COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

BACKGROUND INFORMATION ON KNOWN LIPOPHILIC SHELLFISH TOXINS

Introduction

1. Diarrhoetic shellfish poisoning (DSP) is characterised by an acute gastrointestinal disturbance following the consumption of shellfish contaminated with either or a combination of okadaic acid (OA), hydroxylated esters of OA; dinophysistoxins (DTX), pectenotoxins (PTX), yessotoxins (YTX) or azaspiracids (AZA). There is good evidence for the diarrhoetic effects of OA, DTXs, and AZAs but the clinical effects of PTXs and YTXs are less clear. However, all these toxins have been grouped together as they can often be found in combination in shellfish and give somewhat similar clinical effects in mouse bioassays.

2. Oral ingestion of the DSP toxins can lead to gastrointestinal disturbances (acute diarrhoea, nausea, vomiting and abdominal pain). Symptoms can begin within 30 minutes of consumption of the toxins, both in animal studies and in human poisoning cases. No human deaths to date have been reported in the literature from cases of DSP, although poisoning may result in hospitalisation.

3. A review of the scientific literature on DSP and the causative toxins is presented. An extensive literature search using the following internet search engines: Medline, PUBMed and CAB (Chemical and Biological abstracts) was carried out using the search terms: *Diarrhoetic shellfish poison, diarrhoetic shellfish poisoning, DSP, shellfish toxins, marine phytoplankton toxins, red tides, okadaic acid, hydroxylated esters of OA, dinophysistoxins, pectenotoxins, yessotoxins and azaspiracids.* Information on international action limits, guidelines and regulations on diarrhoetic shellfish poisoning are also provided.

4. The review is presented as follows:

- Chemistry
- Analysis
- Sources, production, distribution and accumulation
- Absorption, distribution, metabolism and excretion (ADME)
- Toxicology (*in vivo* and *in vitro*)
- Regulations and guidelines
- References

SUMMARY OF THE REVIEW

Chemistry

5. Four classes of lipophilic biotoxins have been isolated from marine biological samples: OA and its DTX analogues, PTXs, YTXs and AZAs. Each class contains a number of structurally similar compounds which are complex polycyclic substances derived from marine algal biosynthesis, although some are the products of additional biotransformation in the digestive glands of shellfish.

Analysis

6. A range of analytical techniques based on chromatography, immunoassay, and biochemical or biological activity have been developed for the detection and investigation of lipophilic biotoxins in shellfish. However, the very limited availability of reference standards for these toxins, precludes the use of many of these methods. Therefore in the UK, routine monitoring involves a mouse bioassay.

Sources: production, distribution and accumulation

7. Lipophilic biotoxins are produced worldwide by a number of marine phytoplankton but mostly by dinoflagellate species growing in the water column, examples of which include *Dinophysis acuta, D. fortii, D. acuminata, D. norvegica, D. mitra,* and *D. caudata*, in addition to being produced by benthic species (growing in the sediment) such as *Prorocentrum lima*. Several different types of biotoxins can be produced by these marine phytoplankton during an algal bloom. The biological function of biotoxin production in dinoflagellates and the environmental conditions or triggers that influence their production are not well understood. The toxins produced by the phytoplankton can bio-accumulate in shellfish. Different shellfish species accumulate and eliminate lipophilic biotoxins to different extents but the reasons for these differences are unclear.

Absorption, distribution, metabolism and excretion

8. There is a paucity of data on the ADME of biotoxins associated with DSP. Through investigation with animals, OA is thought to be absorbed in the jejunum and is distributed systemically to a wide range of organs. OA is eliminated slowly from the kidney, caecum and large intestine. However, no studies of the ADME of the DTX, PTX, YTX or AZA toxins have been performed.

In vivo toxicology

9. Toxicology studies on lipophilic biotoxins are very limited in number and scope. The studies are principally limited by the availability of sufficiently large quantities of purified toxins to conduct detailed studies. Therefore, many studies have used small group sizes, shellfish extracts of unknown or estimated toxin concentrations that possibly contain other toxicologically

active contaminants (possibly other algal biotoxins) as well as parenteral routes of administration to maximise the potency of the test material. In addition, many of the studies have reported clinical symptoms and behavioural responses as an indicator of toxicity rather than more objective toxicological end-points. Very few studies have examined the effects of oral administration of lipophilic biotoxins. The liver and intestine are the main targets of the toxins due to the existence of specific uptake systems in these organs. Diarrhoetic effects have only been sufficiently proven for OA and the dinophysis toxins DTX1 and DTX3.

In vitro toxicology

10. The mechanisms of action of the biotoxins are poorly understood. However OA and DTXs are potent protein phosphatase inhibitors (PPI) and this PPI activity is thought to mediate various mechanisms of toxicity including diarrhoetic effects in intestinal cells, cytotoxicity and tumour promoting activity in various cell types. The research on PTXs, YTXs and AZAs is not as extensive as OA and DTXs. The few studies conducted have not demonstrated they inhibit the PP's tested, (YTX has weak inhibitory action).

Regulations and guidelines

11. The MBA described in EU legislation 2002/225/EC is used in the UK to monitor for lipophilic biotoxins in shellfish.

CHEMICAL STRUCTURES

12. There are four structural classes of phytoplankton-derived lipophilic biotoxins known to accumulate in shellfish within European waters. These are:

- Okadaic acid (OA) and dinophysistoxins (DTXs)
- Pectenotoxins (PTXs)
- Yessotoxins (YTXs)
- Azaspiracids (AZAs)

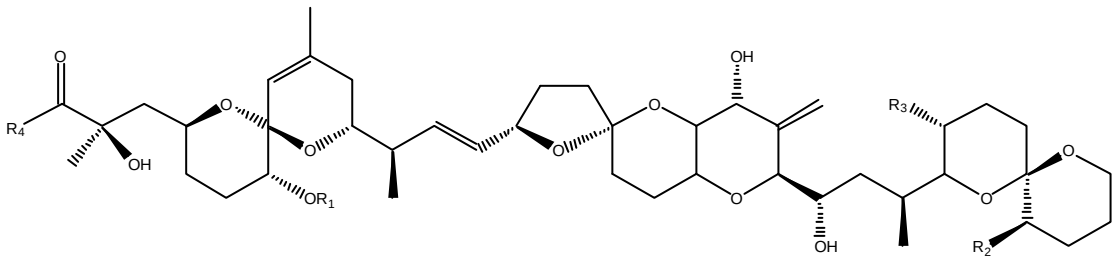
13. All four classes of compound contain polycyclic structures of complex stereochemistry. The compounds within each class share the same basic ring-system but differ in substituents at some ring positions.

14. Many of the structural analogues in each class are referred to by a number, which reflects the chronology of their discovery. Many of the compounds have only been identified in shellfish rather than in phytoplankton. Thus, they may be the result of metabolism within the shellfish from which they were isolated rather than direct products of phytoplankton biosynthesis. A few compounds may also be artefacts produced during extraction of the biotoxins from the biological matrices.

OA and DTXs

15. The structures of OA and the DTXs (analogues of OA) isolated from marine biological material are shown in Figure 1 (James et al. 2000; Vieytes et al. 2000). DTX-3 is comprised of acyl derivatives of OA, some of which are diol esters. DTX-3 compounds are unstable and are degraded under acid and basic conditions to non-toxic compounds (Yasumoto et al. 1985). Sulfated long-chain ester derivatives of OA (DTX5a and b) have also been identified. The esters of OA are unstable and can be hydrolysed to OA. OA and many of its acylated DTX analogues have been isolated from phytoplankton and therefore, appear to be products of algal biosynthesis. However, some may be the result of metabolism in the digestive gland of shellfish.

Figure 1 Chemical structures of OA and DTXs



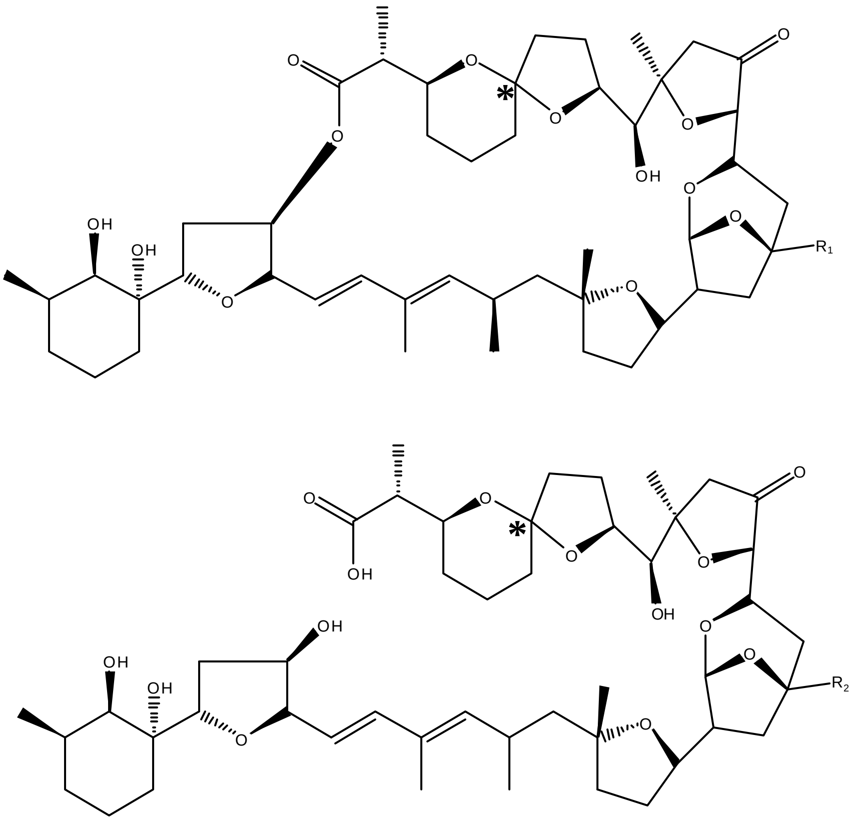
Toxin	R1	R2	R3	R4
OA	CH_3	H	H	OH
DTX-1	CH_3	CH_3	H	OH
DTX-2	H	CH_3	H	OH
DTX-3	Acyl	H or CH_3	H or CH_3	OH
DTX-4	H	H	CH_3	H

PTXs

16. The structures of PTXs isolated from marine biological material are shown in Figure 2 (Draisci et al. 2000; Suzuki et al. 2003). Only PTX-2 and the *seco* acids of PTX-2 (PTX-2SA and *epi*-PTX-2SA) have been isolated from phytoplankton. The other compounds have only been isolated from shellfish samples. Therefore, it has been suggested that PTX-1, -3 to -7, -10 and -11 are formed from oxidative metabolism in the digestive glands of the shellfish. PTX-8 and -9 are thought to be artefacts of acidic extraction of PTX-2 from biological matrices.

17. The melting points of PTX-1 and -3 are 208 and 159^{o}C, respectively. This would suggest that the PTXs are relatively stable to heat. Other than the established acid catalysed conversion of PTX-2 to -8 and -9 there is no further information on the stability of these compounds to acidic or basic conditions although conversion to the seco acids may be expected under basic conditions.

Figure 2 Chemical structures and stereochemistry of PTXs



Toxin	R1	Stereochemistry at *	Toxin	R1	Stereochemistry at *
PTX-1		R	PTX-6	COOH	R
PTX-2	CH_3	R	PTX-7	COOH	S
PTX-3	CHO	R	PTX-10	unidentified	
PTX-4	CH_2OH	S	PTX-11	unidentified	
PTX-5	unidentified				
Toxin	**R2**	**Stereochemistry at ***	**Toxin**	**R2**	**Stereochemistry at ***
PTX-SA	CH_3	R	*Epi* PTX-SA	CH_3 (R2)	S

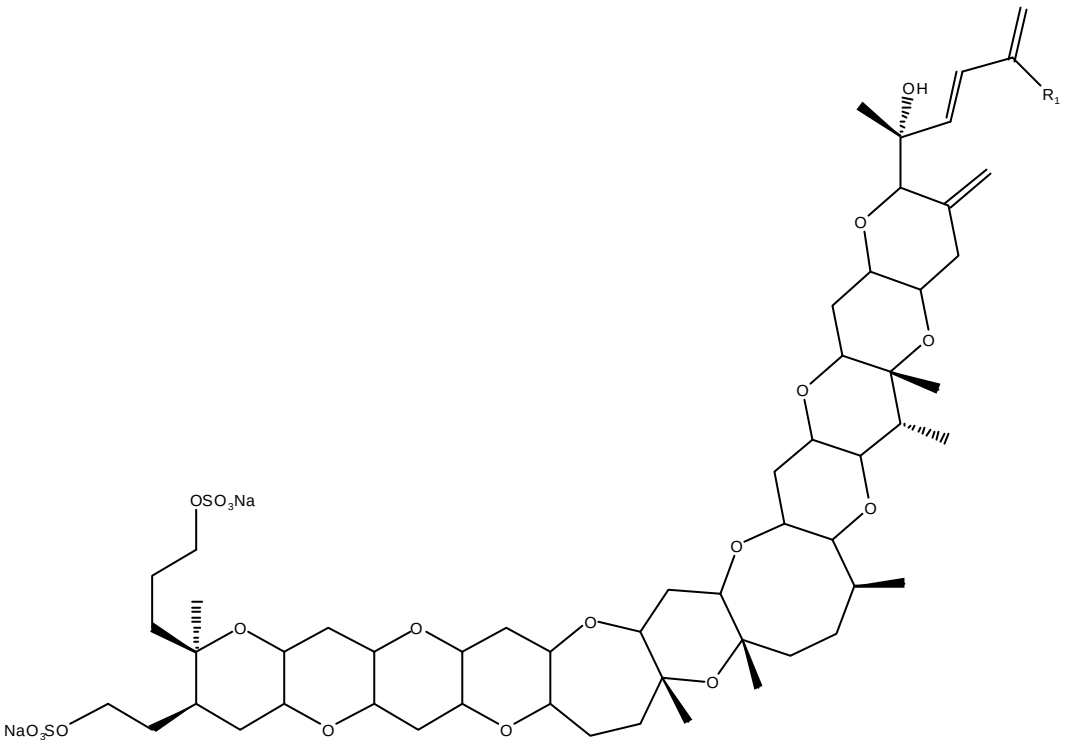
Note: PTX-8 and 9 are similar in structure to PTX-4 and -7, respectively but contain a six-membered ring in place of one of the five-membered rings.

YTXs

18. The structures of five of the six YTXs isolated from marine biological material are shown in Figure 3 (Draisci et al. 2000). Adriatoxin, the sixth compound contains a hydroxyl group in place of the unsaturated side-chain and an additional sulfate group in place of the double bond at the adjacent ring position. YTX and homo-YTX have been isolated from phytoplankton and shellfish however, the hydroxylated derivatives have only been isolated from shellfish and therefore, it is possible that these compounds arise from oxidative metabolism in shellfish. The source organism of adriatoxin is unclear.

19. There is no information available on the thermal or acid-base stability of the YTXs.

Figure 3 Chemical structures of YTXs

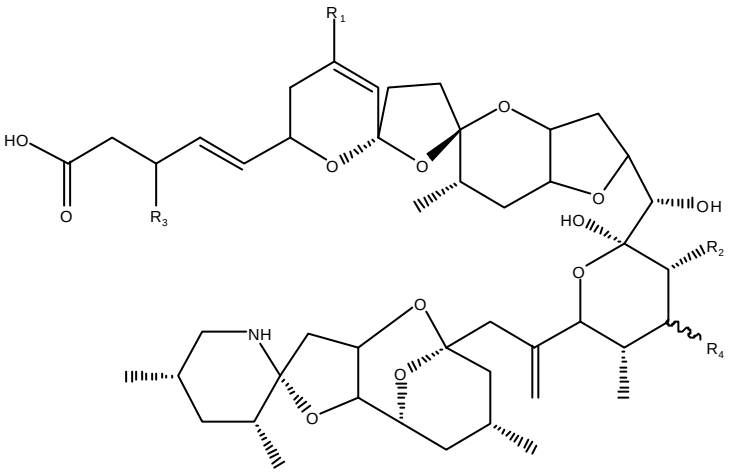


Toxin	R1
Yessotoxin	$CH_2CH_2CH=CH_2$
Hydroxy yessotoxin	$CH_2CHOHCH=CH_2$
Homoyessotoxin	$CH_2CH_2CH=CH_2$
Hydroxy homoyessotoxin	$CH_2CHOHCH=CH_2$
Trioryessotoxin	H

AZAs

20. The structures of the five characterised AZAs isolated from marine biological material are shown in Figure 4 (Ofuji et al. 2001; Ofuji et al. 1999). Six further hydroxylated analogues (AZA-6 to 11) have been tentatively identified (James et al. 2003). Unlike the other classes of lipophilic biotoxins the AZA class includes a basic imine moiety in one ring. Only AZA-1 to -3 have been isolated from phytoplankton and therefore, the other AZAs may be products resulting from metabolism in the digestive glands of shellfish. There is no information available on the thermal or acid-base stability of the AZAs.

Figure 4 Chemical structures of AZAs



Toxin	R1	R2	R3	R4
AZA-1	H	CH_3	H	H
AZA-2	CH_3	CH_3	H	H
AZA-3	H	H	H	H
AZA-4	H	H	H	OH
AZA-5	H	H	OH	H

Summary

21. Four classes of lipophilic biotoxins have been isolated from marine biological samples: OA and its DTX analogues, PTXs, YTXs and AZAs. All are structurally complex polycyclic substances derived from marine algal biosynthesis although some are the products of additional biotransformation in the digestive glands of shellfish.

ANALYSIS

22. A number of analytical methods for the detection of lipophilic biotoxins have been developed (see Table 1), which include:

- High performance liquid chromatography with fluorimetric detection (HPLC-FLD)
- Liquid chromatography with mass spectrometric detection (LC-MS)
- Immunoassays
- Protein phosphatase inhibition (PPI) assays
- Cytotoxicity assays
- Mouse bioassay (MBA)
- Rat oral test (ROT)

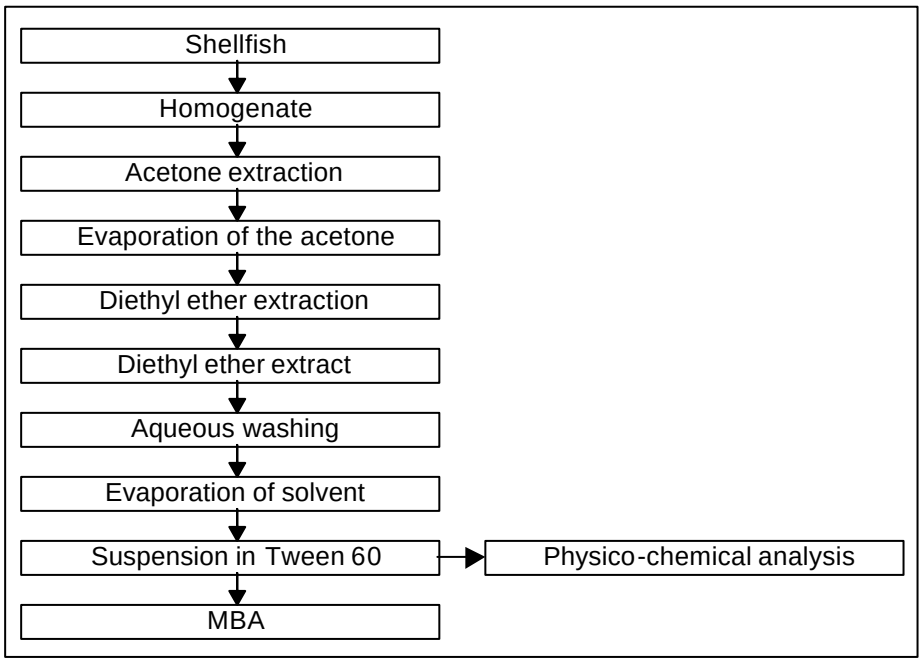
23. The presence of lipophilic biotoxins in shellfish is routinely monitored for in the UK using the mouse bioassay.

Extraction

24. Lipophilic biotoxins are extracted either from whole shellfish or their digestive glands. The extraction process involves a number of sequential steps using organic solvents and/or solid phase separation, and therefore can be costly, laborious and may produce an uncertain yield of toxin.

25. A representative extraction protocol is shown in Figure 5, but numerous variations on this basic method have been developed.

Figure 5 A representative example of an extraction method for lipophilic biotoxins for analysis using the MBA or alternative physico-chemical methods.



Reference standards

26. The availability of reference standards for the wide range of lipophilic biotoxins is limited. The extremely complex chemical structures of the lipophilic biotoxins precludes their large-scale synthesis by chemical methods. It is also very difficult to isolate sufficient quantities of the toxins from their natural sources or from shellfish. Therefore, commercial standards are not available for all of the toxins.

High performance liquid chromatography with fluorimetric detection (HPLC-FLD)

27. HPLC-FLD has been used to detect OA, DTX and YTX toxins. Following extraction, the toxins are derivitized with a fluorescent reagent such as 9-anthryldiazomethane (ADAM) (e.g. Puech et al. 1999), 4-bromomethyl,7-methoxycoumarin (BrMMC) (e.g. Ramstad et al. 2001) or DMEQ-TAD (e.g. Yasumoto and Takizawa, 1997) to facilitate detection.

28. HPLC-FLD is rapid, sensitive and specific. However, free fatty acids in shellfish digestive glands can also produce fluorescent adducts with ADAM, and the low stability of ADAM can lead to incomplete derivitisation of the target analytes (Stabell and Cambella, 1990).

Liquid Chromatography with Mass Spectrometric detection (LC-MS)

29. LC-MS has been used to analyse OA, YTX, DTX, PTX and AZA toxins.

30. This technique has the potential to analyse all lipophilic biotoxins in a single test and is rapid, sensitive, specific and amenable to automation. However, the cost of LC-MS is high, and unless commercial standards are available, the concentrations of lipophilic biotoxins cannot be accurately quantified.

Immunoassays

31. Several antibodies have been developed for lipophilic biotoxin analysis. Detection has involved solid-phase immunobead assay (Park, 1995), competitive binding radioimmunoassay (Levine et al. 1988) and various enzyme-linked immunosorbent assays (ELISAs).

32. Two main test kits are commercially available for some lipophilic biotoxin detection: the DSP-Check ELISA test kit (UBE Industries, Tokyo, Japan) and the Rougier OA ELISA test kit (Rougier Bio-Tech, Montreal). The DSP-Check kit detects OA and certain DTX toxins, and is rapid and convenient. DTX-3 detection requires hydrolysis to DTX-1, which increases the time required for analysis.

Protein phosphatase inhibition (PPI) assays

33. OA and DTX toxins inhibit the protein phosphatases PP1 and PP2A, with PP2A demonstrating the highest sensitivity. Several PPI assays employing radioactive (Luu et al. 1993), bioluminescent (Isobe et al. 1995), colourimetric (Ramstad et al. 2001) and fluorescent (Gonzalez et al. 2002) detection have been developed.

34. PPI assays are sensitive, inexpensive and can be performed in 96-well plates for high-throughput analysis (Tubaro et al. 1996). PPI assays have the scope to detect novel OA analogues, although they cannot detect PTX, YTX or AZA toxins as these exert either no or weak inhibitory effects on PP1 and PP2A.

Cytotoxicity assays

35. Cytotoxicity assays utilising cells such as buffalo-green-monkey (BGM) kidney cells (Croci et al. 2001), human HEp-2 larynx carcinoma cells (Oteri et al. 1998) and mammalian fibroblasts (Diogene et al. 1995) have been developed.

36. Aune et al. (1991) showed that the changes in morphology of rat hepatocytes caused by OA and DTX-1 were different from those caused by PTX-1 and YTX-1, allowing different toxins to be distinguished. However, the assay was time-consuming and analysis of toxin mixtures produced unclear results.

Mouse bioassay (MBA)

37. The MBA for the detection of lipophilic biotoxins involves intraperitoneal (i.p.) injection of shellfish extracts into mice and monitoring survival time within a 24 hour period (Yasamoto et al. 1978).

38. In contrast to physico-chemical methods for lipophilic biotoxin detection, the MBA is capable of detecting a wide range of known lipophilic biotoxins and has the potential to detect unknown biotoxins.

39. However, the method is subject to ethical considerations, lacks sensitivity and quantification, and is unsuitable for high-throughput analysis.

40. Since it was developed in the 1970's, the MBA has been modified to address a number of technical issues. It has been reported that co-extraction of free fatty acids and hydrophilic biotoxins associated with paralytic shellfish poisoning (PSP) has produced false positive results (Takagi et al. 1984; Suzuki et al. 1996; Lee et al. 1987; Gago-Martinez et al. 1996). However, since these reports, the protocols have been modified to prevent interference from fatty acids (Fernandez et al. 1996) and the co-extraction of PSP toxins (Draisci et al. 1998; Draisci et al. 1999; Fernandez et al. 1996).

Rat oral test (ROT)

41. The rat oral test involves feeding shellfish digestive glands mixed with normal feed to rats that have been starved for 24 hours (Kat, 1983). After 16 hours, signs of diarrhoea, faecal consistency, and feed refusal are assessed.

42. The ROT causes less animal suffering than the MBA and is more representative of the route of human exposure. However, because the ROT is based on diarrhoea induction it is unable to detect non-diarrhoetic toxins such as the PTXs and YTXs (Aune et al. 2002; Kat, 1983; Gucci et al. 1994), and only has a weak response to AZA toxins. Additionally, the current ROT protocol was developed to include ingestion of the hepatopancreas only, as this tissue generally contains the highest levels of lipophilic biotoxins. However, for routine monitoring it would be impractical to remove the hepatopancreas from some small shellfish species such as cockles. Testing of such species would therefore require use of the whole shellfish, leading to a relatively less concentrated toxin preparation. Furthermore, the rat may select other parts of the shellfish in preference to the hepatopancreas, and therefore consume a lower, inconsistent proportion of the total lipophilic biotoxin content.

Summary

43. A range of analytical techniques based on chromatography, immunoassay, and biochemical or biological activity have been developed for the detection and investigation of lipophilic biotoxins in shellfish. However, the very limited availability of reference standards for these toxins, precludes the use of many of these methods. Therefore in the UK, routine monitoring involves a mouse bioassay.

Table 1 Representative examples of techniques used for the analysis of lipophilic biotoxins.

Method		Toxin(s) analysed	Reference
HPLC	ADAM or BrMMC derivitisation	OA, DTX-1	(Ramstad et al. 2001)
	ADAM derivitisation	OA, DTX-1, DTX-2	(Puech et al. 1999)
	DMEQ-TAD derivitisation	YTX, nor-YTX, 45-OHYTX	(Yasumoto & Takizawa, 1997)
LC-MS	LC electrospray ionization MS	AZA-1, AZA-2, AZA-3	(Magdalena et al. 2003)
	LC with multiple tandem MS	AZA-7-11	(James et al. 2003)
	LC electrospray ionization MS	OA, DTX-2	(Vale and Sampayo, 2002)
	LC electrospray ionization MS	YTX, 45-OHYTX	(Fernandez et al. 2002)
	LC electrospray ionization MS	DTX-1, PTX-6	(Suzuki & Yasumoto, 2000)
	LC ionspray ionization MS	YTX, OA, DTX-1, DTX-2, DTX-2B, DTX-2C, PTX-2, PTX-2SA, 7-epi-PTX-2SA,	(Draisci et al. 1999)
Immuno-	DSP-Check ELISA test kit	OA, DTX-2	(Carmody et al. 1995)
assays	Rougier ELISA test kit	OA	(Fremy et al. 1994)
	Solid-phase immunobead assay	OA	(Park, 1995)
	ELISA	OA, DTX-1, DTX-3	(Matsuura et al. 1994)
	Radioimmunoassay	OA	(Levine et al. 1988)
PPI	Fluorescent detection	OA	(Gonzalez et al. 2002)
assays	Colourimetric detection	OA, DTX-1	(Ramstad et al. 2001)
	Bioluminescent detection	OA	(Isobe et al. 1995)
	Radioactive detection	OA, DTX-1	(Luu et al. 1993)
Cyto-	BGM kidney cells	OA	(Croci et al. 2001)
toxicity	HEp-2 larynx carcinoma cells	OA, DTX-1	(Oteri et al. 1998)
assays	Mammalian fibroblasts	OA	(Diogene et al. 1995)
	Rat hepatocytes	OA, DTX-1, PTX-1, YTX	(Aune et al. 1991)
MBA		OA, YTX, homo-YTX, 45-OHYTX	(Tubaro et al. 2003)
		OA, DTX-1	(Aune et al. 1991)
		PTX-1, PTX-2	(Yasumoto et al. 1985)
ROT		OA	(Gucci et al. 1994)

SOURCES OF LIPOPHILIC BIOTOXINS

44. Lipophilic biotoxins are produced by marine phytoplankton, which grow in seawaters all over the world. There are several thousand species of marine phytoplankton but only around 60-80 species are known to produce toxins, of which around 75% are dinoflagellate species (Tibbetts, 1998).

45. Dinoflagellates are unicellular phytoplankton, ranging in size from 20 to 200 μm in diameter. Many forms are able to photosynthesize by incorporation of chloroplasts within their structures. Many dinophysis species have proven to be difficult to maintain in culture and thus experimental studies have used wild sources of lipophilic biotoxins collected during algal blooms or extracted from contaminated shellfish. Some examples of dinoflagellates known to produce lipophilic biotoxins are shown in Table 2.

Occurrence and global distribution of phytoplankton blooms

46. Marine phytoplankton can undergo rapid phases of proliferation, known as algal blooms, which can occur at certain times of the year under appropriate environmental conditions. During blooms, algae can become so dense in cell number that they can discolour the sea producing what is termed a 'red tide' (Hallegraeff, 1987). It should also be noted that not all the toxic dinoflagellates produce floating algal blooms as some phytoplankton are benthic species which live in the sediment of the water bed and thus only shellfish living on the sea bed such as cockles will accumulate toxins produced by benthic phytoplankton. The incidence of algal blooms has been reported to have increased world-wide over the last few decades (Anderson, 1989; Hallegraeff, 1987; Hallegraeff, 1993; Anonymous1989; Shimizu, 1989; Tibbetts, 1998; Wong, 1989).

47. Only a small proportion of dinoflagellate species produce toxins, and thus algal blooms can be toxic or non-toxic depending upon the predominant species of phytoplankton within the bloom. Additionally, several lipophilic biotoxins can be produced simultaneously in a single bloom (Draisci et al. 1998; Pan et al. 1999).

48. Algal blooms usually occur in temperature stratified and stable waters where there are sufficient nutrients and organic matter available to support phytoplankton growth. The characteristics of an algal bloom such as species dominance and diversity as well as toxin production can alter during growth, and this is thought to be caused by changes in light intensity, temperature, water movement, chemical composition of the water, and biological factors such as competition between the differing species (Aubry et al. 2000; Carmody et al. 1996; Edebo et al. 1988; Haamer, 1995; Hallegraeff and Reid, 1986; Morlaix and Lassus, 1992; Roelke, 2000; Roelke and Buyukates, 2001).

49. There is evidence of global migration of toxic species between different countries possibly as a result of shipping (Anderson, 1989; Hallegraeff, 1992; Hallegraeff, 1993; Hallegraeff, 1998). Dinoflagellate cysts are able to survive

long journeys in the dark and cold ballast tanks, before being released into seas when the ships dump their ballast water prior to harbour entry. Treatment regimens have subsequently been introduced to kill dinoflagellate cysts in ships' ballast water prior to their arrival in new harbours (Hallegraeff, 1998).

Phytoplankton life cycle and lipophilic biotoxin production

50. The production of lipophilic biotoxins is reported to vary depending upon the stage in the growth cycle of the phytoplankton (Pan et al. 1999). It has also been suggested that the phytoplankton are in fact themselves subject to the toxicity and have their own metabolising enzymes to combat and detoxify the biotoxins throughout their different cycles (Aguilera et al. 1997; Baden, 1989; Baden et al. 1989; Windust et al. 1996).

51. The effects of nitrogen and phosphorus concentrations as well as temperature on cell growth and lipophilic biotoxin production has been investigated in laboratory cultures of the phytoplankton *P. lima* (McLachlan et al. 1994; Morlaix and Lassus, 1992). Nitrogen concentrations had a stimulatory effect on cell growth, up to a threshold value above which the growth decreased. When growth was limited, increases in biotoxin production were evident. Maximum concentrations of toxins occurred during the stationary phase of the cell cycle.

52. Dinoflagellate phytoplankton originate from benthic (sedimentary) cysts that form during winter months or at times that are not suitable for growth, and can remain dormant for many years in the sediment of the ocean floor. Blooms can occur when these cysts are initiated to germination. The triggers for germination are not fully understood, but may involve factors such as water temperature and nutrient availability. It is also not known if shellfish consuming these dormant fish may be exposed to toxins that may be present in the cysts.

53. Individual phytoplankton cells have been shown to produce varying quantities of toxins with the factors determining amount produced not being fully understood. For example, laboratory cultures of *P. lima* have shown concentrations of OA production to range from 0.37 to 6.6 fmol/cell and DTX-1 to range from 0.04 to 2.6 fmol/cell (Pan et al. 1999). *D. norvegica* has been found to produce OA in concentrations ranging from 0.78 to 6.08 mu-g OA g-1 dry plankton (Rao et al. 1993) and another example is the production of DTX-1 from *D. Fortii* ranging from *5-252* pg/cell (Suzuki et al. 1996).

Accumulation and depuration of lipophilic biotoxins in shellfish

54. Phytoplankton are consumed by filter feeding shellfish and thus, lipophilic biotoxins accumulate within the shellfish where they may be metabolised to other toxic or non-toxic compounds. Generally, biotoxin accumulation in shellfish is dependent on the size and composition of the algal bloom, the type of shellfish and where these shellfish live and feed in the water column. Examples of toxins found in different shellfish can be found in Table 3.

55. Biotoxins preferentially accumulate in the hepatopancreas (the digestive gland) of shellfish but may be distributed to other tissues (Magdalena et al. 2003; Pillet et al. 1995).

56. Shellfish eliminate (depurate) biotoxins. The rate of depuration varies depending upon the concentration of toxins accumulated, the species of shellfish, feeding (accumulation) rates, and the type of biotoxin (Blanco et al. 1999; Croci et al. 1994; Svensson, 2003). For example, the levels of OA fell from 10 to 2 μg OA/g hepatopancreas over 45 days in a study of contaminated mussels (Mytilus galloprovincialis) (Morono et al. 2003). In contrast, in a study in bay scallop (*Argopecten irradians*) 99% of the OA was eliminated in 14 days (Bauder et al. 2001).

Summary

57. Lipophilic biotoxins are produced worldwide by a number of marine phytoplankton (mostly dinoflagellate species). Several different types of biotoxins can be produced by these marine phytoplankton during an algal bloom. The environmental conditions or triggers that influence the production of biotoxins are not well understood. The toxins produced can bio-accumulate in shellfish and may be altered by metabolism in the shellfish. Different shellfish species accumulate and eliminate lipophilic biotoxins to different extents but the reasons for these differences are currently unclear.

Table 2 Algal sources of lipophilic biotoxins and their worldwide distribution.

Toxin	***Species***	**World Distribution**	**Reference**
OA	*Dinophysis acuta* *D. acuminata*	Japan	(Lee et al. 1989b)
	Prorocentrum lima	Japan	(Murakumi et al. 1982)
	Prorocentrum lima	Lab culture	(Pan et al. 1999)
	Prorocentrum lima	Atlantic coast of Nova Scotia	(Fernandez et al. 2003; Marr et al. 1992; Norte, 1994)
	D.fortii	Japan	(Lee et al. 1989a; Lee et al. 1989b)
	D.fortii	Northern Adriatic Sea of Italy	(Draisci et al. 1996)
	D. norvegica	Eastern Canada	(Rao et al. 1993)
	Phalacroma rotundatum	southern Adriatic coast of Puglia (Italy)	(Caroppo et al. 1999)
DTXs	*D. fortii* *D. norvegica* *D. mitra* *D. rotundata*	Japan Norway Japan Japan	(Lee et al. 1989b)
DTX-1	*P. lima*	Lab culture	(Pan et al. 1999)
	P. lima	Lab culture	(Suarez-Gomez et al. 2001)
	D. acuta	Portugal, Ireland, Italy	(Draisci et al. 1998; James et al. 1999b; Vale and Sampayo, 2000)
DTX-1	*D.fortii*	Japan	(Suzuki et al. 1996)
DTX-5a and DTX-5b	*P. maculosum*	Lab culture	(Macpherson et al. 2003)
PTX-2	*D. acuta*	Ireland and New Zealand	(James et al. 1999a; Suzuki et al. 2001)
PTX-2	*D. fortii*	Northern Adriatic Sea of Italy	(Draisci et al. 1996; James et al. 1999a; Suzuki et al. 2001)
PTX-2	*D. fortii*	Japan	(Lee et al. 1989a; Lee et al. 1989b; Suzuki et al. 1998)
PTX-2-SAs	*D. acuta*	Australia	(Daiguji et al. 1998; Draisci et al. 1999; James et al. 1999a)
	D. acuminata	Australia	(PhD thesis of V. Burgess, 2003, Griffith University)
	D. caudata	Australia	(Geoff Eaglesham, QHSS oral communication).
YTXs	*Protoceratium reticulatum*	Laboratory culture (New Zealand)	(Satake et al. 1997)
	Lingulodinium polyedrum	Italy (Adriatic sea)	(Draisci et al. 1999)
AZA	*genus, Protoperidinium,*	Ireland	(James et al. 2003)

Table 3 Examples of biotoxins found in different shellfish

Toxin	**Shellfish species**	**Predominant algal species found in gut or in bloom area**	**Country**	**Reference**
OA	Oysters *(Crassotrea virginica)*	*D. caudata*	Mexico	(Dickey et al. 1992)
OA	Mussels (Mytilus galloprovincialis)	*Gonyaulax polyedra*	Italy (Adriatic Sea)	(Draisci et al. 1999)
YTX	Mussels (Mytilus galloprovincialis)	*Gonyaulax polyedra*	Italy (Adriatic Sea)	(Draisci et al. 1999)
DSP (not specified)	Mussle (Mytilus edulis Linne 1758)	*Dinophysis Ehrenberg 1839* and *Prorocentrum Ehrenberg 1833*	German Bight (North Sea)	(Klopper et al. 2003)
AZA (total)	Mussels (Mytilus edulis)	Not detailed in paper	Ireland	(Furey et al. 2003)
AZA (total)	Oysters (Crassostrea gigas)	Not detailed in paper	Ireland	(Furey et al. 2003)
AZA (total)	Scallops (Pecten maximus),	Not detailed in paper	Ireland	(Furey et al. 2003)
AZA (total)	Cockles (Cardium edule)	Not detailed in paper	Ireland	(Furey et al. 2003)
AZA (total)	Clams (Tapes phillipinarium),	Not detailed in paper	Ireland	(Furey et al. 2003)
DTX-2	Mussels (Mytilus edulis)	*D. acuta* (bloom sample)	Ireland	(Carmody et al. 1996)
PTX-2SA	Clams (Donax trunculus)	Not stated	Portugal	(Vale and Sampayo, 2002)

ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION (ADME) OF LIPOPHILIC BIOTOXINS

58. The ADME of DTX, PTX, YTX and AZA toxins has not been examined. However, two studies on the absorption, distribution and excretion of OA have been performed.

59. In a study by Ito et al. (2002), male ICR mice (4 weeks old) were administered a single oral dose of 0.150 OA mg/kg bw with mice being sacrificed at 5, 10, 15, 30 and 45 minutes (all n=1) and 1 (n=8), 2 (n=2), 4 (n=2), 6 (n=2), 8 (n=2), 16 (n=1), 24 (n=2), 48 (n=1) and 72 (n=1) hours, and at 3, 4, 7 and 14 days (all n=1) and at 4 (n=2), 8 (n=2), and 12 weeks (n=2). OA was detected by immunostaining with an anti-OA monoclonal antibody, although this method did not allow precise quantification of OA. Five minutes after administration, OA was detected in all samples tested (i.e. the lung, liver, heart, kidney, and the tissues and contents of the stomach, caecum and small and large intestine), indicating that OA is well distributed. The site of absorption of OA was identified as the jejunum. OA was detected in the liver and blood for up to 2 weeks. Excretion in urine and from the caecum and large intestine began 5 minutes after administration, and faecal excretion continued for 4 weeks.

60. Matias et al. (1999) administered a single oral dose of 50 μg [^{3}H]-OA/kg bw to adult Swiss mice (n = 6). This dose did not give rise to a diarrhoetic effect. Twenty-four hours after administration, the amount of radiolabel and the concentration of OA was analysed in organs and other biological samples by scintillation counting and HPLC-fluorometric detection (OA in the samples was derivatised with a fluorescent label), respectively. The quantities of radiolabel and OA detected by both methods were similar. OA was detected in all samples tested, as follows: intestinal content (36.3 % of given dose) > urine (11.6 %) > skin (8.3 %) > faeces (6.6 %) > blood (4.3 %) > muscle (3 %) > intestinal tissue (2.6 %) > liver and gallbladder > stomach > kidney > brain > lung > spleen > heart (all 1.0 %). In a further experiment, 90 μg unlabelled OA/kg bw was administered to a further 6 animals. This dose gave rise to a diarrhoetic effect. However, other than small differences noted in the stomach and intestinal tissues, the distribution of OA was similar to that of the non-diarrhoetic dose.

Summary

61. There is a paucity of data on the ADME of biotoxins associated with DSP. Through investigation with animals, OA is thought to be absorbed in the jejunum and is distributed systemically to a wide range of organs. OA is eliminated slowly from the kidney, caecum and large intestine. However, no studies of the ADME of the DTX, PTX, YTX or AZA toxins have been performed.

IN VIVO TOXICOLOGY STUDIES WITH LIPOPHILIC BIOTOXINS

Human toxicology

62. Data on the toxicological effects of lipophilic biotoxins in humans are very limited. However, suspected DSP outbreaks based on symptomology following consumption of shellfish subsequently found to be contaminated with lipophilic shellfish toxins have been reported globally. There are no human data on concentrations of toxins in biological samples from poison victims.

63. The classical clinical symptoms of DSP include vomiting and diarrhoea, which is usually resolved within 2-3 days from consumption of contaminated shellfish, have been reported in many countries around the world. Examples include the UK (Scoging and Bahl, 1998), Italy (Ammazzalorso et al. 1991), the Netherlands (Kat, 1983), Ireland (Ofuji et al. 1999), Portugal (Vale and Sampayo, 2002), Japan (Yasumoto et al. 1984), Australia (Quilliam et al. 2000), and Canada (Quilliam et al. 1993).

64. In Japan, incidents of DSP were recorded and monitored between 1976 and 1982 (Yasumoto et al. 1984). Frequency of signs and symptoms were reported as diarrhoea (92%), nausea (80%), vomiting (79%), abdominal pain (53%) and chill (10%). Symptoms were found to usually commence between 30 minutes to 4 hours (70% of cases) with onset rarely exceeding 12 hours following consumption of contaminated shellfish. The minimum amount of toxins to induce symptoms in a human was estimated be 12 mouse units (one mouse unit being defined as the minimum amount of toxin required to kill a mice by i.p. administration within 24 hours), as determined by analysis of leftovers from meals consumed by affected individuals. From this data, the amount of lipophilic biotoxin sufficient to induce gastroenteritis in a human was estimated to be 32μg DTX-1 equivalents when eaten (Yasumoto et al. 1984), and on the basis of figures for human in takes of shellfish, any shellfish containing 2μg OA or 1.8μg DTX-1 per gram of hepatopancreas were considered unfit for human consumption (Lee et al., 1987).

65. An 8 year study conducted in France attempted to investigate whether there was a link between digestive cancer mortality rates and consumption of shellfish that were harvested from beds that had recently been cleared from shellfish harvesting bans (Cordier et al. 2000). The study hypothesised that residual levels of lipophilic biotoxins (specifically OA), below the statutory cut off level, remained in shellfish from harvesting beds that were subsequently opened, could potentially cause health affects in regular consumers of shellfish if sustained over a period of time. The authors claimed they found some evidence from a trend in a proxy estimate of lipophilic biotoxin consumption and increased stomach and pancreatic cancers in women and colon cancer in men ($p < 0.5$), but they stated that these conclusions provided were very tentative.

In vivo toxicology in animals

Okadaic acid

Single dose lethal toxicity

66. LD_{50} values for i.p. administration of OA to mice have been reported as 0.192 mg/kg bw when isolated from the marine sponge *Halichondria okadai Kadota* or approximately 0.12mg/kg bw when isolated from another marine sponge *H. melanodocia (Tachibana et al. 1981)*, (group numbers were not detailed in this paper). Later studies reported an LD_{50} value of purified OA for i.p. administration of 0.2mg/kg bw (Yasumoto et al. 1989), with the same concentration being reported for LD_{50} via i.v. administration (Yasumoto et al. 1985), and for the LD_{99} via i.p. administration (Yasumoto and Murata, 1990). None of these studies detailed mouse numbers strains or dose ranges administered. It has been found that the oral lethality of OA in 10 day old BOM:NMRI female mice was 25-50 times lower than that seen from i.p. dosing (Aune et al. 1998). Only one mouse per dose group was employed in this study which investigated oral dosing at concentrations of 50X and 25X the i.p. lethal toxicity for two purified extracts of OA from mussels, and 10X and 1X i.p. doses for two other purified extracts of OA from other mussels.

67. In a comparative study of the lethal potency (the minimum dose necessary to kill 2 of 3 mice in 24hr) of OA and some of its conjugates in male ddY mice (16-20g), the lethality was determined to be 0.036mg/kg bw for OA, 0.1mg/kg bw for palmitic-OA and linoleoyl-OA, and 0.01mg/kg bw for docosahexaenoyl-OA (Yanagi et al. 1989).

68. Lethal dose concentrations in other species have not been reported for OA.

Diarrhoetic effects of OA

69. Diarrhoetic effects (intestinal fluid accumulation) have been observed in several suckling mice studies. Orally administered OA at doses ranging from 0.05, 0.1, 0.2, 0.5 and 0.8 MU/mouse (mouse units) administered to 3-5 mice per group showed that 0.1MU caused diarrhoea in 4-5 day old CD1 mice (Hamano et al. 1986). In the Hamano et al study 1 MU was defined as the minimum amount of toxin required to kill 2/3 mice within 24 hours. Intestinal fluid accumulation was also demonstrated in suckling male ddY mice at oral doses of commercially sourced OA in a 1% Tween 60 solution of 0.1μg OA/mouse (Ogino et al. 1997). In this study doses were administered at levels of 0.08, 0.1, 0.12 and 0.14mg/kg bw to 3 mice per dose group. Another study found intestinal fluid accumulation following oral adminstration of OA or OA acyl analogues at 0.125 μg/mouse to 5 suckling CD-1 mice (Yanagi et al. 1989).

70. OA has also been shown to cause diarrhoea when administered i.p. in mice (Yasumoto and Murata, 1990), unfortunately, no details of mice strain, numbers or dosing regime were provided in this review paper.

71. OA from mussels (purity of extract was not provided) induced significant fluid accumulation in intestinal loops of 2 male Sprague-Dawley rats (7 weeks old) when 0.5μg OA/animal was injected directly into the intestine (Edebo et

al. 1988b) and again when injected directly into the intestinal loops of 5 female CD-1 mice at a dose of 2µg/animal (Yanagi et al. 1989). Further, oral administration of OA at 0.075mg/kg bw was shown to cause fluid accumulation in the intestine of 4 week old male ICR mice (Ito et al. 2002). Diarrhoea was observed in 6 adult Swiss mice following a single oral dose of 0.09mg/kg bw (Matias et al. 1999). When the dose of OA was increased from 0.05 to 0.09 mg/kg bw, the concentrations of the toxin in the intestinal content and faeces increased proportionally, but diarrhoea was not observed at the lower dose of 0.05mg/kg bw. Additionally, OA was present in liver and bile, in all organs including skin and also found in the blood.

Intestinal toxicity

72. The target organ of OA has been shown to be the intestine (all areas) in studies employing radio-labelled OA or immuno-staining methods. One such study employed oral administration of 0.05 mg/kg bw radio-labelled OA to adult Swiss mice and found the highest concentration of activity to be located in the intestine compared to all other organs (Matias et al. 1999).

73. Previously, several studies had identified pathological changes in the intestine following oral or i.p. administration of OA to animals, a few key studies are summarised below.

74. Pure extracts of OA caused intestinal damage in the duodenum and the upper portion of the jejunum following both oral and intraperitoneal doses of 0.75 mg/kg bw (n=9) and 0.375 mg/kg bw (n=6) respectively in 4-week old male ICR mice (Ito and Terao, 1994). The following effects were observed for both i.p. and oral administration: within 5 min of dosing, the basal portion of the covering epithelium became homogeneous and peeled from the lamina propria, while the upper portion containing microvilli remained intact. There were two types of villous injury and recovery: When the injuries were limited to the villi new cells from the crypts moved upward and differentiated into columnar cells; when injuries progressed into the glands of Lieberkuhn, clusters of crypt cells were exposed to the intestinal lumen, and in the most severe case they were completely separated. Villous fusion was often seen in the recovery process of the progressed injuries and recovery from all injuries was usually completed within 2 days. The minimum dose of OA that was found to induce these injuries by i.p. administration was 0.2 mg/kg bw.

75. In a further study employing an immuno-staining method, intestinal cell oedema and light erosion of surface epithelial cells with degenerated parietal cells were seen in the stomach, duodenum, jejunum and ileum sections of the intestine in 4 week old male ICR mice orally administered pure extracts of OA at oral doses of 0.075 (n=12), 0.150 (n=36) 0.250 (n=3) mg/kg bw with one mouse being sacrificed at different time points to observe for pathology (Ito et al. 2002). In these studies pathological changes were observed at the lowest dosing concentration of 0.075 mg/kg bw within 60 minutes of dosing. At the higher doses changes were detectable in intestinal tissues within 5 minutes of dosing and toxic responses (as detailed above) were observed within 45 minutes of dosing that could last for up to 12 weeks (Ito et al. 2002).

Hepatotoxicity of OA

76. Information on the DSP toxins has been reported over the last decade has suggested that some of these toxins are hepatotoxic and potent tumour promoters in laboratory animals (Arias et al. 1993; Fessard et al. 1994; Takai et al. 1987).

77. In the liver, histochemical studies employing immuno-staining methods found OA to be located around nuclei of hepatocytes within 5 minutes of dosing when oral doses of pure OA in the range of 0.075-0.25 mg/kg bw were given to male ICR mice (n=12 per dose group). OA appeared to move into the cytoplasmic vesicles by 15 minutes and by 45 minutes vesicles in the hepatocytes had disappeared (Ito et al. 2002). However, prominent liver injury was not observed in this study at any of the doses administered.

Neurotoxicity of OA

78. OA has been shown to cause neurotoxic effects (Arias et al. 1998; Arias et al. 2002). Commercially sourced OA was administered by stereotaxic injection (1μl) directly into the dorsal rat hippocampus at doses of 0, 25, 50, 150 and 300 ng/animal. A notable neurodegeneration occurred in the CA1 subfield, the dentate gyrus, and the hilus 3 hours after injection of 300 ng OA/animal. Neuronal death was more evident at 24 hours and a dose-dependent increase in neurotoxicity was evident at doses 50ng/animal (Arias et al. 1998).

Tumourigenicity of OA

79. Several studies have demonstrated that OA induces ornithine decarboxylase (ODc) production in different tissues following *in vivo* administration. Induction of ODc in the mucosa of rat glandular stomach was demonstrated in a study involving oral intubation of 10 μg/animal of pure OA to rats and the tumour promotion capabilities of OA was determined in the glandular stomach of rats initiated with N-methyl-N'-nitro-N-nitrosoguanidine in a two-stage carcinogenesis experiment (Suganuma et al. 1992). OA in drinking water, 10 μg/rat/day from weeks 9-55 of the experiment, and 20μg/rat/day from weeks 56-72, significantly enhanced development of the neoplastic changes in the glandular stomach ($P < 0.05$). The neoplastic changes included adenomatous hyperplasias and adenocarcinomas.

80. In another carcinogenesis study, OA was found to cross the cell membranes in mouse skin and induce ODc 4 hours following topical application. Further studies by the same group with 8 week old female CD-1 mice (n=15) determined that OA is a potent non-TPA-(12-0-tetradecanoylphorbol-13-acetate)-type tumour promoter in mouse skin after twice-weekly topical applications of 10 μg of purified OA (in 0.1mL acetone) to mouse skin for 30 weeks (Suganuma et al. 1988).

Reproductive and developmental toxicity of OA

81. No reported data are available on the reproductive or developmental toxicity of OA, but commercially sourced purified OA has been shown to be able to cross the placental barrier to the foetus 24 hours following oral administration 0.05 mg/kg bw OA to pregnant female Swiss-Webster mice

(n=3) at day 11 of gestation (Matias and Creppy, 1996). It was shown that OA has high affinity for the foetus with a higher percentage of OA being detected in the foetus (6%) compared to the liver (2%) or kidney (3%) of the mother.

Dinophysistoxin (DTX)

Single dose lethal toxicity

82. The i.p. lethality of DTX-1 and DTX-3 in 1% Tween 60 solution to mice (17-20g) was found to be 0.166 mg/kg bw and 0.5 mg/kg bw respectively (Yasumoto et al. 1984). DTXs were purified from samples of shellfish but experimental conditions of the mouse bioassay such as mouse strain, sex or group numbers were not detailed in the paper. The lethality of DTX-3, purified from the marine sponge *H. okadai* and administered by i.p. injection was found to be 0.1 mg/kg bw when tested in 3 male ddY mice with lethality being determined as the amount of toxin sufficient to kill 2/3 mice in 24 hours (Yanagi et al. 1989).

83. The oral lethality of DTX-1, purified from scallops, was assayed in male ddY mice (20 ± 0.5g) with doses ranging from 0.1- 0.4 mg/kg bw DTX-1 (n=4-5 mice/dose group) (Ogino et al. 1997). Observations were made up to a maximum of 48 hours. A dose-dependent increase in lethality was observed at 0.3 mg DTX-1/kg bw (2 of 4 mice died within 6 hours) and at a lower dose of 0.1 mg DTX-1 /kg bw, only 1/5 mice died within 30 hours of dosing.

Diarrhoetic effects of DTXs

84. The diarrhoetic effects (intestinal fluid accumulation) of purified extracts of DTX-1 and DTX-3 were observed in suckling CD-1 mice orally administered 0.1 and 0.05 mouse units respectively (animal numbers were not detailed for this experiment in the paper). (Hamano et al. 1986). Another study in suckling male ddY mice showed that orally administered purified DTX-1 to cause fluid accumulation in the intestine at the lowest dose administered of 0.1μg DTX-1/mouse (n=3) (Ogino et al. 1997). Fluid accumulation was also observed in suckling CD-1 mice following oral doses of semi-synthetic OA acyl analogues (DTX-3) at 0.125 μg/mouse (n=5) and also following injection into intestinal loops of CD-1 mice at doses of 2μg/mouse (n=5) (Yanagi et al. 1989).

85. Following i.p. administration, DTX-1 has been shown to produce severe diarrhoetic symptoms within one hour in mice administered with 0.3mg/kg bw (Terao et al. 1986). Further studies with DTX-1 with i.p. dosing of 0.375 mg/kg bw or oral doses of 0.750 mg/kg bw showed that within 15 minutes the duodenum and upper portion of the small intestine were distended and held mucoid (Terao et al. 1993).

Intestinal toxicity of DTXs

86. Purified DTX-1 has been shown to produce severe intestinal injury in the duodenum and upper portion of the small intestine within one hour of i.p. administration to BALB/c mice (Terao et al. 1986). In these experiments, mice were allocated to groups 1-6 (n=12/group) and dosed with 0.5, 0.4, 0.3,

0.2, 0.1 and 0.05 mg/kg bw of DTX-1, respectively. Three mice in groups 1-3 were sacrificed at 5, 10, 30 and 60 min and the mice in groups 4 and 5 were likewise killed at 30, 60, 90 and 120 minutes. No effects were observed at doses <0.3mg/kg bw. At a dose of 0.3mg/kg bw congestion was seen in the villous and submucosal vessels. At the histological level, a marked oedema was seen in the lamina propria of villi and at higher dose levels intracellular vacuolisation of the mucosal epithelium occurred. Ultrastructural changes were also seen including extravasion, degeneration of the absorptive epithelium, desquamation of the mucus epithelium from the villous surface, and synapses within certain regions of the digestive tract were swollen and occasionally myelin fibres could be seen within the synapses.

87. The injuries and repair processes in the duodenum and upper sections of the jejunum of male ICR mice induced by purified DTX-3 and DTX-1 have been investigated (Ito and Terao, 1994). DTX-3 impaired intestinal villi by the oral route only when administered at doses of 0.75 mg/kg bw, whereas DTX-1 caused intestinal injury with both oral and intraperitoneal doses of 0.75 mg/kg bw and 0.375 mg/kg bw, respectively (n=9/dose group). Mice were sacrificed and examined for injury at 5, 15, 20 and 30 minutes as well as 1, 2, 4, 6 and 24 hours post dosing. The character of the lesions caused by DTX-1 and DTX-3 and the recovery processes were highly similar. Within 5 min of dosing, the basal portion of the covering epithelium became homogeneous and peeled from the lamina propria, while the upper portion containing microvilli remained intact. There were two types of villous injury and recovery: 1) when the injuries were limited to the villi new cells from the crypts moved upward and differentiated into columnar cells; 2) When injuries progressed into the glands of Lieberkuhn, clusters of crypt cells were exposed to the intestinal lumen, and in the most severe case they were completely separated. Villous fusion was often seen in the recovery process of the progressed injuries. Recovery from any injuries was usually completed within 2 days.

Hepatotoxicity of DTXs

88. Hepatic injuries of fat droplet accumulation and necrotic foci were reported following oral doses of 0.75 mg/kg bw DTX3 to male ICR mice and Wistar rats 24 hours after administration. Following i.p. administration of 0.375 mg/kg bw of DTX-1 and DTX-3, vacuoles appeared in hepatocytes with DTX-3 inducing more severe effects of degeneration and necrosis of hepatocytes in the mid-zone of the liver (Terao et al. 1993).

Tumourgenicity of DTXs

89. Purified DTX-1 has been shown to be an inducer of ODc and a potent tumour promoter in a two stage carcinogenesis experiment on the skin of 8-week old female CD-1 mice (n=15) that were topically administered 5μg DTX-1 (dissolved in 0.1mL acetone) twice a week for up to 30 weeks (Fujiki et al. 1988).

Neuro-, reproductive and developmental toxicity

90. No in vivo studies have been reported for the neurotoxicity or the reproductive and developmental toxicology of DTXs.

Pectenotoxins

Single dose lethal toxicity

91. Oral and i.p. LD_{50} for various purified PTXs are summarised in Table 4. No lethality was observed for PTX-7, -8 and -9 up to maximum doses of 5mg/kg bw, but details of the bioassay such as mouse strain, sex and numbers were not made available in the publication (Sasaki et al. 1998). A review paper has stated that the LD_{99} in mice (i.p.) for PTX-1 to 6 ranged from 0.106-0.77 mg/kg bw but did not define which PTX isomer caused the greatest toxicity, detail any pathologys observed nor provide information on mouse strain or numbers used in the experiment (Yasumoto and Murata, 1990). No lethal toxicity data have been published on the PTX-2 seco acid isomers to date.

Diarrhoetic effects of PTXs

92. Mild diarrhoetic effects caused by oral administration of purified PTX-2 were observed in 20g male ddY mice 3-6 hours following a dose of 2.50 mg/kg bw (n=1), 2 mg/kg bw (n=1), 1 mg/kg bw (n=5), or 0.25 mg/kg bw (n=1) (Ishige et al. 1988). No diarrhoetic effect was seen after i.p. administration of PTX-1 with dosing levels of 1 (n=30), 0.7 (n=10), 0.5 (n=10), 0.25 (n=10), and 0.15 mg/kg bw (n=10) in suckling BALB/c mice (Terao et al. 1986) or observed in suckling CD-1 mice (n=3-5) orally administered PTX-1 up to doses of 0.4 mouse units (1 MU being defined as the quantity of toxin necessary to kill 2/3 mice in 24 hours) (Hamano et al. 1986). No published data are available for the diarrhoetic effects of the PTX-2-SAs.

Intestinal toxicity

93. An oral toxicity study with purified PTX-2 was conducted where it was observed that PTX-2 caused severe mucosal injuries in the small intestine between 30 to 360 minutes post doses of 0.25mg kg (n=1) (lowest dose administered), 1mg/kg bw (n=5), 2mg/kg bw (n=1) and 2.5mg/kg bw (n=1) (Ishige et al. 1988). No pathological changes in the large and small intestines were observed following i.p. doses of PTX-1 at 1 (n=30), 0.7 (n=10), 0.5 (n=10), 0.25 (n=10), and 0.15 mg/kg bw (n=10) in suckling BALB/c mice (Terao et al. 1986).

Hepatotoxicity

94. Purified PTX-1 administered by i.p. injection was shown to be hepatotoxic causing rapid necrosis of hepatocytes, in particular to periportal regions of hepatic lobules in suckling BALB/c mice (Terao et al. 1986). Dosing was performed at levels of 1 (n=30), 0.7 (n=10), 0.5 (n=10), 0.25 (n=10), and 0.15 mg/kg bw (n=10). The most severe observations were seen in mice treated with an i.p. dose of 1 mg PTX-1/kg bw which caused severe congestion of their livers, and the surface of the livers appeared finely granulated within 60 minutes of i.p. injection. Multiple large vacuoles appeared around the periportal regions of the lobules. The vacuoles did not stain with neutral red, which indicates changes in neutral lipids, and several of the vacuoles located in the subcapsular region contained erythrocytes. Hyaline droplets were seen

in the hepatocytes that developed vacuoles. Electron microscopic observation showed flattening of the microvilli on the hepatocytes in the periportal region and also showed the plasma membrane to be invaginated into its cytoplasm. Terao et al. (1986) found that PTX-1 caused damage to the hepatocytes in the periportal area of the lobule within 10 minutes of i.p. doses of 0.5 mg/kg bw by electron microscopy. At doses below this only slight dilatation of the space of Disse occurred with small vacuoles in the periportal region. One hour following a higher dose of 1 mg/kg bw many fat droplets could be seen within the cytoplasm of hepatocytes and mitochondria often appeared swollen.

95. A decrease in hepatic microsomal protein content was observed in a study where male ICR mice were repeatedly dosed by i.p. injection with 0.02 or 0.1 mg/kg bw/day (n=7-8 per group) with purified PTX-2 for 1 or 2 weeks (Mi and Young, 1997). However, the total P450 content, cytochrome b5, NADPH-cytochrome c reductase and aminopyrine N-demethylase activities were not affected. Glutathione content was also unaltered, but elevated serum enzyme levels of alanine aminotransferase, aspartate aminotransferase, and sorbitol dehydrogenase were found (Mi and Young, 1997).

96. Hepatic injuries were observed in a study with male ddY mice orally administered purified PTX-2 at doses of 0.25mg kg (n=1), 1mg/kg bw (n=5), 2mg/kg bw (n=1) and 2.5mg/kg bw (n=1) (Ishige et al. 1988). Injuries included hyaline droplet degeneration in hepatocytes around peripheral zones of the hepatic lobule and spongiosis lesions of hydropic vacuolar degeneration could be seen in the intermediate zone. Dilatation of the sinusoids and hydropic swelling of the endothelial cells were also observed. Severity of lesions were described as dose-dependent and could be seen with the lowest dose administered of 0.25mg/kg bw.

Other toxicology

97. No *in vivo* studies have been reported for the tumourigenicity, neurotoxicity or reproductive and developmental toxicity of PTXs.

Yessotoxins

Single dose lethal toxicity

98. The highest i.p. toxicity for lipophilic shellfish toxins is seen with the YTXs with an LD_{50} of 0.1 mg/kg bw for purified YTX and 45-homo-YTX (Yasumoto and Murata, 1990). A later study confirmed this finding. The minimum amount of YTX required to kill a mouse was determined to be in the range 0.08 – 0.1 mg/kg bw when assayed in male ddY mice (15 ± 0.5g) by i.p. injection with 3 mice per dose group being dosed with purified YTXs (Ogino et al. 1997). Doses of 0.3 mg/kg bw of purified YTX have been shown to kill male ICR mice within 3 hours of i.p. administration, whilst de-sulfated YTX at a dose of 0.301 mg/kg bw when administered to male ICR mice did not cause lethality until 48 hours post dosing (Terao et al. 1990).

99. The oral lethality of purified YTX was determined in male ddY mice (20 ± 0.5g; 4-5 mice per dose group). All mice dosed via the oral route survived following doses up to the maximum testing dose of 1 mg/kg bw (Ogino et al.

1997). A later study found that female NMRI, BOM mice (n=3) survived following oral doses of purified YTX up to maximum doses of 10mg/kg bw (Aune et al. 2002).

Diarrhoetic effects of YTX's

100. Oral and i.p. studies in mice found purified yessotoxins did not induce diarrhoetic symptoms or cause any notable intestinal pathology (Tubaro et al. 2002, oral communication 10th International Conference on Harmful Algal Blooms, Florida, Ogino et al. 1997; Terao et al. 1990; Yasumoto et al. 1989).

Target organ toxicity

101. Purified YTX was found to target cardiac muscle cells and cause intracytoplasmic oedemas in male ICR mice (n=5) administered an i.p. dose of 0.3 mg/kg bw YTX (Terao et al. 1990). A later study confirmed these results in female NMRI, BOM mice and dose dependent pathological changes in the heart were evident following oral doses of 2.5, 5 and 10mg/kg bw to groups of 2 mice per dose and i.p. administered doses of 0.1-1mg/kg bw to 3 mice in each group. The lowest oral dose where effects were seen was 2.5mg/kg bw and for an i.p. dose was 0.75 mg/kg bw (Aune et al. 2002).

102. Purified desulfated YTXs were found to target the liver and pancreas with 0.3 mg/kg bw causing dose dependent severe fatty degeneration and intracellular necrosis within 24hr of i.p. injection to groups of 5 male ICR mice (Terao et al. 1990).

103. Purified YTX was found not to cause hemolysis at concentrations of 50ppm in a suspension of red blood cells of male ddY mice (n=1) (Ogino et al. 1997; Terao et al. 1990). In another study, purified YTXs were found not to cause alterations in plasmic enzymes or leucocyte percentages following oral administration of various YTXs up to a dose of 2mg/kg bw in female CD-1 mice (Tubaro et al. 2003).

104. Oral and i.p. studies with the YTX have reported that no hepatotoxicity was observed with these toxins following studies with various strains of mice (Aune et al. 2002; Terao et al. 1990).

Other toxicity

105. No in vivo neurotoxicity or reproductive and developmental toxicology of YTXs has been reported in the literature.

Azaspiracids (AZAs)

Single dose lethal toxicity

106. Concentrations of i.p. lethality for various purified AZAs are summarised in Table 4. All the LD_{99} lethality data for i.p. dosing reported in table 4 were for male ddY mice (14-17g). Oral dosing studies with purified AZAs have been conducted with male ICR mice, with lethality being found to be between 0.5-0.6 mg/kg bw (Ito et al. 2000).

Diarrhoetic effects of AZAs

107. There are no animal data on the diarrhoetic effects of AZAs but symptoms of AZP in humans are similar to DSP with nausea, vomiting, severe diarrhoea and stomach cramps. This diarrhoetic effect was reported following human exposure in the Netherlands through consumption of contaminated mussels harvested in Ireland (McMahon and Silke, 1996).

Target organ toxicity

108. Purified AZAs have been found to target the liver, pancreas, thymus, spleen and gastrointestinal tract (Ito et al. 2002; Ito et al. 2000). Oral doses in the range of 0.3 mg/kg bw (n=5), 0.5mg/kg bw (n=7), 0.6mg/kg bw (n=6) and 0.7 mg/kg bw (n=2) 0.9mg/kg bw (n=2) were administered to male ICR mice. Following oral administration of 300 μg/kg AZA necrosis in the lamina propria of the small intestine and in lymphoid tissues such as thymus, spleen and the Peyer's patches were observed. Additionally, both T and B lymphocytes were effected following a dose of 0.5 mg/kg bw (n=5) and fatty change was noted in the liver 1 hour following oral administration of 0.3 mg/kg bw of AZAs. (Ito et al. 2000).

109. Recent chronic studies involved observation of recovery processes from severe injuries following repeated administration of purified AZA to male ICR mice twice a week at doses of 0.05mg/kg bw (n=10), 0.02 mg/kg bw (n=10), 0.005 mg/kg bw (n=5), and 0.001 mg/kg bw (n=6) (Ito et al. 2002). When AZA, even at the lowest dose of 0.001 mg/kg bw, was administered twice a week up to 40 times to four groups of mice many mice became so weak that they were sacrificed before completion of 40 injections. All these mice showed interstitial pneumonia and shortened small intestinal villi. Most importantly, lung tumours were observed in four mice, one out of ten mice at 0.005 mg/kg bw and three out of ten mice at 0.020 mg/kg bw. Tumours were not observed in 11 mice treated at lower doses and in 19 control mice. Hyperplasia of epithelial cells was also observed in the stomach of six mice out of ten administered at 0.02 mg/kg bw. Slow recoveries from injuries were found including erosion and shortened villi persisting in the stomach and small intestine for more than 3 months, oedema, 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.

110. AZA has been shown to induce a slow progressive paralysis in mice (Satake, IOC proceedings of the 9th International conference on Harmful algal blooms, 2000).

111. No data are available on the reproductive and developmental toxicity of AZAs.

Crude DSP extract studies

112. The diarrhoetic effects caused by a crude extract of DSP toxins from scallops were observed in suckling CD-1 mice (n=3-5 mice) following oral administration (Hamano et al. 1986). Quantitative determination of concentrations of lipophilic biotoxins in the crude extract was not provided but measured in mouse units (MU) of 0.025, 0.05, 0.1, 0.2 and 0.4 MU, one mouse unit being defined as the amount required to kill 2/3 mice within 24 hours. Diarrhoetic effects were also observed following injection of the crude extract directly into isolated rabbit and mice intestinal loops. For rabbit studies 5 loops were isolated from each of 6 rabbits, into which a suspension of crude lipophilic DSP extract was injected in doses of 0, 1, 2, 4 or 8 MU. Fluid accumulation was observed in 1/3 loops after 18 hours of injection of 4 MU of crude DSP. Mouse studies were conducted in female CD-1 mice with 6 loops were isolated in each of 3 mice with doses of 0.1, 0.2, 0.4, 0.8 or 1.6MU per isolated loop. In this study 0.4MU was the lowest dose found to cause fluid accumulation and was observed within 6 hours of administration.

113. Diarrhoetic effects were shown to peak within 2 hours following an injection of a crude extract from Blue Mussels directly into the intestine of male Sprague-Dawley rats (7 weeks old) (Edebo et al. 1988a; Edebo et al. 1988b). Seven extracts from different organs of the toxic mussels were tested in single intestinal loops. A dose-repose effect of fluid accumulation was observed when serial dilutions of extracts were made and injected into intestinal loops, but determination of concentrations of lipophilic toxins in the crude extract was not performed.

114. An additional toxic extract, along with OA, has been separated from mussels in France (Bansard et al. 1995). This toxic extract was found to produce neurotoxic symptoms in mice such as convulsive spasms, jumping and gasping within 5-30 minutes of administration. Experimental details such as dose concentration or mode of administration, mice strain and number of animals was not provided in the paper. The additional toxic extract was found to have a lower polarity than OA. In the investigations of Bansard et al, OA was found to induce a cyanosis whereas the newly identified toxin was found to cause cardiac arrest in addition to the neurological symptoms. It was proposed that the cardiac arrest was caused by inhibition of the calcium current by the new extract, whereas normally OA increases calcium current by the inhibition of protein phosphatase activity (Bansard et al. 1995). It may be possible that this particular toxin was a known shellfish toxin but the authors did not sufficiently purify the substance to identify it.

Summary

115. Toxicity studies on lipophilic biotoxins are very limited in number and scope. The studies are principally limited by the availability of sufficiently large quantities of purified toxins to conduct detailed studies. Therefore, many studies have used small group sizes, shellfish extracts of unknown or

estimated toxin concentrations that possibly contain other toxicologically active contaminants (possibly other algal biotoxins) as well as parenteral routes of administration to maximise the potency of the test material. In addition, many of the studies have reported clinical symptoms and behavioural responses as an indicator of toxicity rather than more objective toxicological end-points. Very few studies have examined the effects of oral administration of lipophilic biotoxins. The liver and intestine are the main targets of the DSP toxins due to the existence of specific uptake systems in these organs. Diarrhoetic effects have only been sufficiently proven for OA and the dinophysis toxins DTX-1 and DTX-3.

Summary of acute lethality studies

Table 4 A summary of LD_{50} and LD_{99} toxicity's via i.v., i.p. and p.o. administration for DSP lipophilic toxins in mice are summarised below.

	Toxin class	*LD_{50} toxicity mg/kg bw*	*Reference*
i.v. dosing			
	DSP (extract)	0.192	(Tachibana et al. 1981)
	OA	0.2	(Yasumoto et al. 1985)
	DTX-1	0.166	(Yasumoto et al. 1985)
	YTX	0.1	(Yasumoto and Murata, 1990)
i.p. dosing			
	OA	0.2	(Yasumoto et al. 1989)
	OA	0.192	(Tachibana et al. 1981)
	DTX-1	0.16	(Yasumoto et al. 1984)
	DTX-3	0.5	(Yasumoto et al. 1985)
	PTX-1	0.25	(Yasumoto et al. 1984)
	PTX-2	0.26	(Yasumoto et al. 1984)
	PTX-2	0.25	(Jung et al. 1995)
	PTX-3	0.35	(Murata et al. 1986)
	PTX-4	0.77	(Yasumoto et al. 1989)
	PTX-6	0.5	(Yasumoto and Murata, 1990)
	YTX	0.1	(Ogino et al. 1997; Yasumoto and Murata, 1990)
	YTX	0.512	(Tubaro et al. 2003)
	45-OH YTX	Approx. 0.1	(Yasumoto and Murata, 1990)
	Hydroxy YTX	0.5	(Satake et al. 1996)
	Homo YTX	0.1	(Satake et al. 1997)
	Homo YTX	0.444	(Tubaro et al. 2003)
	Hydroxy-homo-YTX	0.5	(Satake et al. 1997)
	NorYTX	0.22	(Satake et al. 1996)
	CarboxyYTX (adriatoxin)	0.5	(Ciminiello et al. 1998)
	AZA	0.2	(Satake et al. 1998)
	AZA-2	0.11	(Ofuji et al. 1999)
	AZA-3	0.14	(Ofuji et al. 1999)
	AZA-4	0.47	(Ofuji et al. 2001)
	AZA-5	1.0	(Ofuji et al. 2001)
p.o. dosing			
	DTX-1	0.3 (50% lethality in 6 hrs)	(Ogino et al. 1997)
	PTX-2	0.3 (lethality in 2/5 mice in 6 hrs)	(Ogino et al. 1997)
	YTX	>1.0	(Ogino et al. 1997; Tubaro et al. 2003)
	YTX	>10	(Aune et al. 2002)
	AZA	0.5-0.6	(Ito et al. 2000)

Toxin class	*LD_{99} Toxicity mg/kg bw*	*Reference*
i.p. dosing		
OA	0.2	(Yasumoto and Murata, 1990)
DTX-1	0.16	(Yasumoto and Murata, 1990)
DTX-3	0.5	(Yasumoto and Murata, 1990)
PTX1-6	0.16-0.77	(Yasumoto and Murata, 1990)
YTX	0.1	(Yasumoto and Murata, 1990)

IN VITRO TOXICOLOGY AND MECHANISMS OF TOXICITY FOR LIPOPHILIC DSP TOXINS

Okadaic acid

Phosphorylation of proteins by okadaic acid

116. Many studies have identified OA as a very potent inhibitor of protein phosphatase 1 (PP1) and PP2A and have shown this mode of action is linked to the mechanisms involved in diarrhoea, degenerative changes in various cells, alterations in glycogen and lipid metabolism, tumour promotion, changes in oncogene expression, cytotoxicity and neurotoxicity. A summary of *in vitro* studies with OA is given in Table 5.

Diarrhoetic effects of OA

117. The diarrhoetic effects of OA are thought to be caused by a similar mechanism to that caused by the bacteria *Vibrio cholerae*. This mechanism causes activation of adenylate cyclase leading to a cascade of events of increased cAMP that activates cAMP-dependent protein kinase and certain proteins that regulate intestinal sodium secretion which lead to diarrhoetic effects as reviewed in (Cohen et al. 1990). Additionally, research has shown that OA increases paracellular permeability of intestinal epithelia (Tripuraneni et al. 1997).

Tumour promotion

118. Tumour promoters of the OA class have been reviewed in several articles and are considered to be non-PTA type tumour promoters and their actions are found to not be mediated through the actions of protein kinase C (Fujiki et al. 1988a; Fujiki et al. 1988b; Fujita et al. 1999; Yatsunami et al. 1992).

Neurotoxicity

119. Protein phosphatases dephosphorylate serine and threonine residues in the cytosol of mammalian cells and as a result of this, OA is cytotoxic to cultured nerve cells (Bagu et al. 1997; Bezvenyuk et al. 2000; Davis and Maher, 1994; Fernandez et al. 1993; Fernandez et al. 1991; Li et al. 1996; Suuronen et al. 2000; Yagami et al. 2002; Zeevalk et al. 2001).

Cytotoxicity

120. Numerous studies have been conducted on the cytotoxicity of OA in cellular systems (Ganapathi, 1992; Kiguchi et al. 1994; Matias et al. 1999; Oesch et al. 1997; Oteri et al. 1998; Traore et al. 2001) and increased cytotoxicity has been observed with the addition of metals to culture media (Traore et al. 2000; Traore et al. 1999).

Changes in cell cycle regulation

121. Changes to cell cycle cause by OA are complex, because research has shown OA can affect the cell cycle directly and indirectly and at multiple levels, in particular through its action on protein phosphatases. An example of this effect in the cell cycle is the capability of OA to cause phosphorylation of H2A in HeLa cells throughout the cell cycle leading to a mitosis-like state (Ajiro et al. 1996).

Dinophysis toxins

122. DTX toxins cause protein phosphatase inhibition (Honkanen et al. 1996a; Honkanen et al. 1996b), and thus may act in similar mechanisms to OA in their tumour promoting, cytotoxic and diarrhoetic activity. Key *in vitro* studies are summarised in Table 6.

Pectenotoxins

123. PTXs have been described as hepatotoxins, tumour promoters and to cause apoptosis but do not act though mechanisms of protein phosphatase inhibition. Studies on the PTXs are not as extensive as those of the OA toxins. Key *in vitro* studies are summarised in Table 7.

Yessotoxins

124. The mechanism of action of YTX remains poorly understood. YTX exposure alters the surface morphology of rat hepatocytes, and studies in HeLa and BE(2)-M17 neuroblastoma cells have shown that the toxin activates caspases involved in apoptotic cell death. In the presence of Ca^{2+}, YTX is thought to activate phosphodiesterases (PDEs), leading to increased cAMP hydrolysis. YTX also increases the cytosolic Ca^{2+} concentration of human lymphocytes, possibly by interacting with calcium channels. Key *in vitro* studies are summarised in Table 8.

Azaspiracids

125. Only one *in vitro* study of AZA1 has been performed. In excitable neuroblastoma cells, AZA1 did not modify mitochondrial activity or cell membrane potential (Roman et al. 2002). However, the concentration of F-actin was decreased, indicating that the cytoskeleton may be an important target of AZA1. AZA1 increased the level of cytosolic calcium and cAMP. The increase in cytosolic calcium may have been dependent on both the release of calcium from intracellular Ca^{2+} pools and calcium influx from extracellular media through Ni^{2+}-blockable channels. The AZA1 induced Ca^{2+} increase was negatively modulated by cAMP increasing agents, protein kinase C (PKC) activation and the inhibition of protein phosphatases 1 and 2A.

Summary

126. In general, OA and DTXs are known protein phosphatase inhibitors and this PPI is thought to mediate the mechanisms of toxicity including tumour promoting activity, cause diarrhoetic effects and have cytotoxic action on various cell types such as hepatocytes and neuronal cells. The research on PTXs, YTXs and AZAs is not as extensive as OA, most likely due to lack of availability of purified samples of toxins. From the few studies conducted, these toxins are not known to cause inhibition of PP2A, with YTX only being weakly inhibitory, but are thought to act through other mechanisms.

127. Below, Tables 5, 6, 7 and 8 summarise a selection of key studies investigating the different mechanisms discussed above. It should be noted that the tables of studies are not exhaustive, especially for OA (Table 5).

Table 5 Selection of invitro studies with Okadaic acid

Cell line/organ toxicity	Observations/ conclusion	**Reference**
Mucosa	OA strongly inhibited protein phosphatases 2A IC50, 0.07 nM and protein phosphatase 1 to a less extent with an IC50 of 3.4 nM.	(Suganuma et al. 1992)
Guinea pig taenia coli	OA produced a dose-dependent inhibition of myosin phosphatase and induced contractions in the coli fibers following exposure of OA at 5μM.	(Takai et al. 1987)
Rat cortical neurons and human neuroblastoma cells	OA was shown to induce changes in microtubule-associated protein 2 and τ phosphorylation prior to neurodegeneration and caused astrocyte morphology in cultured rat cortical neurons to change within 5-30 minutes following doses of 250nM.	(Arias et al. 1993).
MCF-7 breast cancer cells	The activation of P-53 has been demonstrated in with 50 nM okadaic acid.	(Rossini et al. 1997)
Chorioallantoic membrane of the chick embryo.	OA induced a dose-dependent angiogenesis (the minimum effective dose: 5 fmol/egg, effective dose for 50% induction: 90 fmol/egg.	(Oikawa et al. 1992)
Mammalian fibroblast BHK21 cells	A 1% solution of OA induced neutral red uptake and caused the formation of square-shaped cells followed by cell rounding with micro-blebs appearing on the cell surface.	(Fessard et al. 1994; Fessard et al. 1996).
Isolated Rat hepatocytes (male Wistar)	Rapid (within 2 hours) morphological changes to cells with order of potency being OA (1μg/mL) > DTX-1 > PTX1 > YTX (25 μg/ml).	(Aune et al. 1991)
neuroblastoma cells	A reduction in cellular glutathione transferase, glutathione reductase and catalase activity with an increase in lipid peroxidation was observed 2 hours following 50 nM okadaic acid.	(Montilla et al. 2002)
Intestinal Caco-2 cells	Protein synthesis was inhibited and induced modified bases in DNA following treatment with 15 ng/ml OA for 24 h. The combination of OA and cadmium was more cytotoxic and increased lipid peroxidation (MDA).	(Traore et al. 2000)
intestinal Caco-2 cells	Lipid peroxidation was observed with dose-dependent increases in rates of 8-OH-dG and m(5)dC formation causing CG to AT transversion mutations and gene deregulation following doses of 0.75-7.5 ng/ml OA.	**(Creppy et al. 2002)**

CHO-K1 cell line	Induction of centromere-positive micronuclei nuclei was observed after a 24 h treatment with 20 (68.9%) and 30 (77.0%) nM OA.	(Le Hegarat et al. 2003)
Vero cell	OA induced lipid peroxidation and inhibited protein synthesis in a concentration-dependent manner (IC50=27 ng/ml). Vitamins E and C prevented the lipid peroxidation induced by OA in cells, indicating that the formation of oxygen free radicals could be a mechanism of OA induced toxicity.	(Matias and Creppy, 1996).
Oestrogen-primed rat uterus	OA induced a transient contraction of the uterus with OA concentrations of 20 uM.	(Arteche et al. 1997)
Cultured colon epithelium T-84 cell monolayers	OA at a concentration of 0.06 μmol/L decreased trans-epithelial electrical resistance in a polarised fashion increasing the paracellular permeability of intestinal epithelia.	(Tripuraneni et al. 1997)
IEC-6 (cultured rat intestinal cells)	Following doses of 0.25, 0.5 and 1 μM OA, a dose- and time-dependent effect on F-actin was observed in intoxicated cells.	(Fiorentini et al. 1996)

Table 6 Selection of invitro studies with Dinophysis toxins

Cell line/organ toxicity	Observations/ conclusion	**Reference**
L1210 mice leukemia cells	Cytotoxic responses were observed in cells following doses of 3 analogues of DTX-3 with ED50 concentrations being 0.13μg/ml for OA, 1.5 μg/ml for 7-O-palmitoyl, 1.2μg/ml for 7-O-linoleoyl, and 0.78μg/ml for 7-O-docosahexaenoyl okadaic acid.	(Yanagi et al. 1989)
HEp-2 (larynx) and Caco-2,	Dose-related effects of DTX-1 on cell viability were seen from doses of 10ng/mL and on cell morphology/cytoskeleton structure at concentrations of 80 mg/mL and above.	(Oteri et al. 1998)
Isolated Rat hepatotcytes (male Wistar)	Dose-dependent changes in cell shape were observed with blebs on the cell surface appearing within 2 hours of incubation with 2.5μg/ml DTX-1	(Aune et al. 1991)
Human MCF-7, AU-565, and MB-231 breast tumour cells	DTX-1 induced differentiation and apoptosis in tumour breast cells.	(Kiguchi et al. 1992)

Table 7 Selection of invitro studies with Pectenotoxins

Cell line/organ toxicity	Observations/ conclusion	Reference
Isolated rat hepatocytes	PTX-1 doses from 7.5µg/ml caused vacuoles in the cytoplasm, small groves on the cell surface and invagination in the cell membrane.	(Aune et al. 1991)
Primary cultures of hepatocytes from chick embryos	PTX-1 doses ranging from 0.05-50µg/ml induced a loss in number and radial arrangement of microtubules with disruption of stress fibres and accumulation of actin at the cellular peripheries.	(Zhou et al. 1994)
KB cells	PTX-2 was cytotoxic at a dose of 0.05µg/mL.	(Daiguji et al. 1998)
Human lung (A-549), colon (HT-29) and breast (MCF-7) cancer cell lines,	PTX-2 was potently cytotoxic to various cell lines: ovarian (EC_{50}=6.16x10^{-8}), renal (EC_{50}=1.91x10^{-8}),lung (EC_{50}=2.51x10^{-8}), CNS (EC_{50}=7.77x10^{-8}), melanoma (EC_{50}=4.73x10^{-6}), and breast cancer (EC_{50}=6.27x10^{-8}).	(Jung et al. 1995)
Isolated rat and salmon hepatocytes	Extreme nuclear and cellular shrinkage and chromatin hypercondensation were observed within one hour of dosing 2 µM PTX-1.	(Fladmark et al. 1998)
Neuroblastoma cells (BE(2)-M17).	PTX-6 caused a dose-dependent depolymerisation of F-actin at concentrations above 1mM.	(Leira et al. 2002a)
A10 cells	PTX-2 inhibited the velocity and the degree of actin polymerization in a concentration dependent manner in the actin stressfiber center of the cell.	(Hori et al. 1999)

Table 8 Selection of invitro studies with Yessotoxin

Cell line/organ toxicity	Observations/ conclusion	Reference
Isolated rat hepatocytes	YTX, at concentrations from 10 μg/ml, induced the formation of small blebs on the cell surface in a dose-dependent manner.	(Aune et al. 1991)
Isolated rat hepatocytes	25-50 μg/ml YTX induced the formation of small blebs on the cell surface.	(Aune, 1989)
Cultured BE(2)-M17 neuroblastoma cells	1 μm YTX induced caspase-3-mediated apoptotic cell death.	(Leira et al. 2002b)
Cultured HeLa cells	Sub-nanomolar concentrations of YTX caused loss of intact poly(ADP-ribose)-polymerase and an 85 kDA fragment, caused activation of pro-caspase-3 and pro-caspase-7 and induced apoptotic cell death between 48-96 hours.	(Malaguti et al. 2002)
Isolated human lymphocytes	1 μM YTX produced a Ca^{2+}-dependent decrease in cAMP from 52.81 ± 3.66 to 44.53 ± 4.5 fmol.	(Alfonso et al. 2003)
Isolated human lymphocytes	1 μM YTX increased the cytosolic calcium concentration 1.3-fold by influx through nifedipine- and SKF 96365-sensitive channels.	(De la Rosa et al. 2001)

REGULATORY PROCEDURES FOR THE DETECTION OF LIPOPHILIC SHELLFISH TOXINS IN THE UK

128. The Food Standards Agency (FSA) has responsibility for monitoring shellfish harvesting areas for toxic phytoplankton and shellfish for the occurrence of algal toxins that may be harmful to health. Statutory monitoring for shellfish biotoxins is laid down EU (91/492/EC and 2002/225/EC). Shellfish samples are tested by mouse bioassays and decisions to close shellfish beds are based on the outcome of this bioassay. Recent EU legislation (2002/225/EC) also allows chemical testing with maximum permitted levels of biotoxins shown in table 9.

Table 9 Legislation for maximum permitted levels of lipophilic biotoxins stated in EU legislation 2002/225/EC

Toxin	**Maximum Permitted level**
OA, DTXs and PTXs	160µg/kg (combined and expressed as OA equivalents)
AZAs	160µg/kg
YTXs	1 mg/kg YTX (expressed as equivalents)

Summary

129. The MBA described in EU legislation 2002/225/EC is used in the UK to monitor for lipophilic biotoxins in shellfish.

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