyle, 2001). Sensitivity for STX was reported to be equivalent to that of the Na channel binding assay (2 µg STXeq/litre). The results of the assay in several biological specimens were reported to show good correlation to other assays. However, these were rather unique samples, such as crabs and cyanobacteria. In the analysis of shellfish samples, the correlation was 0.84, but the method underestimates toxicity of many samples, because the affinity of saxiphilin to some other analogues was reported to be different from that of the Na channel (Llewellyn *et al.*, 2001). More characterization is necessary before this can be utilized as a general method to evaluate shellfish toxicity.

4.2.2.1.3 Live cell-based RBA

Binding of the toxin to Na channels can be observed indirectly by measuring cytotoxicity to Na-channel-rich cells. Dose-dependent protective effects of the toxin to mouse neuroblastoma cells, which had been challenged by veratridine (Na channel activator) and ouabain (inhibitor of Na pump, Na/KATPase), were first reported by Kogure *et al.* (1988). The original report was based on microscopic observation, and the method has since been improved by several authors, especially in the determination of cell viability. Jellett *et al.* (1992) used crystal violet staining and employed a microplate reader for automated determinations of the stained neuroblastoma cells. The detection limit was found to be as low as around 10 ng STX eq/ml of extract (= 0.02 µg STX eq/g shellfish tissue). The method provided results virtually identical to those obtained with MBA ($r > 0.96$), when compared on 10 acid extracts of dinoflagellates and 47 extracts of shellfish tissues prepared according to the AOAC procedure (Jellett *et al.*, 1992). Gallacher and Birkbeck (1992) used neutral red staining for the measurement of cell viability. Truman and Lake (1996) also compared results of the neuroblastoma assay and the MBA. Twenty-nine extracts of shellfish gave negative results in both assays. Fifty-seven extracts gave positive results in at least one assay. In spiking studies with shellfish extracts, the neuroblastoma assay showed a good response to added STX. The correlation between the assays in shellfish was 0.876. More recently, Nicholson *et al.* (2002) developed the method based on rhodamine 6G as a probe for depolarization using freshly prepared mouse brain synaptoneurosomes. Data sets of 120 mussels and 29 other shellfish species indicated correlation $r^2 = 0.84$ and 0.86 ($r = 0.92$, 0.98). No false negatives were observed and the cell bioassay tended to show higher toxicity than the MBA as reported in the other method. The authors emphasized shorter test time as an advantage over the other neuroblastoma assays. In principle, the Na channel binding assay and the neuroblastoma cell assay could be good alternatives to MBA for testing shellfish for PSP toxins.

4.2.3 Other biochemical assays

Fairey, Edmunds and Ramsdell (1997) reported a cell-based assay for STX using c-flos-luciferase reporter gene expressed in mouse neuroblastoma cells. Luminescence response generated by treatment of BTX (PBTX-1) was inhibited by STX in a concentration-dependent manner at 3.5 ng/ml of half the maximum effect. The sensitivity of this method is close to that of receptor binding and cytotoxicity assays.

Kerr, Briggs and Saba (1999) investigated *in vitro* rat hippocampal slice preparations as a means of detecting STXs together with the BTX and domoic acid (DA) by observing toxin-specific electro-physiological signatures. Shimojo and Iwaoka (2000) found that red blood cells from the tilapia (*Sarotherodon mossambicus*) were haemolized by the treatment with veratridine and ouabain, and haemolysis was protected by addition of STX. The authors reported the test was able to detect STX in concentrations at 0.3 µg/ml, which is slightly above the limit of detection of MBA. No information was provided about its value in screening shellfish in practice. Cheun *et al.* (1998) developed a tissue biosensor system consisting of a Na+ electrode covered with a frog bladder membrane integrated within a flow cell. The Na+ transfer, from the internal to the external side of the membrane, was blocked by Na+ channel blockers. The tissue sensor responded to STX analogues (GTX1-GTX4, neoSTX, dcSTX) by Na+ ion decrease. Comparing the results with those obtained by the standard MBA showed good agreement except for GTX2. Lee *et al.* (2000) used the method to examine the toxicity in cultured *Alexandrium tamarensis* strains under various environmental conditions. The tissue biosensor system was able to measure very small quantities of PSP toxin within an individual plankton cell (5 femtograms). More characterization is necessary in order to determine the usefulness of these methods for routine shellfish monitoring.

4.2.4 Immunoassays

Initially polyclonal antibodies have been obtained from rabbits injected by a STX-protein immunogenic conjugate (Carlson *et al.*, 1984; Yang *et al.*, 1987; Renz and Terplan, 1988; Usleber, Schneider and Terplan, 1991; Cembella and Lamoureux, 1993; Usleber *et al.*, 1994, 1997; Huang, Hsu and Chu, 1996). Using the same type of immunogen, monoclonal antibodies were also produced (Dietrich *et al.*, 1996). Other polyclonal antibodies have been obtained from conjugates with other compounds than STX, such as neoSTX (Huang, Hsu and Chu, 1996; Bürk *et al.*, 1995), or GTX2,3 (Frémy *et al.*, 1997). Regarding specificity, because the STX group can be divided according to the molecular formula in two subgroups, cross-reactivity figures are different accordingly: antibodies obtained from a conjugate with STX cross-react with the subgroup involving STXOL, dcSTX, GTX2,3 and N-sulphocarbamoyl-STX (B1); and antibodies obtained from a conjugate with neoSTX cross-react with the subgroup involving the N1-hydroxy STX compounds (neoSTX, GTX1,4) (Usleber *et al.*, 2001). For this reason, it is difficult to expect a general quantitative immunoassay specific to all STX group toxins by using only one type of antibody. Moreover, it has been noticed that monoclonal antibodies have a lower affinity than polyclonal. The lowest sensitivity obtained by an ELISA test was 0.2 ng STX eq/g of shellfish tissue (Usleber *et al.*, 2001). Based on this principle a test kit is commercially available (R-Biopharm) and in-house validated.

Some other screening test kits have been developed using membrane principle, such as the MIST Alert™ by Jellett *et al.* (1992) and Jellett, Doucette and Belland (1998) with a detection limit of 0.4 µg STX eq/g in less than 20 minutes. Field trial results indicate that MIST Alert™ could also be used by shellfish farmers as a shellfish management tool and by processors in end-product testing (Jellett *et al.*, 2002). Because it is believed that different geographic areas have predominant STX group toxin profiles, initial evaluations are proceeding in many countries to look at false positive rates of the Rapid Test, which did vary between 2 and 15 percent in the initial validation trials (Jellett *et al.*, 2002). In the validation trials, LC showed that the majority of the false positive samples resulted from the presence of significant amounts of B and C analogues in the sample extract. Unlike MBA, which does not recognize these analogues as toxic, the Rapid Test detects these analogues in nM concentrations in the same range as STX, neoSTX, GTXs and the other members of this toxin family that are recognized by the

MBA as toxic (Laycock *et al.*, 2001). The B and C toxins are the main forms found in phytoplankton and, therefore, blooms developing offshore and coming onshore are more likely to cause false positive Rapid Test results, as the shellfish will contain many undigested toxic cells in their guts. Blooms developing *in situ* are less likely to cause false positive Rapid Test results, as shellfish have more time to digest the algal cells, at which time the B and C analogues are converted to the more toxic analogues that are detected in the MBA. Most of the "false positive" Rapid Test results were shown by LC in the validation trials to contain between 0.2-0.4 µg STX eq/g of toxins, and with many having B and C analogues as the majority of the toxin profile. This has made the Rapid Test for PSP useful in both phytoplankton monitoring and in early bloom detection. The United Kingdom Food Standards Agency has completed the validation of the Rapid Test for PSP (MacKintosh *et al.*, 2002; MacKintosh and Smith, 2002) in its laboratory in Scotland, and has proceeded to implement it there as of spring 2004.

4.2.4.1 Comparative studies

The main purpose of immunochemical assays development is the screening of general toxin contamination as an alternative to animal tests. For this reason, comparative studies involving the MBA are unavoidable. Chu *et al.* (1996) compiled data from comparative studies between mouse tests and ELISA tests specific to STX and neoSTX performed on 1 540 positive or negative samples. A good correlation was found for toxin contents higher than 0.4 µg STX eq/g. This correlation becomes poor when data from ELISA tests for STX and from ELISA tests for neoSTX are compared separately with data from the mouse tests. This observation confirms the necessity of using immunochemical assays specific to both STX and neoSTX subgroups to detect the general toxicity of all toxins belonging to the STX group. However, combining ELISA tests specific to the two subgroups could save the performance of 80–85 percent of mouse tests (Chu *et al.*, 1996). When interlaboratory studies were conducted for the validation of STX certified reference materials, data from ELISA tests were above the mean content estimated from data obtained by the other methods. This could be because of the detection by cross-reactivity of other compounds than STX present in the naturally contaminated samples. A similar overestimation was noticed by Usleber *et al.* (1997) in comparing data from the ELISA test and from the mouse bio test for contents below 0.8 µg STX eq/g, and the opposite figure was noticed for highly contaminated samples.

In another study, the ELISA test specific to STX subgroup, the LC method and MBA were conducted by O'Neill, Gallacher and Riddoch (1998) on scallop and mussel samples coming from the east coast of the United Kingdom of Great Britain and Northern Ireland. The toxin content was underestimated by the ELISA test in mussels because the LC chromatograms showed the presence of neoSTX and GTX1,4. These toxins belonging to the second subgroup were not detected by this ELISA test. However, ELISA tests, because of their high sensitivity and despite their respective specificity to the two subgroups of toxins, can evaluate the general toxin contamination around the sensitivity level of the mouse test (Usleber *et al.*, 2001).

4.3 Chemical assays

4.3.1 Introduction

Saxitoxins show no specific UV absorption nor fluorescence that can be utilized for the detection. Colorimetric measurement targeted to guanidium or carbamoyl moieties is not realistic because of the required concentration level of 1 uM for the health risk assessment and large amount of contaminants coexisting in the extracts. During structure determination of STX, an iminopurine compound was identified as the product of alkaline oxidation by H_2O_2. Fluorescent intensity greatly increased in acidic solution, probably because of formation of lactum ring. The reaction is very much

specific to the toxin group and later was used for the detection of other analogues on TLC or electrophoresis during purification without actual knowledge on the structures of fluorophores. This reaction was also adopted for the measurement of toxin level in shellfish, and soon it was recognized that H_2O_2 oxidation does not yield enough fluorescent derivatives in solution from N-OH toxin group, such as neoSTX, GTX1 and GTX4. Another oxidation reagent was tested, such as tert-butylhydroperoxide. At present, periodate seems the most suitable for the chemical detection of the wide range of toxin analogues. However, it should be noted that fluorescence yield varied greatly with slight change of oxidizing conditions (species and concentration of oxidants, pH, temperature and reaction time). Even 11-hydroxysulphate epimers (e.g. GTX2/GTX3) show different responses to the oxidation conditions. Another difficulty of chemical analysis is the large number of target molecules with different chemical natures, especially the wide range of charged states from -1 to +2 at pH 7, and the wide variation of specific toxicity. The toxin profiles (relative abundance of analogues) of contaminated shellfish greatly differ according to the toxin profiles of the causative organisms, and also by the time after accumulation.

4.3.2 PSP toxins standards

For the application of both precolumn and postcolumn derivatization LC, a whole set of STX analogues is essential as external standards. In the papers cited above, authors used standards of various sources, either personally prepared or donated (Sullivan and Iwaoka, 1983; Oshima, 1995b), or mixing commercially available toxins or those obtained from the European Community (EC) or the National Research Council of Canada (NRC) (Hummert *et al*., 1997). At the moment, certified standards of STX, neoSTX, GTX1/4, GTX 2/3, B1(GTX5), C1/C2, dcGTX2/3, dcSTX and dcneoSTX are commercially available (Laycock *et al.*, 1994; NRC, 2011) from the NRC, Canada. An STX reference material for calibration is distributed by the Center for Food Safety and Applied Nutrition, Food and Drug Administration of the United States of America (US FDA CFSAN). Two mussel tissue certified reference materials (CRMs) are available (Joint Research Centre [Directorate-General of the European Commission]/ Institute for Reference Materials and Measurements, Geel, Belgium). Researchers need to be careful when changing from one standard to another because a discontinuity of data may occur. Concentration differences up to 20 percent have been noticed between STX concentrations of three different suppliers (Quilliam *et al*., 1999).

4.3.3 Fluorometric techniques without separation

The alkaline oxidation of the toxins yields fluorescent products, allowing simple determination using fluorometric techniques (Bates and Rapoport, 1975; Bates, Kostriken and Rapaport, 1978, Shoptaugh *et al*., 1981a, 1981b). Later, a semi-automated method, applying a derivatization system for postcolumn oxidation, was developed by Jonas-Davies *et al*. (1984), and an automated method, using flow injection analysis with automatic correction of background fluorescence, was developed by Hungerford, Lee and Hall (1991). Good correlation was observed (r around 0.8), when compared with MBA on 149 shellfish samples, especially when shellfish samples from the same area were compared. However, a false negative result was observed in a sample that contained mainly highly toxic neoSTX, yet gave a low fluorescence response (Jonas-Davies *et al*., 1984). The method might be useful for rapid screening of shellfish samples for the presence of the toxins in an area where the information on toxin constituents in shellfish is well known.

4.3.4 Precolumn-derivatization LC

Lawrence *et al*. (1991) developed a method to separate and detect the fluorescent compounds formed by the oxidation of toxins. The so-called "precolumn oxidation

method" is instrumentally much simpler than the postcolumn derivatization method described below, by using a single reverse phased column chromatography system. However, in some cases, multiple products are produced from single toxins. This can lead to difficulty in interpreting the chromatograms. Also, some toxin epimer pairs (11-hydroxysulphate toxins (GTX2/GTX3, GTX1/GTX4 and C1/C2) could not be distinguished from each other, even by the improvement of analysis reported afterwards (Lawrence, Wong and Ménard, 1996; Lawrence and Niedzwiadek, 2001). The detection limits ranged from 3 to 12 ng/g depending on toxins with a correlation coefficient with the MBA of 0.95 for 16 samples from the east coast of the United States of America and Canada (Lawrence and Ménard, 1991). A modification of the method (Lawrence, Niedzwiadek and Ménard, 2004) was submitted to an interlaboratory study involving 18 laboratories from 14 different countries. The 13 PSP toxins studied were STX, neosaxitoxin, GTX2,3 (together), GTX1,4 (together), decarbamoyl STX, B-1 (GTX5), C-1 and C-2 (together), and C-3 and C-4 (together). B-2 (GTX6) toxin was also included, but for qualitative identification only. The samples (mussels, clams, oysters and scallops) included shellfish tissue from Spain, New Zealand and the east and west coasts of Canada and contained a variety of PSP toxin patterns. Collaborators were able to quantify STX, NEO, dcSTX, GTX2,3, GTX1,4 and B1 at individual concentrations down to between one-tenth and one-twentieth of the common regulatory guideline level of 800 µg/kg (80 µg/100 g) STX equivalents. The C toxins were successfully quantified at levels down to about one-fiftieth to one-hundredth of the regulatory level in terms of STX equivalents although in terms of µg/kg concentration units they were the least sensitive. The HORRAT values were less than 2.0 in most cases, indicating acceptable interlaboratory reproducibilities for all the toxins at the concentration levels examined. The results of this study are currently being evaluated by AOAC International for approval as an AOAC International official method. The method is also useful for the screening of shellfish samples to reduce the number of analyses by MBA, as exemplified by Vale and Sampayo (2001).

4.3.5 Postcolumn-derivatization LC

During the last decade, considerable effort has been applied to the development of an LC method for the complete analysis of the toxins. The idea of continuous analysis of the toxins by postcolumn oxidation and subsequent detection of fluorescence was first proposed by Buckley, Oshima and Shimizu (1978). Separation of the toxins was achieved to some extent with ion-exchange (Oshima *et al.*, 1984) and cyano-bonded silicagel columns (Sullivan and Iwaoka, 1983). Sullivan and Wekell (1984) reported complete separation of the major ten toxins by an ion pair chromatography on a reversed phased column. They used a polystyrene divinylbenzene polymer column with gradient elution of mobile phase containing hexane and hepatanesulphonic acids (as counter ion) and methanol as modifier. The C toxin group (C1-C4), which has both a N-sulphonate and an 11-hydroxysulphate, was not resolved by this condition. Application of periodide for the postcolumn oxidation enabled much better fluorescent response for the N-1 hydroxyl toxin group (neoSTX, GTX1/GTX4) than H_2O_2 previously used. For the better resolution of toxins, especially for the decarbamoyl toxins from corresponding carbamoyl toxins, Oshima (1995b) and Oshima, Sugino and Yasumoto (1989) used silica based C8 column with isocratic elution of two sets of mobile phases containing heptanesulphonic acid as an ion-pair reagent. Gradient or stepwise elution was abandoned because of long re-equilibration time needed to restart the analysis. Separation of C1-C4 toxins could be achieved by adopting another mobile phase containing tert-butyl ammonium as a counter ion. It was later shown to be applicable to Sullivan's polymer column (Boczar *et al.*, 1988). Thus, at least two sets of analysis are required for the complete analysis of whole toxin groups. Other authors reported modified methods using a different combination of columns (silica based C18)

and mobile phases, but essentially on the same principle using ion pair chromatography with postcolumn derivatization and fluorescent detection (Nagashima *et al*., 1987; Thielert, Kaiser and Luckas, 1991; Franco and Fernandez-Vila, 1993; Yu *et al*., 1998; Chen and Chou, 2002). Some authors reported electrochemical oxidation for the postcolumn reaction and yielded almost identical fluorescence response to the chemical reaction (Janiszewski and Boyer, 1993; Boyer, Janiszewski and Hu, 1998; Goddard and Boyer, 1999; Gregory, Goddard and Boyer, 2001). Detection limits of postcolumn derivatization LC varied greatly according to the chemical nature of toxins, but were generally an order of magnitude lower than the mouse assay.

Postcolumn derivatization fluorescent detection LC has been utilized extensively for the research of toxin chemistry in dinoflagellates as well as in shellfish, but there are only a few reports that compare the results with those of MBA. The estimated total toxicity of the sample was calculated by summation of toxicity of each of the analogues detected by LC. Sullivan, Wekell and Kentala (1985) reported r=0.92 on 40 toxic samples from the Pacific coast. Salter *et al*. (1989) analysed 94 shellfish from New England, the United States of America, by Sullivan's system and found high correlation (r=0.94) in overall samples, but lower correlation (r=0.53) on the low toxic samples. Oshima, Sugino and Yasumoto (1989) found r=0.84 for 20 scallop samples from Japan, but recognized that LC tended to give higher values than MBA in the analysis of low toxic samples, probably because of the "salt effect" that underestimates toxin content by the mouse assay. Chen and Chou (2002) reported essentially the same phenomenon on the analysis of 20 purple clam samples from Taiwan Province of China (r=0.94).

4.3.6 Capillary electrophoresis (CE)

Analysis of STX and some analogues was resolved by CE (Thibault, Pleasance and Laycock, 1991; Buzy, Thibault and Laycock, 1994; Locke and Thibault, 1994). Applicability of the method for the shellfish extract was not tested much and was rather questionable because of the limit sample loading and low sensitivity.

4.3.7 LC with mass spectroscopic detection (LC-MS)

Mass spectroscopy has been used as the fundamental tool for the structure elucidation of STX analogues as well as for the identification of purified toxins. There are some reports on the utilization of MS coupled with LC for the quantitative analysis of STXs. However, most of them are preliminary studies using mainly pure toxins and have not been proved useful for practical analytical methods of STXs in shellfish.

Quilliam *et al*. (1989) reported the determination of STX by LC-MS applying an ion-spray ionization technique. The detection limit of pure STX was estimated to be 0.1 uM (1 ul injection) by flow injection analysis, which is about five times more sensitive than the AOAC MBA. Pleasance *et al*. (1992) achieved separation and detection of GTX2, GTX3, neoSTX, STX by ionspray MS coupled with PRP-1 column, and C1 and C2 with amino-bonded column. However, reproducibility and sensitivity were not satisfactory. Quilliam and Janecek (1993) reported the LC-MS analysis of periodate oxidation products of toxins. This study provided useful information on the structure of the derivatives, but sensitivity was much lower than fluorescent detection. Lagos *et al*. (1999) used ionspray MS coupled with ODS column and mobile phase containing volatile ion-pair reagent for the analysis of STX and analogues in cyanabacteria as a confirmatory procedure.

Jaime *et al*. (2001) introduced splitting after postcolumn oxidation of LC equipped ion exchange column, and detected oxidation products by ionspray MS. Detection limit for each toxin was equivalent to florescence detection for N1-OH toxin group (GTX1/4, neoSTX), but 20–150 higher (less sensitive) for N1-H toxin group (GTX2/3, STX). Recently, Dell'Aversano, Hess and Quilliam (2003) reported hydrophilic interaction

liquid chromatography coupled with electrospray ionization mass spectrometry. Not all the toxins could be completely separated, but with the additional selectivity provided by mass spectrometry, quantitation of all the nine major toxins tested was possible. Detection limits of most of the toxins were higher than postcolumn LC, but close to required levels for practical analysis of the toxins in shellfish (0.05–1 uM). This method is promising because of simple chromatographic conditions, which enable the analysis within 30 minutes.

5. EVALUATION[3]

5.1 Toxicology

Saxitoxins are a group of toxins that have caused severe human illness from time to time, when blooms of causative algae have become widespread leading to an uptake of toxins by various species of shellfish in those areas. The main adverse effect of STXs in animal species and humans is neurotoxicity observed shortly after exposure. There are no animal data available on short- or long-term toxicity, carcinogenicity, genotoxicity, reproductive toxicity or developmental toxicity.

There is a considerable amount of data on exposures that have resulted in acute human illness. Large interindividual differences in susceptibility were noted, related in part to pharmocokinetic and pharmacodynamic differences, as well as possible misreporting. Thus, some people who ate similar amounts of a meal became seriously ill, while others eating the same meal were much less or not affected. However, in many individual case series, the observed responses were related to dose. In addition, differences in apparent susceptibility between various case series may have arisen from a lack of precision in exposure assessments because of problems with sampling, considerations of the effect of processing and analysis of contaminated shellfish at the time of intoxication. Whereas many studies referred to the effect of cooking on lowering toxin levels, this information was lacking in some studies and, if ignored, may have resulted in overestimates of dose in some studies.

There is a need for better data on patient description, food consumption, symptomatology, follow-up and exposure assessment for individual case histories, because these data form the basis for a derivation of the acute reference dose (ARfD). It would help if internationally there were agreement on a medical history form regarding further investigation of such illnesses.

Because of the large person-to-person variation in susceptibility, average doses are not very informative for the purpose of deriving a safe dose estimate. One therefore needs to focus on the limits of the range in sensitivity and particularly the minimum dose that may induce illness. At what level should the effects be considered "adverse", and what level is the actual no observable adverse effect level (NOAEL) and the lowest observable adverse effect level (LOAEL)? Because the data on PSP represent a range of individuals with varying susceptibilities (occupation, age and sex), a tenfold factor to take into account differences in susceptibility may not be needed. Based on the overall epidemiological data, a dose of 2 µg STX eq/kg b.w. may be considered as the LOAEL. Because mild illness at lower doses is readily reversible, applying a safety factor of 3 to the LOAEL would establish a provisional ARfD of 0.7 µg STX eq/kg b.w.

Further effort is needed to evaluate epidemiological data fully or to assess the effects of cooking or processing for deriving the ARfD and provisional guidance levels/ maximum levels for the STX group.

[3] It must be pointed out that, as a result of the Expert Consultation, the Evaluation Section of the Report differed from that of the Saxitoxins draft chapter. The Evaluation Section of the present Background Document has been adapted to fit the outcome of the Expert Consultation Report. In particular, the effect of cooking on STX level has not been considered.

5.2 Analytical methods

The AOAC protocol for MBA is widely used and has provided health protection for many years when used within a biotoxin monitoring programme. However, performance was highly variable in an interlaboratory study involving nine European laboratories (Earnshaw, 2003). Some tightening of the protocol (e.g. pH adjustment of the extract) has been recommended to improve the reproducibility (European Community Reference Laboratory [CRL], Asia-Pacific Economic Cooperation [APEC]). Accurate conversion from MU to mg/kg requires calibration of mouse strain sensitivity to STX according to the AOAC protocol. The detection limit of the MBA is 0.4 mg/kg STX.2HCl equivalents and considerable uncertainties exist at levels close to this limit. The method may underestimate true levels, e.g. when levels of about 0.8 mg/kg are detected by the MBA, the actual concentration present may range from 1.2 to 2.1 mg/kg (because of salt effects). Ethical issues, relating to the use of live animals, affect the acceptance and use of MBA in many Member States.

The RBA, using tritiated-STX and rat brain membrane preparations with shellfish extracts prepared by the AOAC protocol, has shown excellent correlations to the MBA (van Dolah, 2005). The limit of quantitation (LOQ) is 0.001 mg/kg STX.2HCl equivalents. Interlaboratory trials are proceeding to establish the full performance characteristics. Availability of the labelled reagent and use of radioisotopes are ongoing issues for routine use of this RBA. Other functional assays are at a preliminary stage of validation.

Antibodies are not available with binding characteristics that match the toxicity spectrum of all the STXs. Therefore immunoassays cannot deliver quantitative toxicity data for mixtures, particularly over wide geographical regions and different algal species. The PSP strip test (Jellett Rapid Testing) has delivered promising data in extensive studies in North America and Europe (the United Kingdom of Great Britain and Northern Ireland) with a low false negative rate in comparisons to the MBA (detection threshold set at 0.8 mg/kg). This has led to its approval as a screening test within biotoxin monitoring programmes in several countries.

For LC detection, oxidative conversion of STXs to fluorescent derivatives is required. Because the fluorescence yield is compound dependent, individual toxin calibration is required and toxicity equivalence factors (TEFs) are required to calculate the total STX equivalents. The Lawrence precolumn LC-FL method can provide a full coverage of STXs. The method has been interlaboratory studied in Europe (mussel and two toxins) and approved by the Comité Européen de Normalisation (European Committee for Standardization, CEN [2004]). A collaborative study with a wider scope (4 matrices and 12 toxins) has been submitted to AOAC for approval. Accuracy and precision were good, and correlation to MBA data was high. The LOQ is about 0.1 mg/kg STX equivalents, dependent on the composition of toxins. The Oshima postcolumn LC-FL method is widely used but has not had a full interlaboratory study. Correlations with MBA have been given favourable results. Other instrumental assays such as LC-MS are at a tentative stage.

5.3 Regulatory limits

A regulatory level of 80 µg STX eq/100 g (0.8 mg STX eq/kg) or the equivalent value of 400 MU/100 g shellfish has been in existence in North America since the middle of the last century, and was established on the basis of exposure data from earlier PSP outbreaks in North America. Since then, many countries have adopted this level. Because toxin levels can rise rapidly, routine monitoring in Canada is increased when toxin values exceed the detection limit by MBA of 40 µg STX eq/100 g shellfish. Most shellfish poisonings have happened when PSP levels in raw shellfish exceeded the 80 µg/100 g level at least several-fold, i.e. > 250 µg/100 g. Illnesses generally have been the result of "illegal" harvesting in closed areas, or in areas that had not yet been

suspected of having PSP toxins in shellfish. Thus, the monitoring programme appears to be working.

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Yessotoxins

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1. BACKGROUND INFORMATION[1]

The presence of yessotoxins (YTXs) in shellfish was discovered because of their high acute toxicity in mice after intraperitoneal (i.p.) injection of lipophilic extracts. They are much less potent via the oral route, and they do not induce diarrhoea. There are no reports of human intoxication caused by YTXs. Consequently, YTXs should be regulated separately from the okadaic (OA) toxin group (diarrhoeic shellfish poisoning [DSP] toxins). At present, the regulation of YTXs is based on results from animal experiments. The analysis of YTXs poses considerable potential problems because of the large number of analogues produced by the algae and their extensive metabolism in shellfish. Yessotoxins are persistent in shellfish tissues and, therefore, depending on the regulatory significance, may require long-term monitoring in management programmes.

2. ORIGINS AND CHEMICAL DATA IN SHELLFISH

2.1 Production of YTXs by dinoflagellates

2.1.1 Production of YTXs by Protoceratium reticulatum

Yessotoxin was originally demonstrated as a metabolite of an isolate of *Protoceratium reticulatum* (formerly *Gonyaulax grindleyi*) from New Zealand by Satake, MacKenzie and Yasumoto (1997). Production of YTX has since been demonstrated in *P. reticulatum* isolates from Japan (Satake *et al.* 1999); Italy (Ciminiello *et al.*, 2003); Norway (Samdal *et al.*, 2004); Spain and the United States of America (Paz *et al.*, 2004); Chile and eastern Canada (M. Quilliam, personal communication). Typical levels of YTX reported are 3–12 pg/cell. In addition to YTX, *P. reticulatum* has been shown to produce trinoryessotoxin (Satake *et al.*, 1999), heptanor-41-oxoyessotoxin, 1a-homoyessotoxin, a carboxyyessotoxin and a hydroxyyessotoxin (Ciminiello *et al.*, 2003). A wide range of other YTXs have recently been identified from *P. reticulatum*, including 41a-homo-, 41a-homo-9-methyl-, dihydroxyamido-, dihydroxy- and 41-oxo-yessotoxins (Figure 1) (Miles *et al.*, 2004a, 2004b, 2005a, 2005b). Also produced are 32-*O*-mono- and -di-glycosides of YTX (Miles *et al.*, 2006), as well as oxidized glycoside analogues (Cooney, Jensen and Miles, 2004). Konishi *et al.* (2004) recently characterized 32-*O*-mono-, -di-, and -tri-arabinosides of 1a-homoyessotoxin from cultures of an alga resembling *P. reticulatum*. These compounds were highly cytotoxic and appear to be the homo analogues to the glycosides of YTX from *P. reticulatum*. In the majority of isolates, *P. reticulatum* produces mainly YTX and related analogues

[1] Corresponds to the "Background Information" section of the Expert Consultation Report.

without 1a-homoyessotoxins, but in some cases, particularly from the Adriatic Sea, 1a-homoyessotoxins predominate.

Satake (2000) has demonstrated the principle features of YTX biosynthesis by incorporation experiments using isotopically labelled acetate. The degree of flexibility of biosynthesis in *P. reticulatum* has only recently become fully apparent, with Miles *et al.* (2005a, 2005b) revealing the presence of an array of more than 90 YTX analogues in one isolate, the majority as yet unidentified.

2.1.2 *Production of YTXs by* Lingulodinium polyedrum

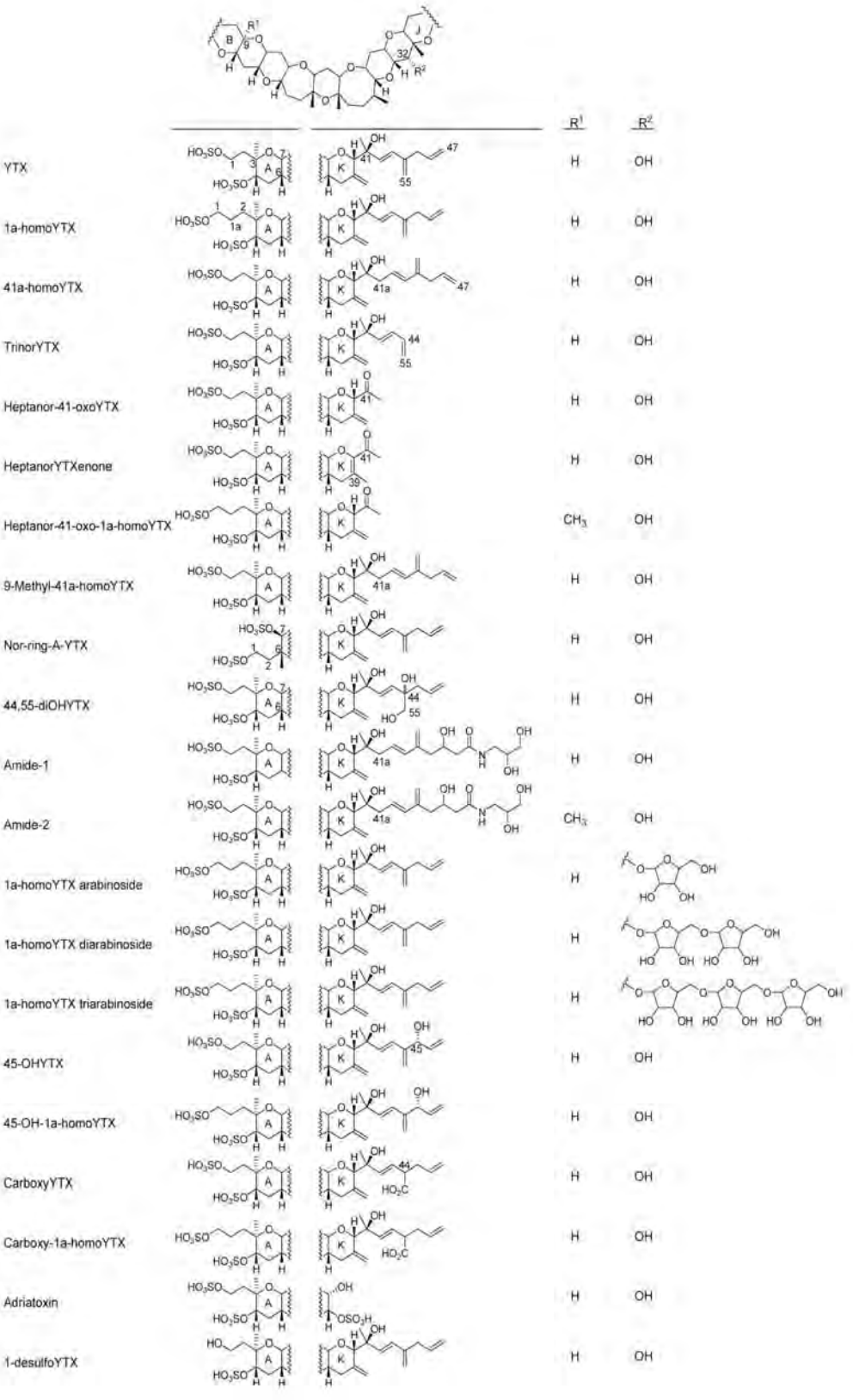
1a-Homoyessotoxin was identified in algal blooms in which *Lingulodinium polyedrum* was the predominant alga (Draisci *et al.*, 1999b), and evidence that this organism can indeed produce yessotoxins was provided by identification of YTX in cultures of a Spanish isolate (Paz *et al.*, 2004). Recently, YTX, but not homoYTX, was found in New Zealand coastal waters and shellfish contaminated by a bloom of a closely related dinoflagellate *Gonyaulax* cf *spinifera* (Rhodes *et al.*, 2004).

2.2 Uptake and metabolism of YTXs by molluscan shellfish

Yessotoxin and analogues are readily accumulated by filter feeding molluscs. Most of the compounds found in the alga (Section 2.1) have also been found in contaminated shellfish tissues, either by isolation and full structural characterization (Murata *et al.*, 1987; Satake, Mackenzie and Yasumoto, 1997; Ciminiello *et al.*, 1997, 1999, 2000a, 2000b, 2001a, 2001b); or by liquid chromatography with fluorescence detection (LC-FL) and liquid chromatography with mass spectrometry (LC-MS) analyses (Lee *et al.*, 1988; Draisci *et al.*, 1999a; Mackenzie, Suzuki and Adamson, 2001, Mackenzie *et al.*, 2002; Ciminiello *et al.*, 2002a, 2003; Aasen *et al.*, 2005; Finch *et al.*, 2004; Samdal *et al.*, 2005). Morohashi *et al.* (2000) confirmed that C45 has the *R*-configuration in 45-OHYTX. Three other YTX analogues not reported from algae have been found in significant amounts in contaminated mussels – adriatoxin (Ciminiello *et al.*, 1998), 1-desulphoyessotoxin (Daiguji *et al.*, 1998) and a putative hydroxylated-carboxyYTX (Samdal *et al.*, 2005).

The extent to which the oxidized or degraded analogues of YTXs in shellfish arise from direct uptake or by metabolism of YTX and analogues within the shellfish is a matter for debate and further research. Evidence that metabolism plays a major role includes the much higher proportions of 45OHYTX and carboxyYTX relative to YTX in shellfish compared with that in *P. reticulatum* and the variation in the proportions of metabolites between different shellfish species. In Norwegian blue mussel (*Mytilus edulis*) concentrations of YTX, 45-OHYTX and carboxyYTX were of similar magnitude (Aasen *et al.*, 2005) while in Greenshell™ mussel (*Perna canliculus*) YTX predominated over these metabolites (Mackenzie, Suzuki and Adamson, 2001; P. McNabb, personal communication). Yessotoxins are relatively persistent in shellfish tissues (Ramstad *et al.*, 2001a; Mackenzie *et al.*, 2002; Aasen *et al.*, 2005; Samdal *et al.*, 2005) and, at least initially, are concentrated in the digestive gland (Yasumoto and Takizawa, 1997). The half-life for YTX in Greenshell™ mussel was estimated at 49 days (Mackenzie *et al.*, 2002). As discussed in Section 4.3, enzyme-linked immunosorbent assay (ELISA) analyses of contaminated shellfish extracts have shown much high levels of immunoreactive YTXs than could be accounted for by LC-MS analyses that included YTX and several metabolites (Samdal *et al.*, 2005). Thus, similar to the causative algae *P. reticulatum*, contaminated shellfish contain a range of YTX analogues and metabolites including many with unknown structure or toxicity.

FIGURE 1
Summary of the structures of YTX and known analogues or metabolites



Source: Hess and Aasen, 2007.

3. BIOLOGICAL DATA IN MAMMALS

3.1 Biochemical aspects

3.1.1 Absorption, distribution and excretion

The majority of an oral dose of YTX administered to mice was recoverable from the faeces (Munday *et al.*, 2004), indicating a very low degree of absorption of the toxin from the gastrointestinal (GI) tract.

No data are available on the distribution of YTX in tissues of higher animals. No data regarding the excretion of YTX in vertebrates are available.

3.1.2 Biotransformation

No detailed data on metabolism of YTX in animals are available.

3.1.3 Effects on enzymes and other biochemical parameters

Inhibition of protein phosphatase

The effects of YTX at the molecular level have been originally investigated with reference to those already defined for the protein phosphatase inhibitor OA (Bialojan and Takai, 1988), because both toxins could be found in acetone extracts from contaminated shellfish (Ogino, Kumagai and Yasumoto, 1997).

Ogino, Kumagai and Yasumoto (1997) analysed whether YTX could inhibit the protein phosphatase isoform 2A, which has been shown to be sensitive to OA concentrations of 10^{-9} M, and showed that high concentrations (3×10^{-4} M) of YTX were needed for 50 percent inhibition of this enzyme under cell-free conditions. These data showed that YTX cannot be considered a protein phosphatase inhibitor, and the authors concluded that YTX should not be included in the category of diarrhoetic toxins (Ogino, Kumagai and Yasumoto, 1997). The consensus is that YTX is not a protein phosphatase inhibitor.

Effects of YTX on calcium movements across cell membranes

Based on the fact that YTX structurally resembles BTXs (Murata *et al.*, 1987), it was checked whether YTX might bind to voltage-gated sodium channels, and it was found that YTX does not interact with site 5 of voltage-gated sodium channels in rat brain synaptosomes (Inoue *et al.*, 2003).

De la Rosa *et al.* (2001a, 2001b) reported that YTX induces an increase in the cytosolic calcium concentrations of about 70 nM in isolated human lymphocytes. This effect is caused by 10^{-6} M concentrations of YTX, is completed within 2 minutes of toxin addition to isolated cells and depends on the presence of Ca^{2+} in the medium bathing the lymphocytes. Thus, YTX induces the influx of extracellular Ca^{2+} in human lymphocytes. Cell pre-incubation with YTX in Ca^{2+}-free medium, instead, prevents the influx of Ca^{2+} once the ion is added back to the medium (de la Rosa *et al.*, 2001a).

Attempts were made to identify the calcium channel(s) involved in these phenomena, by the use of inhibitors of different calcium conductive pathways. Among the inhibitors tested, $LaCl_3$ had no effect on the YTX-induced calcium entry in lymphocytes, whereas nifedipine and SKF 96365 could inhibit the YTX-induced calcium entry in human lymphocytes (de la Rosa *et al.*, 2001a). The authors, therefore, concluded that both L-type voltage-activated and depletion-activated calcium channels could be activated by YTX.

On the basis of the inhibitory effect of YTX on calcium entry observed in Ca^{2+}-free medium, experiments were carried out to ascertain whether YTX could affect the thapsigargin-induced calcium entry in human lymphocytes in Ca^{2+}-containing medium. The data obtained showed that YTX addition to lymphocytes before or together with thapsigargin strongly inhibited the influx of extracellular calcium induced by

thapsigargin in a Ca^{2+}-containing medium (de la Rosa *et al*., 2001a). A second effect of YTX, therefore, is the inhibition of capacitative calcium entry in human lymphocytes.

The data by de la Rosa *et al*. (2001a) show that YTX could interact with different calcium channels and interfere with their gating properties.

The capacity of YTX to interact with calcium channels and interfere with calcium movements across the plasma membrane was further analysed by investigating the effect of YTX on the calcium entry induced by maitotoxin (MTX) in human lymphocytes (de la Rosa *et al*., 2001a). Maitotoxin is a marine toxin whose mode of action involves both voltage-activated and receptor-activated calcium channels (Yasumoto and Murata, 1993). When human lymphocytes were pretreated with micromolar concentrations of YTX, the addition of MTX to the Ca^{2+}-containing medium determined an ion influx that resulted in intracellular calcium concentrations 25–30 percent higher than those found in cells treated with MTX alone (de la Rosa *et al*., 2001a). The calcium channels involved in this phenomenon were investigated by the use of the Ca^{2+} channel antagonists nifedipine and SKF 96365, and it was found that only the latter compound could reduce the calcium entry induced by YTX upon MTX addition to human lymphocytes. If these results are matched with those obtained in the previous study, as the authors note, it is apparent that MTX and YTX do not share the same mechanism of action and do not activate the same calcium entry pathway (de la Rosa *et al*., 2001a).

Support to the contention that YTX exerts some of its effects by the modulation of calcium entry in sensitive cells comes from a different study on possible effects of YTX on contaminated shellfish. Mussel immunocytes are activated by the treatment with the peptide N-formyl-Met-Leu-Phe (fMLP) (Malagoli and Ottaviani, 2004). Yessotoxin does not share the activating capacity of fMLP when administered alone to isolated mussel immunocytes, but it enhances the activating response to fMLP when immunocytes are treated with the toxin before the addition of fMLP (Malagoli and Ottaviani, 2004). The effective concentrations of YTX in this system were between 10^{-7} and 10^{-6} M. By the use of inhibitors of components in signal transduction pathways, it was shown that YTX effects are inhibited by verapamil and 2',5'-dideoxyadenosine, but were not affected by H-89, calphostin C and wortmannin. On the basis of those observations, the authors proposed that YTX could bring about its effect by intervening in L-type Ca^{2+} channel opening through a cAMP-dependent and PKA-independent pathway (Malagoli and Ottaviani, 2004). This study also confirmed that YTX effects can involve changes in intracellular cAMP concentrations (see below).

In another study, changes in the intracellular concentrations of Ca^{2+}-binding proteins and modification in some cytoskeletal components have been detected by an immunocytochemical method in Purkinje cells from the cerebellum of mice receiving an i.p. dose of 420 µg YTX/kg (Franchini *et al*., 2004a). The authors concluded that changes in intracellular Ca^{2+}-binding protein level that could impair calcium homeostasis may be considered an early indicator of neurological disorders induced by acute YTX toxicity. Direct conclusions regarding a primary role of Ca^{2+} in the mechanism by which YTX induced alterations in Purkinje cells after i.p. injection into mice cannot be drawn from this type of study.

An effect of YTX on the movement of calcium ions across cell membranes also has been shown in isolated mitochondria, targeting a voltage-dependent calcium channel. Bianchi *et al*. (2004) have shown that YTX opens the permeability transition pore (PTP) of rat liver mitochondria at concentrations of 10^{-7} M. The effect requires the presence of micromolar concentrations of calcium and is inhibited by cyclosporin, implying a direct effect of YTX on the PTP. Yessotoxin can induce opening of the PTP also *in situ*, as the addition of 10^{-7}–10^{-6} M YTX to intact hepatoma cells induced mitochondria depolarization.

Available data indicate that YTX, at 10^{-6} M concentrations, can affect several calcium channels, leading to altered calcium fluxes across cell membranes.

Effects of YTX on phosphodiesterases and intracellular cAMP concentrations

The incubation of isolated human lymphocytes with 10^{-6}–10^{-5} M YTX was found to induce a biphasic change in the intracellular concentrations of cAMP, consisting of an initial increase followed by a relative decrease, within minutes of toxin addition to cells in a calcium-containing medium (Alfonso *et al*., 2003). If YTX was added to Ca^{2+}-free medium, instead, sustained levels of cAMP were detected in treated lymphocytes. Yessotoxin was found to ablate the forskolin-induced increase in cellular cAMP in human lymphocytes. Because YTX could stimulate an initial rise in intracellular cAMP, its capacity to prevent the effect of forskolin was interpreted in terms of activation of some phosphodiesterase (PDE), rather than as an inhibition of adenylate cyclases (Alfonso *et al*., 2003).

By the use of inhibitors of different PDE isoforms, an attempt was made to identify the enzyme(s) selectively activated by YTX. The recorded responses indicated that several PDE isoforms could be targets of YTX (Alfonso *et al*., 2003).

Based on those results, the authors speculated that the most likely target of YTX could be the Ca^{2+}/calmodulin-dependent type I PDE, and proposed that the mechanism leading to PDE activation by YTX involves an initial increase of calcium influx followed by a relative rise in cytosolic calcium available for calcium-dependent type I PDE, with a consequent decrease in intracellular cAMP concentrations (Alfonso *et al*., 2003). The capacity of YTX to interact directly with PDE is implied by the observation that 10^{-6}–10^{-5} M YTX caused a 15–25 percent increase in the activity of PDE from bovine brain under cell-free conditions (Alfonso *et al*., 2003). The function of lymphocytes is believed to be inhibited by agents that increase the intracellular concentrations of cAMP (Sheth *et al*., 1997). The effect of YTX on interleukin-2 release was evaluated, and it was found that the toxin concentrations causing a decrease in intracellular cAMP caused an increase in interleukin-2 production in lymphocytes after a 20-hour treatment, lending support to the notion that YTX might affect the functioning of lymphocytes (Alfonso *et al*., 2003).

Available evidence indicates that 10^{-7}–10^{-5} M concentrations of YTX can induce changes in the intracellular levels of cAMP in cultured cells, but it remains to be established whether the effects of YTX involve either a decrease (Alfonso *et al*., 2003) or an increase (Malagoli and Ottaviani, 2004) in cellular concentrations of cAMP.

Effects of YTX on cell adhesion and related proteins

Yessotoxin has long been recognized to induce cell detachment from culture dishes (Ogino, Kumagai and Yasumoto, 1997).

E-cadherin belongs to a large family of proteins responsible for Ca^{2+}-dependent cell-cell adhesion (Tepass *et al*., 2000). The effects of YTX on E-cadherin in the human breast cancer cell line MCF–7 have been investigated by Rossini and his collaborators, after they found that 10^{-10}–10^{-9} M concentrations of YTX cause E-cadherin fragmentation (Pierotti *et al*., 2003), and a collapse of the E-cadherin system was detected after 2–5 days of treatment with the toxin (Ronzitti *et al*., 2004). Fragmentation of E-cadherin was observed in other epithelial cells, such as human intestine Caco-2 and canine MDCK cells, after treatment with YTX, but the toxin could not induce accumulation of fragments of other members of the cadherin family, such as N-cadherin and K-cadherin (Ronzitti *et al*., 2004). The E-cadherin fragment accumulated after YTX treatment lacks the intracellular domain of the protein, including the binding sites of catenins, which mediate the link between E-cadherin and the intracellular microfilament network of actin (Ozawa, Baribault and Kemler, 1989). The accumulation of the E-cadherin fragment was accompanied by reduced levels of β- and γ-catenins bound to E-cadherin. Taken together, those results showed that YTX induces disruption of cell adhesion by altering the E-cadherin-catenin system in cultured cells (Ronzitti *et al*., 2004).

Therefore, YTX disrupts the E-cadherin-catenin system in cultured cells at 10^{-10}–10^{-9} M concentrations.

Mechanisms of action of YTX

A general mechanism of action of YTX cannot currently be derived from data obtained with *in vitro* and cell-free systems, and available information provides only a frame for temporary evaluations and indications regarding future studies.

The observations made with cultured cells are summarized in Table 1. Yessotoxin disrupts cell adhesion and causes E-cadherin fragmentation at low concentrations (10^{-10}–10^{-8} M), whereas it modulates intracellular calcium movements and cAMP levels at higher concentrations (10^{-7}–10^{-5} M). The cytotoxic effects and the apoptogenic activity of YTX, in turn, are caused by YTX concentrations spanning five orders of magnitude (10^{-10}–10^{-5} M) (see below).

In a study aimed at establishing the structure-activity relationship of YTX, it was found that removal of the C_9 terminal chain, or an increase in its size and polarity, results in loss of activity, and the authors concluded that this portion of the YTX molecule is involved in the formation of appropriate interactions with an YTX receptor (Ferrari *et al*., 2004). Based on effective doses of YTX in cultured cells, it was concluded that both high affinity ($K_D \approx 10^{-10}$ M) and low affinity ($K_D \approx 10^{-7}$ M) receptors of YTX exist (Ferrari *et al*., 2004).

Based on available data, YTX appears to exert its effects in living systems by multiple mechanisms of action.

A comparison of effective concentrations of YTX in cultured cells (Table 1) and in mice after i.p. injection (Table 2) shows that some cell responses can be triggered *in vitro* by very low doses of YTX.

TABLE 1
Effective concentrations of YTX in cultured cells

Effects	Cell lines and species	Concentrations (M)	References
Cytotoxicity and apoptosis	Glioma C 6 (rat)	10^{-5}	Ogino, Kumagai & Yasumoto, 1997
	HeLa S_3 (human)	10^{-10}	Malaguti & Rossini, 2001
	BE(2)-M17 (human)	10^{-8}	Leira *et al*., 2002
	L6 myoblasts (rat)	10^{-7}	Suarez Korsnes *et al*., 2004
	BC3H1 myoblasts (mouse)	10^{-7}	Suarez Korsnes *et al*., 2004
Disruption of cell adhesion	Glioma C 6 (rat)	10^{-8}	Ogino *et al*., 1997
	HeLa S_3 (human)	10^{-10}	Malaguti *et al*., 2002a, 2002b
	BE(2)-M17	10^{-8}	Leira *et al*., 2002
	MCF-7 (human)	10^{-9}	Ronzitti *et al*., 2004
Modulation of calcium movements	Lymphocytes (human)	10^{-6}	De la Rosa *et al*., 2001a
	Immunocytes (mussel)	10^{-7}	Malagoli & Ottaviani, 2004
	MH1C1 (rat)	10^{-7}	Bianchi *et al*., 2004
Modulation of cAMP concentrations	Lymphocytes (human)	10^{-6}	Alfonso *et al*., 2003
	Immunocytes (mussel)	10^{-7}	Malagoli & Ottaviani, 2004
E-cadherin fragmentation	MCF-7 (human)	10^{-9}	Pierotti *et al*., 2003
	MDCK (dog)	10^{-9}	Ronzitti *et al*., 2004

TABLE 2
Acute toxicity of YTX and derivatives to mice by i.p. injection

Compound	Strain	Sex	Body weight (g)	Parameter	Acute toxicity (µg/kg body weight)	References
YTX	?	?	?	MLD?	100	Murata *et al.*, 1987
YTX	?	?	?	MLD?	100	Satake, MacKenzie & Yasumoto, 1997, Satake *et al.*, 1997
YTX	ddY	Male	15 ± 1	LD_{50}	Between 80 and 100	Ogino, Kumagai & Yasumoto, 1997
YTX	ICR	Male	23–25	LD_{50}	286**	Terao *et al.*, 1990
YTX	NMRI	Female	15–19	LD_{50}	Between 500 and 750	Aune *et al.*, 2002
YTX	CD-1	Female	18–20	LD_{50}	444 (312–618)*	Tubaro *et al.*, 2003
YTX	Swiss albino	Female	18–22	LD_{50}	111.8 (95.5–130.9)*	Munday *et al.*, 2004
YTX	C57Bl		18–22	LD_{50}	135.9 (111.5–156.6)*	Munday *et al.*, 2004
45-HydroxyYTX	?	?	?	MLD?	~500	Satake *et al.*, 1996
DesulphoYTX	?	?	?	MLD?	~500	Daiguji *et al.*, 1998
DesulphoYTX	ICR	Male	23–25	LD_{50}	301	Terao *et al.*, 1990
HomoYTX	?	?	?	MLD?	~100	Satake *et al.*, 1997
Homo TYX	CD-1	Female	18–20	LD_{50}	512 (315–830)*	Tubaro *et al.*, 2003
45-Hydroxy-homoYTX	?	?	?	MLD?	~500	Satake, MacKenzie & Yasumoto, 1997, Satake *et al.*, 1997
45-Hydroxy-homoYTX	CD-1	Female	18–20	LD_{50}	> 750***	Tubaro *et al.*, 2003
45,46, 47-tri-NorYTX	?	?	?	MLD?	~220	Satake *et al.*, 1996
CarboxyYTX	ddY	Male	20	MLD?	~500	Ciminiello *et al.*, 2000a
CarboxyhomoYTX	?	?	?	MLD?	~500	Ciminiello *et al.*, 2000b
1,3-enone isomer of heptanor-41-oxoYTX	Swiss albino	Female	18–22	LD_{50}	> 5 000***	Miles *et al.*, 2004a
Trihydroxylated amides of 41-a-homoYTX	Swiss albino	Female	18–22	LD_{50}	> 5 000***	Miles *et al.*, 2004b

* Figures in brackets indicate 95% confidence limits.
** This was the LD_{50} at 3 hours after dosing. Mice receiving YTX may die many hours after injection; therefore, the true LD_{50} in this experiment may be below that indicated.
*** No effects were recorded at this dose-level.

3.2 Toxicological studies

3.2.1 Acute toxicity

3.2.1.1 Toxicity by i.p. injection

Results of acute toxicity studies with YTX and analogues following i.p. injection are summarized in Table 2. The reported values for the acute toxicity of YTX itself differ substantially, ranging from ~100 µg/kg to between 500 and 750 µg/kg. The reason or reasons for such discrepancies are not known. Several strains of mice have been employed, and strain differences could be important in the response to YTX. A sex difference is also possible.

The 45-HydroxyYTX, desulphoYTX, homoYTX, carboxyYTX, carboxyhomoYTX and 45,46,47-trinorYTX are of similar acute toxicity to YTX. With 45-hydroxyhomoYTX, the data are conflicting. Satake *et al.* (1997) reported lethal effects at 500 µg/kg, while Tubaro *et al.* (2003) found no effects from an i.p. dose of 750 µg/kg. Recent studies have shown that the 1,3-enone isomer of heptanor-41-oxoYTX and the trihydroxylated amides of 41-a-homoYTX are of low acute toxicity, with no effects being recorded at an i.p. dose of 5 000 µg/kg (Miles *et al.*, 2004a, 2004b).

Overall, the data indicate that the YTX analogues that have been investigated up to the present are either of similar toxicity to YTX or are less toxic.

Clinical signs and macroscopic pathology

Five week-old male mice (b.w. 23–25 g) showed normal behaviour for several hours after i.p. injection of 300 g YTX/kg. After this, dyspnoea occurred and the mice died (Terao *et al*., 1990). Similar results were seen in a later study with lethal dose-levels of YTX, although flicking of the hind legs was observed shortly before death (Munday *et al*., 2004).

In contrast, Tubaro *et al*. (2003) found that mice treated with YTX were restless and, at lethal doses, dyspnoea and jumping were recorded before death. Similarly, Aune *et al*. (2002) noted shivering in mice injected with high doses of YTX, which turned into vigorous jumping and cramps just before death.

No diarrhoea has been observed in mice injected with YTX, and no accumulation of fluid was recorded in suckling mice receiving this substance, even at lethal doses (Ogino, Kumagai and Yasumoto, 1997). No macroscopic changes were recorded at necropsy of mice dosed at up to 750 µg/kg YTX (Tubaro *et al*., 2003; Aune *et al*., 2002).

Light microscopy

By light microscopy, YTX, at a dose of 300 µg/kg i.p., caused no discernable changes in the liver, pancreas, lungs, adrenals, kidneys, spleen or thymus of mice (Terao *et al*., 1990).

No changes were recorded in the lung, thymus, liver, pancreas, kidneys, adrenals, jejunum, colon and spleen of mice given YTX at 250, 500 or 1 000 µg/kg. Slight intracellular oedema was recorded, however, in the hearts of animals receiving the two highest doses of the toxin (Aune *et al*., 2002).

No histological changes were observed in the liver, heart, lungs, kidneys, spleen, stomach, duodenum, jejunum, colon, rectum, pancreas, thymus, uterus, ovaries, skeletal muscle, brain or spinal cord of mice receiving injection of YTX between 265 and 750 µg/kg (Tubaro *et al*., 2003). No apoptotic changes were recorded in the myocardium of treated animals using *in situ* TUNEL staining (Tubaro *et al*., 2003).

Franchini *et al*. (2004a) observed damage to cerebellar Purkinje cells in mice injected with YTX at 420 µg/kg. The cells were shrunken, and the intensity of staining in the cytoplasm was increased. Immunoreactivity to the family of calcium-binding proteins, S100, was increased, while reactivity toward calbindin D28-K, β-tubulin and neurofilaments was decreased.

In a subsequent study, no alterations were recorded in the large neurons of the cerebral and cerebellar cortex of mice injected with YTX at 10 or 420 µg/kg, and no alterations were seen in immunoreactivity to S100, calbindin D28-K or neurotubule and neurofilament proteins (Franchini *et al*., 2004b). Infiltration of blood cells between the epithelial cells of the duodenum was observed at a dose of 420 µg/kg. Changes in immunoreactivity toward IL-6, IL-8 and TNF–α were also recorded in this tissue. Morphological changes were recorded in the thymus, involving death of thymocytes, apoptosis and increased mitotic activity, particularly in the cortex. The severity of the effects in the thymus was greater at 10 µg/kg YTX than at 420 µg/kg. It was concluded by these authors that the thymus is the tissue that is most susceptible to the toxic effects of YTX.

Electron microscopy

Electron microscopy of the hearts of mice injected with YTX at 500 µg/kg showed swelling and degeneration of the endothelium of capillaries. Cardiomyocytes were swollen, and mitochondria became rounded. Bundles of myofibrils, sarcoplasmic reticulum and T-systems were separated (Terao *et al*., 1990).

Aune *et al*. (2002) recorded swelling of cardiomyocytes and separation of organelles, particularly near capillaries, in mice injected at 1 000 µg/kg YTX. Vacuolation of cardiac cells was also observed.

YTX analogues

Fewer data on the acute i.p. toxicity of YTX analogues are available. Male mice given chemically prepared desulphoYTX at 300 µg/kg b.w. survived 48 hours. DesulphoYTX caused only slight deposition of fat droplets in the heart muscle. On the other hand, effects in liver and pancreas were seen. Within 12 hours after an i.p. dose of 300 µg/kg b.w., livers were pale and swollen. By electron microscopy, fine fat droplets were demonstrated in the periphery of hepatocytes. Almost all mitochondria were slightly swollen and showed reduced electron density. Pancreatic acinar cells also showed degeneration. Disarrangement of the configuration of the rough endoplasmic reticulum was prominent within six hours (Terao *et al*., 1990).

HomoYTX and 45-hydroxyhomoYTX caused ultrastructural changes in the heart of the same type as those seen with the parent compound (Tubaro *et al*., 2003).

3.2.1.2 Toxicity by oral administration

Clinical signs and macroscopic pathology

Mice receiving YTX orally at 7 500 and 10 000 µg/kg were noticeably calm after dosing (Aune *et al*., 2002). No changes in behaviour or macroscopic appearance were observed in mice dosed p.o. with YTX at 1 000 or 2 000 µg/kg (Tubaro *et al*., 2003, 2004).

Administration of YTX at 2 000 µg/kg/day for 7 days by oral intubation caused no alterations in weight gain or behaviour of mice, and no macroscopic alterations were noted (Tubaro *et al*., 2004).

No deaths, changes in behaviour or growth, or macroscopic abnormalities were recorded in mice given YTX at 50 000 µg/kg (Munday *et al*., 2004).

Biochemistry and haematology

Oral administration of YTX at 1 000 or 2 000 µg/kg caused no changes in plasma activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) or creatine kinase (CK). Leucocyte percentages were also unaffected (Tubaro *et al*., 2003).

Light microscopy

No pathological signs were observed by light microscopy in the kidney, intestine, adrenals, spleen or thymus of mice given YTX by oral intubation at a dose of 500 µg/kg (Terao *et al*., 1990), or at 1 000 or 2 000 µg/kg given as a single dose (Tubaro *et al*., 2003) or by repeated administration over a 7-day period (Tubaro *et al*., 2004).

Electron microscopy

No discernable ultrastructural changes were recorded in the hearts of animals dosed at 500 µg/kg (Terao *et al*., 1990).

Swelling of cardiomyocytes was recorded in mice dosed at 2 500, 5 000 and 10 000 µg/kg YTX, and protrusions into the pericardial space were noted. The severity of these changes increased with increasing dose (Aune *et al*., 2002). Similarly, Tubaro *et al*. (2003) observed protrusions of cardiac muscle cells into the pericapillary space, with rounding of mitochondria and alterations of fibrillar structure in mice receiving YTX at 2 000 µg/kg/day. In a follow-up study, YTX (2 000 µg/kg/day) were given to female CD–1 mice by gastric intubation on 7 consecutive days (Tubaro *et al*., 2004). Again, changes in myocardial muscle cells near capillaries were observed, with no changes in plasma levels of ALT, AST, LDH and CK. These authors concluded that seven times repeated exposure of mice, via the oral route, at approximately 100 times the maximum human exposure yield no symptoms of intoxication in mice. The only effects seen were ultrastructural changes near capillaries

in myocardial cells. No cumulative toxic effects were observed. In the study by Aune *et al*. (2002), the lowest observable effect level (LOEL) of YTX was 2 500 µg/kg, and a no observable effect level of 1 000 µg/kg was given.

At the ICMSS04 in Galway, Ireland, 14–18 June 2004, a study from the Norwegian School of Veterinary Science with repeated oral exposure of mice for YTX was presented (Espenes *et al*., 2006). They exposed NMRI mice (14 g b.w. at start) for YTX seven times within 21 days by oral intubation, at doses of 1 000, 2 500 or 5 000 µg/kg. Three mice were used in each group, while five control mice received vehicle (1 percent Tween 60 with 1.25 percent ethanol). The mice were killed three days after last treatment. The following organs were studied by light microscopy: myocardium, lung, liver, kidney, small intestine, spleen, thymus, pancreas, brain, testes and adrenal gland. The myocardium was also studied by transmission electron microscopy. No clinical symptoms were observed in any of the groups exposed to YTX. Furthermore, there were no differences in b.w. gain between treated mice and controls. No pathological effects were observed by light microscopy. By TEM, some vacuoles were observed in the myocardium in mice at the highest dose, but their significance remains to be investigated. Compared with their previous study, with only one exposure to YTX, fewer morphological changes were observed in this study. One possible explanation for this is that the mice in the repeated oral exposure study were killed three days after last treatment, which may have allowed for repair mechanisms to operate. In any case, the authors conclude that the lack of toxic effects even after seven times repeated oral exposure at a range exceeding maximum human exposure by several hundred times indicate that YTX presents a small, if any, problem for humans via consumption of shellfish.

YTX analogues

Mice treated orally with 500 µg desulphated YTX/kg b.w. developed fatty degeneration of the liver (Terao *et al*., 1990).

Mice receiving 1 000 µg/kg homoYTX or 45-hydroxyhomoYTX, either as a single dose or as repeated doses over a 7-day period, showed the same ultrastructural changes in the heart as seen with YTX at the same dose-level (Tubaro *et al*., 2003, 2004).

3.2.2 Long-term toxicity studies and carcinogenicity studies

No data are available.

3.2.3 Genotoxicity studies

No data are available.

3.2.4 Reproduction/teratogenicity studies

No data are available.

3.2.5 Special studies

3.2.5.1 In vitro *toxicity*

Original studies on the cytotoxicity of several lipophilic phycotoxins showed that treatment of freshly prepared rat hepatocytes with 25-50 µg YTX/ml culture medium induced very tiny blebs on the cell surface without changing the general spheric appearance of the cells (Aune, 1989). Later, freshly prepared hepatocytes from rats were exposed for 2 hours to YTX at concentrations from 7.5 to 50 µg/ml (Aune, Yasumoto and Engeland, 1991). Morphological effects were seen at 10 µg/ml or above, and increased in a dose-dependent manner. The cells were also studied by scanning electron microscopy. The cells kept their spherical shape, but their surface

was studded with round blebs. No significant increase in LDH leakage from the cells was observed.

The first indication of a cytotoxic effect of YTX was obtained with rat glioma C6 cells (Ogino, Kumagai and Yasumoto, 1997). In this study, it was also shown that, as opposed to its weak cytotoxic effect (micromolar concentrations), YTX could cause cell detachment from culture plates at concentrations around 10^{-8} M.

The first information regarding the molecular bases of cytotoxic effects of YTX was provided by Malaguti and Rossini (2001), studying the death response induced by the toxin in the HeLa S3 cell system. At concentrations comprised between 10^{-10} and 10^{-9} M, YTX induced HeLa cell death after a 48–96 hour treatment, loss of intact poly(ADP-ribose)-polymerase (PARP) and detection of the 85 kDa fragment of this enzyme, showed that apoptosis of HeLa cells was induced by YTX (Malaguti and Rossini, 2001; Malaguti *et al*., 2002a, 2002b). PARP fragmentation is indicative of caspase activation (Thornberry and Lazebnik, 1998), and measurement of caspase activity using substrates for caspase-3/7 and caspase-2 isoforms showed that the former isoforms were the most active in YTX-treated cells (Malaguti and Rossini, 2001; Malaguti *et al*., 2002a, 2002b). The activation of caspase-3 and caspase-7 isoforms in these experimental conditions was confirmed by immunoblotting procedures, which showed that accumulation of the active subunits of the enzymes occurred in YTX-treated HeLa cells (Malaguti and Rossini, 2001; Malaguti *et al*., 2002b). It was also shown that cell death induced by YTX is accompanied by some DNA degradation, but no DNA laddering was detected (Malaguti *et al*., 2002b). The capacity of YTX to induce cell detachment from culture dishes was confirmed by this study, and the effective concentrations were in the subnanomolar range (Malaguti *et al*., 2002a).

With regard to the mechanism by which YTX triggers the death response and brings about the activation of effector caspases in the HeLa cell system, Rossini *et al*. (2003) have shown that the activator caspase isoform 9, rather than caspase 8, is involved.

The apoptogenic activity of YTX has been also shown with the BE(2)-M17 neuroblastoma cell line by Leira *et al*. (2002). Yessotoxin treatment of neuroblastoma cells induced a decrease in the mitochondrial membrane potential, an increase in the levels of active caspase-3 and an increase in annexin V binding to the cells, indicating that the toxin caused apoptosis in this experimental system (Leira *et al*., 2002). A slow response (48–72 hours) to YTX was also recorded in this system, and the YTX effects were quantitatively similar for toxin concentrations comprised between 10 and 1 000 nM (Leira *et al*., 2002), indicating that maximal responses had been already attained following cell treatment with YTX concentrations in the 10^{-8} M range. In keeping with previous observations, YTX was found to induce cell detachment (about 25 percent after a 24-hour treatment) from culture dishes and cell death (Leira *et al*., 2002).

Based on the detection of PARP cleavage and processing of the pro-caspase-3 and pro-caspase-9 isoforms, an apoptotic response has been also shown to occur in rat and mouse myoblasts treated with 10^{-7} M YTX (Suarez Korsnes *et al*., 2004).

The detailed mechanism of the YTX-induced apoptosis has not been characterized yet. In the light of the capacity of YTX to alter calcium movement across cell membranes, it has been proposed that YTX could induce cell death through mechanisms involving alteration of calcium levels in cellular compartments (Leira *et al*., 2002; Bianchi *et al*., 2004), leading to activation of caspases. In some experimental systems, however, this mechanism would not be responsible for triggering apoptosis, because this response can be observed after cell treatment with nanomolar concentrations of YTX (Malaguti and Rossini, 2001; Malaguti *et al*., 2002b), whereas micromolar concentrations of the toxin are needed to alter calcium movement in sensitive systems (De la Rosa *et al*., 2001a; Bianchi *et al*., 2004).

Taken as a whole, available data show that YTX can be both cytotoxic (Ogino, Kumagai and Yasumoto, 1997; Malaguti and Rossini, 2001; Leira *et al.*, 2002; Malaguti *et al.*, 2002; Suarez Korsnes *et al.*, 2004) and cytostatic (Ronzitti *et al.*, 2004) in cultured cells. The effective concentrations of YTX in causing cell death may vary in different systems by about four orders of magnitude (between 10^{-10} and 10^{-6} M). This extended range of effective YTX concentrations, and the variability in cytotoxic activity of YTX imply cell-specific differences in the events set in motion by YTX in cultured cells. The possibility that distinct receptor systems might be responsible for cell-specific differences in molecular responses to YTX (Section 3.1.3) cannot be ruled out at the present time.

3.3 Observations in domestic animals/veterinary toxicology

No data are available.

3.4 Observations in humans

No data are available.

4. ANALYTICAL METHODS

4.1 General

Yessotoxin is included in a review of analytical methods for marine biotoxins (FAO, 2004). Analysis is conventionally divided into extraction and detection phases. Both have proved problematic for YTX and its analogues because of the unusual chemical structures and properties (large lipophilic polyether with hydrophilic sulphate groups). Exhaustive extraction is required for high recoveries from contaminated shellfish tissues and the compounds have unfavourable solvent partitioning properties. The lack of a strong chromophore has hampered use of HPLC-UVD techniques for isolation or analysis.

Yessotoxin contributes to the toxicity of contaminated shellfish prepared using the standard protocols for DSP toxins that produce crude lipophilic extracts for i.p. injection into three mice (Yasumoto, Oshima and Yamaguchi, 1978; Hannah *et al.*, 1995). Even relatively low levels of YTX cause rapid death times and give misleading indications of high levels of true DSP toxins of the okadaic acid group (OA, dinophysistoxins [DTXs]). These responses led to the initial discovery of YTX (Murata *et al.*, 1987). Mouse bioassays (MBAs) in various forms remain the basis for screening of DSP toxins in many shellfish monitoring programmes. However, the relatively non-specific nature of the toxic response by the i.p. route cannot reliably distinguish YTXs from other lipophilic toxins such as pectenotoxins (PTXs), the true DSP toxins, or azaspiracids (AZAs). This has led to a wide range of research into more specific detection techniques for YTXs including instrumental analysis, *in vitro* bioassay and ELISA. Concomitantly, the diverse chemistry and metabolism of YTXs has been revealed and more detailed toxicological studies completed. The low acute oral toxicity has led to uncertainty regarding the significance of YTX and its analogues for human health. This has created problems in defining analytical methods, target analytes and regulatory levels. In addition, reference materials including analytical standards have been lacking, which severely hampered earlier work on methods (Draisci, Lucentini and Mascioni, 2000).

Despite these difficulties, the European Commission (EC, 2002) set a limit in shellfish tissues of 1 mg/kg for YTX plus homoYTX and their 45-hydroxy metabolites. Mouse bioassay is defined as the reference method for enforcement, although the protocol is not specified. The annex to the EC directive states that a diethylether partition cannot be used to extract YTXs. Dichloromethane is also ineffective. Although these solvent partitions lead to low recoveries of YTX, the high i.p. toxicity means that the results can still be positive in the mouse assay and death times are likely to be highly

variable. A rat bioassay is also permitted for DSP toxins by the European Commission (EC, 2002), but only for the truly diarrhoeic toxins (OA, DTXs, and AZAs) and not for YTXs. The standard DSP protocols for extraction of shellfish and MBA have not been validated for quantitative determination of YTXs and certainly do not meet the Codex Alimentarius performance criteria for reference methods of analysis. Therefore, there is a strong need for alternative analytical methods.

4.2 Screening tests

4.2.1 Mouse bioassay (MBA)

Mouse bioassay screening methods for DSP toxins in shellfish are based on the Yasumoto protocol using acetone extraction and diethyl ether partition (Yasumoto, Oshima and Yamaguchi, 1978; Yasumoto, 1981; Stabell *et al*., 1991). The alternative of dichloromethane partition is used in New Zealand (Hannah *et al*., 1995). These methods have not been validated for YTXs. The double extraction with acetone is assumed to be effective at recovering most of the YTXs along with other lipophilic toxins and lipid material. However, no performance data has been reported including recovery of YTX from fortified samples or extractability from contaminated tissues. Partitioning YTX from aqueous solution requires polar solvents. Butanol was used in the initial preparative studies of YTX (Murata *et al*., 1987) and by Yasumoto (2001). Recoveries of YTXs in the partitioning steps with diethyl ether or dichloromethane are likely to be low and variable (Ramstad, Larsen and Aune, 2001; Yasumoto, 2001) with the level and composition of shellfish coextractives affecting emulsions at the interface.

Higher recoveries for YTX from mussel tissues have been reported but not specified using a chloroform partition following diethyl ether in a modified MBA (Stabell, Yndestad and Heidenreich, 1991; Ramstad *et al*., 2001; Ramstad, Larsen and Aune, 2001). The detection limit for YTXs using the chloroform extract was about 0.5 mg/kg digestive gland. However, chloroform will not recover the key metabolite 45-hydroxyyessotoxin (45-OHYTX) from aqueous solutions (Goto *et al.*, 2001). To reduce matrix effects, MBA methods often test the dissected digestive gland of the shellfish rather than whole flesh (WF). The assumptions that the digestive gland contains all the toxins and that application of a factor of 0.2 will give the concentration in WF are not well established for YTXs in a range of shellfish species or at lengthy times after intoxication.

Mice subjected to i.p. injections of YTX were restless and exhibited jumping prior to death (Aune *et al*., 2002; Tubaro *et al*., 2003). Although these symptoms are different from those of classic DSP toxins, other neurotoxins and fatty materials can cause similar symptoms. Occurrence of YTX with other toxins, particularly from *Dinophysis* spp., is generally the norm in shellfish contamination events involving *Protoceratium reticulatum* (Mackenzie *et al*., 2002; Samdal *et al*., 2004). The high variability in the measured i.p. toxicity of purified YTX to mice from different laboratories (Aune *et al.*, 2002; Munday *et al.*, 2001; Ogino, Kumagai and Yasumoto, 1997; Tubaro *et al*., 2003) suggests that there is likely to be a high interlaboratory variability in MBA results on shellfish extracts containing YTXs. Effects of matrix materials and subtle operational factors on the low efficiency of partitioning for YTX are also likely to contribute significantly to sample-to-sample, season-to-season and region-to-region variations in results of MBAs. Therefore, symptoms in MBAs on crude extracts should not be used diagnostically. Any mouse deaths require confirmation by other more specific techniques to establish the types and levels of toxins present.

Two revised MBA protocols (Table 3) for testing whole flesh samples have been proposed that prepare DSP and YTX fractions for separate mouse assay (Yasumoto, 2001). The development study also provided data on the partitioning behaviour of YTX and 45-OHYTX (Table 4) that is useful in considering other test methods. Protocol 1 uses diethyl ether partitioning of crude extracts followed by

butanol partition to provide quantitative recovery of true DSP toxins (OA, DTXs, and AZAs) and PTXs but only partial recovery of YTX (about 50 percent in each fraction). The revised mouse protocol 2 uses 60 percent methanol/dichloromethane partitioning of the crude extract to achieve almost complete separation of the YTXs from the other lipophilic toxins. The dichloromethane fraction is bioassayed after evaporation (three mice). A subsample of the aqueous methanol fraction containing only the YTXs is also bioassayed after evaporation (further three mice). Yessotoxins are detectable to levels below 1 mg/kg WF. The study also provided preliminary data indicating simple independent action in mice dosed i.p. with mixtures of OA, PTX-6 and YTX, e.g. mice survived when dosed with half the lethal dose each of OA plus YTX.

TABLE 3
Summary of revised mouse protocols for lipophilic toxins

	Protocol 1		Protocol 2	
Whole flesh homogenate	100 g		100 g	
Extraction (blend/filter)	300 ml acetone, then 300 ml methanol		300 ml acetone, then 300 ml methanol	
Concentrate	Evaporation to syrup		Evaporation to syrup	
Partition	100 ml diethyl ether / 50 ml water		30 ml dichloromethane / 3 × 60 ml methanol-water (6:4 v/v)	
Treat two phases separately	Diethyl ether phase	Aqueous phase	DCM phase	Aqueous phase
	Evaporate	Partition with 50 ml butanol	Evaporate	To 200 ml with methanol
Fraction for assay	Wash w. 16 ml methanol	Remove 1/5 butanol phase	Wash w. 16 ml methanol	Take 16 ml
	Evaporate	Evaporate	Evaporate	Evaporate
Dosing suspension 1% Tween 60	4 ml	5 ml	4 ml	4 ml
Mouse assay	1 ml i.p. to 3 mice	1 ml i.p. to 3 mice	1 ml i.p. to 3 mice	1 ml i.p. to 3 mice
OA, PTX or AZA > 0.16 mg/kg	2 or 3 mice dead in 24 hours	nil	2 or 3 mice dead in 24 hours	nil ?
YTX > 1 mg/kg	2 or 3 mice dead in 24 hours	2 or 3 mice dead in 5 hours	?	2 or 3 mice dead in 5 hours

Source: Yasumoto, 2001.

TABLE 4
Partitioning of YTXs between solvents with and without the presence of shellfish extractives; percentage in the organic phase by LC-MS

	YTX Water	YTX Mussel ext. Aq. res.	45-OHYTX Mussel ext. Aq. res.	YTX Mussel ext. 60% MeOH	45-OHYTX Mussel ext. 60% MeOH	YTX Scallop ext. 60% MeOH
Diethylether	32%	47%	0%[a]	nt	nt	nt
Dichloro-methane	nt	nt	nt	6%	0%[a]	0%[a]
Ethyl acetate	21%[b]	nt	nt	nt	nt	nt
Chloroform	87%	nt	nt	nt	nt	nt

[a] 100% recovered in aqueous phase.
[b] In other studies, 1.5% (Holland *et al.*, unpublished) and 0.2% (Miles *et al.*, unpublished).
Note: nt = not tested.
Source: Yasumoto, 2001.

Revised mouse protocol 2 has been proposed as a reference method in an Annex to the EC decision on DSP toxins (EC, 2002). The method is in routine use at the Marine Research Institute, Cesenatico, Italy (EU reference laboratory), where it has proved useful in the Italian shellfish monitoring programme because of the common co-occurrence of YTXs with DSP toxins in samples from the Adriatic Sea. There are still some problems with false positives and false negatives that could be overcome if

hepatopancreas rather than whole flesh was used for the assay (T. Yasumoto, personal communication).

4.2.2 In vitro *bioassays*

Microscope studies on the morphology of treated hepatocytes showed some potential to differentiate toxin groups, including YTX (Aune, Yasumoto and Engeland, 1991). However, studies were limited to pure toxins and the practical application to testing of seafood may be limited because extractives from shellfish have been demonstrated to have a range of cytotoxic effects in the absence of marine biotoxins (Malaguti *et al.*, 2002a).

In the course of studies aimed at understanding the molecular bases of YTX effects in cultured cells, Rossini *et al.* (2003) found that the toxin induced a dose-related (0.3–1 nM) accumulation of a 100 kDa fragment of the cell adhesion molecule E-cadherin in MFC-7 cells. Because other lipophilic toxins, such as OA and PTX-6 could not induce this effect, it was exploited to develop a procedure to measure the total biologically active pool of YTXs in contaminated shellfish samples by a functional assay (Pierotti *et al.*, 2003, 2004). Twenty naturally contaminated samples were used in the original study, where it has been shown that the limit of quantification of the procedure is about 100 ng YTX equivalents/g of digestive gland, and preliminary validation of the functional assay gave stable calibrations, but quantitative results for YTXs in shellfish extracts were 40 percent lower than those from HPLC-FL analysis. Structure-activity relationships for production of the E-cahedrin fragment showed that YTX was 15- and 42-fold more potent than 45-OHYTX and carboxyYTX, respectively (Ferrari *et al.*, 2004).

Yessotoxin has also been shown to reduce cAMP levels in exposed cells through enhancing phosphodiesterase activity (Alfonso *et al.*, 2003). This mechanism has been used as the basis for another functional assay for YTXs (Alfonso *et al.*, 2004). Phosphosdiesterase and anthranloyl-cAMP (fluorescent) were incubated with YTX or shellfish extracts and the decrease in fluorescence measured. The rate of cAMP hydrolysis was shown to be linearly correlated to YTX concentration (0.5–10 µM). Two shellfish extracts gave the same results when analysed by the method and HPLC-FL within the errors of the methods. The detection limit of the assay was approximately four times lower than that of MBA. However, fatty coextractives were observed to interfere; therefore, a change in protocol from acetone extraction and dichloromethane partition to 80 percent methanol/water extraction was recommended but not tested with shellfish. A biosensor for YTX has been constructed by immobilizing phosphodiesterase on a resonant mirror (Pazos *et al.*, 2004). A linear relationship for ligand binding was obtained over the range 1–15 µM YTX. Preliminary data for extracts of a mussel h.p. and a dinoflagellate culture spiked with YTX showed high sensitivity and good repeatability (relative standard deviations: 4–15 percent).

At present, there is limited information about the ability of *in vitro* assays to detect other analogues of YTX, and there is no evidence that the biological responses detected *in vitro* are directly related to the rapid toxic effects observed when YTXs are injected i.p. into mice.

4.3 ELISA

An ELISA for YTX was reported as part of suite of immunoassays for comprehensive testing of marine biotoxins (Garthwaite *et al.*, 2001). A more detailed report on the performance of a YTX ELISA has recently been published (Briggs *et al.*, 2004). Polyclonal antibodies were raised in sheep to YTX conjugated on the K ring (non-sulphated "right hand" end). High cross-reactivities were obtained for key analogues YTX (100 percent), 45-OHYTX 159 percent, 1a-homoYTX 39 percent, 45-OHhomoYTX 51 percent (Table 5). Several other analogues with modifications

to the side chain also gave high cross-reactivity while the desulpho analogue gave very low cross-reactivity. The calibration range was 70–1 300 pg YTX/ml. Using a 90 percent methanol extraction, the recoveries for YTX fortifications were 103–118 percent (Table 6). The limit of quantitation in shellfish (diluted methanolic extracts) was 0.12 mg/kg WF.

TABLE 5
Specificity of commercial antibody to YTX analogues

Compound	%CR[a] with 504
Yessotoxin	100
45-Hydroxyyessotoxin	159
Trinoryessotoxin	59
1a-Homoyessotoxin	39
45-Hydroxy-1a-homoyessotoxin	51
1-Desulphoyessotoxin	2
1-*O*-Acetyl-desulphoyessotoxin	0.8
Carboxyyessotoxin	160
DMEQ-TAD yessotoxin	127
Yessotoxin-enone	168
Yessotoxin–amide 2	34

[a] Cross-reactivity = (I_{50} yessotoxin/I_{50} analogue) × 100, where I_{50} is the molar concentration of compound giving 50% inhibition of antibody binding to the coating antigen.
Source: Briggs *et al.*, 2004.

TABLE 6
Recovery of YTX spiked into greenshell mussel homogenate as measured by ELISA

Yessotoxin added µg/2 g	MPL[a] equivalents	Yessotoxin by ELISA (ng/ml)	Recovery of yessotoxin Mean %	n[b]	CV%
2	1	0.67	102.9	8	11.6
1	1/2	0.33	111.9	8	6.5
0.4	1/5	0.13	116.1	8	5.5
0.2	1/10	0.67	118.1	8	4.8

[a] Maximum permitted level for YTX set by European Commission of shellfish at 1 mg/kg.
[b] Number of separate assays run.

Commercialization of this ELISA in a 96-well plate, competitive binding format is proceeding (Kleivdal, Briggs and Miles, 2004). A preliminary interlaboratory study (four laboratories in three countries) has been conducted using pilot kits with fortified shellfish samples. Mean recovery was 111 percent and reproducibility (RSD_R) was 16 percent. The performance parameters for this assay are summarized in Table 7.

TABLE 7
Commercial YTX ELISA kit – performance parameters

Calibration range (I_{20}–I_{80})	80–1 250 pg/ml
Limit of quantitation	0.16 mg/kg for whole shellfish flesh 4 ng/ml for seawater
Specificity	All YTX analogues required to be tested for in Decision 225/2002/EC are recognized (EC, 2002) plus a range of other analogues
Accuracy *Interlaboratory recovery*	 Mean 111% at 0.5–1.5 mg/kg (n=4)
Precision *Within-laboratory repeatability* *Interlaboratory reproducibility*	 RSD_r 8% at 0.1–1.0 mg/kg RSD_R 16% at 0.5–1.5 ng/kg (n=4)
Assay analysis time	1.5 hours
Samples per 1-plate kit	36 duplicates

ELISA data for digestive glands (DGs) from extracts of naturally contaminated Norwegian blue mussel samples (*Mytilus edulis*) gave consistently much higher estimates of YTX equivalents than those from instrumental analysis (LC-MS for YTX) by a factor of greater than ten (Aasen *et al.*, 2005; Samdal *et al.*, 2001, 2005). LC-MS revealed that these samples also contained 45-OHYTX, carboxyyessotoxin and hydroxycarboxyyessotoxin (Aasen *et al.*, 2005; Samdal *et al.*, 2005). However, the levels of these metabolites could still not account for most of the immunoreactivity that is presumed to be because of the presence of a range of other analogues or metabolites of YTX whose identity and toxicological significance have yet to be fully evaluated.

The high sensitivity of the ELISA has been used to advantage in studies on the production of YTXs by *Protoceratium reticulatum* (Samdal *et al.*, 2004). Analyses were conducted on picked cells (1–20 cells) from Norwegian and New Zealand coastal waters and gave yields of yessotoxins of 18–79 pg/cell. Analyses of net-haul samples by LC-MS gave a lower estimated content of YTX per cell than by ELISA for YTXs. *P. reticulatum* is known to produce a range of YTX analogues (Ciminiello *et al.*, 2003; Konishi *et al.*, 2004; Miles *et al.*, 2004a, 2004b, 2005a, 2005b, 2006; Satake *et al.*, 1999) that could account for the higher estimates obtained by ELISA. Metabolism of these analogues by shellfish could also account for the higher results observed by ELISA of mussel extracts.

4.4 High-performance liquid chromatography (HPLC) with fluorescence detection

The formation of a fluorescent derivative with the dienophile reagent DMEQ-TAD is the basis for a useful method for analysis of YTX in shellfish and algal concentrates (Yasumoto and Takizawa, 1997). The method can also detect some other analogues of YTX including 45-OHYTX, 45,46,47-trinoryessotoxin (Yasumoto and Takizawa, 1997) and 1-desulphoyessotoxin (Daiguji *et al.*, 1998). However, the method cannot detect analogues lacking the 1,3-dienyl moiety, such as carboxyYTX and 42,43,44,45,46,47,55-heptanor-41-oxoYTX. Extraction of DG from naturally contaminated shellfish with 80 percent methanol recovered 90 percent of the YTX in the first extraction and 10 percent in the second extraction. Therefore, the protocol uses a single extraction using 1 g DG with 9 ml 80 percent methanol. This extraction procedure has also been validated for OA and some other DSP toxins (Lee *et al.*, 1987). The crude extract was cleaned up by solid phase extraction (SPE) and, after derivatization of the YTX fraction with DMEQ-TAD, a further SPE cleanup was carried out prior to reversed-phase HPLC with fluorescence detection. Each compound yielded two epimeric peaks and the calibration with YTX was highly linear. Blank shellfish extracts gave low interferences with detection limits below 0.1 mg YTX/kg digestive gland. Recovery of YTX from fortified samples was 94 percent (mean, 0.2–20 mg/kg DG).

The repeatability of this HPLC-FL method for determination of YTX has been tested using digestive gland of contaminated Norwegian blue mussel samples (Ramstad, Larsen and Aune, 2001). There was good agreement between duplicates over the range 2–30 mg YTX/kg digestive gland with no significant effect of level on the percentage difference. The data on YTX levels were also compared with MBA results. Bioassay of the diethyl ether partition often failed to detect high levels of YTX. There was also a relatively poor correlation of the YTX levels to mouse deaths using the subsequent chloroform partition, although lower levels of YTX were detectable.

4.5 Liquid chromatography with mass spectrometry (LC-MS)

The power of mass spectrometry for detection and structural elucidation has commonly been applied in the field of novel marine natural products. For YTX, initial experiments used negative ion fast atom bombardment (FAB) (Naoki, Murata and Yasumoto, 1993). Collisional activation of the molecular anion gave fragment ions of structural utility

dominated by loss of sulphate [M-H-80]- (where M is considered to be the sulphonic acid form) and a series of cleavages along the polyether ladder.

The breakthrough for quantitative analysis of YTXs was the development of an LC-MS method for separation, electrospray ionization (ESI) tandem mass spectrometric detection (triple quadrupole analyser) of YTX (Draisci *et al.*, 1998). Use of C18 reversed-phase chromatography with acetonitrile/4 mM ammonium acetate (80:20) mobile phase and negative ion mode ESI provided narrow peaks and high sensitivity for YTX, with the molecular anion ([M-H]-) the main peak in the mass spectrum. The major peaks in the collisional activation spectrum of [M-H]- matched those from FAB with loss of sulphate predominating. Selected ion recording (SIR) on [M-H]- or SRM of the loss of sulphate both provided excellent signal to noise on a crude extract of contaminated mussel DG. The sensitivity was estimated at 4 000 times that of MBA. In a further publication, Draisci *et al.* (1999a) reported the extension of this method to a multitoxin format for direct detection of three groups of "DSP" toxins in shellfish or phytoplankton. Either positive ion or negative ion mode ESI and SIR detection were used, although YTX was not detected in positive ion mode (mobile phase acetonitrile/water + 0.1 percent trifluoroacetic acid). The method was demonstrated using a sample of naturally contaminated mussel, but no performance data such as recovery or precision were gathered.

Japanese workers (Goto *et al.*, 2001) presented the first data on quantitative determination of YTX with other "DSP" toxins in shellfish using LC-MS. Toxins were extracted with 90 percent methanol (18 ml with 2 g tissue) and freed of polar contaminants by partition between chloroform and water. The lipophilic toxins in the chloroform phase were separated into two fractions by silica gel-SPE (fr. 1: PTX1, PTX2; fr.2: PTX6, PTX2 seco acid, OA, DTX1, DTX3, YTX). Yessotoxin and 45-OHYTX were also separately purified from the crude methanolic extract using C18-SPE. The fractions were analysed by LC-MS (quadrupole) using various reversed-phase columns and mobile phases, and electrospray ionisation with SIR detection in positive or negative ion modes. For YTX and 45-OHYTX, the mobile phase was methanol/0.2 M ammonium acetate (80:20) with detection of the molecular anions. Calibrations were highly linear ($R^2 > 0.997$) for all toxins, including YTX and 45-OHYTX over the range 40–1 600 pg injected. Detection limits for these two toxins in scallop were 0.08 mg/kg DG or 0.04 mg/kg adductor muscle. Recoveries of YTX from fortified scallop tissues using the silica gel cleanup were 80–90 percent at 1.6 mg/kg DG and 86–95 percent at 0.8 mg YTX/kg muscle. Recoveries were similar or higher for toxins in the other groups. Recoveries were somewhat lower for YTX and 45-OHYTX using the C18-SPE cleanup (59–79 percent at 1.6 mg/kg DG).

Several groups have reported the use of LC-MS with ion-trap mass analysers for determination of YTX and analogues (Ciminiello *et al.*, 2002a, 2002b, 2003; Fernandez-Amandi *et al.*, 2002; Cooney, Jensen and Miles, 2004). In addition to the high sensitivity of ion-traps in full scan mode, sequential collisional activation experiments (MSn) can provide more structural information than conventional MS-MS experiments. For YTXs, the ability to probe the [M-H-SO3]- ions that dominate the MS-MS spectra has been shown to be of particular utility. A range of novel analogues has been identified in algal extracts of *Protoceratium reticulatum* using these techniques with ion trap LC-MS (Ciminiello *et al.*, 2002b, 2003; Cooney, Jensen and Miles, 2004; Miles *et al.*, 2004a, 2004b, 2005a, 2005b, 2006). MSn can in theory also provide higher degrees of specificity for quantitative analysis. However, in practice, no significant increases in signal to noise ratio have been demonstrated for analysis of YTXs in shellfish extracts over the large increases obtained in moving from SIR (MS) to SRM (MS-MS). Cañás *et al.* (2004) demonstrated the utility of QTOF LC-MS for determination of YTX in picked cells of *P. reticulatum* and obtained structurally useful cleavages with MS/MS of the doubly charged molecular anion.

Ciminiello *et al.* (2002a) extended the range of YTX metabolites tested in shellfish by LC-MS to include 45-OHhomoYTX, carboxyYTX, carboxyhomoYTX and 42,43,44,45,46,47,55-heptanor-41-oxohomoYTX. Liquid chromatography mobile phase conditions were changed from the isocratic acetonitrile/ammonium acetate buffer advocated by Draisci *et al.* (1998, 1999a) to an acetonitrile gradient with formic acid 50 mM/ammonium formate 3.5 mM buffer. A similar gradient system with acidic buffer has advocated by Quilliam, Hess and Dell'Aversano (2001) and McNabb, Selwood and Holland (2005) for separation and LC-MS detection of a wide range of polar and non-polar toxins. Ciminiello *et al.* (2002a) detected all the YTXs with high sensitivity using the ion trap in full scan MS-MS mode on the molecular anions. The strong fragment ion from loss of SO3 was used for quantitation. High linearity was observed over the range 290 pg–71 ng YTX injected with a detection limit of 70 pg YTX injected. The method was used to confirm the types of YTXs present in contaminated shellfish samples from the Italian monitoring programme, but quantitative performance data were not presented.

Similar performance data were reported by Fernandez-Amandi *et al.* (2002) for ion trap LC-MS determination of YTX and 45-OHYTX in shellfish. Shellfish tissues were extracted with 80 percent methanol (1 g + 9 ml) and the supernatant concentrated 20-fold for LC-MS. The preferred LC system was an RP amide-C16 column with mobile phase acetonitrile/water (60:40) containing 0.5 mM ammonium acetate. Linear MS-MS calibrations were obtained for YTX with a detection limit in a mussel extract of 30 pg injected (0.003 mg/kg). The repeatability RSD was 6.3 percent at a level of 0.25 µg/ml YTX in extract. Accuracy and reproducibility data for the method using fortified shellfish tissues were not reported.

Norwegian workers (Aasen, Torgersen and Aune, 2003) have reported a multitoxin LC-MS/MS method that determined YTX, 45OHYTX, carboxyYTX and several other "DSP" toxins. Extraction was based on the 80 percent methanol protocol as used by Yasumoto and Takizawa (1997), but the crude extract was directly analysed without concentration or partition. A C18 column (50 × 2 mm) with water/acetonitrile gradient and acidic ammonium formate buffer, similar to Quilliam, Hess and Dell'Aversano (2001), was reported to give excellent separation of the toxins. Selected ion recording detection with channels for the molecular anions was used to detect the YTXs. Detection limits were reported to be 0.02–0.08 mg/kg but no other validation or performance data were provided.

Matrix effects are common in LC-MS mainly because of coextractives enhancing or suppressing electrospray ionization of analytes. Concentrated crude extracts of scallop tissues (1 g/ml) were reported to suppress ionization of YTX by 40–50 percent (Goto *et al.*, 2001; Ito and Tsukada, 2002). Solid phase extraction cleanup reduced these indirect interferences to acceptable levels (Goto *et al.*, 2001). The use of immunoaffinity columns (IACs) has become common for sample cleanup in the related field of mycotoxins and could also be useful for YTX, as suggested by Briggs *et al.* (2004). Calibration of LC-MS response using YTX standards prepared in extract of a blank scallop did not adequately correct for the suppression of YTX responses in extracts of three other different scallop samples (Ito and Tsukada, 2002). It was concluded that it is not always possible to ensure adequate matching of sample and standard matrices. For this reason, use of such matrix-matched standards is not favoured by the United States Food and Drug Administration in the approval of methods for veterinary drug residues in animal tissues. Standard addition for quantitation of a scallop extract fortified at 0.20 mg YTX/g digestive gland gave 0.213 mg/kg ±0.020 (mean ±SD, n=6) compared with 0.134 mg/kg ±0.014 by external calibration and similar improvements were reported for quantitation of other "DSP" toxins (Ito and Tsukada, 2002). The disadvantages of standard addition are that each extract requires two LC-MS runs, and the addition of appropriate levels of standards becomes complex if several toxins

are present in the extract. An alternative solution is to use more dilute extracts that can be satisfactory, provided acceptable limits of detection can be achieved. The most satisfactory method for correcting for enhancement/suppression effects is use of stable isotope-labelled internal standards, but these are not currently available for YTX or other marine biotoxins.

The reversed phase chromatography of YTX and analogues can be problematic (Fernandez-Amandi *et al*., 2002) with tendencies to variations in retention times and peak broadening. The most stable performance is obtained with heavily end-capped C8 or C18 column packings and neutral mobile phases with ammonium acetate buffer (M.A. Quilliam, personal communication) as originally used by Draisci *et al*. (1998). Use of acidic ammonium formate/formic acid buffers moves YTX to longer retention times, leading to better separation of analogues, increased intensity of the [M-H]- ion at the expense of the [M-2H]2- ion, and is more suitable overall for separation and electrospray ionization of a wide range of other toxins. Therefore, the multitoxin LC-MS method specifies a C18 column (150 × 2 mm) using gradient elution with 5–80 percent acetonitrile plus constant 46 mM formic acid and 4 mM ammonium formate. However, the precise retentions of YTX and analogues have been found to be very sensitive to buffer composition and to subtle column changes over several hours' use. For example, the retention time for YTX linearly increased from 19.2 to 21.7 minutes with decreases in ammonium formate concentration from 4 mM to 3.1 mM (Cawthron Institute, unpublished data).

Based on the very promising performance of multitoxin LC-MS methods (Draisci *et al.*, 1999a; Goto *et al*., 2001; Quilliam, Hess and Dell'Aversano, 2001) an LC-MS/MS screening method has been developed for a wide range of toxins including DA, "DSPs" and YTXs (Holland and McNabb, 2001). This method has been subjected to a comprehensive within-laboratory validation (Holland and McNabb, 2002; Holland *et al*., 2004a, 2004b; Holland, McNabb and Selwood, 2004; McNabb, Selwood and Holland, 2005), taken to interlaboratory study (Holland and McNabb, 2003; Holland, McNabb and Selwood, 2004; McNabb, Selwood and Holland, 2005) and brought into routine use in the New Zealand shellfish regulatory programme (McNabb and Holland 2003, 2004). Features of the method that enable relatively large numbers of samples to be analysed routinely (20–30 per day) for a wide range of toxins with low detection limits and high specificity include the analysis of dilute crude extracts to minimize cleanup steps while reducing matrix effects, use of gradient elution, rapid positive/negative ion switching, MS/MS detection (selected reaction monitoring [SRM]), and a high degree of automation in instrument operation and data processing. Yessotoxins are detected using SRM channels for loss of SO3 from the molecular anions. Quantitation for YTX uses the linear five-point calibration (5–200 ng/ml, equivalent to 0.05–2 mg/kg) carried out with each batch of samples. Additional SRM channels are used to monitor for 45-OHYTX, homoYTX and carboxyYTX. For quantitation of these compounds, in the absence of reliable standards, the SRM response factor for YTX is applied. Some specific aspects of the method performance for these toxins emerging from the within- and inter-laboratory studies are as follows.

The method specifies a single extraction of whole flesh homogenates with 90 percent methanol (ultrasonic blending of 2 g with 18 ml 90 percent methanol) followed by a hexane wash to remove lipids. Extractability of YTXs from contaminated mussel tissues was determined using three successive extractions (Table 8). The relatively large proportions in the second and third extracts indicate that YTXs are difficult to extract fully from mussel tissues and that further residues may remain even following three extractions. However, a single extraction with 90 percent methanol could recover up to 75–80 percent of YTXs. Similar but slightly lower extractabilities were obtained using 80 percent methanol, but this solvent was much less efficient at extracting AZAs

and esters of OA, DTX1 and DTX2 (Holland *et al.*, 2004b; McNabb, Selwood and Holland, 2005).

Accuracy and precision data for the method were gathered using replicate blank shellfish tissues fortified with either YTX standard or aliquots of an extract of highly contaminated mussel digestive gland (Table 9).

TABLE 8
Extractability of YTXs from homogenates of three naturally contaminated mussel tissues

	YTX		45-OHYTX		CarboxyYTX	
	Rec.	±s.d.[c]	Rec.	±s.d[c]	Rec.	±s.d.[c]
Sample 1 1st extn[a]	75.4%	0.8	92.3%	0.3	75.3%	1.3
2nd extn	13.3%	1.0	4.6%	0.1	13.4%	1.6
3rd extn	11.3%	–	3.1%	–	11.3%	–
Total (mg/kg)	4.50	0.13	3.09	0.14	2.24	0.07
Sample 2 1st extn[a]	78.6	3.8	76.4	1.8	NT[b]	
2nd extn	11.8	2.4	13.6	1.2	–	–
3rd extn	9.6	1.7	10.0	0.6	–	–
Total (mg/kg)	0.65	0.05	0.058	0.008	–	–
Sample 3 1st extn[a]	74.4	3.6	75.9	2.4	NT[b]	–
2nd extn	13.5	2.8	13.4	2.7	–	–
3rd extn	12.1	1.1	10.7	0.8	–	–
Total (mg/kg)	2.32	0.29	0.23	0.006	–	–

[a] First extracts further diluted for LC-MS/MS analysis.
[b] NT – not tested.
[c] n = 3.
Notes: Three successive extractions (2 g whole flesh + 18ml 90 percent methanol). Recovery as percentage of total extracted and total as mg/kg whole flesh. Corrected for carryover of solution in centrifuged pellet.
Source: Holland and McNabb, 2002.

TABLE 9
LC-MS/MS determination of YTXs in fortified shellfish tissues

Fortification level	YTX 0.10 mg/kg	YTX 1.0 mg/kg	YTX[a] 0.802 mg/kg	45-OHYTX[a] 0.133 mg/kg
Greenshell mussel	0.088 ±0.0049	0.81 ±0.059	0.69 ±0.031	0.104 ±0.002
Pacific oyster	0.069 ±0.015	0.65 ±0.023	0.70 ±0.039	0.105 ±0.010
New Zealand cockle	0.076 ±0.011	0.84 ±0.074	0.75 ±0.050	0.106 ±0.002
Scallop roe	0.055 ±0.0078	0.43 ±0.062	0.75 ±0.037	0.112 ±0.010

[a] Homogenate fortified with crude digestive gland extract of contaminated mussel. Fortification levels estimated from analysis of equivalent fortified solvent sample.
Notes: Recovery in mg/kg (mean ±SD, n=3).
Source: McNabb, Selwood and Holland, 2005.

This data set and some further recovery data provided the following overall estimates of the method performance for determination of YTX in shellfish tissues (excluding scallop roe) over the concentration range 0.1–1.0 mg/kg: recovery 74.8–76.8 percent; repeatability) 14.2–12.6 percent; reproducibility 15.4–12.6 percent (within-laboratory; different days and operators); limit of detection 0.016 mg/kg; limit of quantitation 0.05 mg/kg (Holland and McNabb, 2003; McNabb, Selwood and Holland, 2005). The precision and detection limits for 45-OHYTX were similar to those for YTX. The low apparent recoveries for YTX and 45-OHYTX were shown to be mainly because of signal suppression by matrix. Further dilution of extracts or use of a recovery factor provided a correction for more accurate results with samples contaminated close to the regulatory limit.

Further information on the performance of the method was provided by an interlaboratory study. Eight laboratories obtained data on a range of toxins in the methanolic extracts of three contaminated mussel samples (Holland and McNabb, 2003; McNabb, Selwood and Holland, 2005). The precision estimates for YTX (present in two samples at levels equivalent to 2.9 and 1.7 mg/kg) were RSD_r (repeatability)

8–12 percent, and RSD_R (reproducibility) 15–22 percent with 1.3 for the HORRAT figure of merit (acceptable). Precisions for most of the other analytes were also within the acceptable range based on the HORRATs. This was regarded as very satisfactory, taking into account the fact that some of the laboratories had no experience with analysis of YTX by LC-MS and that several laboratories used SIR (single quadrupole MS) rather than the more specific SRM (MS/MS) detection.

The overall accuracy of the method for 45-OHYTX and other YTXs is affected by the lack of certified analytical standards. However, the assumption of the same electrospray response factor as for YTX provides a basis for determination of these compounds within a system of rigorous quality control and provides a direct path for quantitative improvement in accuracy as reliable standards come available.

5. LEVELS AND PATTERNS OF CONTAMINATION OF BIVALVE MOLLUSCS

5.1 Results of surveys

Italy

In the DG of mussels from the Adriatic Sea, besides YTX, two new analogues of YTX, homoyessotoxin and 45-hydroxyhomoyessotoxin were identified (Ciminiello *et al.*, 1997). *Gonyaulax polyhedra* was implicated as responsible for the YTX contamination in these mussels (Tubaro *et al.*, 1998).

In 1999, DSP toxins were detected in 350/900 samples of *Mytilus galloprovincialis* from the northern Adriatic. The main problem area was Emilia Romagna. Yessotoxin always dominated over OA (EU-NRL, 2000).

In 2000, DSP toxins were detected in *M. galloprovincialis* with 13 percent of the samples giving positive MBAs. In the Emilia Romagna region, closures were enforced from late August until the end of December; in the Veneto region, closures were enforced from late October until the end of December; and in Friuli Venezia Giulia, closures were enforced in late December. The closures were mainly because of YTXs. In 2001, DSP toxins were detected in *M. galloprovincialis* with 18 percent of samples giving positive bioassay results. Closures were enforced in the Emilia Romagna region in January, February and early March, which was a continuation of the 2000 closures, and also later in the period mid-June–late October. In Veneto, closures were enforced in July, while in Friuli Venezia Giulia, closures were enforced from January to mid-February and again from the start of July to early August (EU-NRL, 2001). In 2002, DSP was detected in the northern Adriatic Sea (Friuli Venezia Giulia, Veneto and Emilia Romagna coast). Harvesting was forbidden. In July, *Pecten maximus* samples from Scotland, the United Kingdom of Great Britain and Northern Ireland, appeared to be positive for DSP (EU-NRL, 2002).

Norway

Yessotoxin was first identified in Norwegian mussels in the late 1980s (Lee *et al.*, 1988), and this toxin group has been responsible for closures of Norwegian commercial shellfisheries. Many of the closures occurred because YTX was extracted together with DSP toxins, contributing to killing mice in the traditional MBA. With the current European Union (EU) regulatory limit of 1 mg/kg of YTX equivalents (EC, 2002), YTXs have caused less of a problem for mussel farmers in Norway. Studies using the ELISA technique have shown that *Protoceratium reticulatum* is an important source organism for YTXs in Norway, similar to observations in New Zealand (Samdal *et al.*, 2004).

Regular sampling of algae and mussels at a research station (Flødevigen) in Norway by LC-MS and ELISA indicates that occurrence of *P. reticulatum* in seawater lead to YTX contamination of blue mussels (Aasen *et al.*, 2005). Yessotoxin is rapidly oxidized

to 45-hydroxyyessotoxin and more slowly to carboxyyessotoxin by blue mussels. Their half-life in mussels is about 3–4 weeks.

In 1999, 135/473 samples gave positive results for DSP. On many occasions, YTX was the dominant toxin, and in approximately 33 percent cases, closures of production areas were because of the detection of YTX (EU-NRL, 2000).

Japan

Yessotoxin was first isolated from the scallop *Pactinopecten yessoensis* and named after the species name of the scallop (Murata *et al*., 1987). In the monitoring project implemented by the Government Fisheries Agency, YTX was monitored first by HPLC (Yasumoto and Takizawa, 1997) and later by the LC/MS method developed by Goto *et al*. (2001). All the data are published only in Japanese, deterring access by scientists in other countries. The situation is briefly summarized as follows.

Yessotoxin and 45-OH-YTX occur mainly in scallops cultured in Mutsu Bay located at the northern tip of Honshu Island (Japan's main island). Scallops cultured in Hokkaido, across the channel, seldom show occurrence of YTX and 45-OH-YTX, at a very low level if they do. Yessotoxin and its analogues have not been detected in other parts of Japan. In Mutsu Bay, which was divided by a small peninsula into the east and west bays, YTX levels are always higher in the east bay than in the west bay. In the east bay, YTX levels start rising in April, rise higher in June and July, and reach the maximum in September, which last in some years until the end of September. From August to late September, when *Dinophysis fortii* is no longer seen in the water, YTX and 45-OH-YTX account for nearly 90 percent of the mouse toxicity, resulting in banning harvest solely on the YTX presence. The highest level of YTX in the past five years was about three mg/kg. Yessotoxin comprises 80–90 percent and 45-OH-YTX the remaining 10–20 percent. No other analogues occurred at a detectable level.

5.2 Distribution and annual variation

Blooms of *P. reticulatum* in New Zealand coastal waters typically occur in early summer for relatively brief intervals of 1–2 weeks in conjunction with more persistent blooms of *Dinophysis acuminata* or, less commonly, *D. acuta*. *P. reticulatum* cells proliferate over a few days and reach peaks of 30–120 cells/ml (Mackenzie *et al*., 2002, 2004). At these levels, YTX contamination of mussels occurs rapidly, reaching flesh concentrations of over 2 mg/kg and then slowly declining. At monitoring sites in the Marlborough Sounds, the annual pattern of blooms of *Dinophysis* with *Protoceratium* has been a recurring one for more than a decade. Low levels of shellfish contamination can occur when *P. reticulatum* levels are less than 1 cell/ml, which can be difficult to detect with the standard Utermohl chamber. This, combined with the often very rapid increase in cell numbers, means phytoplankton monitoring may not give early warning of potential high-level contamination of shellfish. Similarly, in Norway, high and persistent levels of YTXs were found in blue mussels contaminated by *P. reticulatum* (Ramstad *et al*., 2001; Aasen *et al*., 2005; Samdal *et al*., 2004). *P. reticulatam* blooms were sharp and brief, similar to in New Zealand (Samdal *et al*., 2005) with bloom maxima of 2 cells/ml leading to 3 mg/kg YTX + 45-OHYTX + carboxy YTX in mussel tissues by LCMS (Aasen *et al*., 2005; Samdal *et al*., 2005), which then decreased with a half-life of about 25 days. Following the initial contamination, YTX levels decreased while 45-OHYTX and then carboxyYTX levels increased in mussel tissues. The ratio of YTXs by ELISA to LC-MS was about 4 during the bloom but rose to about 10 over the next 8 months presumably because of formation of more unknown metabolites.

Solid phase adsorption toxin tracking (SPATT) has shown great promise for tracking low levels of YTXs and other lipophilic toxins (Mackenzie *et al*., 2004). Adsorption of toxins onto the sachets of resin deployed at coastal sites provided time integrated sampling that gave high correlations with cell counts for toxic algae and contamination

of shellfish. The high sensitivity enables forecasting of harmful algal bloom (HAB) events.

5.3 Effects of processing

A high proportion of YTXs are concentrated in the DG (Yasumoto and Takizawa, 1997) and, therefore, shellfish products where the DG is removed will have substantially lower levels of YTXs. Steam treatment of contaminated mussels has been used as part of sample preparation for analysis (T. Aune, personal communication; M. Quilliam, personal communication). Recent research (P. Holland, personal communication) comparing sample preparation methods using a bulk sample of contaminated mussels gave levels of YTX by LC-MS of 84 µg/kg (SEM 2.6 percent) for fresh processing versus 48 µg/kg after steaming (correcting for loss of moisture). This indicates that levels of available YTX might be reduced by some treatments involving heat typically used in processing whole shellfish.

6. DOSE RESPONSE ANALYSIS AND ESTIMATION OF CARCINOGENIC RISK

6.1 Contribution of above data on assessment of risk

Although the toxicity of YTX and its analogues should be indicated separately, at present, these data are only partly available for the acute lethal effect. Available data indicate that the YTX analogues are either of similar toxicity to YTX or are less toxic. The changes observed in animals dosed with YTX were similar to those seen with homoYTX and 45-hydroxyhomoYTX, whereas the effects of sulphoYTX were quite different. If more data become available, the toxicity of the different analogues could be expressed relative to YTX, similar as it is done in case of paralytic shellfish poisoning (PSP) where the toxicity of saxitoxin (STX) analogues are expressed as STX. So far, the biological activity of some YTX analogues with reference to the parent compound has been determined only in one *in vitro* system involving cultured cells (Ferrari *et al*., 2004). The results of this study show that the effective doses of YTX and some of its analogues in cultured cells differ widely from those observed in mice that received the tested compounds by i.p. injection.

Yessotoxin itself is very poorly absorbed from the GI tract of mice, which would account for the gross differences in acute toxicity between i.p. injection and oral administration. By injection, YTX is toxic at between 100 and 750 µg/kg, while no deaths have been recorded in mice given YTX orally at doses up to 50 000 µg/kg.

Acute administration of YTX by i.p. injection leads to ultrastructural changes in the heart, which appear to be dose-dependent. It has been reported that no changes in the liver, pancreas, lungs, adrenals, kidneys, spleen or thymus, stomach, duodenum, jejunum, colon, rectum, uterus, ovaries, skeletal muscle, brain or spinal cord are induced in mice dosed with YTX (Terao *et al*., 1990; Aune *et al*., 2002; Tubaro *et al*., 2003). In contrast, Franchini *et al*. (2004a) described damage to Purkinje cells, although in a later study no alterations were recorded in the large neurons of the cerebral and cerebellar cortex of mice injected with YTX (Franchini *et al*., 2004b). The latter authors also described lesions in the duodenum and thymus of treated animals, and suggested that the thymus was particularly vulnerable to the toxic effects of YTX. Previous studies, however, revealed no alterations in the thymus or duodenum, and these differences cannot be reconciled at present.

Ultrastuctural changes in the heart have also been observed after oral administration of YTX. The alterations comprised swelling of cardiomyocytes and protrusions into the pericardial space, and these changes were dose-related. No necrosis was observed, which is consistent with the observation that activities of ALT, AST, LDH and CK, enzymes that are elevated following muscle necrosis, were not altered in animals dosed with YTX. If the observed ultrastructural changes reflect cellular changes that

ultimately lead to cell death, it would be expected that necrosis of cardiomyocytes would be seen after prolonged administration of YTX. Furthermore, such changes would be seen even after cessation of treatment, because cardiac tissue does not readily regenerate. However, the severity of the ultrastructural changes did not appear to increase after multiple doses of YTX, and no necrosis was recorded. Indeed, as reported by Espenes *et al.* (2006), no ultrastructural changes were seen in mice receiving high doses of YTX seven times in three weeks. These animals were killed for examination three days after the last dose, and the absence of any ultrastructural change at this time suggests that these are fully reversible. The significance of the cardiac changes induced by YTX requires further investigation.

Direct searches for apoptotic nuclei in the myocardium showed that no differences could be recorded between control and YTX-administered animals (Tubaro *et al.*, 2003). These data do not exclude the possibility that YTX might induce apoptosis of some selected cell population(s) *in vivo*, particularly in the case of repeated exposure to the toxin, but available data indicate a low risk of apoptotic induction in the intact animal after oral ingestion of YTX.

In vitro, changes can be induced in cells by very low (10^{-10}–10^{-8} M) concentrations of YTX. Based on available data, two effects can be induced by those low concentrations of YTX: cell demise by apoptosis and disruption of cell adhesion.

The disruption of cell adhesion, in general, and of the E-cadherin-catenin system, in particular, are other sources of risk, as alterations of cell-adhesive properties are linked to tumour spreading and metastasis formation in most cancers (Birchmeier and Behrens, 1994; Beavon IRG, 2000), and E-cadherin plays a role of tumour suppressor (Christofori and Semb, 1999).

The observation that YTX induces an alteration of E-cadherin, which has the potential to disrupt its tumour-suppressive role, raises the possibility that YTX might favour tumour cell invasion and metastasis formation *in vivo*. This type of risk deserves careful consideration. No data regarding the capacity of YTX to disrupt the E-cadherin-catenin system *in vivo* have been obtained so far, and studies in this area are urgently needed in order to ascertain whether the results obtained *in vitro* are replicated *in vivo*.

7. EVALUATION[2]

7.1 Toxicology of YTXs and regulatory limits

Absorption, distribution, metabolism and excretion

Limited absorption of the toxin from the GI tract has been observed, but no further data on absorption, distribution, metabolism and excretion are available.

Mechanism of action

Based on available data, YTX appears to exert effects in living systems by multiple mechanism of action, but detailed information on the mechanism(s) of toxic action is not available.

Toxicity in animals

Acute toxicity data, based on i.p. administration, are available for nine YTX analogues. Of these, seven were of similar toxicity to YTX itself, with median lethal dose (LD_{50}) values between 100 and 750 µg/kg b.w. Two analogues were much less toxic, with no effects being recorded at a dose of 5 000 µg/kg b.w. The oral toxicity of YTX in mice

[2] It must be pointed out that as a result of the Expert Consultation, the Evaluation Section in the report differed from the Yessotoxins draft chapter. The Evaluation Section of the present "Background Document" corresponds to the Yessotoxins Section of the Expert Consultation Report.

is much lower, with no effects observed at an acute dose level of 50 mg/kg b.w. Data from one short-term gavage study in mice revealed no toxicity of YTX at 5 mg/kg b.w.

No data are available on the long-term toxicity, reproductive toxicity, carcinogenicity or genotoxicity of YTX.

Toxicity in humans

There have been no reports of ill-effects in humans attributable to YTX.

Evaluation

By applying a safety factor of 100 to the dose of 5 mg YTX/kg b.w. that showed no toxicity in an oral short-term mouse study, and in the absence of human data, the Expert Consultation established a provisional acute reference dose (ARfD) of 50 µg/kg b.w.

The Expert Consultation found that, because of insufficient data on the chronic effects of YTX, no tolerable daily intake (TDI) could be established.

The consumption of 250 or 380 g shellfish meat would lead to a derived guidance level of 12 and 8 mg/kg, respectively.

Gaps in the data

Low concentrations of YTX (0.5 ng/ml) cause the disruption of the tumour suppressor E-cadherin *in vitro*, calling for attention with regard to the risk that YTX might favour tumour spreading and metastasis formation *in vivo*. Studies on the long-term effects of YTX in animals when given by the oral route are urgently needed, as are studies on the mechanism of action of YTX.

Information on absorption, distribution and elimination of YTX is required.

7.2 Analytical methods for YTXs

This evaluation is predicated on the need for analytical methods that can assist regulation of shellfish to meet the current limits set by the EU and in several other countries of 1 mg/kg YTX, 45OHYTX and their 1a-homo-analogues. Analytical methods available for the determination of YTXs in shellfish are summarized in Table 10. Only methods that have either widespread use or documented method performance are included. Important issues relating to the metabolism of YTXs, the analogues and metabolites accumulated in shellfish, their oral mammalian toxicity and risks to human health remain unresolved. These issues have major ramifications for future testing requirements of seafood products for YTXs.

The imminent availability of a commercial certified reference standard for YTX will facilitate establishing alternative test methods to MBA (Institute for Marine Biosociences, NRC, Halifax, Canada).

The difficulty of extracting YTX and analogues from shellfish tissues has not been well acknowledged, and only recently have the problems been fully recognized arising from the weak partitioning of YTX and 45-OHYTX into organic solvents. Of the various solvent systems proposed, only 90 percent methanol has been rigorously tested for efficiency of extraction.

Standard MBA protocols using diethyl ether or dichloromethane partitioning to isolate lipophilic toxins give low recoveries of YTXs. However, presence of quite low levels of YTX in the extracts will confound the bioassay results for other toxins. Therefore, these methods can only act as crude screens, and require extensive confirmatory tests before regulatory decisions can be made. The modifications proposed by Yasumoto (2001) enable mouse assays of two separate fractions, one containing most of the YTXs and the other most of the other lipophilic toxins. However, similar to the original MBAs for DSP toxins, the revised protocol 2 has given relatively high rates of false negatives and false positives during routine use.

Furthermore, the extraction procedure is quite tedious and more mice are required. No data for interlaboratory testing of a range of shellfish species are available. These factors do not recommend it for use in routine screening of shellfish. Under the Codex definitions of types of test methods, the Yasumoto revised protocol 2 can only be rated as Type IV (Tentative Method) for YTXs.

Functional assays have been developed based on the specific effects induced by YTX on the E-cadherin-catenin system in mammalian cells and the activity of phosphodiesterases (PDEs). These have the potential to give an integrated response that may be related to the mechanisms of toxic action. The PDE assay has a relatively straightforward procedure, and some limited validation data has been obtained. This promising method requires further validation using a reliable extraction technique and a range of shellfish species. Currently, these functional assays can only be rated as a Codex Type IV methods.

TABLE 10
Analytical methods used to determine YTXs in shellfish tissues

Method reference	Detection	Test portion	Extraction	Clean-up	LOD mg/kg	Recovery YTX	Precision
Yasumoto, 2001 (revised protocol 2)	Mouse bioassay 3 mice i.p.	100 g WF	Acetone 300 ml; Methanol 300 ml	Partition: DCM/aq. methanol	1	94%	NR
Briggs *et al.*, 2004	ELISA	2 g WF	90% methanol 18 ml	Dilution 1:200	0.16	103–118%	RSD_r 8% RSD_R 16%
Yasumoto & Takizawa, 1997	HPLC-FL	1 g DG	80% methanol 9 ml	SPE, DMEQ-TAD deriv., SPE	0.1	94%	NR
Goto *et al.*, 2001	LC-MS	2 g WF	90% methanol 18 ml	SPE	0.05	90%	RSD_r 8%
McNabb, Selwood & Holland, 2005	LC-MS/MS	2 g WF	90% methanol 18 ml	Hexane wash	0.01	76%	RSD_r 8–12% RSD_R 16–22%

Note: HPLC: high-performance liquid chromatography; FL: fluorescence detector; LC-MS: combined liquid chromatography mass spectrometry; LOD: limit of detection (estimated); RSD_r: relative standard deviation for repeatability; RSD_R: relative standard deviation for reproducibility; WF: whole flesh; DG: digestive gland; SPE: solid phase extraction; NR: not reported.

The ELISA test for YTXs (Briggs *et al.*, 2004) that is reaching commercialization has some impressive performance characteristics and has been subjected to a preliminary interlaboratory study (Kleivdal, Briggs and Miles, 2004). The ELISA has high cross-reactivity to a wide range of YTX analogues, including 45-OHYTX and carboxyYTX, and appears very suitable for initial screening of shellfish. However, the ELISA has given apparent levels of YTXs in shellfish about tenfold higher than the levels of YTX plus 45-OHYTX determined by LC-MS (Samdal *et al.*, 2005). This creates difficulties in using the kit for regulating shellfish to the current EU limit. Further research is required to confirm the observed relationship between ELISA responses and the levels of YTX entities currently requiring regulation (Samdal *et al.*, 2004) and, for the future, of those entities of toxicological concern. If this can be achieved and further satisfactory interlaboratory precision data gathered, then the method could move from Codex Type IV to Type III or Type II status.

The HPLC-FL method (Yasumoto and Takizawa, 1997) has been brought into routine use in a number of countries, especially for confirmatory analyses. The method provides good recoveries and low detection limits for YTX, 45-OHYTX and some analogues (but not carboxyYTX) in the DG. It has not been validated for whole flesh. The sample workup procedure is relatively tedious, especially if 45-OHYTX is to be included, and it does not fit comfortably into a monitoring programme where

a large number of samples must be screened for a range of toxins. The performance characteristics appear quite favourable but no formal interlaboratory studies have been conducted. If laboratories using the method pooled their validation and quality control data to demonstrate satisfactory performance, then it could meet the requirements for a Codex Type III method (Alternative Approved) for use on digestive gland samples. Development of an IAC for YTXs (Briggs *et al*., 2004) could lead to simplified cleanup procedures for this method.

The power and utility of LC-MS as a confirmatory technique for YTXs and other marine biotoxins has been clearly demonstrated by a number of laboratories. The high sensitivity, high specificity and multitoxin capabilities also make LC-MS particularly attractive as a frontline screening tool with the capability to form the basis for a Reference Method (Codex Type II). However, until recently, there has not been a strong effort put into establishing all the performance characteristics of particular methods that would enable their use in quantitative analysis of YTXs and other toxins in shellfish. Many reports only constitute demonstration of potential, and progress on quantitative aspects, especially for metabolites, has often been limited by lack of reference standards.

The LC-MS method of Goto *et al.* (2001) received a within-laboratory study that demonstrated its suitability for determining a range of toxins in scallop. However, the accuracy and precision data were limited and not convincing for the fraction containing both YTX and 45-OHYTX. Another drawback to the method is the relatively complex workup of the crude extract into several fractions, each requiring separate LC-MS conditions. Further validation of this method with a wider range of shellfish species and an interlaboratory study would be required in order to move it from a Type IV Codex method to a Type III one.

The LC-MS method developed at the Cawthron Institute, New Zealand (Holland *et al*., 2004a, Holland and McNabb, 2002, 2003; McNabb and Holland, 2004; McNabb, Selwood and Holland, 2005), has been subjected to a thorough within-laboratory validation and a limited interlaboratory study. It has been used routinely in New Zealand for three years, with several thousand samples tested. The quality assurance/quality control data gathered further support the performance of the method as a quantitative method broadly meeting the requirements for Codex Type II. For example, the RSD for spike recovery quality control samples run with batches of monitoring samples over a 4-month period gave a recovery for YTX at 0.1 mg/kg of 84 ±15% (mean ±RSD, n=38; McNabb and Holland, 2004). The sample extraction and workup are simple and were demonstrated to give consistent recoveries of YTX and 45-OHYTX, along with a range of other toxins, from the flesh of several species. Further interlaboratory study is desirable, especially to test more widely the performance of single quadrupole and ion-trap instruments. The reliance on the YTX calibration for quantitation of 45-OHYTX is a weakness that will be resolved when a reliable standard for this metabolite becomes available. Good results from a larger collaborative study would enable the method to move to Reference (Type II) status.

Currently, there are no methods for YTXs with proven performance characteristics to meet Codex Reference (Type II) standards. However, the LC-FL method (Yasumoto and Takizawa, 1997), ELISA method (Briggs *et al.*, 2004) and LC-MS method (McNabb, Selwood and Holland, 2005) best fulfil current needs as Type III for screening and confirmation of YTXs in shellfish, and each could move to Reference Method status following further collaborative study.

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Summary of the FAO/IOC/WHO Expert Consultation on biotoxins in bivalve molluscs

1. APPROACH TAKEN

1.1 Risk assessment

The Expert Consultation was asked to perform risk assessments for a number of biotoxins that are present in bivalve molluscs. Because exposure generally involves only occasional consumption, and because most of the available toxicological data concerns only acute and short-term studies, priority was given to the establishment of an acute reference dose (ARfD). Although more frequent exposure may also occur, the Expert Consultation could not establish tolerable daily intake (TDI) values because of the lack of appropriate toxicological data. The risk assessments for the individual toxin groups were performed in a stepwise fashion, including hazard identification, hazard characterization, exposure assessment and risk characterization.

An adverse health effect is more likely in susceptible individuals who consume large amounts of contaminated shellfish. Occurrence data were not available to allow the consultation to conduct a probabilistic risk assessment. The Expert Consultation recognized that regulatory limits already implemented within existing monitoring plans contribute to maintaining the probability of adverse health effects at an extremely low level.

1.2 Intake and exposure

Because of large seasonal variations, the frequency of consumption and the number of consumers should be determined on a one-year basis. Within the whole population, 35 percent consume bivalve molluscs, both in Norway (Meltzer, Bergsten & Stigum, 2002) and in France (AFSSA, 2009). With shorter surveys, this percentage is 11 percent in France (7 days); 8 percent in Italy (7 days); 4 percent in the United States of America (2 days); 3 percent in New Zealand (1 day); and 2 percent in Australia (1 day). In France, the frequency of consumption for those consumers is 4.2 eating occasions per year. In the United States of America, the frequency of consumption is 8.6 eating occasions per year (Sherwood Hall, personal communication). In Norway, 33 percent of consumers eat bivalve molluscs between 1 and 11 times a year, and 2 percent of consumers eat these molluscs between 1 and 8 times a month.

Short-term dietary intake assessment should be carried out to obtain the estimated toxin intake over a single day or for a single eating occasion. The procedure used by the Joint Meeting on Pesticide Residues in Food and the Environment (JMPR) for acute toxicity of pesticide residues employs the database of the World Health Organization/ Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (WHO/GEMS Food), which has compiled the highest reported 97.5th percentile consumption figures for "eaters only" for each single food category. For bivalve molluscs, this large portion corresponds to 380 g for adults (the Netherlands). The conservatism of this figure is confirmed by additional information received from Member States about the 97.5th percentile consumption figures for edible shellfish portions by adults, which are, respectively, 133 g in Japan, 181 g in Australia, 225 g in the United States of America and 263 g in New Zealand. A consumption of

182 g has been reported in Norway as a maximum level of consumption. For children, the highest reported 97.5th percentile consumption figure is 70 g for (Australia), and 27 g was reported for Japan.

It should be noted that the standard portion of 100 g, which is sometimes used in risk assessment, is not adequate to assess an acute risk; a portion of 250 g would cover 97.5 percent of the consumers of most countries for which data were available. Three simulations were done using, respectively, portion sizes of 100, 250 and 380 g.

1.3 Occurrence and concentrations of toxins in bivalve molluscs

Because of insufficient data, the occurrence and the concentrations of toxin in bivalve molluscs were not fully evaluated during the consultation. However, for the purpose of exposure assessment, the Expert Consultation developed the typical range of toxin levels that may lead to closure of the harvesting area and maximum reported level in shellfish (Table 1).

TABLE 1
The typical range of toxin levels that may lead to closure of the harvesting area and maximum reported level in shellfish

Toxin group	Typical level when toxins occur at levels that may lead to closure of the area (mg/kg)	Maximum reported level (mg/kg)
AZA	0.16–0.3	1.4
Brevetoxins	0.8 mg/kg (as PbTx-2)	40
Cyclic Imines	0.1	2
DA	20–200	1 280
OA	0.16–1	36
PTX	LOD–0.2	0.9
STX	0.8–10	800
YTX	1–2	8

2. GENERAL CONSIDERATIONS ON ANALYTICAL METHODOLOGY

Contamination by marine biotoxins often involves more than one toxin group, and monitoring programmes typically cover a range of toxins. Mouse bioassays (MBAs) have been the traditional means of overcoming these complexities. However, these assays have severe technical and ethical limitations and generally lack adequate validation for Codex purposes. Therefore, multitoxin instrumental methods are required for more cost-effective screening and these increasingly utilize liquid chromatography with mass spectrometry detection (LC-MS). However, many toxin groups also encompass numerous analogues that may be impracticable to be individually measured. Choice of suitable marker compounds can ease the analytical burden, but positive samples will generally require more detailed follow-up analyses. Functional assays based on the common biochemical activity of a group of toxins are attractive alternatives to multitoxin methods, but few thoroughly validated assays are widely available. Rapid screening tests, generally based on immuno techniques, are already in widespread use for the saxitoxin (STX) group and can be effective management tools. However, an alternative quantitative test is generally required for opening closed shellfish growing areas. All areas of marine biotoxin method development, validation and testing require certified calibration standards and reference materials. There is an urgent need to expand the currently available certified reference materials (CRMs), and Codex should encourage Member States to fund the necessary efforts.

For Codex purposes, the preference is for methods to be validated by collaborative study according to the harmonized protocol of the International Union of Pure and Applied Chemistry/Association of Official Analytical Chemists/International Organization for Standardization (IUPAC/AOAC/ISO). Laboratories must demonstrate adequate performance of their methods through proficiency testing.

However, there is a lack of interlaboratory studied methods and proficiency testing schemes for phycotoxins. Therefore, there should be particular emphasis on thorough within-laboratory validations and internal quality control (QC) procedures (Thompson and Wood 1995). Codex has issued guidelines for single laboratory method validation based on IUPAC recommendations. For the management of the analytical result it is particularly important to establish the uncertainty of measurement as outlined in ALINORM 04/27/23.

In principle, it is preferable to analyse the part of the shellfish that is considered edible (normally whole tissue). Only the parts analysed should be marketed, except when there are possible analytical method interferences or detectability issues, or other practical issues, e.g. large scallop species with fibrous tissues. In these cases, the most contaminated tissues (e.g. the digestive gland [DG]) may be dissected and analysed, and the result should then be converted to an edible tissue basis. Data should be obtained to determine an appropriate conversion factor, based on weights of dissected parts and the extent of transfer of toxin into other tissues. As shellfish toxins actively accumulate in the DGs of bivalves, the toxin portion in the DG represents typically > 95 percent of the total toxin present in the bivalve. For reasons of sensitivity, test methods in monitoring, such as MBAs, have traditionally focused on the DG as a test portion. Other test methods, including LC-MS could also benefit from this approach.

Several procedures to detoxify shellfish have been developed to mitigate the negative economic impact of toxic contamination. Initial efforts had limited success and were hampered by factors such as operational costs, depuration characteristics of different shellfish species and the effects of treatments on the organoleptic properties of shellfish (Anderson *et al.*, 2001). The concentration of toxins in DGs has enabled commercial evisceration procedures for several shellfish species, principally scallop, to produce edible portions with acceptable toxin levels. This is especially applicable to lipophilic toxins. In contrast, concentrations of water-soluble toxins and/or heat-labile toxins in shellfish are decreased by thermal treatment through their loss in cooking fluids or their destruction. Procedures combining evisceration and conventional canning can successfully reduce the levels of the STX group and domoic acid (DA) group in a number of shellfish species of commercial interest without affecting organoleptic and quality properties. Some countries have issued exceptions to harvest action limits for lots destined for such processing, thereby shortening closure periods. During the Expert Consultation, it was noted that there were insufficient data on the effects of processing. Also, there was insufficient time to complete the literature survey on this topic. Subsequently, a review of this topic was carried out by the European Food Safety Authority (EFSA, 2009). Accordingly, there is recent information clarifying the phenomena of the effects of processing on lipophilic toxins, in particular OA-group and azaspiracid (AZA)-group toxins. During heat treatment processes, these toxins are typically concentrated by factors of 1.25 to 2 in shellfish.

However, to ensure public health safety, it is still necessary to determine the specific effects of post-harvest processing on toxin levels, interconversions and redistribution. In addition, all processed lots should be subjected to final product testing before marketing.

Appendix 1 presents the concepts of marker compounds and relative response factors (RRFs). The concept of a marker residue has been elaborated to enable regulation of contaminants in food where it is not practical on a routine basis to fully determine the levels of all residues present. A related issue is the use of RRFs for calibration of instrumental assays for particular toxins where certified reference standards are not available. In Appendix 1, the definitions, practicality and limitations in use of marker compounds and RRFs are examined in the context of analysis for marine biotoxins in shellfish.

TABLE 2
Summary of methods for analysis of marine biotoxins and recommended reference methods

Toxin group	Animal assays	Functional assays	Immunoassays	Analytical tests
Azaspiracid	MBA/RBA	Cell morphology	Non-applicable (N/a)	*LC-MS***
Brevetoxin	APHA-MBA	Na-channel RBA, Neuroblastoma	ELISA	*LC-MS***
Cyclic imine	MBA	N/a	N/a	*LC-MS***
Domoic acid	N/a	RBA	ELISA, Immunobiosensor	*LC-UV**, LC-FL, LC-MS, TLC
Okadaic acid	(S)MBA/RBA	PP2A, PP1, F-actin	ELISA	*LC-MS***, LC-FL
Pectenotoxin	MBA	F-actin	N/a	*LC-MS***, LC-FL, LC-UV
Saxitoxin	AOAC-MBA	Na-channel RBA Saxiphilin RBA, Neuroblastoma	ELISA, FLIC	*LC-FL**, LC-MS, FIFLD
Yessotoxin	MBA	E-cadherin fragmentation PDE-enhancement	ELISA	*LC-MS***, LC-FL

* Recommended as reference method.
** Recommended as reference method after completion of successful collaborative trial.

3. MONITORING

3.1 The role of microalgal monitoring in marine biotoxin management

Microalgae (including planktonic and benthic organisms) are the primary source of biotoxins in bivalve molluscs.

A marine biotoxin management programme should be described in a marine biotoxin management plan. This plan should include marine biotoxin action plans for growing areas containing, for example, sampling strategy and requirements (frequency, sample size and composition), analyses to be carried out, and management action to be based on monitoring results and expert judgement.

Toxicity monitoring cannot be replaced solely by microalgae monitoring. Information from microalgal monitoring, especially if it is carried out regularly (for example, weekly during harvesting), as part of a bivalve mollusc biotoxin management programme, has particular strengths, including:

- Generally, observable concentrations of toxic microalgae precede critical levels of toxins in bivalve molluscs and, therefore, microalgal monitoring allows management options to be considered, such as precautionary closures, intensified monitoring or depth-specific sampling.
- It can also help focus shellfish testing, for example, on likely toxins, at the right location, at the appropriate time and when new toxin-producing species of microalgae are found in an area.
- As part of an integrated biotoxin management programme, it is cost-effective and operationally efficient.
- It may be used to investigate unknown, unusual or atypical toxic events.
- It may be used to provide information to set or use switching factors. These may activate associated management options.
- It may provide information not only on the onset of a toxic event but also on the duration of any intensified management action.

Therefore, for early warning purposes and direct risk-management activities, it is recommended to have a programme to monitor growing areas for species of toxin-producing microalgae. The programme should also include evaluation of other environmental conditions, for example, wind, water temperature and salinity, which may suggest upwelling, stratification or mixing. These conditions may indicate that favourable conditions for a toxic event are developing.

However, the weaknesses of such a system may include:

- Microalgal observations may not accurately reflect the actual level of toxins in shellfish. In part, this may be because of significant inter- and intra-species variability in toxin profile and toxin content for many microalgal species even from the same area and over a short period.
- While microalgae are the primary source of toxicity in shellfish, the toxins may remain in shellfish long after the toxic microalgae are gone. Thus, the absence of toxic microalgae cannot be taken as an indication that the shellfish are safe.
- Microalgae are not always distributed uniformly in either time or space. "Patchy" distribution of microalgae may make representative sampling difficult.
- The logistics of sampling offshore or remote areas, where scallops or clams, for example, are fished, may make microalgal monitoring less cost-effective.
- Special monitoring arrangements may be necessary to address the problems posed by benthic species of toxic microalgae, for example, *Prorocentrum lima.*

In conclusion, decisions made on the safety of shellfish can only be based on the direct measurement of toxins in shellfish flesh. However, an integrated shellfish and microalgal monitoring programme is highly recommended to provide expanded management capability and enhanced consumer protection.

Furthermore, recent developments indicate that microalgal monitoring coupled with operational oceanographic, meteorological, and remote sensing data, including modelling and other measurements, may be used to base advice on the imminent onset of harmful events.

3.2 Indicator microalgal species

TABLE 3
Examples of source indicator organisms for some of the toxin groups

Toxin group	Genus	Example species
Azaspiracid	Azadinium	Spinosum
Brevetoxin	Karenia	Brevis
Cyclic imines	Alexandrium	*Ostenfeldii* (for spirolides)
Domoic acid	Pseudo-nitzschia	*Australis, seriata, pungens, multiseries*
Okadaic acid	Dinophysis Phalachroma Prorocentrum	*Acuta, acuminata, sacculus, fortii, caudata* Rotundatum Lima
Saxitoxin	*Alexandrium,* *Gymnodinium,* *Pyrodinium*	*Tamarense, minutum, catenella* *Catenatum* *Bahamense*
Yessotoxin	Protoceratium Lingulodinium	Reticulatum Polyedrum

3.3 Indicator shellfish species

The selection of an indicator shellfish species for each toxin group is problematic because the rate of toxin uptake and depuration is unique to the combination of species, toxin and geographic location.

It is important to note that, using an indicator shellfish species, the absence of toxicity in the indicator species is assumed to imply the absence of toxicity in other species in the growing area. This implication must be verified for each shellfish species and for each group of toxins before defining a particular shellfish species as an indicator for that growing area.

3.4 Sampling

A microalgal and shellfish sampling protocol over time and space should include the adequate location and number of sampling sites. Sampling frequency must be sufficient

to address spatial–temporal changes in microalgae, toxins in shellfish and to cover the risks of rapid rises in shellfish toxicity.

Spatial representational sampling

The selection of sampling stations for both benthic and suspended culture should be based on sites that have historically presented toxicity in the early stages of a toxic event. It is recognized that sampling, generally, cannot be carried out in a statistically valid way without excessive cost. In order to protect public health, the selection of sampling stations should give appropriate coverage of the extent of a toxic event or the likely "worst case scenario" in a growing area. This should be based on expert judgement using the following factors:

- hydrography, known upwellings, fronts, current patterns and tidal effects;
- access to sampling stations in all weather conditions during harvesting;
- desirability of toxin and microalgal sampling at the same sampling station;
- in addition to primary (routine) stations, the need for secondary (complementary) and offshore stations;
- existence of *in-situ* growth (for example, toxic microalgae from cyst beds);
- the advection of offshore toxic microalgal blooms into growing areas.

Routine sampling for microalgae will generally mean taking an integrated sample from the water column. When a toxic event is in progress or developing, targeted, depth-specific sampling should be considered.

Sampling for shellfish grown in suspension should at least involve an integrated sample composed of shellfish taken from the top, middle and bottom of the lines.

Temporal representational sampling

Minimum weekly sampling frequencies are adopted by most monitoring programmes in areas where toxicity is prevalent and where harvesting is taking place or about to take place. Decisions on the frequency of sampling should be based on risk evaluation. Inputs into the decision may include factors such as seasonality (toxicity and/or harvesting), accessibility, historical baseline information, including toxin and microalgal data, and the effects of environmental factors such as wind, tide and currents.

Sampling frequency and the factors that may lead to it being changed should be described in a "marine biotoxin action plan" for the growing area.

Shellfish sample size

There is no internationally agreed sample size for different shellfish species. There may be high variability of toxicity among individual shellfish. The number of shellfish sampled should be sufficient to address this variability. For this reason, the number of shellfish in the sample, rather than the mass of the shellfish flesh, should be the determining factor for the sample size. In addition, the size of the sample should be sufficient to allow the test or tests for which the sample is being taken to be carried out, and the shellfish sampled should be of the size marketed.

Appendixes 2 and 3 present more detailed considerations about the marine biotoxin action plan and the role and design of phytoplankton monitoring in harmful algal bloom (HAB) programmes, from the documents collated by Working Group 3 of the FAO/IOC/WHO Expert Consultation in 2004.

4. REPLIES TO SPECIFIC QUESTIONS POSED BY THE CODEX COMMITTEE ON FISH AND FISHERY PRODUCTS (CCFFP)

Q1. Provisions of scientific advice for the establishment of safe upper limits:

Review of toxicological information and provisional scientific advice to define which toxins belong in which toxin group, and recommendations for the establishment of upper safety limits for the following toxin groups: PSP-, DSP-, ASP-, AZP- and NSP-toxins, and YTXs and PTXs.
Please find the Evaluation Section in each toxin-specific section.

Q2. Provide advice on management of "new toxins" and "newly discovered analogues of existing toxins" where either:

i. There is no epidemiological evidence of illness resulting, or
ii. Where epidemiological evidence exists.

New classes of compounds
The Expert Consultation envisaged three situations in which new toxins may be identified (Figure 1):

1. An outbreak of poisoning in humans that is not associated with known toxins.
2. The identification of a new species or strain of algae.
3. Unusual clinical signs in MBA.

In the case of human intoxication, the Expert Consultation recommends that every effort should be made to identify the symptoms and clinical changes in affected individuals, in order to give information on the target site of the new toxin. Samples of the material associated with the intoxication should be gathered and stored.

Initial evaluation of new toxins should be via oral administration in mice. Subsequently, the major toxin(s) should be separated and identified. These should then be evaluated for toxicity again by the oral route in order to establish acute RfDs and TDIs.

New analogues of existing toxins
Concerning toxins for which adequate structure-activity data are available (Figure 2), a decision with regard to regulation can be made on the basis of structure. If no adequate information is available, the Expert Consultation proposes that new analogues present in shellfish at less than 5 percent of the parent toxin should not be regulated against. Compounds present at a concentration greater than 5 percent of the parent compound should be isolated, characterized and then toxicological properties investigated in order to establish an ARfD and TDI.

Q3. Provide guidance on the application of different methods of analysis concerning each toxin group:

- **Bioassays, analytical instrumental methods (high-performance liquid chromatography [HPLC], LC-MS ...), immunological methods, other rapid methods – which methods should be considered reliable for each toxin group to ensure safety of product.**
- **Recommend choice of reference method in case of conflicting results.**
- **Discuss needs for standards and reference materials.**
- **Suggest management of analytical results, concerning precision, standard deviation, acceptance levels, etc.**

Please find the information described in each toxin-specific section.

FIGURE 1
Proposed process for the management of new toxins

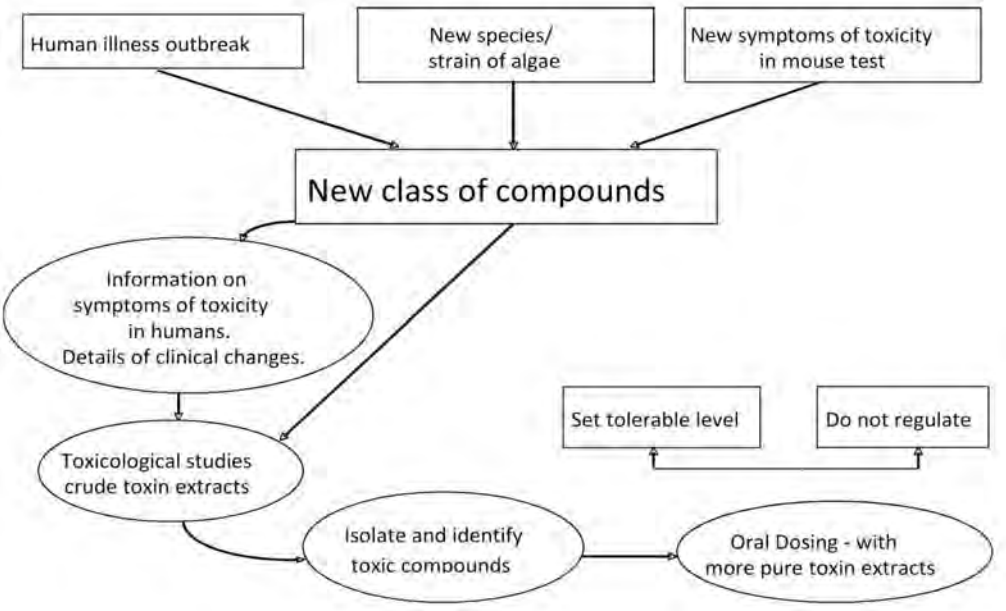
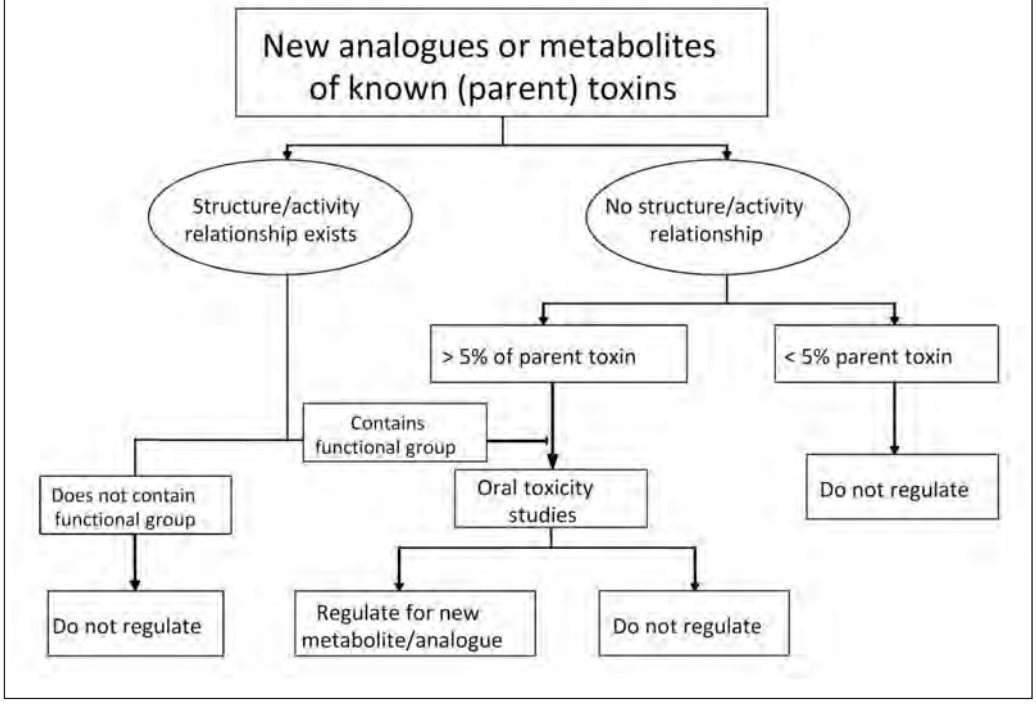


FIGURE 2
Proposed process for the management of newly discovered analogues of existing toxins



Q4. Monitoring:

- **Provide guidance on which part of the seafood (shellfish or other) should be used for analysis (whole meat, different edible parts, digestive organs …).**

Please find the information described in Sections 3.3, 3.4.

- **Provide guidance on sampling methods; suggest minimum representative sampling (size of sample, number of samples, different depths, frequency, etc.).**
- **Provide guidance on use of phytoplankton monitoring (strengths and weaknesses) as part of a shellfish biotoxin control programme.**
- **Provide guidance on indicator organisms for the different toxin groups.**

Please find the information described in Sections 2.0, 3.4.

Q5. Geographic distribution:

Provide information on the existence of biotoxin forming marine algae in various geographical regions of the world.

Microalgae responsible for the production of the toxins within the major toxin groups, saxitoxins (STXs), domoic acid (DA) and okadaic acid (OA), have a worldwide distribution. Some species have restricted geographical distribution but toxic representatives from the different genera are known worldwide.

Microalgae responsible for the production of the rest of the toxins within the defined toxin groups, listed in Table 3, have a more restricted geographical distribution, such as *Karenia brevis*, which is mainly reported from the Gulf of Mexico.

Although previously the heterotroph *Protoperidinium crassipes* had been associated with AZAs, recent studies including a statistical review of Irish monitoring data (Moran *et al*., 2007) could not corroborate this hypothesis. Instead, a small dinoflagellate was discovered to produce AZAs in the field and in culture, *Azadinium spinosum* (Krock *et al*., 2009; Tillmann *et al*., 2009). Because of its small size (5 × 15 µm) and its fragility to iodine-based fixing agents, this organism may not have been identified in other regions. However, it is noted that *A. spinosum* has also been found in Danish waters (Krock, Tillmann and Cembella, 2009). In addition, AZAs have been reported to occur in shellfish from France, Morocco, Portugal and the United Kingdom of Great Britain and Northern Ireland (Amzil *et al*., 2008; EFSA, 2008; Taleb *et al*., 2006; Vale, Bire and Hess, 2008). Finally, Azaspiracid-2, a major analogue of Azaspiracid-1 also in Irish shellfish, has most recently been discovered in a sponge collected from Japanese waters (Ueoka *et al*., 2009). Therefore, a significantly wider geographical distribution than initially proposed must be assumed.

It is suggested that representative microalgal species responsible for producing toxins from all defined toxin-groups are regarded as potentially worldwide.

5. RECOMMENDATIONS

5.1 To Member States, FAO, WHO

- Encourage Member States to implement public health programmes that ensure that shellfish poisonings are captured in a more systematic way:
  - reportable disease (physicians);
  - public awareness programmes;
  - rapid outbreak-response teams (timely sample capture + analysis and predefined communication channels, questionnaire).
- Encourage Member States to generate more toxicological data to perform more accurate risk assessments.

- Promote increased international effort for the production of certified reference materials and calibration standards.
- Encourage Member States to improve and validate toxin detection methods in shellfish.
- Promote toxicological studies conducted according to the guidelines the Organisation for Economic Co-operation and Development (OECD).
- Encourage studies to clarify the mechanism of action for a number of toxin groups.
- Encourage Member States to implement an integrated shellfish and microalgae monitoring programme.
- Consider the position of developing countries regarding implementation of chemical analytical methods.
- Encourage Member States to determine the relationship between quantitative occurrence of toxin-producing microalgae (planktonic and epiphytic) and the accumulation of biotoxins in bivalve molluscs.
- Encourage Member States to develop operational models for forecasting blooms of toxin-producing microalgae in time and space.

5.2 To Codex

- Codex should continue to work on risk management recommendations (e.g. Standards and Code of Practice) to address issues related to biotoxins in bivalve molluscs.
- When selecting detection methods, consideration should be given to the situation in developing countries.

5.3 To FAO, WHO

- Establish a standing expert panel to periodically review scientific data and information at the international level. This panel should be convened soon to review epidemiological and cooking/processing data so as to derive guidance levels/maximum levels more accurately for some toxin groups.

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APPENDIX 1

Marker compounds and relative response factors – issues for screening natural toxins in food

Discussion document for the Joint FAO/IOC/WHO ad hoc Expert Consultation on marine biotoxins in molluscan shellfish

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1.1 INTRODUCTION

The transition to instrumental analysis and immunoassays from animal bioassays for marine biotoxin testing of seafood products has brought to the fore issues relating to identification of the toxic residue to be regulated, setting regulatory limits, establishing suitable analytical methods for enforcement and calibration of the assays.

The range of toxins produced by hazardous algae is generally complex and varies by species and location. Metabolism of the toxins in shellfish can lead to enhancement or reduction in their mammalian toxicity and the paths and rates for this metabolism are dependent on shellfish species. Overall, these factors can lead to complex and often poorly defined mixtures of toxins and their derivatives in contaminated shellfish samples. For example, yessotoxins (YTXs) are currently regulated by the European Union (EU) and New Zealand at 1 mg/kg for YTX plus its 1-homo analogue and their respective 45-hydroxy metabolites. However, recent research has shown that: a) the acute toxicity of YTX by the oral route is low (Tubaro *et al*., 2003); b) other YTX analogues are produced by the contaminating algae *Protoceratium reticulatum* (Ciminiello *et al*., 2007; Konishi *et. al*., 2004; Loader *et al*., 2007; Miles *et al*., 2004; Suzuki *et al*., 2007); c) other significant metabolites are formed in shellfish including carboxyYTX (Ciminiello *et al*., 2000); and d) a wider range of immunoreactive YTX species are present in the algae and contaminated shellfish (Samdal *et al*., 2004). Certified standard material is currently only available for YTX (IMB-NRC).

Similar issues regarding the definition of the toxic residue and analytical methods for regulation of set limits have had to be addressed in the regulation of other contaminants in food, particularly mycotoxins, pesticides and veterinary drugs. The concept of a marker residue has been elaborated to enable regulation of contaminants in food where it is not practical on a routine basis to fully determine the levels of all residues present. The levels of the marker in a food sample may be used to estimate the total toxic residues present and decide whether a regulatory limit has been exceeded.

A related issue is the use of relative response factors (RRFs) for calibration of instrumental assays for particular toxins where certified reference standards are not available. In this discussion paper, the definitions, practicality and limitations in use of marker compounds and RRFs are examined in the context of analysis for marine biotoxins in shellfish.

1.2 MARKER COMPOUND

Definition

Marker residue (veterinary drug): Residue whose concentration is in a known relationship to the concentration of the total residue in the last tissue to deplete to its permitted concentration. The marker residue can be the sponsored compound, any of its metabolites, or a combination of the residues for which a common assay can be developed. The target tissue and marker residue are selected so that the absence of marker residue above a designated concentration R(M) will confirm that each edible tissue has a concentration of total residue at or below its permitted concentration. (US-FDA 2004).

For veterinary drug residues in animal products, application of the concepts of marker residue and target tissue requires an experimental determination of the quantitative relationships among the residues that might serve as the marker residue in each of the various edible tissues that might serve as the target tissue. Because these relationships may change with time, the depletion of potential marker residues in potential target tissues must be measured starting after the last treatment with the drug and continuing until the residue has reached the permitted concentration for that tissue. The sponsor may use the results from the total residue depletion and metabolism studies carried out with the radio-labelled drug to determine the marker residue, target tissue, and R(M).

Marine biotoxins are regulated on the basis of risks to human health – other concepts do not apply, such as Good Agricultural Practice, which can lead to regulation of veterinary drugs or pesticides as "no detectable residues" or at other levels below limits based solely on safety. Thus, for use in managing health risks from shellfish, the marker compound and its R(M) must have a demonstrated relationship to the regulatory limit, however expressed. Other differences and limitations that affect application of the marker compound approach to regulatory testing of marine biotoxins in shellfish include the following:

1. Hazardous algae generally produce complex mixtures of marine biotoxins that vary even within species. Shellfish are exposed to mixtures of toxins, including minor analogues, some of which may not have been characterized or taken into account when setting regulatory limits.
2. Experimental studies using radio-labelled toxin are generally not available to accurately determine metabolism and depuration kinetics. Even data from non-labelled studies may be limited in quantity, cover only one or two shellfish species and be largely obtained from field exposures with associated uncertainties.
3. Toxicological studies on marine biotoxins are often very limited in scope and precision compared with those required to be carried out for new drugs or pesticides. This is because of the limited range and quantities of purified toxins and their metabolites that can be produced and the expense of the associated toxicology. Often, only acute toxicity data in mice or rats are available. This paucity of reliable and comprehensive data leads to major difficulties in setting regulatory limits that are securely related to risks from human consumption of contaminated shellfish. The uncertainty in the quantitative relationship between levels of a marker compound and the potential human toxicity of contaminated shellfish samples will have a large component from this source.

These factors make it difficult to establish a quantitative relationship between levels of a nominated marker and those of all the toxicologically relevant compounds in shellfish tissues. Even for diarrhoeic shellfish poisoning (DSP) toxins, where a sound EU regulatory limit of 0.16 mg/kg has been set, it is not possible to use okadaic acid (OA) as a quantitative marker for total DSP (OA, DTX1, DTX2 and their esters)

because of the large variations in the profiles of these toxins produced by algae and accumulating in shellfish (species/time). It is important to note that regulatory limits set for classes of toxins, using levels of individual compounds, such as for DSP, already embrace the marker compound concept because the limits implicitly include a range of other related metabolites and analogues that are not measured in enforcing the limits, i.e. the marker for DSP toxicity is the sum of OA, DTX1 and DTX2 in hydrolysed samples. However, the situations where a marker compound that does not comprise the major proportion of the overall toxicity and could be used to directly manage human risk are likely to be limited to a few very well-researched scenarios, e.g. *Karenia brevis* contamination of Eastern oyster with BTXs.

An alternative scenario, not envisaged in the veterinary drug definition, is the use of marker compounds to screen for presence or absence of particular toxin classes. A positive result for the screening test would require an alternative quantitative test to determine whether the sample exceeded the regulatory limit. In the absence of a known relationship between level of marker and total regulated residue, it will be sufficient to show that the sensitivity of the screening method for the marker compound is high enough to avoid false negatives, i.e. all samples containing total residues above the regulated limit would be detected. This screening approach can be particularly useful when instrumental analyses are being used to test shellfish regularly where the majority of samples are expected to test negative, e.g. as part of large monitoring programmes. Some examples follow of where this screening concept could be useful in application of liquid chromatography with mass spectrometry detection (LC-MS) analyses to regulatory testing.

1. DSP esters/DTX3. *Dinophysis* spp. produce OA and dinophysis toxins (DTX1, DTX2) in proportions that vary widely by species and location (Yasumoto, 2005); other isomers may be present (James *et al.*, 1998) and a significant proportion of the toxins may be in esterified forms (Suzuki *et al.*, 2001; Mackenzie *et al.*, 1998). Further transformations with respect to esterification take place after ingestion by shellfish. Shellfish contaminated by DSP contain free toxins and a proportion in ester forms such as DTX3, which must be released by hydrolysis to estimate overall compliance with the regulatory limit. Certified standards are only available for OA although a certified reference material (CRM) mussel tissue contains both OA and DTX1. Contamination of shellfish by DSP toxins is almost exclusively caused by *Dinophysis* species, which also produce much higher levels of pectenotoxins (PTXs), mainly PTX2. Rapid hydrolysis of PTX2 in shellfish leads to PTX2 seco acid production, which can be sensitively detected by LC-MS (Draisci, Lucentini and Mascioni, 2000; Mackenzie *et al.*, 2002; Miles *et al.*, 2004; Suzuki *et al.*, 2001). Use of PTX2 seco acid as a marker compound for the onset of DSP contamination enables LC-MS screening of shellfish using a single run per sample with a second run of the hydrolysed sample only required when PTX2 seco acid is detected.
2. BTX/NSP. The spectrum of brevetoxins (BTXs) produced by *Karenia brevis* is dominated by PbTx-2 but a range of other BTXs are generally present at lower levels (Baden, 1989). Brevetoxins undergo a complex metabolism in shellfish with oxidations, reductions and conjugations of the terminal side chain leading to a wide range of derivatives, many toxic (Plakas and Dickey, 2010; Ishida *et al.*, 2004; Morohashi *et al.*, 1995). Certified standards are available for PbTx-1, PbTx-2, PbTx-3 and PbTx-9 but not for BTX-B5 or any of the conjugates that dominate the toxic residues in contaminated shellfish. Parent PbTx-2 and the minor parent toxin and primary metabolite PbTx-3 have been shown to be present in shellfish contaminated by *Karenia* species (Abraham *et al.*, 2006; Ishida *et al.*, 2004; McNabb *et al.*, 2004). Although levels of

PbTx-2 and PbTx-3 are relatively low, the sensitivity of LC-MS is such that samples contaminated well below the regulatory limit for neurotoxic shellfish poisoning (NSP) are readily detected. Thus, LC-MS screening for NSP using these marker compounds can reduce the amount of mouse testing using the standard APHA-ether protocol without compromising public safety.

3. AZAs. AZAs can be readily detected in shellfish using LC-MS. However, they are a complex class of toxins with three major analogues and a range of minor analogues known (Satake *et al.*, 1998; Ofuji *et al.*, 1999, 2001). They are also relatively rare internationally. A standard is available (Rehmann *et al.*, 2008; McCarron *et al.*, 2009) for azaspiracid-1. Therefore, it is convenient for multitoxin screening of shellfish to set up the LC-MS method to detect this toxin. In the event of detection of this marker compound, samples can be rerun by LC-MS or other technique to determine a fuller range of the AZAs present and, thus, enforce the regulatory limit.

Conclusion

Use of marker compounds to give quantitative estimates of the total toxic residue has limited application to the direct enforcement of regulatory limits for marine biotoxins in shellfish because of the highly variable and complex nature of shellfish contaminations. However, marker compounds do have strong utility for screening, especially in routine monitoring programmes where they can reduce the complexity and cost of the testing while protecting public safety.

1.3 RELATIVE RESPONSE FACTORS

Definition

Relative response factor (RRF). This is the ratio of instrument responses (generally peak areas for chromatographic methods) for a compound of interest to that of a reference compound at equal concentration, or the ratio of the slopes of the linear calibration equations. The concentrations of the compound of interest in unknown samples can be estimated following application of the previously established RRF to the calibration equation established for a reference compound.

Relative response factors are useful for calibration of instrumental assays for particular toxins where certified reference standards are not available for routine use. Two situations can be recognized:

1. Only small quantities of a quantitative standard are sufficient to establish the RRF to a reference toxin using a few instrument runs. This is a very viable approach, but the accuracy of the RRF is dependent on the accuracy of the concentration of the standard, which is likely to be poor for small quantities of relatively rare toxins. The precision of the RRF can be checked by re-determination at intervals if sufficient quantities of the standard are available in a stable form.
2. No quantitative standard is available. This is the situation for many toxins and their metabolites, although crude standards or contaminated shellfish extracts are generally available to establish retention times and spectroscopic characteristics. The application of an RRF must then rely on chemical similarities between the toxin and chosen reference compound such that an assumption of equimolar responses, i.e. RRF = 1, can be taken as a reasonable approximation.

Relative response factors should only be applied where the toxin and its reference have close chemical similarity and behaviour in the analytical system, i.e. there are only small differences in detector response factors and retention times. Generally, the concept has been applied where a parent toxin, e.g. OA or YTX, is available as a

certified standard and an assumed RRF of 1 is taken for an isomeric toxin or a simple analogue or metabolite, e.g. DTX1 (35-methyl OA) or 45-OHYTX. This assumption should be valid if the structural changes are minor and not close to the chromophore where the detection system is spectroscopic (ultraviolet [UV] or fluorescence), e.g. the high-performance liquid chromatography and fluorescence (HPLC-FL) methods for OA, DTX1 and DTX2, and for YTX and 45-OHYTX, which are calibrated using OA and YTX, respectively. In the absence of any independent calibration data, such estimated RRFs cannot be used to obtain quantitative data. However, trends can be monitored, e.g. accumulation and depuration of toxins in shellfish.

Application of RRFs to LC-MS methods is more problematic because electrospray ionization (ESI) efficiency is highly dependent on chemical structure and the ionizing environment (solvent composition, coeluting coextractives). It is more difficult *a priori* to justify the assumption of equal response factors. This may be a good approximation in groups of large polyether toxins where ionization can be assumed to be mainly at a common moiety relatively remote from the structural changes, e.g. the positive-ESI of AZAs with the common ionisable N-centre or negative-ESI of YTXs with the common electronegative sulphate groups. However, other subtle effects on ionization efficiency may arise from changes in surface tension, etc. in the electrospray droplets. There are also likely to be shifts in RRF in moving from selected ion recording (SIR) of molecular species, e.g. MH^+ or $[M-H]^-$ to MS/MS with multiple reaction monitoring because of effects of structure on the relative intensities of the key ions in the collisional activation spectra. However, the RRF can be corrected for these changes by comparing responses of the toxin and reference in separate runs using single ion and multiple reaction monitoring Absolute concentrations are not required; thus, crude standards or contaminated shellfish are adequate for this determination.

The advantages of RRFs are that concentration data can be obtained for a wide range of toxins and analogues in shellfish based on a limited set of standards for the parent toxins and suitable contaminated shellfish extracts. Precision data can be gathered and general analytical quality assurance/quality control maintained. Although the use of RRFs increases the uncertainty of determination, the data sets are consistent and trends can be accurately followed. For monitoring programmes, these are significant advantages and public health risk can be assured by reference to a relatively limited amount of alternative testing, e.g. MBA. Uncertainties arising from use of these instrumental RRFs will often be less than those in the toxicological data used to establish the no observable effect levels (NOELs), and will generally be covered within the safety factors, especially where the proportion of analogues is relatively low. Use of RRFs is accepted in other natural toxin areas, e.g. testing for fumonisin mycotoxins in cereals by HPLC-FL or LC-MS where a range of analogues are known but quantitative standards are only available for fumonisin-B1 and –B2.

The lack of credible alternative analytical methods for the analysis of marine biotoxins is a strong argument for use of instrumental methods with RRFs as a transitional measure. The poor performance of the traditional mouse assays is becoming well documented, and most of the proposed *in vitro* assays have not yet produced precision/accuracy of analytical quality. Enzyme-linked immunosorbent assays (ELISAs) with excellent precision characteristics are coming available for some toxin groups, but the issues with establishing accurate cross-reactivities for analogues are very similar to those for calibration of instrumental analysis.

Conclusion

Relative response factors are a logical approach in a system of continuous improvement for instrumental methods. Multitoxin method validations can be accomplished with a limited range of standards and contaminated tissues. As a wider range of quantitative standards become available: a) limited quantities can be used to establish better RRFs

to the primary toxin; and b) larger quantities can be fully incorporated into methods as routine calibrants. Full revalidation of the methods should not be needed.

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APPENDIX 2

Marine biotoxin action plan

A simple action plan, modified from Andersen, Enevoldsen and Anderson (2003), is shown in Figure 1.

The marine biotoxin action plan describes the marine biotoxin management actions the Competent Authority should take when marine biotoxin activity occurs in a growing area.

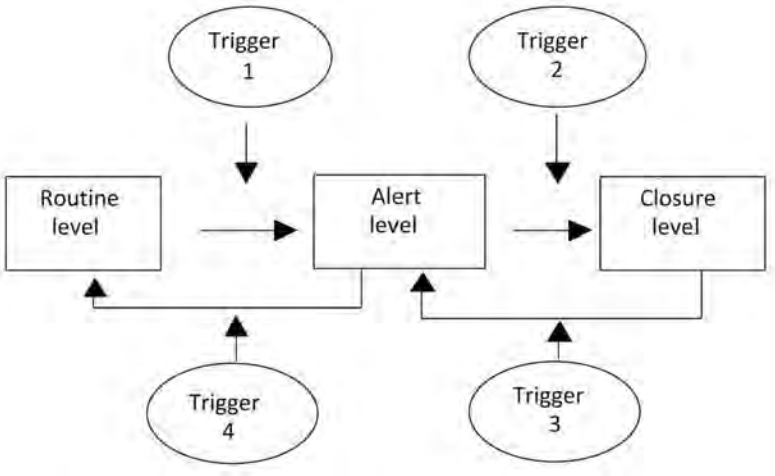
Routine level means the levels detected during routine (non-event) monitoring and during periods when there is no marine biotoxin activity.

Alert level means the levels detected when trigger levels are exceeded or levels of biotoxins are detected in shellfish below the maximum permitted level and above the background level.

Closure level means the biotoxins are observed in concentrations close to or exceeding maximum limits or when delays in obtaining samples or sample results occur.

The change from one level to another must be triggered by specific observations or combinations of observations already identified as triggering factors or triggering scenarios (Figure 1).

FIGURE 1
Marine biotoxin action plan



Trigger 1: biotoxins detected above the background level but below the maximum permitted level; or toxic phytoplankton present above trigger level.
Trigger 2: biotoxins detected above the maximum permitted level; or delays in sampling or receipt of results.
Trigger 3: biotoxins detected above the background level but below the maximum permitted level; or toxic phytoplankton detected above the trigger level.
Trigger 4: toxic phytoplankton detected below the trigger level; or biotoxins not detected or detected at a background level.
Source: Modified from Andersen, Enevoldsen and Anderson, 2003.

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Andersen, P., Enevoldsen, H. & Anderson, D. 2003. Harmful algal monitoring programme and action plan design. *In* G.M. Hallegraeff, D.A. Anderson & A.D. Cembella. *Manual on harmful marine microalgae*, pp. 627–655. Monographs on Oceanographic Methodology. UNESCO Publishing.

APPENDIX 3

The role and design of phytoplankton monitoring in HAB programmes

3.1 INTRODUCTION

Phytoplankton are sometimes regarded as an ancillary measurement to toxin monitoring programmes, and the full potential of this useful tool has not been fully realized in most national harmful algal bloom (HAB) programmes. The analysis of phytoplankton is much more cost-effective than shellfish testing, and the analysis is rapid, often by two or more days, compared with toxin analysis. In many programmes, the incorporation of phytoplankton into the decision-making might allow the status of a production area to be determined while waiting for the shellfish analysis. The use of phytoplankton as an early warning, therefore, merits particular attention in the setting up and reviewing of HAB monitoring plans.

During open periods, the onset of toxicity may sometimes be forecast by the increase in toxic species, in a shift from diatom to dinoflagellate dominance, or in a change in population diversity. When this information is coupled with basic oceanographic, meteorological and other measurements, it may be used as a valuable model on which to base advice on the imminent onset of a harmful event. This may also yield information not only on the onset of the event but on the length of time that it is likely to remain in the area.

In future, coupling phytoplankton results with real-time measurements of ocean temperature and wind may be useful to help predict HAB events. The real-time data may be acquired by deploying probes on existing aquaculture platforms, such as salmon cages and mussel longlines, to gather crucial environmental data related to harmful algal events around the coast. The measurement of phytoplankton is critical to the development of these forecast systems, and only by having sufficient data is it possible to use hind-casting techniques to run scenarios and develop models of the dynamics of toxin flux in the environment.

In designing suitable monitoring programmes for the monitoring of phytoplankton, a number of areas need to be considered. The programme can be broken down into three main areas: sampling, analysis and reporting. The design of a suitable sampling protocol is an area that merits significant consideration. The distribution of phytoplankton in a given area is subject to huge field variability and as such, obtaining a representative sample can be difficult. In many cases, several samples must be taken to reduce the variability, but even this may not be sufficient if a tidal or current regime in the area results in rapid species community changes. This is the nature of the planktonic habitat, and there are as many estimations of the intensity of sampling and frequency of sampling as there are micro, meso and macro habitats, compounded by such features as bathymetry, stratification, salinity, temperature, nutrient, currents and any other number of physical and physico-chemical variables. The design of harmful algal monitoring programmes and the implementation of action plans are described in Anderson, Enevoldsen and Anderson (2003).

3.2 SAMPLING

3.2.1 Identification of production areas

In designing a sampling programme for estimating the presence of HAB species in shellfish growing areas, it is necessary to first have a suitable means of identifying the location, or production area. The drafting of a set of maps is the first task that should be undertaken. These production areas are where the shellfish are actually grown, but it may be wise to also include some offshore locations in certain cases to provide for early warning. The designation of these production areas may be based on natural embayments, the use of Global Positioning System (GPS) coordinates in coastal locations, International Council for the Exploration of the Sea (ICES) rectangles, etc. It may also be desirable to divide the areas up into subareas for micromanagement of the region. This may also include division of a production area based on species being produced where species-specific closures are desired.

The important consideration when drafting the maps is that the production area is contained within the limits and also that it is of a suitable size depending on the local situation. This may be based on a contiguous shellfish farming area, or a natural shellfish bed, but may also take consideration of bathymetry and currents for example.

3.2.2 Nature of production area

In designing a sampling programme for phytoplankton, the means of shellfish production in the area must be taken into account. The main methods of cultivation and harvesting are: dredging, onshore intertidal harvesting, raceways and pumped tank systems or longlines and other forms of suspended or off-bottom culture. The type of production methods in the area will usually have an influence on the sampling methods to be used, and the location of sampling points.

3.2.3 Hydrography and oceanography

Currents and stratification are important features for the locating of a sample point and choice of sampling method. Coastal currents are features of many areas where shellfish are produced. They may carry with them HABs and act as a vector for the transport of these from one area to another. Where there is a well-established coastal current, it may advect either an established population or a seed inoculum of a harmful species into a production area. In these cases, it may be possible to site sample points upstream of the production area to attempt to forecast the onset of HAB events. Stratification occurs when the upper layer of water heats up from solar radiation and this results in the layering of water masses of different densities on top of each other. This stratified water can result in favourable conditions for the growth of particular species over others, and also may result in subsurface thin layers.

3.2.4 Selection of sampling sites

The selection of suitable sample sites must be identified using a number of pieces of information. The optimal location and number of sample sites may be based on variability of plankton populations, which may be derived from a baseline study, or on historical data. In most cases, the sample site will be co-located with the shellfish site. Information that may be useful in selecting a suitable site includes any information on phytoplankton distribution and variability in the area, bathymetry, effect of wind and rivers, whether the area is prone to stratification, and presence or absence of cyst beds. The risk of the presence of known HABs should be established by examining historical or survey data. In addition, there may often be a trade-off between the most desired site and what is practical. The best site from a scientific perspective may be difficult or impossible to reach. Accessibility may be only possible during harvest periods if a boat is required. In some cases, hydrographic models may have a role in predicting the

most appropriate site location, but appropriate expert judgment should also always be taken into account.

3.2.5 Frequency of sampling

Decisions on the frequency of taking samples should be based on risk, and input into the decision could include factors such as seasonality, accessibility, periods of harvesting, historical baseline data and models of wind/tide/currents. In many cases, the frequency of sampling may be adjusted according to the time of the year, for instance, the winter months in temperate areas may be subject to reduced levels of phytoplankton and, therefore, sampling may be reduced accordingly. At other times of the year (particularly threshold periods such as spring and autumn), the frequency should be increased to account for rapid changes in the presence of HAB species. There is no hard and fast rule about the appropriate frequency of sampling; however, the usual minimum adopted by most monitoring programmes is one sample per week during high-risk periods and reducing to once per fortnight or month during less risky times of the year. Apart from seasonality, the frequency of sampling may be increased during harvesting periods and decreased if there is no harvesting activity. However, a year-round programme should be in place to build up data during all times of the year. The change in HAB populations may be influenced by currents, tides and wind and, therefore, the merit of incorporating the use of models is encouraged to observe these potential changes.

3.2.6 Offshore sites

For some shellfish species, such as scallops that are fished offshore, there are particular difficulties in sampling. In most cases, the locations are mainly inaccessible and difficult to sample. The shellfish are located on the bottom, which will most probably have a different phytoplankton population from that in the water column. However, the use of commercial fishing boats or naval vessels may be a useful means of obtaining samples from these areas. In addition, in the case of species of HABs that cause discoloration, there may be merit in using aircraft or satellites for early warning.

3.2.7 Sampling methods

The field sampling of phytoplankton is critical to obtaining appropriate samples depending on the type of subsequent analysis that will be performed. For qualitative work, the types of samplers that are typically used are phytoplankton nets that are either dragged up through the water or towed horizontally. It is important to use the appropriate mesh size for the target species. Another means of taking qualitative samples is to pump water through a "bongo" filter and backwash the filtered material into a small container. This is particularly useful for the study of thin layers of phytoplankton as the pump inlet may be deployed at a suitable depth where phytoplankton are known to accumulate.

For quantitative measurements, it is usually more appropriate to use sampling bottles and/or surface buckets to take the sample and avoid filtering through a mesh. Typical bottle samplers include Niskin, Van Dohrn, and NIO bottles (water samplers designed by F.E. Pierce, D.I. Gaunt and R. Dobson at the National Institute of Oceanography, the United Kingdom of Great Britain and Northern Ireland). The sample may also be taken by means of a tube sampler to integrate the sample to the depth of the water column. Once the sample has been taken, it is either preserved of left unpreserved, again depending on the requirements of the laboratory. The typical preservatives used are Acidic, Neutral or Alkaline Lugol's Iodine, which has the advantage of staining and preserving the cells, and formaldehyde, which may be a better fixative for certain species but must be used with care in a fume hood as it is a potential carcinogen.

3.2.8 Transport to the laboratory

It is essential that the sample is transported to the laboratory as quickly as possible and in the most effective manner to prevent damage or deterioration, and steps must be taken to ensure that it arrives in an identifiable manner in the laboratory. This involves the attachment of a label to the sample, including details about where it was taken and date. The sender's details and any other relevant details about the sample should be included. In some cases, it may be possible to take the sample directly to the laboratory, and this is preferable for fresh samples. These unpreserved samples begin to deteriorate as soon as they are taken and they should be analysed as soon as possible after they are taken. Preserved samples, on the other hand, are stabilized, but it is important to turn around the information on HAB species as quickly as possible, so it is desirable to expedite them through the laboratory system, and transporting should be carried out as quickly as possible.

3.2.9 Training in sampling techniques

Training of samplers is most important in the implementation of a monitoring programme. In some cases, the samplers will have a clear idea of how to take a good sample, but in other cases it is necessary to demonstrate the techniques. In both cases, however, it is best to have a standard operating procedure written down so that everyone is clear about what is expected. The training should always be carried out by staff that are familiar with the equipment and technique.

3.3 ANALYSIS – PHYTOPLANKTON LABORATORY

The role of the phytoplankton laboratory is to provide the personnel carrying out the work with facilities to enable them to do the analytical work. The laboratory will provide results that may then be used to generate actions depending on levels of HAB in the water. These might include preclusion of harvesting, changing to a more frequent sampling routine or a more geographically intensified monitoring. In most cases, the senior laboratory personnel will issue the recommendations based on the analysis of the samples. The evaluation of these results may take into account recent trends and results from adjacent areas in order to form a better picture of the situation.

3.3.1 Selection of laboratory

In selecting a suitable laboratory, the location of the laboratory is important to ensure that the sample can be delivered to the laboratory and preclude delays. In addition, the facilities available must be of a standard that is adequate to carry out the analysis to a high standard. Laboratory accreditation and qualifications of the staff should also be investigated, and only laboratories that operate to a high standard of quality should be employed.

3.3.2 Laboratory role in supply of sampling equipment

The laboratory may also be used as the contact point for the distribution of sampling equipment, containers, etc. This is often an efficient means of ensuring that the samples are preserved, packed and labelled properly because it means that the personnel in the laboratory and samplers meet occasionally and any problems can be addressed.

3.3.3 Sample logging and tracking in laboratory

The traceability of samples is of utmost importance, and the laboratory should have a secure system of tracing samples through the laboratory. The most usual method is by the assigning of a laboratory test identification code number on each sample as it arrives in the laboratory, and this follows that sample through each process right through to reporting. The benefit of this system is that it provides a strong tracking capability in the laboratories, but also hides the location of the sample from the analyst

and, thereby, may prevent any bias in the analysis. Where possible, the adoption of a laboratory information management system should be employed. This computerized system assists in the sample flow through the laboratory and ensures that every stage of the analysis is carried out. It also provides an automatic assigning of test sample codes and provides the template of the report once all the analysis is complete. Where these sophisticated systems are not in place, data should be stored (including the data forms and final report) in a readily accessible format.

3.3.4 Analysis methods

The method of analysis of the phytoplankton sample is dependent on the type of sample and the reasons for analysing it. The phytoplankton samples may be quantified or a qualitative investigation carried out to see what species are present. The microscopic methods used to evaluate these are diverse, and many different chambers for settling and filtering the samples may be employed. The choice of the method depends on the concentration of the cells in the sample, as some may be harmful at low concentrations. It is necessary to choose a technique that will detect these low numbers.

Prior to analysis, it is essential to keep preserved samples in the dark to prevent deterioration of Lugol's iodine. This may be topped up if it is noticed that the samples are going clear over time. The samples must then be concentrated or in some cases diluted to provide a suitable concentration that can be evaluated under the microscope. The settling chambers that are commonly employed are 10, 25 or 100 ml, and the relevant concentration factor must be noted depending on the volume concentrated for subsequent calculations back to cells per litre. Counting strategies and methods are described in Anderson and Throndsen (2003) in detail and the calculations necessary to calculate the concentrations back to cells per litre are demonstrated. New technologies in the analysis of phytoplankton should be kept abreast of, in particular the role of gene probes is promising in the rapid analysis of select species in a sample.

3.3.5 Qualitative vs quantitative

It may be desirable to obtain a comprehensive idea of the presence or absence of a particular HAB species in a region. In these cases, a quick qualitative survey may give valuable insight into the extent of a bloom, or the absence of a particular species. While qualitative surveys have their place, the issuing of risk-based advice on the closure of a region of shellfish owing to the presence of an HAB species is more likely to be based on the evaluation of quantitative data. The observation, either temporal or spatial, of the trends is clearer to observe in quantitative samples, and subtle changes may be seen.

3.3.6 Training in laboratory techniques

Similarly to the training of sampling staff, the training of analysts is of utmost importance to provide quality laboratory analytical services. In most cases, this will involve formal training in phytoplankton identification and counting, either in college or at a specialized training course. The identification and enumeration of HABs is a skill that comes through experience and training. It is also important to have a standard operating procedure written down to provide guidance on the chosen methods. The training in this method should also be documented, and any changes to the method should be notified to the analysts and re-training scheduled to account for this.

3.4 REPORTING – COMMUNICATION OF RESULTS

It is imperative that the results from these samples are turned around in the laboratory in a quick and efficient manner, and this includes the reporting of the final results. Once the data have been assembled, it is necessary to evaluate them, normally the senior person in the laboratory will do this, and then distribute them to the approved distribution list. This will vary from place to place. In some cases, it is distributed to

the shellfishers themselves directly from the laboratory, and in other cases it is the food safety authority or other competent agency that receives the results for dissemination. It is the responsibility of the laboratory to ensure that unambiguous results are provided in a clear and easy to understand format. In addition to the data, it is sometimes requested that interpretation of the data is provided and even recommendations on what action to take. In these cases, clear guidance must be available to allow the laboratory staff and management to make these decisions.

3.4.1 Design of reports

The reports must contain the critical data, including the report number/date, production area, sample location, sample date, species counts, predominant organism and biomass. The comments should be completed to indicate locations where HABs are present and what action plans are going to be adopted. Maps may be incorporated to show the affected areas and, most importantly, a contact person should be identified for further enquiries.

3.4.2 Means/speed of transfer

Reports should be transmitted electronically (e-mail/fax) where possible, and, if necessary, a follow-up telephone call made to alert the relevant persons if there is an HAB event. It is now common to place results, particularly those that affect human health, on a Web site. This can provide a very effective means of dissemination of the results to a wide population.

3.5 ARCHIVING

As reports are issued, they should be systematically archived in a format that will allow their subsequent retrieval for many years. This is important for the analysis of multiannual trends or to deal with other queries. It is most efficient if the results are placed on a database and this can allow for a variety of statistical analyses and summaries to be produced. The archiving of samples is sometimes precluded by space limitations, but permanent slides of important or unusual species can be prepared and stored in very little space. The short-term storage of samples should be allowed for, in case there is a query on the results.

3.6 CONCLUSION

The establishment of a suitable monitoring programme is dependent on the implementation of many diverse elements. The shellfish monitoring programme may be supplemented effectively by the implementation of a phytoplankton monitoring programme. The phytoplankton may often provide an early warning of potential biotoxin contamination, often with results available before the shellfish tests. It can, therefore, act as a pre-emptive tool to trigger an action plan to delay harvest, or to close an area and thereby protect human health.

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