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EPISODIOS TÓXICOS EN MARISCOS, PRODUCIDOS POR FICOTOXINAS LIPOFÍLICAS: UNA VISIÓN GLOBAL

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ABSTRACT

The actual knowledge on the diarrhoeic shellfish poisoning and lipophilic phycotoxins (DSP,yessotoxins,pectenotoxins and azaspiracids) is reviewed in this work. The most relevant aspects of the problem are taken into account, including chemical structure of the toxins, detection methods, toxicological properties, producer organisms and their biology, processes involved in their accumulation and transformation in shellfish, regulation and mitigation estrategies.

RESUMEN

En el presente trabajo se revisa el estado actual de conocimiento de la intoxicación diarreica por mariscos y de las ficotoxinas lipofílicas (DSP, Yessotoxinas, Pectenotoxinas y Azaspirácidos). Se contemplan los aspectos más relevantes del problema, que incluyen la estructura química de las toxinas, los métodos de detección, las propiedades toxicológicas, los organismos productores y su biología, los procesos involucrados en la su acumulación y transformación en los mariscos y las estrategias de regulación y mitigación.

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INTRODUCTION

The increase in the human population and the general overexploitation of the fisheries are contributing to a surge in the development of aquaculture. Additionally, the renewable resources in the coastal areas are being increasingly subjected to specific plans designed to attain sustainable exploitation, so that this activity lies more within the domain of extensive aquaculture than the traditional fisheries. An important fraction of these two activities is focused on molluscs and more specifically on bivalves.

Bivalves feed on the organic matter in suspension in the water. Taking into account that the main component of this matter is phytoplankton, it becomes clear that the interactions between bivalves and phytoplankton are very strong. In most cases the interaction is positive for the bivalves and also, indirectly, for man who consumes or exploits them, since phytoplankton usually represents good quality food for these molluscs. Notwithstanding, in some cases, these interactions can be harmful ingestion of phytoplankton, may produce different kinds of toxic or noxious effects. Phytoplankton development in the sea has a seasonal character both quantitatively and qualitatively. Phytoplanktonic populations are subjected to an annual cycle through which abundance and species composition change continuously. Many species generally contribute to the annual cycle but only a few of these produce adverse effects in bivalves or are toxic to man. When one of these species blooms, it has a strong negative impact on fisheries, aquaculture and, in extreme cases, on human health. Bivalves and other organisms accumulate the toxins produced by toxic phytoplankton, serving both as vectors of the toxins and as amplifiers.

Human intoxications derived from mollusc consumption have been known for a long time (i.e. Cabeza de Vaca in the 16th century (edition by Ferrando 1984) and Vancouver in the 18th century), but they acquired real scientific interest at the end of the past century, especially in the seventies. There are several types of intoxications derived from phytoplankton, the main ones being the paralytic, diarrhoeic and amnesic type (PSP, DSP and ASP, respectively). Among these intoxications, DSP is the one that has least dramatic consequences as no fatalities are known to have been caused by the consumption of organisms contaminated with toxins of this kind. Notwithstanding, the economical losses that they incur are of the same order of magnitude -or even greater- that those produced by the other groups of toxins. This fact, together with their very likely chronic effect as tumour promoters and carcinogens, make the DSP group of toxins one of the most relevant among the toxins of phytoplanktonic origin.

In this review we summarise the state of the art of the knowledge of the various aspects involved in diarrhoeic intoxications associated with the consumption of shellfish contaminated with phycotoxins, from the development and distribution of the causative species to the effects on humans, including detection techniques, toxin production, mitigation strategies and the development of predictive capability.

HISTORICAL NOTES

In 1961 Korringa and Roskam described an intoxication with symptoms of DSP after the consumption of mussels from The Netherlands but the causative agent was not identified. Guzmán and Campodonico (1975) described a similar episode which affected the inhabitants of a Chilean fjørd, in 1970, and found a relationship with a large bloom of *Dinophysis*. However, these observations were overlooked by most of the scientific community. In June 1976, in Japan, forty-two people suffered severe vomiting and diarrhoea after eating mussels (*Mytilus edulis*) or scallops (*Patinopecten yessoensis* and *Chlamys nipponensis akazara*) harvested from the Miyagi and Aomori prefectures. The same kind of intoxication took place in

1977 in the same season and area, with 122 persons affected. Yasumoto et al. (1978; 1979) managed to isolate two fat-soluble toxins and to implement a mouse bioassay that allowed the quantification of this kind of toxicity. The main toxin was identified by Murata et al. (1982) as okadaic acid, a compound that had been previously isolated and described from the sponge *Halichondria okadai* by Tachibana et al. (1981). Furthermore, a number of previous incidences were identified in several geographic areas, namely the Netherlands (Kat, 1979), Spain (Campos et al., 1982) and Norway (Underdahl et al. 1985). The causative organism remained unknown until Yasumoto et al. (1980) related DSP toxins to the planktonic dinoflagellate *Dinophysis fortii* and Murakami et al. (1982), to the benthic *Prorocentrum lima*. Later studies identified DSP episodes in different areas mostly linked to the appearance of *Dinophysis* populations in local plankton (summarised for Europe by Van Egmond et al. 1993) but, in some rare cases, to the benthic dinoflagellate *Prorocentrum lima* and other species of the same genus. Nowadays this toxicity is known to be distributed worldwide (FIG.5).

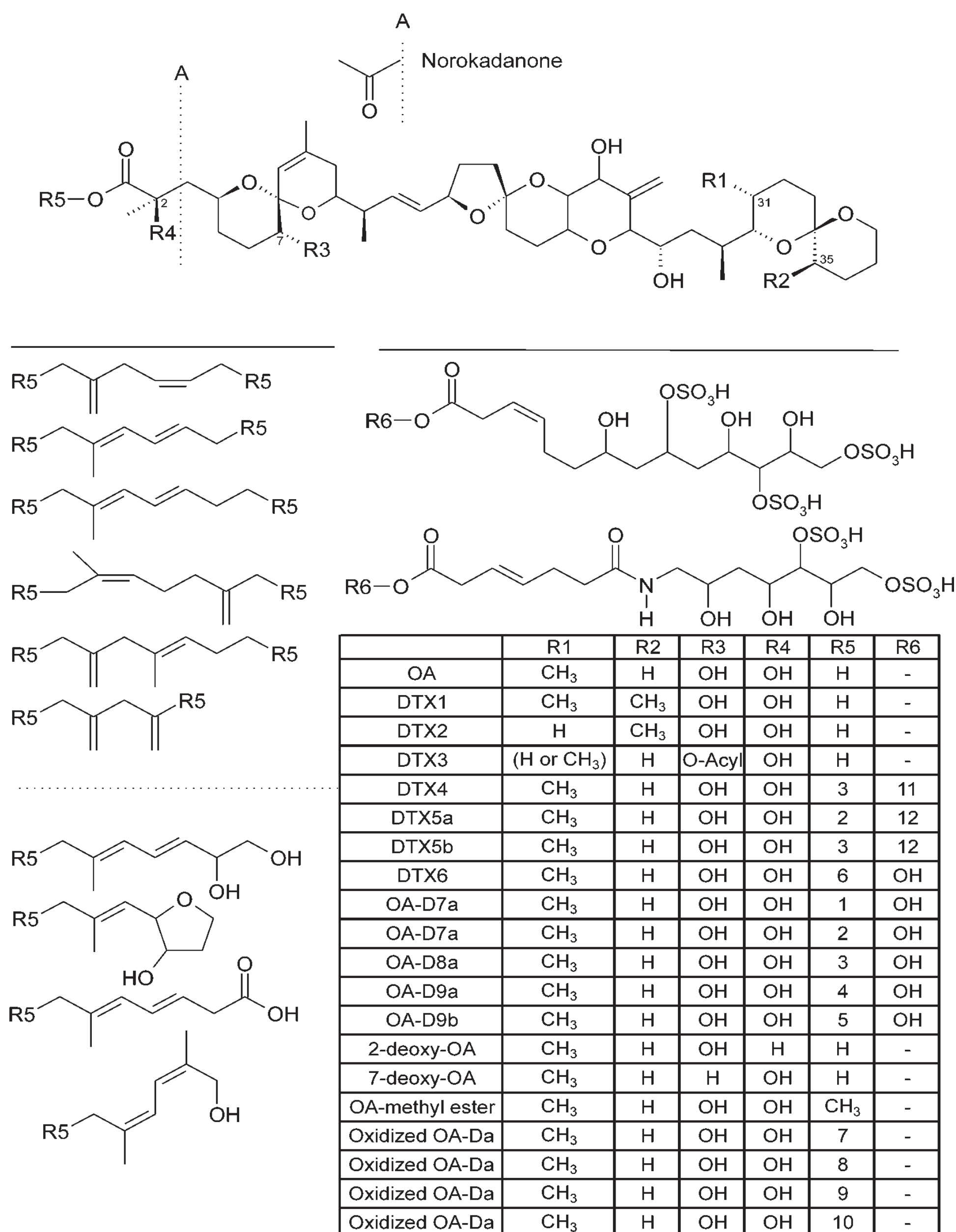
In 1985 and 1987, two new groups of toxins were discovered, pectenotoxins (Yasumoto et al., 1985) and yessotoxins (Yasumoto et al., 1987). These toxins were extracted by the same procedure as okadaic acid (OA) and killed the mice when injected intraperitoneally in the mouse bioassay used to detect DSP. Consequently, they were included in the DSP class, before their toxicity was sufficiently understood. Both toxins were isolated from the Japanese scallop *Patinopecten yessoensis* and frequently co-occur with the typical DSP toxins and are probably responsible for some of the discrepancies found between the toxicity in bivalves estimated by bioassay and by chemical analysis of the DSP toxins of the okadaic acid group.

In 1995 some intoxications with the typical DSP symptoms were detected in The Netherlands after the consumption of mussels from Ireland. As the mussels contained only traces of okadaic acid and DTX2, the presence of unknown diarrhoeic toxins was suspected. As a consequence of subsequent research, a new group of toxins, the azaspiracids, were found to be the causative compounds (Satake et al., 1998). Recently, an organism of the heterotrophic genus of dinoflagellates *Protoperidinium* has been identified as the causative agent (James et al., 2003)

CHEMISTRY

Four chemically different groups of toxins have been historically associated with DSP. The toxins of the okadaic acid group are considered to be the true DSP toxins because they were responsible for most of the registered intoxications and also because their effects coincide exactly with the typical DSP syndrome. Pectenotoxins and Yessotoxins have been considered to be DSP toxins because they can be detected with the extraction and bioassays traditionally used for the toxins of the okadaic acid group. Azaspiracids have never been included in the DSP group, but they cause diarrhoea in humans.

The main toxins in the group, okadaic acid and its analogues DTX1 and DTX2, are long chain compounds containing polyether rings and an ?-hydroxycarboxyl function, the difference between them being only the number or position of the methyl groups they contain. Their molecular masses are around 810. Some isomers have been found but their precise structure has not yet been elucidated (Quilliam, 2003). Several derivatives have also been found both in bivalves and plankton, differing from the original toxins mainly in: a) the esterification of the hydroxyl group in the C_7 position with fatty acids of a different chain length but typically C_{14} to C_{18}, saturated or unsaturated, to produce DTX3 compounds; b) the formation of diol-esters with C_7 to C_9 unsaturated diols; c) oxidation of the diol part of the molecules of the diol-esters; d) esterification of the diol-esters with sulphated chains, which may or may not include an amide function in the polar side chain, to produce DTX4 and DTX5, respectively; and e) the lack of the hydroxyl group in C_2 or C_7 (FIG. 1).

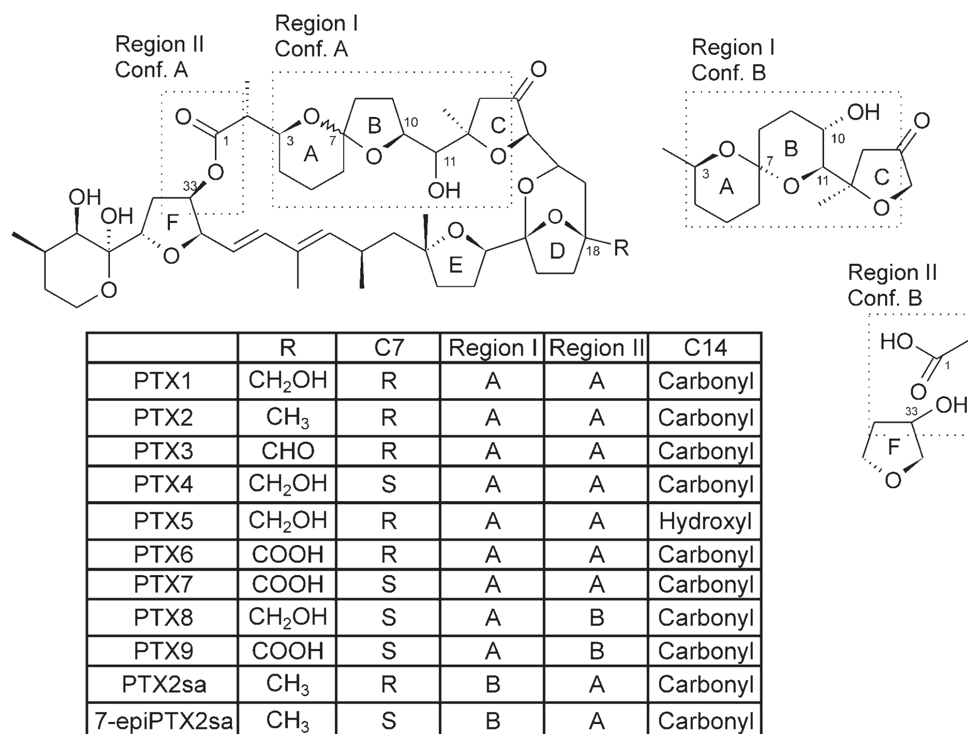


	R1	R2	R3	R4	R5	R6
OA	CH_3	H	OH	OH	H	-
DTX1	CH_3	CH_3	OH	OH	H	-
DTX2	H	CH_3	OH	OH	H	-
DTX3	(H or CH_3)	H	O-Acyl	OH	H	-
DTX4	CH_3	H	OH	OH	3	11
DTX5a	CH_3	H	OH	OH	2	12
DTX5b	CH_3	H	OH	OH	3	12
DTX6	CH_3	H	OH	OH	6	OH
OA-D7a	CH_3	H	OH	OH	1	OH
OA-D7a	CH_3	H	OH	OH	2	OH
OA-D8a	CH_3	H	OH	OH	3	OH
OA-D9a	CH_3	H	OH	OH	4	OH
OA-D9b	CH_3	H	OH	OH	5	OH
2-deoxy-OA	CH_3	H	OH	H	H	-
7-deoxy-OA	CH_3	H	H	OH	H	-
OA-methyl ester	CH_3	H	OH	OH	CH_3	-
Oxidized OA-Da	CH_3	H	OH	OH	7	-
Oxidized OA-Da	CH_3	H	OH	OH	8	-
Oxidized OA-Da	CH_3	H	OH	OH	9	-
Oxidized OA-Da	CH_3	H	OH	OH	10	-

Figure 1.- Structures of okadaic acid, dinophysistoxins and some of their derivatives. The diolesters of okadaic acid are designated as OA-D#x, where # is the number of carbon atoms in the diol chain, and x is an arbitrary assignation. DTX6 is adiol ester that has received that specific designation. Oxidized OA-Dx are some derivatives produced by oxidation of diol esters. Norokadanone is a possible precursor of OA found in *Prorocentrum lima*.

Okadaic acid and its analogues are lipophilic compounds, which are highly soluble in organic solvents such as methanol, acetone, chloroform or dichloromethane. Their derivatives have different polarities and consequently their solubilities in organic solvents are extremely variable. Acyl-derivatives (DTX3) and diol-esters are less polar than their original toxins. On the other hand, oxidised derivatives of diol-esters, DTX4 and DTX5, are more polar than their corresponding toxins and their solubility in some organic solvents such as dichloromethane or hexane is reduced, especially in the case of the two latter types, DTX4 and DTX5, which are water-soluble.

Pectenotoxins are cyclic polyether lactones (FIG. 2), which differ structurally from each other mainly because of: a) the different degrees of oxidation at C_{18}, from methyl to carboxylic acid; b) the arrangement or epimerisation of the spiroketal ring system in rings A and B; and c) the opening of the large lactone ring in C_1-C_{33} (Burgess and Shaw, 2001; Quilliam, 2003). They are lipophilic and soluble in organic solvents, but some of them, such as aqueous acetonitrile, can produce transformations of the original toxins (Sasaki et al., 1998). They are also easily destroyed under strong alkaline conditions, but detailed stability studies have not been performed.



	R	C7	Region I	Region II	C14
PTX1	CH_2OH	R	A	A	Carbonyl
PTX2	CH_3	R	A	A	Carbonyl
PTX3	CHO	R	A	A	Carbonyl
PTX4	CH_2OH	S	A	A	Carbonyl
PTX5	CH_2OH	R	A	A	Hydroxyl
PTX6	COOH	R	A	A	Carbonyl
PTX7	COOH	S	A	A	Carbonyl
PTX8	CH_2OH	S	A	B	Carbonyl
PTX9	COOH	S	A	B	Carbonyl
PTX2sa	CH_3	R	B	A	Carbonyl
7-epiPTX2sa	CH_3	S	B	A	Carbonyl

Figure 2.- Structures of pectenotoxins (PTX) and pectenotoxin seco-acids (PTXsa)

Yessotoxins are ladder-shaped cyclic polyethers similar to brevetoxins, with molecular masses of around 1150. Yessotoxin is characterised additionally by the presence of two sulphate esters and a C_9 side chain (FIG. 3). The known analogues differ from yessotoxin in one or two of these characteristics, mainly by desulphation, hydroxylation, carboxylation or by changes in the length of the carbon chain (Satake et al., 1997; Tubaro et al., 1998). Adriatoxin is also a very closely related compound that differs from yessotoxins in that it lacks one ring and the lateral carbon chain (Ciminiello et al., 1998). The presence of sulpho-esters make these molecules more polar than most others in the DSP group, and it is easy to find large amounts of them in the culture medium (enriched or artificial seawater) when the producer dinoflagellates are grown (Franco, pers. comm). They can be adequately extracted with aqueous methanol (Yasumoto and Takizawa, 1997).

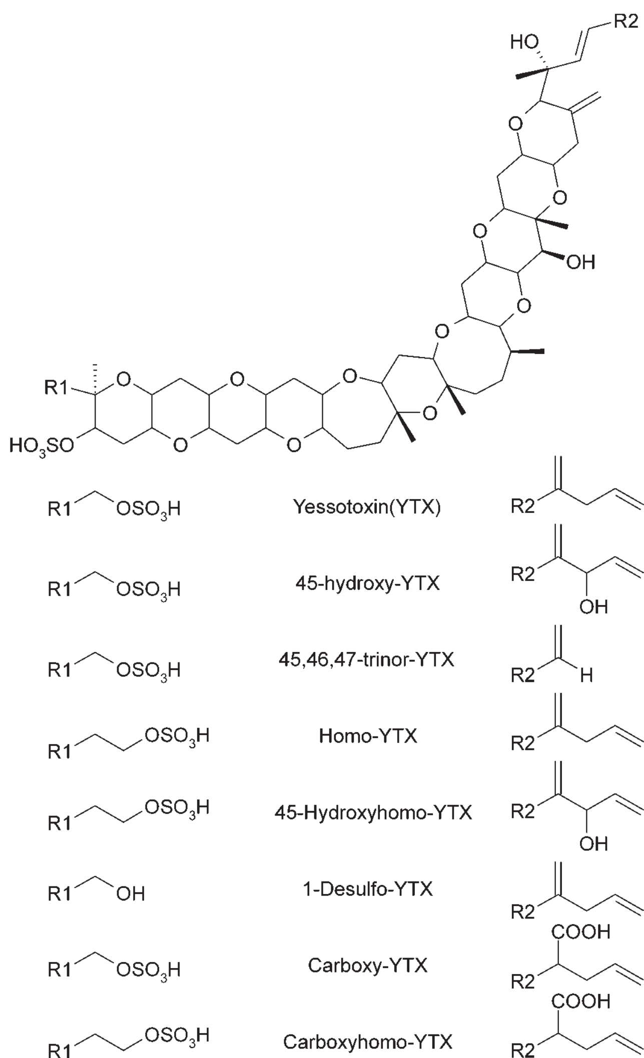
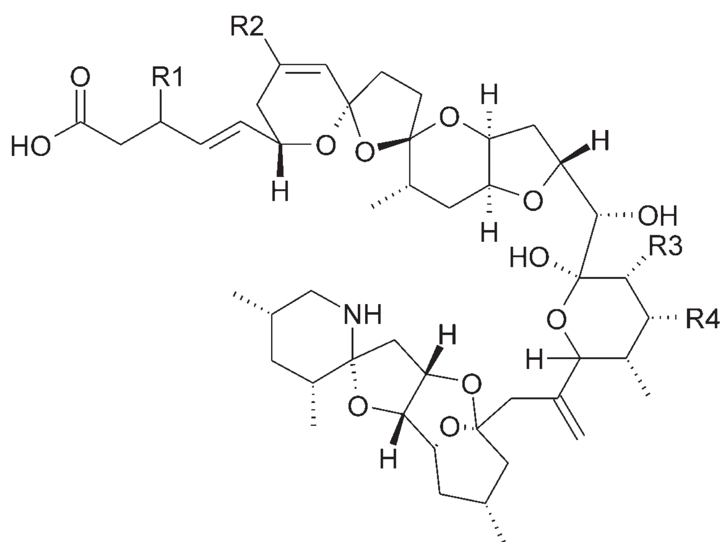


Figure 3.- Structures of yessotoxins and adriatoxin

Azaspiracids are a group of toxins characterised by an unique structure that includes a trispiro ring assembly, an azaspiro ring fused with a 2-9-dioxabicyclo[3.3.1]nonane and a terminal carboxylic acid group (Daiguji et al., 1998). Their molecular masses are around 840. The differences between them are due the methylation of C_8 and C_{22}, and to the hydroxylation of C_3 and C_{23}. Azaspiracids are less polar than what would be expected from the presence of an amine and a carboxylic acid, because these two functions appear to form an intramolecular ion pair (Quilliam, 2003). They are soluble in organic solvents but unstable in some of them, such as chloroform, under slightly alkaline conditions, and or during chromatography on silica-based supports (James et al. 2000)



R1	R2	R3	R4	
H	H	CH_3	CH_3	Azaspiracid (AZA)
H	CH_3	CH_3	H	AZA2
H	H	H	H	AZA3
OH	H	H	H	AZA4
H	H	H	OH	AZA5

Figure 4.- Structures of azaspiracids (AZA)

TOXICOLOGY

These four groups of lipophilic toxins affect humans in different ways. The typical DSP intoxication, caused mostly by the free acid forms of the okadaic acid group of toxins, is char-

Table 1.- Toxicity of some lipophilic toxins applied to mice by intra-peritoneal injection (modified from Fernández et al 2003c).

Group	Compound	LD50 (pg kg-l)	Reference	Pathology
Okadaic acid	OA	200	Yasumoto et al. (1989)	Diarrhoea; tumour promotion and induction
	DTX1	160	Yasumoto et al. (1989)	=OA
	DTX3	500	Yasumoto et al. (1989)	=OA
Pectenotoxins	PTX1	250	Yasumoto et al. (1989)	Hepatotoxic
	PTX2	230	Yasumoto et al. (1989)	Hepatotoxic; diarrhoea
	PTX3	350	Yasumoto et al. (1989)	*
	PTX4	770	Yasumoto et al. (1989)	*
	PTX6	500	Yasumoto (2000)	*
	PTX7	>5000	Sasaki et al. (1998)	*
	PTX8	>5 000	Sasaki et al. (1998)	*
	PTX9	>5000	Sasaki et al. (1998)	*
Yessotoxins	YTX	100	Yasumoto et al. (1989)	Cardiotoxic
	HydroxyYTX	500	Satake et al. (1996)	*
	TrinorYTX	220	Satake et al. (1996)	*
	HomoYTX	100	Satake et al. (1997)	*
	45-hydroxyhomo YTX	500	Satake et al. (1997)	*
	Desulfo YTX	500	Daiguji et al. (1998)	*
	CarboxyYTX	500	Ciminiello et al. (2000)	*
Azaspiracids	AZA	200	Satake et al. (1998)	Diarrhoea
	AZA2	110	Ofuji et al. (1999 a,b)	Diarrhoea
	AZA3	140	Ofuji et al. (1999 a,b)	Diarrhoea
	AZA4	470	Ofuji et al. (2001)	Diarrhoea
	AZA5	1 000	Ofuji et al. (2001)	Diarrhoea

acterised by diarrhoea, nausea, vomiting and, in some cases, abdominal pain, which starts from 3 to usually less than 12 hours after the ingestion of the contaminated organisms. In the first Japanese episode (Yasumoto et al. 1978) vomiting and diarrhoea (which were detected in 92% and 79% of the cases, respectively) occurred with a mean frequency of 4 times/day reaching 12 times/day in the most severe intoxications. Most individuals had recovered completely in about three days. There have been no human fatalities due to DSP intoxication recorded to date but obviously, the debilitating effects of this type of poisoning may last for several days. The toxic potency in mice is toxin-specific and associated with the presence of the free carboxylic acid function, as the forms in which this function is esterified have much less toxic power (Hu et al. 1992a; 1995) (TABLE 1). Notwithstanding, intoxications produced by okadaic acid esters (mostly water soluble) have been recorded (Vale and Sampayo, 2002c).

The minimum dose of okadaic acid required to produce symptoms in human adults has been estimated to be 48µg. OA main target organs for oral administration and intraperitoneal injection are different. If administered orally to rats, okadaic acid causes intestinal damage, diarrhoea and death, but has no detectable effect on the liver, while, when administered intravenously, it has little effect on intestinal function but affects the liver substantially (Berven et al, 2001).

Apart from these acute effects, the okadaic acid group of toxins seems to have some important chronic effects. They have been found to be potent tumour promoters (Fujiki et al., 1988) and the possibility that they are also inducers has recently been suggested (Ten-Hage et al., 2000; Creppy et al., 2002). Although existing observations of human populations are not conclusive, there is some evidence of associations with digestive cancer (oesophagus, stomach, colon and liver in men; and stomach and pancreas cancers in women) (Cordier et al., 2000). Additionally, in the green turtle *Chelonia midas* the appearance of a neoplasic disease, fibropapilomatosis, has also been linked to the abundance of *Prorocentrum lima*, a producer of DSP toxins of the okadaic acid group (Landsberg et al., 1999).

Okadaic acid and its analogues are inhibitors of the serine/threonine protein phosphatases, especially PP2A to a lesser extent, PP1B and minimally, PP2B. The ser/thr protein phosphatases are important molecules that perform the dephosphorylation of the phosphoserine, phospho-threonine residues of substrate proteins, and are critically involved in the regulation of cellular metabolism, DNA replication, transcription, RNA splicing, cell-cycle progression, differentiation and oncogenesis (Vanden Heuvel, 2002). Most of the effects of the okadaic acid-type toxins appear to be linked to the inhibition of these phosphatases. Diarrhoea is most likely associated with the hyperphosphorylation of ion channels (among other proteins), in the epithelial cells of the intestine, impairing the water balance (Cohen et al., 1990). Serine/threonine phosphorylations seem also to be related to DNA damage and genomic instability (Ten-Hage et al. 2000).

Few cases of pectenotoxin intoxications have been recorded. In December 1997, 100 people were poisoned in Australia after consuming *Donax deltoides*. At least 56 cases required hospitalisation. Nausea, vomiting and diarrhoea were the main symptoms of the intoxication. In March 2000, also in Australia, another intoxication was recorded, with the vector being, once again, the same species. The main toxin involved, as in the previous intoxication, was PTX2seco acid. One elderly woman became seriously ill and it was calculated that she had ingested approximately 150 µg of PTX2sa, which represents a dose of ca. 2µg of toxin kg^{-1} of body weight. This dose is much lower than what was found to be toxic by oral assays with mice (250 to 2000 µg kg^{-1}, Ishige et al., 1988), suggesting that perhaps human sensitivity to these kinds of toxins is much higher than that of mice and, consequently, questioning the efficiency of mice as model organisms in the study of human intoxication with these toxins (Burgess and Shaw, 2001). Notwithstanding, at least in the first of these episodes in Australia,

the presence of okadaic acid esters was observed (Quilliam et al. 2000), raising doubts about the real origin of at least part of the detected toxicity and, consequently, about the real toxicity of PTXs.

Pectenotoxins do not cause diarrhoea in mice when injected intraperitoneally, but they do when administered orally (Ishige et al. 1988). After intraperitoneal injection, serious damage is observed in the intestine and liver of mice. At the cellular/molecular level, they do not inhibit protein phosphatases but show a potent cytotoxicity (Terao et al., 1990, 1993), and may induce apoptosis (Fladmark et al., 1998). Micromolar concentrations of PTX6 have been shown to induce the depolarization of F-actin in neuroblastoma cells. As no other effect was found on specific signal transduction pathways or cell survival, cytoskeletal disruption is assumed to be a key mechanism in the toxicity (Leira et al., 2002) of this compound and very likely of the other PTXs.

To date Yessotoxins have not caused any human intoxications. This group of toxins has been shown to kill mice when injected intraperitoneally but, due to the presence of sulphate groups in the molecule, these compounds are not readily absorbed or metabolised in the digestive tract when administered orally. Consequently, they do not produce diarrhoea and have a high LD_{50} (TABLE 1). According to recent studies on oral toxicity in mice (Espenes et al. 2004), these toxins do not appear to be a serious risk to human health. Mouse intraperitoneal toxicity is high (100 µg kg^{-1}) mainly because these toxins produce severe damage to the heart muscle (Terao et al., 1990, 1993). At the cellular level, YTXs induce apoptosis in human neuroblastoma, but this effect is quantitatively lower than what was observed in okadaic acid (Alfonso et al., 2003). The action mechanism used by YTXs is not known but it is clear that they do not inhibit the activity of the serine/threonine phosphatases, as other lipophilic toxins do. It is also clear that their action differs from that of two other groups of lipophilic toxins which are structurally similar to YTXs -maitotoxins and brevetoxins- and this action is related to calcium modulation (Alfonso et al. 2003). Recent studies point to an increase in the activity of phosphodiesterases (PDE) as the main mechanism of action, but both the type of PDE involved and the precise way of interaction require additional studies (Alfonso et al. 2003). If the target were PDE III, which is abundant in heart muscle, cardiotoxicity would be easily explained.

Azaspiracids (AZAs) are a recently identified group of shellfish toxins that are responsible for an emerging type of shellfish intoxication in Europe, called azaspiracid poisoning (AZP) (Ofuji et al. 1999a,b). Cases of AZP were first reported in the Netherlands in November 1995, caused by the ingestion of mussels from Killary Harbour, on the west coast of Ireland (Daiguji et al. 1998; Marcaillou-Le Baut et al. 1998). Human symptoms caused by AZP toxins are similar to those caused by OA and DTXs, and include nausea, vomiting, severe diarrhoea and stomach cramps. Neurological disorders have not been reported. *In vivo* studies with mice have shown that the target organs of AZAs are the digestive tract, liver, pancreas, thymus, spleen and lungs. Data on chronic exposure of mice to AZA strongly suggest that this compound induces the formation of lung tumours without the contribution of an initiator (Ito et al. 1998, 2000; 2002). In relation to okadaic acid, injuries appear later but recovery is also much slower (Ito et al. 2002). The symptoms recorded in mice after ip injection were different from those of the okadaic acid group of toxins: aberrant neurological symptoms, such as respiratory difficulties, spasms, slow progressive paralysis of the limbs and, at higher doses, death within 20 minutes. One of the main cellular targets seems to be the citoskeleton, specially F-actin. In spite of the fact that it shares this capability with DSP toxins, AZA differs from the those in that it does not induce apoptosis and, consequently, the changes detected in the citoskeleton cannot be attributed to this degenerative process. It also increases the intracellular calcium and cAMP. The molecular action mechanisms are not known, but these changes do not

depend on extracellular calcium and are insensitive to okadaic acid, indicating that both toxins might interact at the same transduction level (Román et al., 2002).

METHODS FOR DETERMINATION OF OAS, PTXS, YTXS AND AZAS IN SHELLFISH

As in the case of other marine toxins, two different approaches may be used to address the determination of these toxin groups depending on the type of information required: assay methods and analytical methods. Assay methods measure an integrated biological or biochemical response which is usually converted into equivalents of a representative toxin of the family on the basis of a previous dose/response curve, and this correlates with overall toxicity. Assays for marine toxins comprise *in vivo* bioassays, using live animals, and *in vitro* assays. The latter may be further categorised into functional and structural assays. Functional assays measure a response linked to the action mechanism of the toxin (e.g. phosphatase inhibition assays) and therefore correlate well with real toxicity. Structural assays (e.g. immunoassays) are based on the measurement of the interaction between the antibodies and specific toxin structures that are not necessarily related to the biological activity of the toxin, so, correlation with actual toxicity is not always as good as in the case of functional assays.

Analytical methods generally involve a preliminary toxin separation step and further identification and quantification of the individual toxins by measuring an instrumental response that is proportional to the concentration of the toxin. This requires the previous calibration of the instrumental equipment using toxin standards of each one of the toxins to be quantified. The response should be converted to toxicity values on the basis of specific conversion factors and the overall toxicity is determined as the sum of the individual toxicities. This category of methods includes High Performance Liquid Chromatography (HPLC) with different detection methods (Monochromatic and Spectral UV, Fluorescence, Mass Spectrometry) and Capillary Electrophoresis.

Apart from economical considerations, the choice of method mainly depends on the objectives of the study. Analytical methods are mostly applied for research or confirmatory purposes when the identification and quantification of each one of the toxins present is required. For public health protection purposes, assays are usually applied, as the priority is to determine the potential global toxicity.

The determination of the lipophilic toxins dealt with in this review, is specially complex and challenging because a complex mixture of toxins, from the same and/or different families, exhibiting common solubility properties but with different modes of action, coexist in phytoplankton and shellfish.

Assays

In vivo assays

In vivo bioassays, and specifically, mouse bioassays, have been applied worldwide for the determination of OA and DTXs.

Due to common solubility properties, most of these procedures can also detect PTXs, YTXs and AZAs, although the range of substances detected, sensitivity and toxin recovery depend largely upon the selection and ratio of theorganic solvents used for extraction and cleanup steps as well as on the anatomical part used as the analytical sample. In spite of its widespread use, so far none of these procedures has been evaluated in a formal collaborative study and therefore, performance characteristics, such as accuracy and precision, are not fully known.

Intraperitoneal mouse bioassays

The original mouse bioassay procedure described by Yasumoto et al. (1978) has been applied in monitoring programmes worldwide. Although, as originally established, the procedure involves the calculation of i.p. toxicity in mouse units (1 MU is defined as the minimum amount of toxin required to kill 2 out of 3 mice in 24 hours following i.p. injection), most regulatory laboratories use mouse survival time for the evaluation of DSP toxicity with no general consensus on the appropriate observation period -acceptable criteria may vary from "two out of three mouse deaths in less than five hours" to "two out of three mouse deaths in less than 24 hours". According to this method, a sample of homogenized digestive gland is extracted thrice with acetone and the filtrates combined. The solvent is removed by rotary evaporation under reduced pressure and the residue is resuspended in a small volume of Tween 60. An aliquot of this solution is injected intraperitoneally into each of three mice weighing between 18 and 20 g. The mice are observed for a period of time ranging between 24 and 48 hours, and toxicity determined on the basis of survival time. The detection limit of the assay depends on the specific conditions of the procedure, but it could be about 0.2 MU g-1 or 800 mg OA equivalents kg-1 digestive gland. This procedure detects the presence of OA and DTXs, as well as the PTXs, YTXs and AZAS groups, although in many cases it is difficult to discriminate which substances cause the death of the mice. Other lipophilic toxins, such as spirolides, gymnodimine, brevetoxins and ciguatoxins are co-extracted, if present in shellfish. Fatty acids may yield false positives (Takagi et al.,1984, Lawrence et al., 1994) and interference by low levels of PSP and ASP toxins, even at concentrations undetectable by the AOAC (1995) mouse bioassay, may also present problems. Among all the bioassays for DSP detection, this method is the least selective, and when positive results are obtained, it is not always feasible to assign the response to the presence of known toxins. However, after many years of routine application throughout the world, it has proven to be very effective to screen for the presence of DSP and other lipophilic toxins for public health protection purposes.

Several versions of the mouse bioassay including additional purification steps have been further developed for the purpose of eliminating interferences from PSP and salts (Yasumoto et al., 1984) and free fatty acids (Le Baut et al., 1990; Lee et al. 1987) or for using whole shellfish tissues as analytical samples (Hannah et al., 1995). The monitoring and control of the whole group of toxins currently regulated in EU Decision 225/2003 (OA, DTXs, PTXs, YTXs and AZAs) with a single mouse assay, implies the use of whole shellfish tissues for the assay. because azaspiracids, at least, are distributed throughout the whole body. Yessotoxins may yield positive results in most mouse bioassay procedures even at levels far below the limit regulated in Decision 225/2002. The control of all these toxin groups by means of a single mouse assay procedure, at the levels currently regulated in the EU, represent a huge analytical challenge as yet not thoroughly resolved, although attempts to develop such a complex procedure are ongoing (Yasumoto, unpublished data).

The rat bioassay

The rat bioassay (Kat, 1983) is an oral toxicity test in which the presence of DSP toxins is determined by assessing and qualitatively scoring the faeces consistency (diarrhoeic or soft) of pre-starved Wistar rats fed with shellfish digestive glands mixed with the diet. A faeces consistency that is not considered to be normal suggests the presence of DSP toxins in the sample and, in this case, shellfish is usually considered to be unsafe for human consumption. The detection limit of the assay is ca. 800 mg OA equivalents kg-1 of digestive gland.

The rat bioassay simulates the mode of human intoxication and does not involve the extraction of toxins. However, the assay is only semi-quantitative and the dynamic range is poor. The precision is also poor, due to broad individual variation in sensitivity and symptomology

among rats, particularly when few animals are assayed (typically one to three rats). The assay is able to detect the presence of OA, DTXs and some PTXs, but other PTXs and YTXs are not detected by this method. Other diarrhoeic agents including intestinal pathogens might complicate the interpretation of faeces consistency. Azaspiracids produce diarrhoeic effects, therefore the rat bioassay may be used to detect them. Unfortunately, given the absence of quantitative standards for toxicity testing, the detection limit of this oral test has not yet been determined.

Other in vivo *assays*

Other in vivo assays as an alternative to mouse bioassays include the suckling mouse bioassay, which has been used to test the enteropathogenicity of OA, DTX1 and DTX3 (Hamano et al., 1986) and the Daphnia magna bioassay (Vernoux et al. 1993) based on the measurement of the inhibition of Daphnia mobility due to the presence of OA.

Mammalian bioassays have been comprehensively reviewed by Fernández et al. (2003b).

***In Vitro* Functional Assays**

Phosphatase inhibition assay (OA and DTXs)

OA and certain analogues (DTX1, DTX2) are potent and specific inhibitors of the activity of serine and threonine protein phosphatases 1 and 2 (PP1 and PP2A), two of the major phosphatases in the cytosol of mammalian cells, and this action results in the rapid accumulation of phosphorylated proteins (Bialojan and Takai, 1988). This property was used by Holmes et al. (1991) to develop a sensitive functional radioassay based on the inhibition of PP1 by OA and on the use of a labelled substrate, 32 P-phosphorylase. The assay includes a previous toxin fractionation by liquid chromatography and exhibits a detection limit of 0.1 pg OA equivalents kg-1 tissue. It was further applied by Chen et al. (1993) for the detection of microcystins, another class of phycotoxins produced by some cyanobacteria and capable of inhibiting PP1. Also based on a radiolabelled substrate, Honkanen et al. (1996) developed a rapid protein phosphatase assay that was used to detect OA in oyster extracts with a detection limit of 0.2 µg g-1 of shellfish tissue. In spite of the high sensitivity of the radioactive protein phosphatase assays, they have not been extensively applied by regulatory laboratories, because of the high cost of radiollabed compounds and the difficulties associated with their handling and management. Additional useful versions of the PPase inhibition assay based on colorimetric and fluorimetric detection were successfully developed and refined. Thus, Takai and Mieskes (1991) explored the application of p-nitrophenol phosphate as a substrate for PP2A and suggested that could applied in the colorimetric detection of OA in shellfish. Based on the same substrate, Simon and Vernoux (1994) proposed an enzyme assay involving the use of a semi-purified preparation of PP1 and PP2A obtained from rabbit muscle. The lowest concentration detected was 100 ng g-1 when tested with mussel tissues. Based on Takai´s method, Tubaro and co-workers developed a simple colorimetric microplate assay using commercial PP2A. After toxin extraction, a previous purification step using liquid/liquid partitioning is required. The detection limit is 0.063 ng mL-1 or 2 ng g-1 shellfish digestive gland (Tubaro et al., 1996).

Vieytes and collaborators (Vieytes et al., 1997) developed a fluorescent microplate assay using commercial PP2A and tested different fluorescent substrates such as: 4-metil-umbelliferyl phosphate and fluorescein diphosphate. The assay requires a fluorescence plate reader and is very quick and simple. After toxin extraction with 80% methanol, further purification steps are not required. The detection limit is 3.2 pg OA mL-1. A study comparing the colorimetric and fluorimetric versions of the assay (Mounfort et al. 1999) concluded that the fluorimetric assay offered better sensitivity and precision. The PP inhibition assay can detect OA, DTX1, DTX2 and presumably other analogues of these toxins. Other toxins from the group,

such as DTX3, diol esters or DTX4 like compounds, do not inhibit the enzymes, but their presence can be detected after hydrolysis (Mounfort et al., 2001).

PTXs, YTXs and AZAs are not protein phosphatase inhibitors and therefore cannot be detected with this type of assay.

Cytotoxicity assays

These functional assays are based on the measurement of the cell response after toxin exposure by microscopic examination of changes in cell morphology or, in most cases, by the determination of cell viability after incubation with reagents and toxins.

Aune et al. (1991) developed a cytotoxity assay based on the microscopical examination of morphological changes in freshly prepared rat hepatocytes exposed to DSP toxin extract and purified OA. Changes in cell morphology such as "blebbing" and surface irregularities are observed microscopically. The assay makes it possible to differentiate between OA and DTX1, which induce irregular-shaped cells with surface blebs, PTX1, which produces dose-dependent vacuolisation and YTXs, which do not induce changes in the shape of the cells but produce tiny blebs on the surface. The assay is qualitative, as the cell changes produced by the toxins are not dose dependent.

The high toxicity of OA in KB cells, a human cell line derived from epidermoid carcinoma, was used to develop a method based on the determination of the minimal active concentration that induces changes in cell morphology (Amzil et al., 1992). The results obtained with the KB cell assay were found to correlate well with HPLC and mouse bioassay results. Also utilizing KB cells, Tubaro and co-workers (Tubaro et al., 1996) developed a quantitative colorimetric assay based on the metabolic conversion of a tetrazolium dye (MTT) yielding a blue-coloured formazan product which can be read for absorbance with a microplate scanning spectrophotometer. The assay was shown to be effective in detecting OA in mussel samples at a detection limit of 50 ng g-1 digestive gland in a 24 hour endpoint assay. 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. It was concluded from the results, that the technique, based on the determination of the minimal active concentration of DSP toxic extracts inducing morphological changes, is specific for OA and preferable to the determination of a 50% inhibition concentration (IC50) by a cell culture method.

Morphological changes caused by OA in fibroblasts were the basis of a cell assay (Fessard et al., 1994) that was further investigated with a more specific assay based on changes in the actin cytoskeleton (Diogène et al., 1995).

Recently, a functional assay for YTX detection was developed based on the accumulation of fragmented E-cadherin in MCF-7 cells induced by YTX. Concentration-dependent increases of a 102.1± 3.1 kDa (n = 11) protein are detected by Immunoblotting using anti- E-cadherin antibodies. The assay is very sensitive (detection limit around 100 ng YTX equivalent g-1 of digestive gland) and specific, but the complexity of the process required to obtain the final results may restrict its further application by monitoring laboratories.

Cytoxicity assays have proven to be valuable tools in pharmacological research and they appear to be promising as rapid screening methods. However, their application in toxin detection with regulatory purposes is still limited and requires additional validation efforts through collaborative testing.

Other functional assays

A quick assay specific for YTX determination has recently been developed by Alfonso et al. (2003). YTX decreases, in a dose-dependent manner, the intracellular adenosine 3´, 5´-

cyclic monophosphate (cAMP) levels by increasing the activity of the phosphodiesterases (PDEs). The assay utilizes the fluorescent derivative of cAMP, anthranyloyl-cAMP whose fluorescence decreases in time owing to the hydrolysis effect of PDEs and is measured in a plate reader. The linear response rate of hydrolysis from different concentrations of YTX was between 0.1 and 10 µM. This fluorescent assay can detect YTX at concentrations over 0.5µM.

In Vitro Structural assays

Immunoassays

Several immunoassays with different configurations have been developed for OA and DTX detection using antibodies prepared against OA with some cross reactivity against certain analogues. Levine et al. (1988) developed a radioimmunoassay (RIA) based on the competitive binding of OA from the samples with 3H-OA and on the measurement of inhibition by scintillation counting. The method is very sensitive, with a detection limit of about 0.2 pg OAmL-1 but its complexity and the use of radioactive compounds limit its application by monitoring laboratories.

Two enzyme-linked immunosorbent assay (ELISA) test kits, the DSP Check and the Rougier Bio-Tech have been developed for rapid screening of OA, although only the former is available commercially at the present time. The DSP-check (R-Biopharm) utilizes monoclonal antibodies developed by Usagawa and co-workers (Usagawa et al., 1989). The assay is very quick and simple and the detection limit is about 20 ng OA g-1. Antibodies show a cross reactivity of 70% with DTX1 (Usagawa et al., 1989), 40% with DTX2 (Carmody et al., 1995b) and do not cross react with DTX3, which would have to be hydrolysed to obtain at least qualitative results. PTXs and YTXs are unreactive.

The Rougier Bio-Tech kit was based on the use of monoclonal antibodies against OA and an anti-idiotipyc antibody (Shewstowsky et al., 1992), linked to the solid phase that competes with the OA from the samples for binding sites on the antibody. The sensitivity of the antibody for DTX1, DTX2 is considerably lower than that exhibited for OA (10-20 fold) and DTX3 is not detected. However, certain OA derivatives such as diol esters, DTX4 and DTX5 present an affinity similar to that of OA (Lawrence et al., 1998). PTXs and YTXs are not detected.

Major progress has been made recently in the detection of YTX and PTXs by ELISA methods and antibodies against YTX and PTX have been developed (Garthwaite et al., 2001). Recently, a combination of immunoassay and detection with disposable screen printed electrodes was developed (Tang et al. 2003).

Immunoassays present important advantages in toxin detection. They are highly sensitive, and some versions can be developed in the format of quick, easy-to-use test kits that can even be applied in the field. However, upon application, it should be always taken into account that the cross reactivity of the antibodies is limited to components with compatible epitopic sites and therefore not all the naturally occurring toxin analogues will necessarily be detected, with the subsequent risk of false negatives.

Chemical Methods

Liquid chromatography- fluorescence detection(HPLC-FD)

Among chemical methods, High Performance Liquid Chromatography with fluorimetric detection (HPLC-FD) has been widely used for many years in the determination of OA and DTXs. Due to the lack of chromophores exhibited by these molecules, most methods are based on precolumn chemical derivatization of the toxins utilizing fluorescent reagents and further separation and detection of the fluorescent ester derivatives. A number of derivatization compounds that react with the carboxyl group of OA and certain DTXs (DTX1, DTX2 and isomers) have been described. Among these, the most widely used, because of

its selectivity and sensitivity, is 9-anthryldiazomethane (ADAM) (Lee et al., 1987). The quantification limit is about 100 ng g^{-1} tissue or 10-20 ng g^{-1} if digestive glands are used in the analysis. Acyl-derivatives of OA and DTXs are difficult to separate with the Lee´s method due to its high molecular weight and lipophilicity, but its presence can be investigated by converting DTX3 to its corresponding parent compound after alkaline hydrolysis of the extracts (Yasumoto et al., 1989. Fernández et al., 1996). Diol esters, DTX4 and DTX5 cannot be directly investigated due to the esterification of the carboxyl moeity but, as in the case of DTX3, they can be indirectly determined after alkaline hydrolysis.

The ADAM procedure and its critical steps have been extensively investigated and some modifications have been introduced to improve the reliability of the method (Quilliam et al. 1995).The use of in situ produced ADAM has been proposed (Quilliam et al. 1998). Although poor stability of the ADAM reagent and the possibility of toxin losses, during the silica column clean-up step required after derivatization, have been described as the main drawbacks of the method, a thorough evaluation of HPLC methods showed the ADAM method as the most reliable (Ramstad et al, 2001a,b). Other investigated reagents include 1-bromoacetylpyrene (BAP) (Dickey et al., 1993) that showed better stability but lower selectivity and sensitivity than ADAM, several cumarin derivatives (Shen et al., 1991, Luckas et al., 1992, Marr et al. 1994), 2-(anthracene-2,3-dicarboximido) ethyl-trifluoromethanesulfonate (AE-Otf) (Akasaka et al., 1996b) which can also be applied for DTX3 determinations (Akasaka at al, 1996 a) and luminarin-3 (James et al. 1998).

Regarding pectenotoxins, congeners having a carboxyl group such as PTX6 and PTX7 can be determined using the ADAM reagent (Yasumoto et al. 1995). For PTX1 and PTX4, the use of anthrylcarbocianide (Lee et al. 1989a) was proposed. For the determination of the yessotoxins an alternative procedure has been developed, that uses a dienophile reagent DMEQ-TAD (4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione) for fluorescent labelling (Yasumoto and Takizawa, 1997)-. This fluorigenic reagent, has also been proposed to determine PTX2, (Sasaki et al., 1999). So far, no LC-FD methods have been developed for AZA analysis.

Liquid chromatography-mass spectrometry (LC/MS)

The development and application of liquid chromatography-mass spectrometry (LC/MS) for the determination of marine toxins over the last two decades, and the recent developments in multi-toxin analytical methods have led to great progress in the advancement of knowledge and the management of the complex phenomenon of shellfish toxicity associated with lipophilic toxins. LC/MS is currently the most powerful analytical tool to identify and determine multiple toxins. In addition to its high sensitivity, specificity and selectivity, this technique is able to provide valuable structural information in the confirmation of toxin identities as well as in the identification of new toxins and it does not require the complex derivatization and purification steps which are needed for HPLC-FD methods. Although, as in the case of other chemical methods, calibration standards are required for method development and quantitation, LC-MS methods can provide relevant information in the presence of closely related compounds of a known structure, even if the toxin standard for calibration is available only for one relevant toxin of the group. There are different types of mass spectrometers commercially available (single quadruple, triple quadruple, ion traps, time-of-flight…) that differ mainly in sensitivity and capability to provide ion fragmentation for structural information and selective detection. Electrospray ionization (ESI) is the most commonly used Atmospheric Pressure Ionization (API) source for the detection of marine toxins, although atmospheric pressure chemical ionization (APCI) sources have proven useful for some of these. A number of specific LC-MS methods differing in mobile phase, type of buffer, pH, ionic strength, stationary phase, elec-

trospray mode (positive or negative), have been developed for the detection of OA, DTX1 and DTX2 (Pleasance el al., 1990; Quilliam et al., 1995; Draisci et al., 1998a; Suzuki and Yasumoto, 2000), DTX3 and diol esters of OA and DTX1 (Hu et al. 1992b; Marr et al., 1992, Suzuki et al. 2004), Pectenotoxins (Suzuki et al., 1998; Suzuki and Yasumoto, 2000), Yessotoxins (Draisci et al., 1998b) and Azaspiracids (Ofuji et al., 1999b; Draisci et al, 2000). Current existing LC/MS methods for the determination of lipophilic toxins, and the influence of different features and parameters on the performance of LC/MS have been comprehensively reviewed by Quilliam (2003).

An important issue to be taken into account when applying LC-MS is that the ionization eficiency of the analytes may be significantly affected due to matrix components that are accumulated on the LC column after repeated injections. The inclusion of cleanup stages by solid phase extraction have been suggested to remove matrix effects (Ofuji et al. 1999b, Suzuki et al., 2000). Interference with ionization may also vary from matrix to matrix. This effect has been described previously by Ito and Tsukada (2001), who concluded that the suppression of the ionization efficiency due to co-eluting substances may cause variation in the responses, making quantification difficult by using pure standard solution, suggesting the use of standard addition methods for LC-MS quantification of DSP toxins.

LC-MS is being used in the routine testing of DSP and PTX toxins in New Zealand to manage commercial shellfish harvesting (McNabb and Holland, 2003) and is currently applied to complement the mouse assay in monitoring programmes (Hess et al., 2003)

Capillary electrophoresis

Capillary electrophoresis is a technique that provides high resolution separation and requires a very small sample size. Some methods have been developed by Gago Martínez et al (2003 a,b).

TOXIC OUTBREAKS: TOXIN PRODUCTION AND PHYTOPLANKTON DEVELOPMENT

TOXIN PRODUCTION

Two dinoflagellate genera produce toxins of the okadaic group, *Dinophysis* and *Prorocentrum. Prorocentrum lima*, *P. maculosum* and *P. hoffmmanianum,* are photosynthetic species that can be cultured in standard media for autotrophic algae. The toxins in these species are, therefore, products of the photosynthetic activity. On the other hand, no (pigmented) species of *Dinophysis* has been cultured to date, suggesting that they could be heterotrophic or at least mixotrophic organisms (Jacobson and Andersen, 1994). If so, the DSP toxins they contain would either be synthesised by the cells or acquired by ingesting producing organisms.

Little is known about the biosynthesis of these compounds. It would seem that certain amino acids, such as valine, might be among the precursors of these molecules (Souto et al. 2001). Norokadanone (FIG. 1) is probably an intermediate product in the biosynthetic route (Fernández et al., 2003d).

Prorocentrum cells produce a wide range of toxins of the okadaic acid group. Okadaic acid, DTX1, DTX2, DTX4, DTX5 and DTX6 are the main toxins that have been found in phytoplankton cells (Tables 2 and 3). Frequently a large proportion of the DSP toxins are stored in the cells as sulphated esters, mainly DTX4 and DTX5 (Hu et al. 1992a, 1995; Quilliam et al. 1996). These esters can be hydrolysed to diol-esters and to the acidic forms of the toxins. Nevertheless, in some strains only the acidic forms of certain toxins were found, suggesting that these compounds can be synthesised *de novo* (Bravo et al. 2001, Holmes et at 2001). As the esters are much less toxic than the acidic toxins (Hu et al., 1992a; 1995), the storage of these compounds might be used as a strategy to protect the enzymatic system of the cell from the toxicity of the free forms, and they may or may not act together with the isolation in intra-

cellular compartments as found by Franchini et al. (2003). Additionally, diol-esters may be an effective carrier for moving OA between different cellular compartments, because this molecule does not have an ionisable group (Quilliam et al. 1996).

It has been observed that toxin levels did not increase when the dinoflagellates were maintained in dark, with the production starting when the culture was returned to the regular light-dark cycle (Pan et al., 1999). This suggests that the production of toxins may be a metabolic light-mediated process, as supported by the localisation of OA predominantly in the peripheral chloroplasts (Zhou and Frizt, 1994; Rausch de Traubenberg et al., 1995). However, the fact that *D. rotundata*, in which chloroplasts have not been observed, may contain high levels of DTX1 (Cembella, 1989) raises doubts about the fact that the production of toxins is a photosynthesis mediated process.

Aikman et al. (1993) observed that the kinetics of OA production in a culture of *Prorocentrum hoffmannianum* was very similar to that of other compounds produced such as proteins, carbohydrates or chlorophylls, with an increase during the log phase of culture and remaining almost constant during the stationary phase. Rausch de Traubenberg and Morlaix (1995) confirmed that the toxin production rate is very similar to growth rate, although in some cases the production of toxins may stabilise after the cultures enter the stationary phase (Holmes et al., 2001) or continue after the cessation of cell division (Jackson et al., 1993). An increase in OA production or OA cellular content, in response to physiological stress appears to take place. Sharp changes in salinity in *Prorocentrum hoffmanianum* (Morton et al., 1994), or nutrient deficiency in *Dinophysis acuminata* and *D. acuta* cells (Johansson et al., 1996) induced a higher production (the first case), or cellular burden (Dinophysis) than the original populations. However, owing to the similarity between growth and OA production, it is often difficult to differentiate the direct effects of environmental factors, such as light, temperature or salinity, on the kinetics of DSP production, from their general effect on the growth processes (Jackson et al., 1993; Morton et al., 1994).

As it would be expected, the assimilation of nitrogenous nutrients (NH4+, NO3=) does not have any effect on DSP toxin production since they do not contain nitrogen (with the exception of DTX5). Nevertheless, many amino acids (leucine, valine, lysine, threonine or serine) may even double the toxin production of Prorocentrum lima, affecting the toxin profile (Souto et al., 2001). These findings have lead to postulate that valine, at least, is involved in the biosynthesis of the toxins of the OA group (Norte et al., 1994; Souto et al., 2001).

Toxin acquisition by ingestion of producing organisms: bacteria and picophytoplankton

All available data indicate that *Prorocentrum lima* produces OA, independently of the presence of intra and/or extracellular bacteria (Morton et al., 1994; Rausch de Traubenberg and Morlaix, 1995; Rausch de Traubenberg et al., 1995). However bacteria cannot be ruled out as vectors of DSP toxin transfer to the food chain since a low amount of toxin (0.1 to 1 %) has been associated with free-living bacteria, which might be ingested by bivalves and other organisms (Rausch de Traubenberg and Morlaix, 1995).

Several authors have postulated that the DSP toxins in *Dinophysis* cells would not be due to synthesis but rather to the ingestion of picophytoplankton which produces the toxins (Imai and Nishitani, 2000; Nishitani et al., 2002; Imai et al., 2003). This theory is based on the mixotrophy observed in some photosynthetic *Dinophysis species*, such as *D. acuminata* and *D. norvegica* (Jacobson and Anderson, 1994), and in the presence of picophytoplankton attached to the surface of *Dinophysis* cells. This might explain the great differences in toxin content per cell in *Dinophysis*. Notwithstanding, if picophytoplankton were the producer and the cells were large enough to be efficiently retained by the bivalves, the accumulation of DSP toxins by

Table 2.- Toxin content of the okadaic acid group of the cells of several *Prorocentrum* species.

Species	Origin	Sample type	Toxin Content (pg·cell-1)	Reference
P. lima	Spain	1	5.4-26.0 OA; 6.0-14.3 DTX1	Lee et al., 1989
P. lima	Canada	1	1.4-8.0 OA+DTX1	Jackson et al., 1993
P. hoffmannianum (SIU 882a)	US Virgin Islands	1	3.46-20 OA	Aikman et al., 1993
P. hoffmannianum (SIU 882a)	US Virgin Islands	1	4.6-53.8 OA	Morton et al., 1994
P. lima (PL2V)	Guillard-Provasoli Center	1	5.0 OA; 0.6 DTX1	Pillet et al., 1995
P. belizeanum	Belize	1	12.45 OA	Morton et al., 1998
P. lima	Japan	1	0.3-1.3 OA; n.d. DTX1	Koike et al., 1998
P. lima (MARS1)	France	1	1.9 OA; 0.8 DTX1	Barbier et al., 1999
P. lima (PL2V)				
P. lima (MARS1)	Spain	1	14.3 OA; 2.7 DTX1	Barbier et al., 1999
P. lima	Canada	2	8.4 DTX1; trace OA	Lawrence et al., 2000
P. lima (PL2V)	Cíes lagoon (Spain)	1	8.75 OA; 8.11 OAE; 3.02 DTX1	Bravo et al., 2001
P. lima (PL16V)	Cíes lagoon (Spain)	1	12.87 OA; 9.44 OAE; 4.60 DTX1; 0.97 DTX2; 0.70 DTX2E	Bravo et al., 2001
P. lima (PL17V)	Cíes lagoon (Spain)	1	9.70 OA; 7.24 OAE; 3.30 DTX1; 0.49 DTX2; 0.60 DTX2E	Bravo et al., 2001
P. lima (PL20V)	Cíes lagoon (Spain)	1	8.37 OA; 8.78 OAE; 1.84 DTX1; 1.14 DTX2; 1.60 DTX2E	Bravo et al., 2001
P. lima (PL24V)	Cíes lagoon (Spain)	1	6.65 OA; 6.75 OAE; 2.73 DTX1; 0.36 DTX2; 0.52 DTX2E	Bravo et al., 2001
P. lima (PL3V)	Aldán (Spain)	1	1.86 OA; 8.96 OAE; 3.05 DTX1; 0.10 DTX2; 0.68 DTX2E	Bravo et al., 2001
P. lima (PL7V)	Bueu (Spain)	1	4.26 OA; 17.51 OAE; 0 DTX1; 0.17 DTX2; 1.33 DTX2E	Bravo et al., 2001
P. lima (PL4V)	Aldán (Spain)	1	2.40 OA; 2.88 OAE; 11.62 DTX1; 0.33 DTX2; 0.51 DTX2E	Bravo et al., 2001
P. lima (PL9V)	Bueu (Spain)	1	3.62 OA; 1.94 OAE; 12.45 DTX1; 0 DTX2; 0 DTX2E	Bravo et al., 2001
P. lima (PL5V)	Vigo (Spain)	1	1.53 OA; 1.92 OAE; 3.21 DTX1; 0 DTX2; 0.26 DTX2E	Bravo et al., 2001
P. lima (PL11V)	Canido beach (Spain)	1	3.34 OA; 4.91 OAE; 7.63 DTX1; 0.06 DTX2; 0 DTX2E	Bravo et al., 2001
P. lima (PL12V)	Canido beach (Spain)	1	5.11 OA; 7.02 OAE; 12.39 DTX1; 0.35 DTX2; 1.02 DTX2E	Bravo et al., 2001
P. lima (PL13V)	Canido beach (Spain)	1	5.36 OA; 4.65 OAE; 11.88 DTX1; 0.65 DTX2; 0.20 DTX2E	Bravo et al., 2001
P. lima (PL14V)	Canido beach (Spain)	1	5.69 OA; 6.34 OAE; 11.57 DTX1; 0.19 DTX2; 0.53 DTX2E	Bravo et al., 2001
P. lima (PL6V)	Bueu (Spain)	1	0.19 OA; 0.77 OAE; 1.01 DTX1; 0 DTX2; 0 DTX2E	Bravo et al., 2001
P. lima (PL26V)	Areas beach (Spain)	1	2.61 OA; 5.85 OAE; 4.13 DTX1; 0.22 DTX2; 0.80 DTX2E	Bravo et al., 2001
P. lima (PL27V)	Canelas beach (Spain)	1	3.10 OA; 8.82 OAE; 8.26 DTX1; 0 DTX2; 0.99 DTX2E	Bravo et al., 2001
P. lima (PL28V)	Canelas beach (Spain)	1	1.73 OA; 3.00 OAE; 2.70 DTX1; 0 DTX2; 0.55 DTX2E	Bravo et al., 2001
P. lima (PL29V)	Canelas beach (Spain)	1	3.10 ± 0.66 OA; 3.25 OAE; 4.51 DTX1; 0.49 DTX2; 0.04 DTX2E	Bravo et al., 2001
P. lima (P6)	New Caledonia	1	1.1-15 OA; 0.2-1.5 7-Deoxy-okadaic acid	Holmes et al., 2001
P. lima (PRL-1)	México	1	5.2 OA + DTX1	Heredia Tapia et al., 2002

Table 3. Reported concentrations of DSP toxins in *Dinophysis* species. Sample type: 1. sorted cells; 2. raw sample; 3. Sorted cells maintained in culture; 4. fractioning using plankton nets (20-50 µm). Species: DF=*D. fortii*, DM= *D.Mitra*, DT= *D.tripos*, DR= *D.rotundata*, Dat= *D.Acuta*, Dac= *D.acuminata*, DN= *D.Norvegica*, Dspp= *Dinophysis* spp, DK= *D. skagi*, DSa= *D. sacculus*, DI= *D. infundibulum*, DC= *D.caudata*, DR=*D. rudgei*

Species	Origin	Date	Sample type	Toxin content (pg·cell-1)	Reference
DF	Japan	86-88	1	13-191.5 DTX1 42.5 PTX2	Lee et al., 1989
DF	Japan	May-88	1	23.0 PTX2	Lee et al., 1989
DM	Japan	Aug-87	1	10.0 OA	Lee et al., 1989
DR	Japan	Jun-88	1	101.0 DTX1	Lee et al., 1989
DT	Japan	Aug-88	1	36.0 DTX1	Lee et al., 1989
DAt	Spain	Oct-87	1	9.4 DTX1	Lee et al., 1989
DAt	Norway	Aug-87	1	4.0 OA 4.2 DTX1	Lee et al., 1989
DN	Norway	Aug-87	1	0.8 OA 2.5 DTX1	Lee et al., 1989
DN	Norway	Aug-87	1	14.0 DTX1	Lee et al., 1989
DAc	France	Jun-84	1	1.6 DTX1	Lee et al., 1989
DAc	Japan	Jun-84	1	trace OA	Lee et al., 1989
DN	Canada	Jul-89	2	32.6 ±5.2 OA	Cembella, 1989
DAc	Canada	Jul-89	2	25.5 ±6.7 OA	Cembella, 1989
DR	Canada	July-89	2	n.d.	Cembella, 1989
Dspp.	France	Summer-88	2	10 OA	Marcaillou-Le Baut and Masselin, 1990
DAc	Japan	Early Spring	2	0.1 OA	Yasumoto, 1990
DAt	Sweden	Oct-87	2	20 OA	Edler and Hageltorn, 1990
Dspp. (mainly DAt)	Sweden	Oct-88	2	105 OA	Haamer et al., 1990
DS D. cfAc DK	France	Jun-91	2	6.0 ± 3.9 OA	Masselin et al., 1992
DS	France	Jun-91	1	16.5 OA	Masselin et al., 1992
DS DR	France	Jul-91	2	2.1 ± 1.7 OA	Masselin et al., 1992
DS DR	France	Jul-91	1	14.0 OA	Masselin et al., 1992
DS D. cfAc	France	Jul-91	2	5.8 ± 1.4 OA	Masselin et al., 1992
DS D. cfAc	France	Jul-91	1	29.6 OA	Masselin et al., 1992
DS DC DK	France	Aug-91	2	1.0 ± 0.2 OA	Masselin et al., 1992
DS	France	Aug-91	1	12.9 OA	Masselin et al., 1992
DS DI DC DR DK	France	Sept-91	2	13.6 ± 2.6 OA	Masselin et al., 1992
DN	Canada	Jul-Aug-90	2	0.07-54.77 OA	Subba Rao et al. 1993
DAc	Spain	90-93	2	1-37 OA	Blanco et al., 1995
DAt	Spain	90-93	2	0.6-94 OA	Blanco et al., 1995
DS	Spain	Oct-94	3	1.2-5.7 OA	Delgado et al., 1996
DAc	Sweden	Sept-94	3	1.1-23 OA n.d.-trace DTX1	Johansson et al., 1996
DAt	Sweden	Sept-94	3	n.d-14 OA n.d.-6.6 DTX1	Johansson et al., 1996
DAc	Denmark	Jun-Oct-94	2	0-40 OA	Andersen et al., 1996
DF	Japan	94	1	n.d.-57.7 OA n.d.-16 DTX1	Sato et al., 1996
DR	Japan	94	1	20.3 OA	Sato et al., 1996
DF	Japan	May-Aug-95	2	5-252 DTX1	Suzuki et al., 1997
DAt	Ireland	Aug-96	1	58 ± 7 OA 78 ± 14 DTX2	James et al., 1997

Species	Origin	Date	Sample type	Toxin content (pg·cell-1)	Reference
DF	Japan	Jul-96	2	182 ± 4 PTX2 n.d. PTX6	Suzuki et al., 1998
DAt	Ireland	Aug-96	1	58 OA 78 DTX2	James et al., 1998
DF	Japan	Jul-96	4	140 DTX1	Suzuki et al., 1999
DAt	Ireland	Aug-96	4	60 OA 80 DTX2	James et al., 1999
DC	Singapore	97-98	1	7.2·10-2 OA	Holmes et al., 1999
DF + DC	Italy	Aug-97	2	235.4 PTX2	Sasaki et al., 1999
Dspp.	Italy	Oct-96	2	191.7 PTX2	Sasaki et al., 1999
DF	Japan	Jul-96	2	10.1 PTX2	Sasaki et al., 1999
DS	Italy	Mar-98	2	0.1-0.4 OA 8-65·10-3 DTX1	Giacobbe et al., 2000
DAc	Spain	Jun-98	1	7.9 OA 9.9 OA	Fernández et al., 2001
DAc	Spain	Sept-98	1	21.7 OA	Fernández et al., 2001
DAt	Spain	Oct-97	1	6.3-33.1 OA 4.4-22.0 DTX2	Fernández et al., 2001
DC	Spain	Oct-97	1	0.73 OA	Fernández et al., 2001
DF	Japan	Jun-Jul-96	4	189-209 DTX1	Suzuki and Mitsuya 2001
DAc	France	94-95	4	0-57.7 OA	Marcaillou et al., 2001
DAt	New Zealand	Jan-Feb-01	2	1.7-2.7 OA 11.5-19.4 DTX3 81-82 PTX2 1.9-3.0 PTX2-SA 22-47 PTX1i 0.5-1 PTX1i-SA	MacKenzie et al., 2002
DAc DN DAt	Sweden	Oct-Nov-00	2	0.24-38.8 OA	Godhe et al., 2002
DAc DN	Germany	Apr-Oct-00	2	3.3 OA equiv.	Klöpper et al., 2003
DAc	Spain	Jun-91	2	9.2 OA	Fernández et al., 2003a
DAc DAt DC	Spain	Oct-94	2	1.0 OA 0.24 DTX2 0.24 PTX2	Fernández et al., 2003a
DAc DAt DC	Spain	Oct-97	2	3.1 OA 1.54 PTX2	Fernández et al., 2003a
DAc DAt DC	Spain	Oct-01	2	0.4-2.5 OA	Fernández et al., 2003a
DAc	Spain	Sept-Nov 1998	2	n.d-11.3 OA nd.-5.2 CFOA	Moroño et al., 2003

molluscs without the detection of *Dinophysis* cells, would be frequenty recorded, this, actually, does not happen because in most cases *Dinophysis* detection and DSP toxin accumulation in bivalves have syndronyc processes (Yasumoto et al., 1980; Haamer et al., 1990; Blanco et al., 1995; Dahl et al., 1995; Sidari et al., 1995; Vale and Sampayo, 2000). The possibility of production by small picoplankton, which is inefficiently retained by most bivalves, cannot be ruled out.

Toxin profile and toxin contents

The toxic potentiality of a *Dinophysis* or *Prorocentrum* population depends on both cell concentration in the water and toxicity per cell. The first attempts made to find a direct correlation between *Dinophysis* cell concentration and the toxicity accumulated by shellfish have led to suspect that, even when they belong to the same species, cells could have a highly varia-

ble toxicity (Haamer et al., 1990; Dahl et al., 1995). Inter-specific differences were also evident. As toxins have different toxic potency, the differences in cellular toxicities may be attributed to two causes: differences in total toxin contents and differences in the relative contributions of the toxins (toxin profile).

Ever since the first study of toxin contents and toxin profiles of several species of *Dinophysis* (Lee et al., 1989b), both intra- and inter-specific differences were found in the same area. Thereafter, differences of one or two orders of magnitude in toxin content (OA or DTX1) were recorded for populations of individual species from the same area (Lee et al., 1989b; Subba Rao et al., 1993; Blanco et al., 1995; Johansson et al., 1996; Andersen et al., 1996; Sato et al., 1996; Suzuki et al., 1997; Fernández et al., 2001). Differences between geographic populations of the same species also appeared to be very high. *Dinophysis acuminata*, for example, which causes frequent closures in different European countries, only produces minor problems in Japan due to its extremely low toxin content (Lee et al., 1989b; Yasumoto, 1990). Apart from physiological and genetic causes, toxin content also seems to be affected by other factors that are not well-defined (perhaps toxic transformations or methodological deficiencies). Some noteworthy discrepancies in the quantification of the toxin content of *Dinophysis* cells, which seem to be related to the sample treatments performed before toxin analysis, have been observed. Masselin et al. (1992) found that the cells isolated from plankton had a consistently higher toxin concentration (from 3 to 12 times) than their equivalents in unprocessed samples. An increase in the estimated toxicity per cell was also observed with successive dilutions of net haul samples (Blanco et al., 1995). Two factors might affect these differences: analytical interferences due to the presence of many different substances in raw samples or an increase in toxin content due to cell-stress caused by the method of isolation (mostly by means of hydrolysis of esterified compounds by autolythic processes associated with mechanical or chemical cellular stress). Neither of the two have actually been proved, although it has been observed that any factor stressing the cells or simply the maintenance of *Dinophysis* cells under laboratory conditions, may cause an increase in toxin content (Johansson et al., 1996; Fernández et al., 2001).

Toxin profile also appears to be highly variable, but most available results should be interpreted with caution. Apart from the main DSP toxins (OA, DTX1 and DTX2), several derivatives, such as diol esters and water-soluble sulphated compounds (DTX4 and DTX5-type) have been detected in *Prorocentrum lima* and *P. maculosum* (Yasumoto et al., 1989; Hu et al., 1993; 1995). Recently, their presence in plankton populations containing *Dinophysis* has been suggested (Moroño et al. 2003) and their presence in *Dinophysis* acuta demonstrated (Suzuki et al, 2004). The importance of these derivatives has been put forth by Quilliam et al. (1996) who showed that DTX4 was the main toxin (up to 80 %) in a culture of *P. lima*. These derivatives were only found when the cells were treated with a boiling procedure to prevent enzymatic hydrolysis by heat denaturing the enzymes. They also observed that the typical freeze-thaw procedure of extraction promotes a rapid enzymatic conversion of DTX4 to diol esters, which are later hydrolysed to OA at a slower rate. Therefore, many of the toxin composition results obtained to date are very likely the result of a partial/total hydrolysis of the esterified toxins. The observations that follow should therefore be analysed in this framework. *Dinophysis* and *Prorocentrum* species were found to contain OA as the main DSP toxin (Table 2 and 3). However there are some exceptions such as the prevalence of DTX1 and PTX2 in D. fortii from Japan (Lee et al., 1989b; Suzuki et al., 1997; 1998; 1999; Suzuki and Mitsuya, 2001) or of DTX2 in *D. acuta* from Ireland (James et al., 1997; 1998; 1999). In *Prorocentrum hoffmannianum, P. belizeanum* and *P. arenarium* only OA has been detected (Aikman et al., 1993; Morton et al., 1994; 1998; Ten-Hage et al., 2000), while in *P. lima*, in most cases, OA is the main toxin, DTX1 represents a high percent of the total toxin and, in some cases, it also contains a small

amount of DTX2 (Table 2). Bravo et al. (2001) observed different toxin profiles in different strains of *P. lima* isolated from a relatively small area (Southern Rías Baixas, NW Spain).

The increasing use of HPLC-MS as an analytical tool for the determination of these kinds of compounds has made it possible to detect certain toxins such as pectenotoxins or yessotoxins that were not taken into account in the past, mostly because of the lack of adequate analytical techniques or reference material to identify and quantify them. In this sense, species like *Dinophysis* caudata, which had been considered almost harmless in view of their OA toxin content (Holmes et al., 1999; Blanco et al., 2000; Fernández et al., 2001) were found to be clearly noxious owing to their PTX content (Fernández and Reguera 2002).

There is very little information available on other toxins. It has been observed that the content of *Protoceratium reticulatum*, like other toxic species, is highly variable from strain to strain as well as in the same strain (Table 4).

SPECIES DEVELOPMENT

Development of *Dinophysis* species

Until the discovery of *Dinophysis* fortii as the causative organism of the DSP syndrome (Yasumoto et al., 1980), the ecology of the species of the genus *Dinophysis* Ehrenberg had received little attention. Previously, the *Dinophysis* species were not suspected of producing marine biotoxins, in spite of their presence being detected during DSP episodes (Kat, 1979), due largely to the fact that they are not usually dominant species. Populations of *Dinophysis* do not generally surpass a concentration in water of 106 cels·L-1 (Dahl and Yndestad, 1985; Krogh et al., 1985; Lassus et al., 1993; Subba Rao et al., 1993; Santhanam and Srinivasan, 1996) and, in most blooms, the maximum is between 103 and 104 cells·L-1 (Lassus et al., 1985; Haamer et al., 1990; Séchet et al., 1990; Boni et al., 1993; Della Logia et al., 1993; Reguera et al., 1993; Giacobbe et al., 1995; Haamer, 1995). Notwithstanding, at least on one occasion, during a bloom of several *Dinophysis* species, they reached such high concentrations as to cause discoloration of the seawater (ca. 20x106 cells·L-1 of *D. norvegica*, 1 million of *D. acuminata* and *D. acuta* ; Dahl et al., 1996),

The development of *Dinophysis* species usually occurs in late summer or autumn (Sechet et al., 1990; Reguera et al., 1991; Della Loggia et al., 1993; Dahl et al., 1995; Palma et al., 1998; Bernardi Aubry et al., 2000; Moita and Jorge da Silva, 2001; Klöpper et al., 2003; Reguera, 2003). In many cases, it appears to be a succession of species, going from *D. acuminata* or *D. sacculus*, in spring or summer, to *D. acuta* , *D. norvegica* or D. fortii, in late summer or autumn (Sechet et al., 1990; Reguera et al., 1991; 1995; Della Loggia et al., 1993; Dahl et al., 1995; Bernardi Aubry et al., 2000; Reguera, 2003). This sequential development is probably related to changes in the environmental conditions, such as temperature and salinity, that infuence the development of each species (Palma et al., 1998).

Most *Dinophysis* blooms develop by in situ growth associated with stability or stratification of the water column (Reguera et al., 1991; Delmas et al., 1992; 1993; Reguera et al., 1995; 1996; Peperzak et al., 1996; Palma et al., 1998; McMahon et al., 1998; Bernardi Aubry et al., 2000; Gisselson et al., 2002), probably because of their ability to migrate and take up nutrients from sub-superficial water layers. The migration capability of several species of *Dinophysis* has been proved (Durand Clement et al., 1988; Lassus et al., 1990; Delgado et al., 1996). In many cases the effectiveness of the migration to obtain nutrients might be favoured by the presence of the thermocline, (Lassus et al. 1990; Delmas et al., 1992; 1993; Gisselson et al., 2002) which is usually coincident with a nutricline. In other cases, *Dinophysis* populations seem to develop in the open sea, later accumulating on the coast by the effect of the Ekman transport of the water masses induced by the wind (Delmas et al., 1993; Lassus et al., 1993; Pazos et al., 1995; Reguera et al., 1995; 1996; McMahon et al., 1998; Godhe et al., 2002).

Table 4. Concentrations of yessotoxins in *Protoceratium reticulatum* and Lingulodinium polyedra. Sample type indicates the origin of the biological material used in the studies: 1. culture; 2. raw sample.

Species	Origin Date	Sample type	Toxin content (pg·cell-1)	Reference
P. reticulatum	New Zealand	1	3.0 YTX	Satake et al., 1997
P. reticulatum	Japan	1	14.0 YTX	Satake et al., 1999
P. reticulatum	Japan	1	n.d. YTX	Satake et al., 1999
P. reticulatum	New Zealand	1	6 YTX	Satake et al., 1999
P. reticulatum	New Zealand	2	5.5 YTX	MacKenzie et al., 2002
P. reticulatum	New Zealand	1	2-53 YTX	MacKenzie et al., 2002
P. reticulatum	Italy	1	11.4 YTX 0.08 homoYTX 0.19 45-OHYTX 0.37 carboxyYTX 0.15 noroxoYTX	Ciminiello et al., 2003
L. polyedra	Italy	2	1.53 homoYTX 1.1 homoYTX	Tubaro et al., 1998

Although some *Dinophysis* species such as *D. rotundata* have been proved to be heterotrophic (Hansen, 1991), many species, -specially the most toxic ones-, showed numerous chloroplasts (Hallegraeff and Lucas, 1988; Durand Clement et al., 1988; Subba Rao and Pan 1993). However, the presence of digestive vacuoles in the photosynthetic species studied (Jacobson and Andersen, 1994; Gisselson et al., 2002), together with the inability to cultivate them by using typical phytoplankton media (Durand Clement et al., 1988; Sampayo, 1993; Delgado et al., 1996), suggest that they have a mixotrophic nutrition. Additionally, it has been observed that, in some cases, the calculated carbon uptake by photosynthesis is not enough to explain the growth rates observed (Gisselson et al., 2002). Notwithstanding, Delgado et al. (1996) tried to grow *Dinophysis* sacculus by adding several phytoplankton cultures (*Synechococcus sp., Cryptomonas sp., Thalasiossira weisflogii* and *Prorocentrum micans*) to f/2 media but did not observe *D. sacculus* as containing any of these organisms, which that led them to assume that the ingestion of dissolved or finely particulate organic matter was needed by the organisms of this species. Some *Dinophysis* have been kept alive in artificial media for up to four months. (Durand-Clement et al., 1988; Sampayo, 1993) but the cells eventually degenerated. The factor or conditions needed to keep the cells healthy in culture are not known.

The reproduction of *Dinophysis* species is mainly asexual by binary fission with growth rates between 0.09 and 0.65 div·day-1 (Delmas et al., 1993; Reguera et al., 1996; 2003; Garcés et al., 1997; Granéli et al., 1997; Gisselson et al., 1999; 2002), depending on hydrodynamic conditions (Reguera et al., 2003). Several authors have postulated that small *Dinophysis* forms, such as *D. skagi, D. diegensis* or *D. dens*, are gametes (MacKenzie, 1992; Berland et al., 1995; Moita and Sampayo, 1993; Reguera and González-Gil, 2001; Reguera, 2003), but this has not yet been demonstrated due largely to the impossibility of cultivating *Dinophysis* species. The sexual conjugation of gametes would lead to the formation of a zygotic resting cyst. Several authors have observed some *Dinophysis* cyst-like structures, but the existence of cysts has not yet been confirmed (Moita and Sampayo, 1993; Reguera et al., 1995; Pazos et al., 1995; Delgado et al., 1996).

Development of DSP-producing species of *Prorocentrum*

All the *Prorocentrum* species that produce DSP toxins are benthic or epiphytic, and live

attached to or in association with macrophytes, floating detritus, sand, debris or coral reefs (Faust, 1995). Six *Prorocentrum* species are currently known to produce DSP toxins: *P. arenarium* (Ten-Hage et al., 2000), *P. belizeanum* (Morton et al., 1998), *P. faustiae* (Morton, 1998), *P. hoffmannianum* (Aikman et al., 1993), *P. lima* (Murakami et al., 1982), *P. maculosum* (Zhou and Fritz, 1994). The DSP toxicity of *P. concavum* has not been demonstrated due to problems with its correct identification (IOC Taxonomic List of Toxic Plankton Algae).

A factor that may regulate *P. lima* density in natural media is the macroalgal surface area (Bomber et al., 1985; Lawrence et al., 2000). It seems that the association of species such as *P. lima* and *P. concavum* with macroalgae may be determined by the availability of nutrient concentrations (Carlson and Tindall, 1985). In this sense, some authors have postulated a nutritional link between tropical brown macroalgal exudates and their use as preferential substrate (Heil et al., 1998). Protected inshore habitats are considered to be the most suitable for the development of these benthic species (Carlson and Tindall, 1985), but *P. lima* has been shown to be very adaptable to the sediment structure, wave activity or incidence of storms (Bomber et al., 1985).

Prorocentrum species are photosynthetic and, in contraposition to *Dinophysis*, easy to culture in typical phytoplankton media, although some species, like *P. belizeanum* and *P. hoffmannianum* have also been observed to ingest photosynthetic nanoplankton (Faust, 1998). The possibility of culturing benthic *Prorocentrum* has made it possible to study the effect of environmental conditions on growth rate under controlled conditions. It has been observed that increasing temperature or salinity have a positive effect on growth rate, until an optimum point beyond which they would have a negative effect (Morton and Norris, 1990, Morton et al., 1994). It has also been observed that temperature and light intensity, in addition to their positive effect, have a synergistic effect on the growth rate of *P. hoffmannianum* (Morton et al., 1994). *Prorocentrum lima* and *P. hoffmannianum* have a clear preference for $NH4^{+}$ as a source of nitrogen (Aikman et al., 1993; Pan et al., 1999) and inorganic phosphate was shown to be used by *P. lima* (Sohet et al., 1995). Chelating substances (humic acids or EDTA) have a positive effect on *P. lima* growth (Sohet et al., 1995).

Asexual reproduction is mainly carried out by binary fission with growth rates of between 0.05 and 0.7 $div \cdot day^{-1}$ (Morton and Norris, 1990; Aikman et al., 1993; Faust, 1993a; Jackson et al., 1993; Morton et al., 1994; Rausch de Traubenberg and Morlaix, 1995; Pan et al., 1999; Bravo et al., 2001; Holmes et al., 2001; Heredia-Tapia et al., 2002). *Prorocentrum lima* was also reported to have an alternate asexual reproduction process by means of cysts that contain between 4 and 32 cells, equally developed and enclosed in a thin membrane. The cells enclosed in this vegetative division cyst are generated at random by binary fission (Faust, 1993a). Moreover, Faust (1993b) observed isogamous sexual reproduction in cultures of *P. lima* not induced by nitrogen or phosphorous limited conditions.

Development of *Lingulodinium polyedra* (Stein) Dodge

Lingulodinium polyedra is a species that typically blooms during upwelling relaxations (Smayda and Reynolds, 2001). Stability and thermal stratification appear to provide the ideal conditions for its development (Amorim et al., 2001) but, in some cases, a stronger stratification than usual, and especially low temperatures at the bottom, may prevent its development (Marasovic et al., 1995).

This species has a typical dinoflagellate life cycle with asexual reproduction by binary fission and sexual reproduction and in some cases it is even possible to observe all the phases at the same time- vegetative cell, temporary and resting cysts (Amorim et al., 2001).

The association of this species with the presence of YTX toxins in shellfish is relatively recent (Tubaro et al., 1998, Draisci et al., 1999a Paz et al 2004).

Development of *Protoceratium reticulatum* (Claparède and Lachmann) Bütschli (syn. *Gonyaulax grindleyi*)

Little is known about the biology of this species. It is a photosynthetic species that can produce resting cysts, which, in paleontological literature are known as *Operculodinium centrocarpum*. The species have formed dense blooms in South Africa and New Zealand.

Development of *Protoperidinium crassipes*

This species is, to date, the only known azaspiracid producer. It is a heterotrophic dinoflagellate, and consequently belongs to the microzooplankton. It is assumed to prey mostly on phytoplankton using a mechanism described by Jacobson (1999).

DSP- PRODUCING DINOFLAGELLATES AND THEIR GEOGRAPHICAL DISTRIBUTION

Both “diarrhoeic” toxins and organisms related to their production, have a broad geographical distribution. Many *Dinophysis* species such as *D. acuminata* are cosmopolitan, with blooms having been recorded in Japan, Germany, France, Spain, Sweden, Norway, Italy, the Netherlands; Denmark, Ireland; Portugal, Greece, Croatia, Russia, Morocco, Canada, USA, Brazil, Uruguay and New Zealand (Table 5). The area most affected by this species is Northwest Europe, and it occurs sequentially with *D. acuta* , which is especially important, additionally, in Chile and New Zealand (Table 4). Other species, in spite of their wide-ranging distribution, only cause major problems in specific areas, for example *D. fortii* in Japan, D. norvegica in Northern Europe and Canada and *D. caudata* in India and Singapore (Table 5).

Prorocentrum lima is distributed all over the world (Faust, 1991). However, only a few cases of DSP directly associated with *Prorocentrum* species have been reported to date, in Nova Scotia, Canada (Lawrence et al., 1998), Baja California, Mexico (Heredia Tapia et al., 2002) and Southern England (Nascimento et al., 2002).

Lingulodinium polyedra (syn. *Gonyaulax polyedra*), a YTXs producer in the Adriatic Sea and Galicia, is a dinoflagellate with a wide distribution in temperate and tropical waters, and it is known to bloom and even cause water discoloration, reaching concentrations of between 10^5 and 10^7 cells·L^{-1}, in different countries (Yuzao et al., 1993; Marasovic et al., 1995; Tubaro et al., 1998; Amorim et al., 2001).

Protoceratium reticulatum is the first dinoflagellate clearly shown to produce YTXs (Satake et al., 1997). It has been observed that even low cell concentrations (around 10^3 cells·L^{-1}) are enough to promote the accumulation of significant YTX amounts in shellfish (Boni et al., 2001). The association of this species with the presence of YTXs in shellfish has a wider geographical distribution than that of *Lingulodinium polyedra*, including New Zealand, Japan United Kingdom, Adriatic Sea, Norway and Canada (Table 5).

In addition to *Protoceratium reticulatum* and *Lingulodinium polyedra* other YTXs-producer species may exist, since the true origin of these toxins is still unknown in some places such as Norway (Ramstad et al., 2001c).

The heterotrophic dinoflagellate *Protoperidinium crassipes*, known to contain azaspiracids (James et al., 2003) has a wide geographical distribution but its association with AZAs has not yet been adequately studied.

The DSP records in shellfish resemble the geographical distribution of *Dinophysis* populations (FIG. 5, Table 5). Most DSP cases are due to okadaic acid, its analogues and its derivatives. Notwithstanding, since many DSP episodes were detected by means of the mouse bioassay, they may include episodes of YTXs or PTXs. With improved analytical methods of detection it will be possible to pinpoint the geographical distribution of each type of toxin, but owing to the limited use of these new methods, at present the reported cases for the two latter groups

Table 5.- Toxic species and associated toxicities of the "diarrhoeic" type, found in different geographical locations. Species identities coded as follows: DA=*Dinophysis acuminata,* DF= *D. fortii*; DT= *D. acuta*; Dspp= *Dinophysis* spp.; DM= *D. mitra*; DR= *D. rotundata*; DTr= *D. tripos*; DN= *D. norvegica*; DS= *D. sacculus*; DK= *D. skagi*; DC= *D. caudata*; PL= *Prorocentrum lima*.

Type of toxins	Country	Phytoplankton producers	Reference
DSP	Germany	DA	M. Kat, 1979
DST	Japan	DF	Yasumoto et al., 1980
DSP	Spain	DA,DT	Campos et al., 1982
DST	France	DA	Lassus et al., 1985
DST	Norway, Sweden	Dspp	Underdal et al., 1985
OA	France, Germany,		
	The Netherlands, Sweden, Spain	Dspp	Kumagai et al., 1986
YTX	Japan	Not studied	Murata et al., 1987
*OA, DTX1, PTX	France, Japan, Norway, Spain	DF, DM,,DR,,DTr, DT,,DN,,DA,,PL	Lee et al., 1989
OA	Canada	DN,,DA	Cembella, 1989
DSP	Spain	DT	Reguera et al., 1990
OA	Norway	Dspp	Haamer et al., 1990
DSP	Portugal	DT DS,Dspp	Sampayo et al., 1990
OA	Sweden	DT	Edler and Hageltorn, 1990
DST	France	DN,,DA,DS	Marcaillou-Le Baut and
			Masselin, 1990
OA	Sweden	DA,DT	Haamer et al.,1990
DST	Norway	DA,DT,DN	Séchet et al., 1990
*OA	France	DS,DA,DK DC,DR	Masselin et al., 1992
DTX2	Ireland	Not studied	Hu et al., 1992
DSP	Uruguay	DA	Mendez, 1992
OA+DTX1	Canada	DN	Subba Rao et al.,1993
OA, DTX1	Chile	DT,	Zhao et al., 1993
	Italy	Not studied,	
	Ireland	Not studied,	
OA	Spain	DA,DT	Reguera et al., 1993
DTX1	Canada	DN	Quilliam et al., 1993
			Todd et al., 1993
Not studied	Uruguay	DA,DC	Mendez, 1993
DST	New Zealand	DA,DT DF,DTr	Chang et al., 1995
OA	Spain, Portugal	Not studied	Gago et al., 1993
DST	Chile	DT	Lembeye et al., 1993
Not studied	France	DcfA	Lassus et al., 1993
Not studied	France	DS,DA,DR	Delmas et al., 1993
OA+DTX1	Italy	DF	Della Loggia et al., 1993
DST	Italy	Dspp	Boni et al., 1993
DST	France	Dspp	Belin, 1993
Not studied	Portugal	DT DS,DTr	Moita, 1993
DST	Ireland	DA,DT,DR,DN	Jackson and Silke, 1995

Type of toxins	Country	Phytoplankton producers	Reference
OA, DTX	Canada	Not studied	Gilgan et al., 1995
OA, DTX2	Ireland	DA,DT	Carmody et al., 1995
DST	Germany	DA	Nehring et al., 1995
Not studied	Spain	DT	Pazos et al., 1995
*OA, DTX2	Spain	DA,DT	Blanco et al., 1995
OA, DTX1	Norway	DA,DN,DT	Dahl et al., 1995
DST	Italy	DF	Sidari et al., 1995
OA	Italy	DF DS	Giacobbe et al., 1995
OA	Norway	Dspp	Haamer, 1995
OA, DTX1	Japan	DF	Suzuki et al., 1995
DST	Denmark	DA,Dspp	Emsholm et al., 1996
Not studied	Latvia	DA	Balode and Purina, 1996
OA, DTX2	Ireland	DT	Carmody et al., 1996
Not studied	India	DC	Santhanam and Srinivasan, 1996
Not studied	New Zealand	DA	Chang, 1996
Not studied	Spain	DS	Delgado et al., 1996
OA	Norway	DA,DN,DT	Dahl et al., 1996
OA	Sweden	DA,DN,DT	Lindahl & Anderson, 1996
Not studied	The Netherlands	DA	Peperzak et al., 1996
*OA	Denmark	DA	Andersen et al., 1996
OA, DTX1	Japan	DF,DA	Sato et al., 1996
OA, DTX2	Italy	Not studied	Ciminiello et al., 1997
OA, DTX1	Japan	DF	Suzuki et al., 1997
*OA, DTX2	Ireland	DT	James et al., 1997
Not studied	Russia	DA,DF,DN	Orlova et al., 1998
OA, DTX1	Mexico	Not studied	Sierra-Beltrán et al., 1998
YTXs	Italy	LP	Tubaro et al., 1998
PTX2, PTX3	Japan	DF	Suzuki et al., 1998
*OA, DTX2	Ireland	DT	Draisci et al., 1998a
OA, DTX1	Italy	DF	Sidari et al., 1998
Not studied	Portugal	DcfA,DT	Palma et al., 1998
Not studied	Ireland	DA,DT	McMahon et al., 1998
YTXs	New Zealand	PR	MacKenzie et al., 1998
*OA, DTX2	Ireland	DT	James et al., 1998
OA, DTX2	Ireland	DT,DF	Draisci et al., 1998a
OA, DTX1	Italy		
OA, YTXs	Italy	LP	Draisci et al., 1999a
YTXs	Italy	PR,LP	Ciminiello et al., 1999
OA, DTX1	China	Not studied	Zhou et al., 1999
PTXs	Ireland	DT	James et al., 1999
OA, YTX, PTXs	Italy	Not specified	Draisci et al., 1999
OA, DTXs, PTXs	Ireland		
OA, DTX1	Singapore	DC	Holmes et al., 1999
OA, DTXs	Portugal	DT,DA	Vale and Sampayo 1999
AZAs	Ireland	Not studied	Ofuji et al., 1999
*PTX2	Italy; Japan	DF+DC,Dspp,DF	Sasaki et al., 1999
AZAs	Ireland	Not studied	Draisci et al., 2000
OA, DTX2	Portugal	DT,DA	Vale and Sampayo 2000
OA, DTX1, PTX6	Japan	Not studied	Suzuki and Yasumoto 2000

Type of toxins	Country	Phytoplankton producers	Reference
OA, DTX1	Italy	DS	Giacobbe et al., 2000
DST	Spain	DA	Mamán et al., 2000
PTXs	New Zealand	DT	Suzuki et al., 2001
7-epi-PTX-2SA	Croatia	Dspp	Pavela-Vran_i_ et al., 2001
DTX1, DTX3	Japan	DF	Suzuki and Mitsuya, 2001
OA/DTX1	Norway	DA,DT,DN	Ramstad et al., 2001c
YTX	Norway	unknown	Ramstad et al., 2001c
Not studied	Russia	DA,DR,DC	Vershinin and Kamnev, 2001
Not studied	Indonesia	DC,DR	Sidabutar et al., 2001
AZAs	Ireland	unknown	James et al., 2001
*OA, DTXs	Spain	DA,DT DC	Fernández et al., 2001
Not studied	Portugal	DT DTr,DcfA	Moita and Jorge da Silva, 2001
OA	France	DA	Marcaillou et al., 2001
Not studied	Italy	DS DF,DC	Caroppo et al., 2001
DST	Greece	DA,DS	Koukaras and Nikolaidis, 2002
OA, DTXs	Portugal	DA,DT	Vale and Sampayo, 2002 d
AZAs	England Norway	Not studied	James et al., 2002
PTXs	Portugal	DT DF	Vale and Sampayo, 2002b
OA, OA esters, DTX2, PTX2sa	Portugal	Not specified	Vale and Sampayo, 2002c
DSP	Mexico	PL	Heredia-Tapia et al., 2002
*OA, DTX1			
OA, YTXs, PTXs	New Zealand	DT,PR	MacKenzie et al., 2002
OA, DTXs, PTXs	Croatia	DS,DT DC	Pavela-Vran_i_ et al., 2002
Not studied	Sweden	DN	Gisselson et al., 2002
YTXs	Italy	Not studied	Ciminiello et al., 2002
DST	Sweden	DA,DT,DN	Godhe et al., 2002
OA, DTX2	Scotland	DT,DN	Bresnan et al., 2002
PTX1	Norway	DT	Aune et al., 2002
OA	USA	DA	Tango et al., 2002
DST	Brazil	DA	Tavares et al., 2002
DST	England	PL	Nascimento et al., 2002
OA	USA	DA	Marshall et al., 2002
DST	Mexico	DC	Flores et al., 2002
DST	Spain	DA	Moroño et al., 2003
DST	Japan	DF	Imai et al., 2003
DST	Germany	DA,DN	Klöpper et al., 2003
AZAs	France, Spain	Not studied	Magdalena et al., 2003
PTXs	New Zealand	DT	Suzuki et al., 2003
DST	Morocco	DC,DA+DS,LP	Joutei et al., 2003
DST	Uruguay	DA	Medina et al., 2003
*DST, PTXs	New Zealand	DT	Miles et al., 2004

of toxins are very scarce.

YTXs reported distribution is restricted to Japan, New Zealand, the Adriatic Sea, Norway (FIG. 5, Table 4) and Spain (Paz et al 2004). PTX toxins show the same distribution with the addition of Spain and Ireland (FIG. 5). To date AZA records are limited to a few European countries: Ireland, England, Norway, France and Spain (FIG. 5, Table 5).

TOXIN DYNAMICS IN MOLLUSCS

Toxin uptake by molluscs

Shellfish (bivalve molluscs, gastropods, crabs, lobsters and others) accumulate phycotoxins mostly by direct filtration of the producer algal cells or by feeding on contaminated organisms (e.g. carnivores and scavengers). In bivalves, feeding involves several processes. The activity of the gills forces water to flow through them. Most particles in this flow are retained in the gills and transported to the mouth, having been selected (pre-ingestive selection) during the transport to the mouth. Two kinds of particles may escape ingestion: the kind that the gill system is not able to retain and the kind that are rejected because of their physical characteristics, palatability or other reasons. After being ingested, the particles are selected again (post-ingestive selection) in the digestive system and, subsequently, extracellularly intracellularly digested.

Some organisms that produce toxins have been shown to affect filtration or/and selection (reviewed in Blanco-Pérez, 2001), as it is the case of *Alexandrium tamarense* or *A. minutum*. Notwithstanding, no DSP producing organism appears to have this capability. Feeding cultures of *Prorocentrum lima* to the bay scallop *Argopecten irradians* (Bauder et al. 2001) and to the mussel *Mytilus edulis* (Pillet et al. 1995) produced no significant alteration in feeding behaviour, only exhibiting a reduction of the feeding rate at very high cell concentrations. The possible effect of *Dinophysis*, cannot be checked directly as cultures of these species have not been obtained to date. No effect has been observed during *Dinophysis* proliferations but perhaps because in most of them the toxic cells are present at low concentrations in the water. Although selection of *Dinophysis* by mussels has been suggested in view of the differences between the species fed to them and those found in the digestive system (Sidari et al. 1998), these differences are very likely due to different digestibilities and not to selection (MacKenzie, 1998). Svensson and Forlin (1998) reported that okadaic acid caused limited inhibition of protein phosphatase activity in the digestive tissue of *Mytilus edulis*, suggesting the possibility that there may be some effects on the process of digestion. Nevertheless, Bauder et al. (2001) only found a decrease in the absorption efficiency in the bay scallop *Argopecten irradians*, which may be attributed to the low digestibility of the species, implying that this organism may have a protective mechanism to counteract the effects of the toxins. Toxin absorption has been reported to increase with the contribution of *Dinophysis* to the total plankton biomass (Sampayo et al., 1990; Boni et al., 1993). Obviously, this fact goes against the possibility of any adverse effect of DSP toxins on the process of digestion/absorption and may be explained by the same mechanisms that regulate the absorption of organic matter and PSP toxins (Moroño et al, 2001), namely, the ratio between the amount of substances of interest and the volume of matter ingested.

The digestion of the toxic phytoplankton modifies the original toxins by means of diverse transformations. The cellular stress inherent to the retention, ingestion and mechanical disruption involved in the acquisition of resources by the bivalves may trigger autolythic processes which are able to hydrolyse the probable main toxins in the plankton, DTX4 and DTX5 and related compounds, to diol-ester or other derivatives (Windust et al. 1997). The esterases invol-

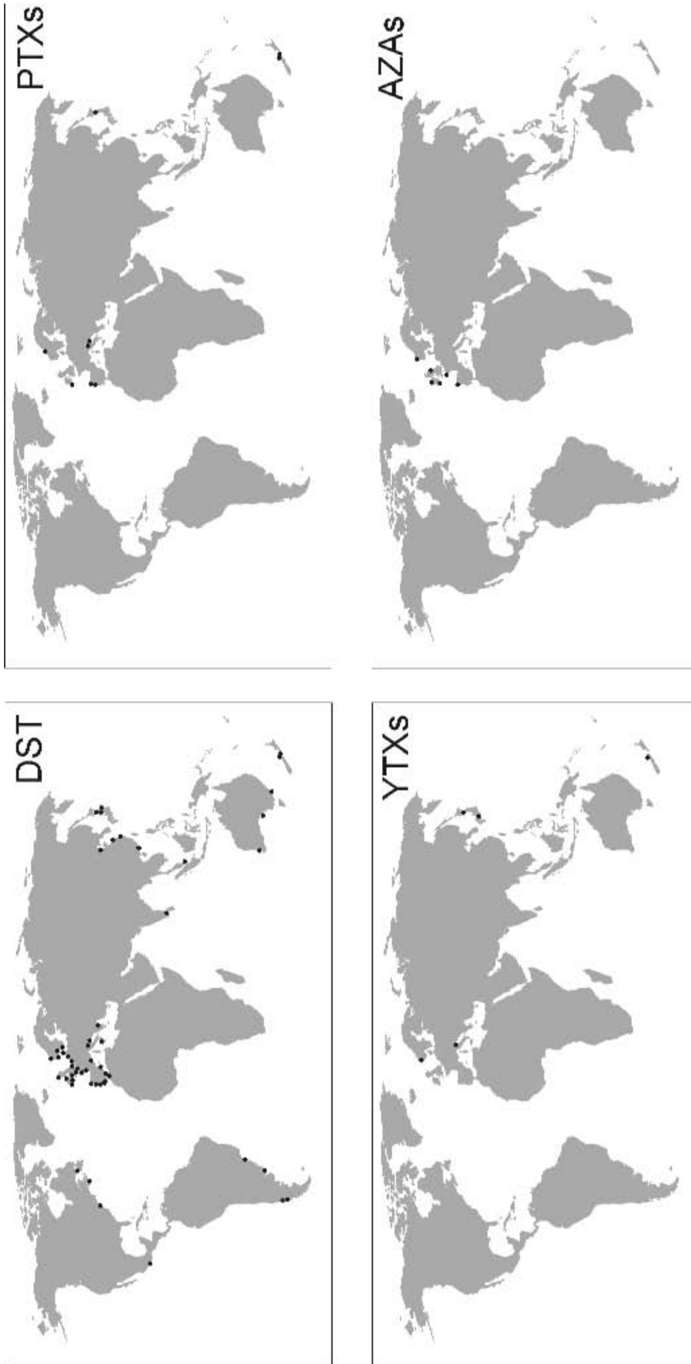


Figure 5.- Geographical distribution of "diahorreic" toxins at the end of 2003.

ved in digestion may very likely continue the same hydrolytic process to yield, at least in part, the free parent toxins such as okadaic acid, DTX2, etc… The product of the above transformations would probably be a spectrum of compounds of different polarities that perhaps have different absorption efficiencies because of their varying ability to pass through cellular membranes.

Little is known about the incorporation of DSP toxins into organisms other than bivalves. The crab *Carcinus maenas* accumulates okadaic acid and its derivatives supposedly by consuming contaminated bivalves (Vale and Sampayo 2002c). The feeding rate of the crustacean (copepod) *Temora longicornis* was not affected by *Dinophysis* cell concentration in a bloom (Maneiro et al. 2002).

Non-feeding incorporation of water-soluble toxins (such as YTXs) or derivatives (such as DTX4 or DTX5) has not been studied but should not be ruled out.

COMPARTMENTALIZATION

The distribution of DSP toxins in shellfish organs or tissues has only been studied sporadically. Early studies on these kinds of toxins coincide in that they are mainly accumulated in the digestive gland (Yasumoto et al., 1978; 1979). This seems to be a frequent situation, given that other authors have found a clearly preferential accumulation in the digestive gland of several species, such as mussels (Pillet et al. 1995; Stabell et al., 1992), the bay scallop *Argopecten irradians* (Bauder et al., 2001) or the king scallop *Pecten maximus* (Hess et al. 2003). Moreover Bauder et al. (2001) showed that the viscera (mainly consisting of the digestive gland) of *Argopecten irradians* depurates the DSP toxins much more slowly than the other tissues. These types of differences in depuration velocity between tissues cause an increase in the percentage of contribution of the digestive gland to the whole shellfish toxin burden as the depuration progresses (in the case of *A. irradians* the percentage increased to 95% after two days of depuration).

Yessotoxins are also predominantly accumulated in the digestive gland, at least in three mussels, *Perna canaliculus, Mytilus galloprovincialis* (Yasumoto and Takizawa, 1997; Suzuki et al., 2001), and M. chilensis (Yasumoto and Takizawa, 1997). A recent study (Franchini et al., 2003) found that yessotoxins are mostly located at the lumen of digestive tubules and ducts inside the digestive gland. The same distribution –predominant accumulation in the digestive gland- was initially shown for azaspiracids (James et al. 2002b; Braña Magdalena et al. 2003), and it would seem to hold in the case of the scallop *Pecten maximus*, at least on some occasions (Hess et al. 2003). However, it was recently discovered that these compounds may be distributed among shellfish tissues in much more balanced proportions than previously assumed (James et al., 2002b)

TRANSFORMATION

Many DSP toxins undergo transformations that produce other toxins of the same group. Frequently, the compounds generated have a toxicity which is different from that of the parent toxin, modifying the toxicity of the organism that produced or accumulated the toxins without any adquisition or loss. Additionally, the polarity of the transformed toxins is frequently different from the parent toxin, making it necessary, in some cases, to use different extraction protocols in order to detect them.

To date, only four kinds of transformations have been documented for the DSP toxins of the okadaic group (FIG. 6): a) hydrolysis of conjugated forms (DTX4, DTX5 type or diol-esters); b) oxidation of diol-esters; c) acylation of free-acid forms; and d) formation of diol esters from the free-acid forms.

DTX4 and DTX5 have been suggested to be the major *de novo* toxin products of

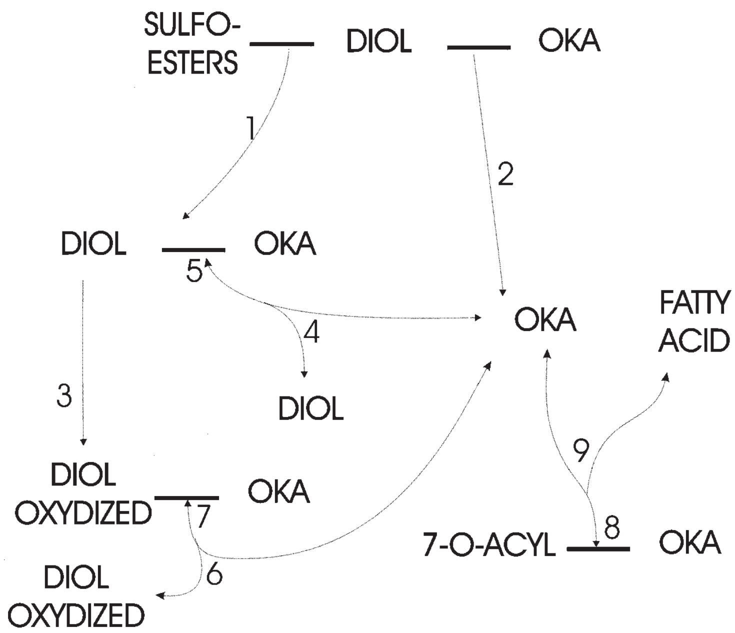


Figure 6.- Transformations between compounds of the okadaic acid group. Okadaic acid (OKA) transformations are shown but they also take place in other toxins of the group, such as DTX1 and DTX2.

Prorocentrum lima. Okadaic acid and diol-esters would only be obtained after hydrolysis of these large sulphated compounds (Hu et al., 1995; Bauder et al., 1996; Windust et al., 2000). DTX4 and DTX5 are relatively polar due to the sulphate groups they contain, they are only partially soluble in chloroform (Hu et al. 1995) and hexane, and are quickly hydrolysed to diol-esters (seconds) and okadaic acid (hours) by the action of the autolytic enzymes of the cells that contain them (Bauder et al. 1996; Windust et al. 2000) and also probably by the enzymes in the digestive tract of the organisms that feed on the toxic dinoflagellate. These circumstances have probably caused them to be overlooked in many studies in which hydrolysis and the liquid/liquid partition were not specifically designed. Recent studies, which do not present these drawbacks, suggest that these kinds of sulphated compounds are also important in *Dinophysis*, (Vale and Sampayo, 1999; MacKenzie et al., 2002; Vale and Sampayo, 2002a; Moroño et al., 2003), and that they are also metabolised in different ways by different invertebrate organisms (Vale and Sampayo, 2002a). The diol-esters produced by the hydrolysis of DTX4 and DTX5 have low polarity, and are able to pass through cellular membranes very easily (Windust et al., 1996; 2000). These diol-esters can be oxidized or hydrolysed. In the diatom *Thalassiosira weissflogii* they undergo some oxidative processes, which only affect the radical diol, yielding different compounds that are more polar than the base toxin (Bauder et al. 1996; Hu et al., 1999; Windust et al. 2000) (known in okadaic acid but probably applicable to DTX1 and DTX2). Several enzymes of the diatom may intervene in these oxidations, such as for example cytochrome p-450 (Windust et al. 2000). Similar transformations may take place in molluscs, as the oxidative processes are important components in their defence strategy against both parasites and xenobiotics.

Diol-esters, and their oxidized derivatives, can also be hydrolysed yielding the main toxin both by the action of the enzymatic pool of the phytoplankton cells when they are broken (Windust et al., 1997) as well as by the action of unspecific esterases (Hu et al. 1999) during digestion in the bivalves, although, as discussed earlier, different invertebrates seem to have different abilities to hydrolyse the esterified derivatives of okadaic acid (Vale and Sampayo, 2002a). In the bay scallop *Argopecten irradians* the dominant forms of DSP, after being fed with *Prorocentrum lima*, were the diol-ester of okadaic acid, in a similar proportion to that found in the dinoflagellate cells, which would suggest that no significant esterase activity took place during the digestive process. Similarly, five out of six bivalves studied in Portugal (*Venerupis pullastra, Ruditapes decussatus, Crassostrea japonica, Cerastoderma edule, Solen marginatus*) seem to have little capacity to hydrolyse okadaic acid esters, since nearly 100% of the accumulated toxin was in this form. In the other species studied, the blue mussel *Mytilus galloprovincialis*, about 50% of the toxin was in the form of free acid (Vale and Sampayo, 2002a). While these differences might be attributed to differences in acylation rate, this possibility seems unlike because previous studies found that this rate is very slow in mussels (Fernández et al., 1998; Moroño et al. 2003), and that the proportion of polar esters decreases when the total DSP toxin stops being incorporated, as can be inferred from the data by Vale and Sampayo (1999). Suzuki and Mitsuya (2001) also found differences, in the same direction, between mussels (*Mytilus galloprovincialis*) and scallops (*Patinopecten yessoensis*) which they attributed to differences in the kinetics of the conversion of DTX1 to the corresponding esters. Notwithstanding, only the hexane fraction was used to quantify the esters and thus, information about the polar esters in both plankton populations and molluscs was lost. In view of the previous information in this section, it would seem likely that most of the esters detected in bivalves belonged to the group of diol-esters, and that these compounds are more readily hydrolysed in mussels than in scallops. *Perna canaliculus* –the greenshell mussel, another mytilid- was found to contain fewer esters of okadaic acid than the plankton population on which it was feeding (MacKenzie et al. 2002), again, supporting the idea that mussels have

more hydrolytic capability than other groups of bivalves.

The hydroxyl group in the C7 of the molecule of the main DSP toxins may be esterified by fatty acids of different lengths, yielding a group of compounds that are less polar than their parent toxins, generically known as DTX3 (Yasumoto, 1985). This acylation was demonstrated for DTX1 in the case of the scallop *Patinopecten yessoensis* (Suzuki et al. 1999) but it probably takes place in other bivalves (as they appear in molluscs and not in the plankton) and in the two other main DSP toxins (Marr et al., 1992; Carmody et al. 1995a). Other studies have not dealt strictly with the 7-O-acyl derivatives, but rather with the low polarity derivatives of the main toxins, (Fernández et al. 1998; Vale and Sampayo, 1999, 2002a; Moroño et al. 2003) which probably include other derivatives such as diol-esters. Some of these studies have been able to discriminate different kinds of compounds through mathematical modelling, and to estimate low acylation rates in the case of the blue mussel *Mytilus galloprovincialis* (Blanco et al., 1999; Moroño et al. 2003).

No evidence of interconversion between the three main toxins, okadaic acid, DTX1 and DTX2, as well as 7-deoxyokadaic acid has been found, and all the information to date would seem to indicate that these transformations do not take place.

There are two main ways in which transformations take place in pectenotoxins. It was initially assumed that the main PTX in plankton is PTX2 and that in bivalves it may undergo a series of transformations that end in PTX6, with PTX1 and PTX3 as intermediate steps, as seen in *Patinopecten yessoensis* (Murata and Yasumoto, 1990; Suzuki et al., 1998).

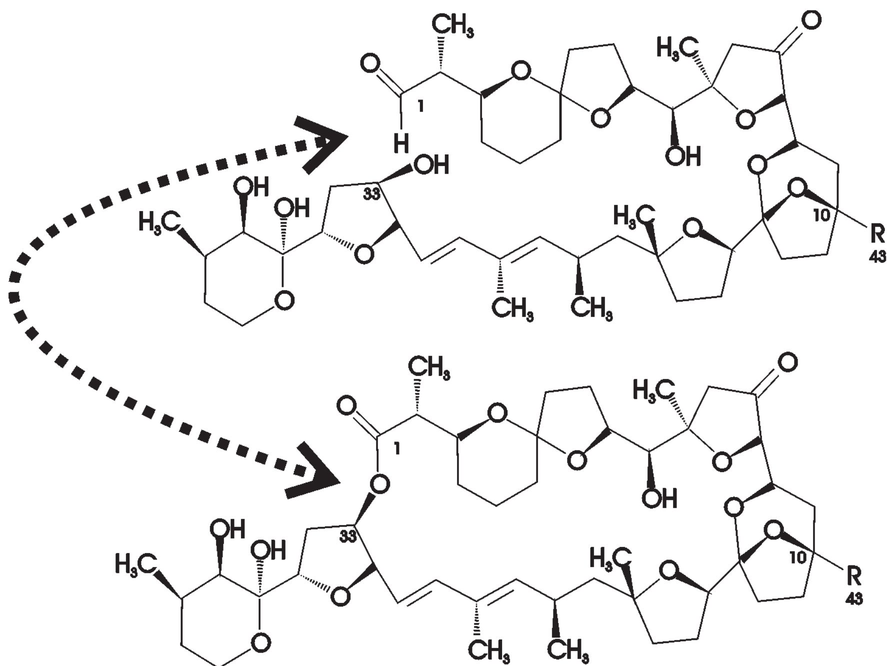


Figure 7.- Transformation of Pectenotoxins in their corresponding seco-acids.

Nevertheless, no PTX6 was found in the mussel *Mytilus galloprovincialis* collected from the same site as the scallops that accumulated this toxin (Suzuki and Yasumoto, 2000), suggesting that the oxidation required is dependent on the enzymatic or biochemical characteristics of each bivalve species. The second known type of transformation is the cleavage of the bound C1-C33, which breaks the large lactone ring converting the PTXs to their corresponding seco-acids (FIG. 7). This is the type that seems to be followed preferentially in the case of *Pecten novaezelandiae*, *Perna canaliculus* (Suzuki et al., 2001c,b,) as well as in other bivalve species (Vale and Sampayo, 2002a), and it appears to be shared by other PTXs such as PTX1i (Daiguji et al. 1998) and 7-epi-PTX2 (Suzuki et al. 2001b,c). Other transformations induced by chemical treatments or by heating may also occur and have been summarised by Burgess and Shaw, (2001).

There is practically no information available on the transformations of yessotoxins and azaspiracids. It was found that yessotoxin can be transformed to 45OH-yessotoxin by the action of the greenshell mussel *Perna canaliculus* (MacKenzie et al. 2002) and *Mytilus galloprovincialis* (Suzuki et al. 2001a), but with better efficiency in the second case, where over 90% of the YTX in the food was converted in 45OH-YTX, *P. canaliculus* transformed less that 10%. In the latter species a number of related substances, probably metabolites of YTX not yet identified, have also been found (MacKenzie et al. 2002).

Recent observations of the distribution of azaspiracids in different organs of the mussel *Mytilus edulis* (James et al. 2002b) showed a dominance of AZA1 in the digestive gland and AZA3 in the remaining tissues, suggesting that some organ-specific transformation might be involved.

Elimination

Okadaic acid group

The organisms do not retain the DSP toxins indefinitely and, some time after the ingestion of toxic cells is interrupted, the toxin burden of the organisms becomes undetectable. As most of the toxin monitoring programmes use, or have used in the recent past, the mouse bioassay, the depuration data of these types of toxins have mostly studied the elimination of toxicity rather than the elimination of the toxins. In simple situations where there is only one toxin or several toxins with similar depuration rates and toxic power, the depuration of toxicity will parallel toxin depuration, but in other –more complex- cases the depuration of the toxicity might relate poorly to that of each particular toxin. Different species depurate DSP toxicity, as well as okadaic acid (the most widely studied toxin of this group), at different rates. Table 6 summarises the depuration rates (computed by assuming a simple exponential decrease) and the time of semi-depuration of several bivalve species.

There is a noticeable variability between species but it does not appear to be as great as that recorded in other groups of phycotoxins. There is no report of species with a very long retention time as happens with PSP toxins in *Saxidomus giganteus* (Kitts et al. 1992) or with ASP in *Pecten maximus* (Blanco et al., 2002a) or *Siliqua patula* (Horner et al., 1993). Similarly, no species has been found to depurate this kind of toxin as fast as most mytilids do with domoic acid (the main ASP toxin), as *Perna canaliculus* (1.97day-1, MacKenzie et al., 1993); Mytilus californianus (0.34-0.55 day-1, Whyte et al., 1995); M. edulis, (0.49-0.99 day1,Novaczek et al., 1992; 2.01-10.59 day-1, in one or two hour experiments, Novaczek et al., 1991; 10.59 and 2.2 day-1 in two hour and 24 hour experiments respectively, Wohlgeschaffen et al., 1992 and M. galloprovincialis, 0.40-0.58 day-1, Blanco et al., 2002b).

Different toxins also depurate at different rates. DTX2 and its low polarity derivatives (DTX3) depurate –in *Mytilus galloprovincialis*- more slowly than OA and OA derivatives.

Table 7.- Depuration rates of “diarrhoeic” toxins of bivalves or body fractions of bivalves.

Shellfish species	Causative species	Body fraction	Depuration rate (day^{-1})	Semi-depuration time (days)	Observations	Reference
Argopecten irradians	Prorocentrum lima	Viscera	0.088	7.9	Experimental depuration	(Bauder et al. 2001b)
		Gonad	0.684	1.0		
		Other	1.137	0.6		
Mytilus edulis	Dinophysis spp.	Whole body	0.016	43.3	Autumn-Winter conditions, Sweden, 6-8ºC*	(Krogh et al., 1985)
Mytilus edulis	Dinophysis spp.	1 week	0.15	4.6	5-day period. Autumn-Winter natural conditions, 8-10ºC	(Haamer et al., 1990)
	Dinophysis spp.		0.12	5.8	Tanks with yeast?	
			0.15	4.6	Tanks without yeast? 5-day periods. Decreasing part	(Haamer et al. 1990)
	Dinophysis norvegica? Prorocentrum lima?	10 days	0.17	4.1	DTX 1. Natural conditions. Canada August	(Quilliam et al., 1993)
Mytilus galloprovincialis	Dinophysis acuminata/ acuta	Digestive gland	0.08	8.7	Natural conditions. Galician Rías. 13-15.2ªC	(Blanco et al., 1995)
		Digestive gland	0.040	17.3	Permanently submerged	(Moroño et al., 1998)
			0.036	19.3	4h/day emerged	
	Digestive gland	0.05	13.8	OA	(Fernández et al. 1998b)	
			0.03	23.1	DTX2	
	Dinophysis acuminata	Digestive gland	0.22- 0.02	3.2	Natural conditions. Galician Rías. 13-15.2ªC	(Blanco et al. 1999)
				34.7		
	Dinophysis spp.	Digestive gland	0.07 0.08	9.9	Mediterranean mussels	
				8.7	Breton mussels, unidentified species (it might be M. edulis) Experimental pond. 16ºC	(Marcaillou-Le-Baut et al., 1993)
	Dinophysis fortii?	Digestive gland	0.52-0	1.3-•	Ozone treatment in experimental tanks. ELISA. 15-18ªC	(Croci et al., 1994b)
	Dinophysis fortii?	Digestive gland	0.21	3.3	Natural conditions. Adriatic sea. 20-25ºC*	(Poletti et al., 1996)

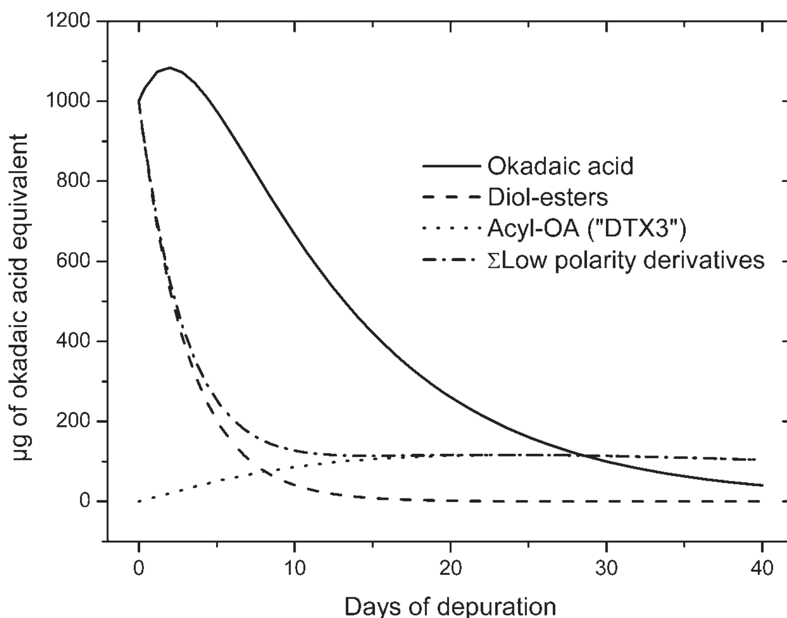


Figure 8.- Expected time course of okadaic acid and some of its derivatives when hydolysis of diol-esters and acylation of okadaic acid take place in bivalves.

Each group of derivatives also has a slower depuration rate than their corresponding parent toxins (Fernández et al., 1998a). The first fact suggests that toxic episodes in which DTX2 is involved last longer than those in which OA is the responsible toxin. The second fact implies that, when acyl-derivatives are produced in large quantities, detoxification will last for a very long time.

Very little information is available on the depuration of DTX4, DTX5 and diol esters and their disappearance from some bivalves is probably due more to their hydrolysis -to yield the corresponding acid toxin- than to their being eliminated from the organism. The combination of transformation and depuration of the toxins may lead to complex or apparently incoherent kinetics of each individual toxin (FIG. 8) and the observations are difficult to interpret if the kinetics of the group of toxins is not modelled.

Other toxin groups

To date, only a few studies have dealt specifically with the depuration of other groups of DSP toxins. Hess et al. (2003), obtained a time series of azaspiracid concentration in the mussel *Mytilus edulis*. Assuming that there is no reintoxication when toxin concentration decreases continuously over the course of three or more consecutive observations, the depuration rates computed range from 0.014 to 0.098 day^{-1}.

The organisms do not retain the DSP toxins indefinitely and, some time after the ingestion of toxic cells is interrupted, the toxin burden of the organisms becomes undetectable. As most of the toxin monitoring programmes use, or have used in the recent past, the mouse bioassay, the depuration data of these types of toxins have mostly studied the elimination of toxicity rather than the elimination of the toxins. In simple situations where there is only one toxin or several toxins with similar depuration rates and toxic power, the depuration of toxicity will parallel toxin depuration, but in other –more complex- cases the depuration of the toxicity

might relate poorly to that of each particular toxin. Different species depurate DSP toxicity, as well as okadaic acid (the most widely studied toxin of this group), at different rates. Table 7 summarises the depuration rates (computed by assuming a simple exponential decrease) and the time of semi-depuration of several bivalve species.

There is a noticeable variability between species but it does not appear to be as great as that recorded in other groups of phycotoxins. There is no report of species with a very long retention time as happens with PSP toxins in *Saxidomus giganteus* (Kitts et al. 1992) or with ASP in *Pecten maximus* (Blanco et al., 2002a) or *Siliqua patula* (Horner et al., 1993). Similarly, no species has been found to depurate this kind of toxin as fast as most mytilids do with domoic acid (the main ASP toxin), as *Perna canaliculus* (1.97day^{-1}, MacKenzie et al., 1993); *Mytilus californianus* (0.34-0.55 day^{-1}, Whyte et al., 1995); *M. edulis*, (0.49-0.99 day^{-1},Novaczek et al., 1992; 2.01-10.59 day^{-1}, in one or two hour experiments, Novaczek et al., 1991; 10.59 and 2.2 day^{-1} in two hour and 24 hour experiments respectively, Wohlgeschaffen et al., 1992 and *M. galloprovincialis*, 0.40-0.58 day^{-1}, Blanco et al., 2002b).

The greenshell mussel *Perna viridis* depurates YTX at a low rate –0.014 day^{-1}- when the process was followed for ca. 150 days (MacKenzie. et al. 2002), but the rate was much higher in a 10-day experiment –0.093 day^{-1}- (Suzuki et al. 2001a). In the same experiment, an analogue of YTX -the 45-OH-YTX- was found to depurate very quickly from the blue mussel *Mytilus galloprovincialis* –0.23 day^{-1}- but the large experimental variance observed and the possibility of the reduction of YTX raise some doubts as to the reliability of the estimation. In a recent study (Franchini et al. 2003) it was found that yessotoxins are mostly located at the lumen of digestive tubules and ducts inside the digestive gland and that they do not seem to be bound to the internal structures of digestive cells, thus suggesting rapid elimination.

Perna viridis, in New Zealand, depurates the main pectenotoxin at a rate of 0.027 day^{-1}, which is very similar to the rate computed (by the method described previously) on the basis of the data from the Aveiro Lagoon, in Portugal (Vale and Sampayo, 2002a), for the cockle *Cerastoderma edule*, 0.025-0.10 day^{-1}.

Notwithstanding, it differs substantially from those obtained for mussels *Mytilus galloprovincialis* (in the same area and study, cited as *Mytilus edulis*) which were much higher, ranging from 0.27 to 0.47 day^{-1}. The latter rates indicate a very fast apparent depuration, suggesting that a transformation process might be involved.

Regulation of the depuration

The process of toxin depuration is presumed to be affected by environmental factors such as temperature, food quantity and others. However, none of these factors has been unequivocally linked to the velocity of depuration. It is generally assumed that low temperatures lead to low depuration rates (Shumway and Cembella, 1993) but there is no solid evidence to back this up. This might be expected even more so if enzymatic processes are involved in the depuration of these toxins, since the Q_{10} of most enzymatic reactions is close to 2. Mussel depuration rates in areas with low water temperatures, such as Sweden and Norway, do not seem to be slower than those in Spanish (Galician), French and Italian (Adriatic Sea) mussels. The attempts made to obtain precise estimates of the temperature effect did not result in any clear conclusions (Blanco et al. 1999) and consequently, it can be inferred that if the effect does indeed exist, it is not substantial. This, in turn, would suggest that okadaic acid depuration is not an enzymatically mediated process. Salinity, also appears to have no effect on depuration.

Differences in depuration rates between mussels maintained in a culture pond and at sea have been reported (Marcaillou-Le-Baut et al. 1993) and attributed to differences in food resources. Observations made by Sampayo et al. (1990) during several DSP episodes on the Portuguese coast produced by *Dinophysis*, suggest that the depuration rate increases with phytoplankton con-

centration, which is consumed as food by bivalves. The same conclusion was drawn by Blanco et al. (1999), with Galician mussels, but the effect was reported to be small. Recently, Svensson (2003) came to the opposite conclusion. She found that mussels that did not receive food depurate okadaic acid faster than those that were fed. Nevertheless, the time course of the depuration of the mussels in this experiment did not follow the typical exponential decrease kinetics, and exhibited an increase at the beginning of the experiment, which would suggest that other processes besides depuration are involved (probably transformations from esterified derivatives). This would mean that the estimates of the depuration rate are of little value and difficult to compare with other rates.

Changing mussels from an aerobic to an anaerobic type of metabolism by means of emersion several hours a day did not alter the depuration of okadaic acid or DTX2 (Moroño et al. 1998), implying that some of the main metabolic processes of mussels do not affect the depuration rate substantially.

Part of the problem regarding this uncertainty about the real effects of environmental factors on depuration lies in the fact that a correct estimation requires sound knowledge, both conceptual and mathematical, of the kinetics of the toxins involved, (as shown by Moroño et al. 1998, Fernández et al. 1998 and Blanco et al. 1999), and this knowledge has only just begun to emerge in recent years with the discovery and demonstration of the real contribution to the toxin pools in bivalves of several compounds (Vale and Sampayo, 2002a, MacKenzie et al. 2002, Moroño et al. 2003).

Allometric aspects of depuration

As a general rule, for consumer protection, the interest is focused on the toxin concentration in the organisms consumed rather than on their burden. This is mandatory, as safety limits must be defined by using a typical food ration based on weight. The toxin concentration of an organism depends not only on the toxin burden but also on the amount of biomass in which the toxins are distributed and, therefore, on the weight of the soft tissues. As a consequence, any alteration of the weight of the soft tissues –if it is not matched by an equivalent modification in the toxin burden– produces changes in the toxin concentration. For DSP contaminated shellfish, the amount of toxin is relatively independent of weight, since weight losses are not paralleled by toxin losses (toxins are not lost at the same rate as biomass). In such cases, when the organism increases its weight, the toxicity decreases because the toxins are “diluted” in a larger biomass. On the other hand, when the weight decreases, the same amount of toxin is concentrated in a smaller portion of biomass, and the toxin concentration of the organism increases. These situations have been reported in several studies carried out both in natural and laboratory conditions (see Bricelj and Cembella, 1995; Blanco et al., 1997; 1999).

Predicting DSP toxin accumulation. Dynamic modelling

The prediction of the amount or concentration of toxin in shellfish requires mathematical modelling. Models have at least two parts: a gain term –which describes the toxin acquired by the organism, starting from the toxin available in the environment– and a loss term which usually pools all possible ways of toxin elimination in a generic depuration term. The gain term includes all the processes that are involved in the acquisition of toxins. In bivalve molluscs, however, it is usually reduced to a few terms describing food acquisition, since the toxins are mainly taken up with the phytoplankton cells that produce them, and consequently other possible uptake mechanisms, such as direct absorption from the water in gills or mantle, may be overlooked. In the models implemented to date, the toxin acquisition process is described with two parameters -the clearance rate and toxin absorption efficiency. The food capture processes, mainly water pumping through the gills, retention in the gills, and pre-ingestive selection, are

pooled into a single parameter -the clearance rate- which describes the rate at which phytoplankton cells are taken up by the bivalves. Once food containing the toxins is captured, it undergoes the processes of post-ingestive selection and digestion with the result that only a portion of the toxin is incorporated by the organism. This is described by toxin absorption efficiency (used only when we are interested in the toxins which are completely internalised by the bivalves). Mathematically, this part of the model can be expressed as:

$$dtox/dt= CR \cdot P_w \cdot T_p \cdot AE \qquad [1]$$

where CR is the clearance rate, P_w the toxic plankton concentration in water, Tp the toxin content per phytoplankton cell, and the product of these three, the amount of toxin retained by the mollusc. AE is the absorption efficiency of the toxin, and its product by the toxin retained yield the toxin absorbed by the mollusc.

The depuration kinetics of several species of shellfish previously affected by DSP episodes have been studied by different authors. In some studies (Marcaillou-Le-Baut et al. 1993; Shumway and Cembella 1993) a two-step depuration curve was found, with high depuration rates during the first few days and much smaller ones afterwards. In other cases (Croci et al. 1994) a single depuration rate appears to exist for the whole depuration period. As with PSP toxins, these two responses can be described by two- and one-compartment models, respectively (Silvert and Cembella, 1995, Blanco et al. 1997). In the depuration of a whole organism, there are several organs or tissues, each with its own depuration rate. An ideal model of the kinetics would use a different compartment for each organ/tissue (defined by the initial amount of toxin it contains, its depuration rate and the transfer rates to/from other compartments). Such complex models are very difficult to implement in real situations because a large number of parameters have to be estimated and, in most cases, this is not feasible. In relatively simple situations, when nearly all the toxin is accumulated in one single organ or when (an unlikely case) all the organs have similar depuration rates, one compartment is usually enough to provide a satisfactory model of the kinetics, which are known as monophasic. A slightly more complex situation, in which the toxin is distributed among organs/tissues, some with high and others with slow depuration rates, can usually be modelled using two compartments, yielding the type of kinetics known generally in the literature as bi-phasic (even though in fact the result of two processes acting simultaneously and not of one process acting differently in two phases).

Taking into account the previous consideration, the loss term can be expressed as follows:

One compartment model

$$dtox/dt= -K \cdot tox$$

where tox is the toxin concentration and K the depuration rate.

Two-compartment model

$$dtox_1/dt= -K_1 \cdot tox_1$$

$$dtox_2/dt= -K_2 \cdot tox_2 - T12 \cdot tox_1$$

Where sub-indices indicate the compartment and T12 is the transfer rate between the first and second compartments.

Blanco et al. (1995), have shown that a very simple one-compartment model can reasonably describe some DSP episodes in Galicia.

When several toxins, which can be transformed into others, are involved, the models are more complex but this may well be the only way to estimate the real depuration rates, because studying the toxins as if they were independent would lead to over or underestimations of the depuration velocity, as can be observed with both DSP (Blanco et al. 1999, Moroño et al. 2003) and PSP toxins (Silvert et al., 1998; Moroño et al. 2003).

When studying the effects of different factors on depuration, it is necessary to choose the appropriate model to be used in the estimation of the rates, as this will substantially affect the conclusions (Moroño et al. 1998; Blanco et al. 1999).

REGULATION FOR HUMAN CONSUMPTION

Among all the groups of marine toxins, those included in the DSP group have been the subject of greatest controversy, and there is no general agreement as to which liposoluble toxins should be regarded as DSP toxins, which ones should be monitored and regulated, what the tolerance levels are, and what the most appropriate testing procedures for regulatory purposes are. Historically, okadaic acid (OA) and its analogues, pectenotoxins (PTXs) and yessotoxins (YTXs) have been included in the DSP group, with a general consensus on the OA group, but conflicting opinions on the other two groups. Azaspiracids have been considered a different group ever since their discovery.

In terms of the sanitary control of these groups of toxins, there are discrepancies in the methods and criteria used for positive results. Although mammalian bioassays have been widely applied in DSP toxicity determination, there are great differences in the performance of the procedures. Bioassay procedures, which are as diverse as the oral dosage rat bioassay (Kat, 1983) and the i.p. injection mouse bioassay, are not equivalent because the former technique quantifies only the diarrhoeic effect of certain DSP toxins whereas the latter assay provides an estimate of total DSP toxicity (Cembella et al., 1995). As far as mouse bioassays are concerned, selectivity, specificity and toxin recovery depend greatly on the selection and ratio of the organic solvents used for extraction.

Regarding regulatory levels, most countries have set the threshold at the detection limit of the analytical method used. The first country to establish a limit was Japan – 5 MU/100 g shellfish tissue, based on an epidemiological study (Yasumoto et al. 1978). This limit has also been established in the Republic of Korea and New Zealand. Chile, Thailand, Turkey, Uruguay and Venezuela regulate DSP on the basis of the Yasumoto mouse bioassay (1978) with a survival time of 24 h as the criterion for a positive result. Canada and the USA have no official regulations on DSP, although Canada may issue informal advice on positive results by mouse assay and/or the fluorescence high-performance liquid chromatography (HPLC) method. Regarding the EU European Union, Directives 91/492/EEC and 91/493/EEC lay down the health conditions for the production and marketing of live bivalves and fishery products respectively.

Directive 91/492 establishes the control of the shellfish production areas and the different activities including harvesting, transport, depuration, storage, processing and marketing, as well as microbiological and chemical parameters and marine toxin content. Regarding DSP control in the European Union, EU Directive 91/492 stipulates that the customary biological method must not yield a positive result for the presence of DSP in the edible part of the molluscs, but it did not clarify the interpretation of positive results and which biological methods should be used.

A Working Group organised by the EU Commission (SANCO) and the EU-Community Reference Laboratory performed a risk assessment on the basis of the available data on okadaic acid and dinophysis-toxins, pectenotoxins yessotoxins (and azaspiracids) with the goal of recommending allowance levels and suitable detection methods. The recommendations and conclusions of this WG were the scientific basis of Decision 2002/225/EC that lays down detailed rules for the implementation of Directive 91/492, as regards the maximum levels and the methods of quantification of OA and Dinophysistoxins, Pectenotoxins, Yessotoxins and Azaspiracids in bivalve molluscs, echinoderms, tunicates, and marine gastropods and the analytical methodology to be used.

Regarding maximum levels, the following was established:

a) The maximum level of okadaic acid, dinophysistoxins, and pectenotoxins, together shall be 160 μg of okadaic acid equivalents kg -1 edible part (the whole body or any part edible

separately).

b) The maximum level of yessotoxins shall be 1 mg of yessotoxin equivalents kg^{-1} of edible part (the whole body or any part edible separately).

c) The maximum level of azaspiracids shall be 160 µg of azaspiracid equivalents kg^{-1} of edible part (the whole body or any part edible separately).

Concerning detection methods, the new Decision states that, in addition to biological testing methods, alternative detection methods such as chemical methods and in vitro assays should be accepted if it is demonstrated that the performance of the chosen method is not less effective than the performance of the biological method and that their implementation provides an equivalent level of public health protection. The compounds to be detected are: OA and Dinophysistoxins, PTX1 and PTX2, YTX, 45 OH-YTX, homo-YTX and 45-OH homo-YTX AZA1, AZA2 and AZA 3. However, if new analogues of public health significance are discovered they should be included in the determinations. Standards must be available before chemical analysis will be possible and total toxicity will be calculated using conversion factors based on the toxicity data available for each toxin. The performance of these methods should be defined after validation following an internationally agreed protocol.

Concerning biological methods, it was stated that a suite of mouse bioassay procedures differing in the test portion and in the solvents used for the extraction and purification steps may be used. Sensitivity and selectivity depend on the choice of the solvents and this should be taken into account, when making a decision on the method to be used, in order to cover the full range of toxins. With regard to the mouse bioassay, it has been established that the death of two out of three mice within 24 hours after inoculation into each one of an extract, equivalent to 5 g of digestive gland or 25 g of whole body, should be considered as a positive result for the presence of one or more of the toxins of the groups regulated in the Decision at levels above those allowed.

The full implementation of this new Decision on the basis of chemical methods and/or in vitro assays still requires a great effort to be made in the development of toxin standards, toxicological studies and validation of alternative methods. Nevertheless it represents an important step towards the harmonisation of phycotoxin control approaches.

A programme leading towards the harmonisation of sanitary controls is being carried out among the countries belonging to the Asia Pacific Economic Cooperation. APEC comprises 18 coastal and archipelago economies with highly diversified marine resources which constitute a significant portion of the regional economic production. Economies belonging to the network are Australia, Brunei, Canada, China, Chinese Taipei, Hong Kong China, Indonesia, Japan, Malaysia, Mexico, New Zealand, Papua New Guinea, the Philippines, the Republic of Korea, Singapore, Thailand and the USA.

The fundamental principle for APEC economies, in seafood certification and trade with respect to marine algal toxins, is the application of performance-based criteria wherever possible. For the DSP groups, a task team on Algal Biotoxin Regulation has proposed that all APEC accept the absence of toxins using bioassays (either rat or mouse) as the limit, subject to change as improvements occur in understanding the toxins or in the availability of detection methods. Furthermore, the use of alternative detection methods is welcomed, insofar as the economy demonstrates that the performance of the chosen alternative is not less than the performance of the bioassay.

MITIGATION

Reducing toxin availability

Exposure of shellfish to phytoplankton that produces DSP toxins can be reduced, at least

in theory, mainly through two procedures: reducing the algal biomass bloom and changing the location of the shellfish population. No method has been used, to date, to try to reduce the toxic algae population, although this approach has been used to eliminate proliferations of *Cochlodinium polykrikoides* in Korea (Kim, 1998).

Other strategies (reviewed in Blanco-Pérez, 2001 and partially in Elbrächter and Schnepf, 1998), such as the use of ultrasounds or chlorine to kill the algal cells or the use of several viral, bacterial, fungal or protozoan parasites that kill the cells, have been tested experimentally, but have not been used in real blooms. The second possible method of action consists of relocating the shellfish population (specially if they are cultured) to places or at depths where the concentration of the harmful population is minimal or restricting the culture to such places (Haamer et al. 1990; Desbiens and Cembella, 1993).

Eliminating toxins from the organisms

Elimination of toxins from living organisms

Various attempts have been made to accelerate the depuration of live shellfish contaminated with DSP. The most obvious method is to transfer shellfish to waters free of the toxic organisms and with environmental conditions that favour depuration. Unfortunately, the minor effect of environmental conditions on the PSP and DSP detoxification rate of the mussel *Mytilus galloprovincialis* (Blanco et al. 1999) would suggest that transplantation is more useful in preventing reintoxication than in increasing the depuration rate. Transplantation is, however, being used in several countries.

Although diverse treatments have been tried to accelerate the elimination of PSP toxins from bivalves (see review in Fernández et al., 2003c), far fewer attempts have been made with DSP toxins. Croci et al. (1994) showed that treatment with ozone had no significant effect on the depuration of this kind of toxins. Temperature and salinity seem to have no effect (Blanco et al. 1999), and food supply was reported to have little positive effect by several authors (Sampayo et al. 1990; Blanco et al. 1999) while starvation was found to increase the apparent depuration rate (see the comments at the beginning of the “elimination” section) by Svensson (2003)

Elimination of toxins by cooking and other treatments

When the contaminated organisms are not to be sold or consumed alive, some processes can be used to reduce or eliminate the amount of toxin they contain. These techniques range from the simple process of selective evisceration to cooking or more complex industrial processing techniques. Some species accumulate toxins preferentially in one or several organs. In these cases an easy way to eliminate most of the toxin is to remove such organ/s (evisceration). Obviously, this procedure can only be used when the size and anatomical configuration of the species allows for the easy dissection of the toxic organs and the species has a commercial value that is great enough to compensate for the high economic cost of dissection, and, also for the additional expense incurred by the special procedures required for extraction from the sea and the quality control of toxin content reduction. This procedure is currently being applied in the EU for the king scallop *Pecten maximus* contaminated with ASP toxins, and may also be used in *Argopecten irradians* with DSP toxins because of its characteristics in the accumulation of these types of toxins (Bauder et al. 2001), but perhaps the shorter depuration time, relative to ASP toxins in the king scallop, make this possibility economically unfeasible.

Boiling mussels in a slightly alkaline medium, during the canning process, has been shown to reduce the OA content between 25 and 75% but the heterogeneity in the results obtained is

high (Vieites Baptista de Sousa et al. 1995).

Recently, the basis for a procedure to eliminate DSP toxins from molluscs by means of critical point extraction has been established by González et al. (2002), presenting an interesting approach for their elimination. The technique requires dehydration of the product and therefore will only be useful in products that require or at least tolerate this process.

PERSPECTIVES

As new analytical techniques are developed and more and more intensive, monitoring systems are implemented, new lipophilic toxins are being discovered raising some doubts as to how to adequately monitor their risk to human consumers. How to monitor an increasingly complex group of toxins, how to regulate their allowable levels and how to harmonise the regulations of different countries, are probably the most relevant problems for human health. The definition of the toxins that must be monitored depends on their real risk to humans, but the risk presented by new toxins, as well as some of those already known, has yet to be precisely determined. The lack of toxicological information may either increase the risk for public health or charge the producers with the cost of overprotective legislation. Toxicological studies are, therefore, strongly needed. Notwithstanding, the large number of toxin analogues or derivatives, probably with different toxic power, will make very difficult -if not impossible- both the assessment of the toxicological characteristics of them all and their individualised detection. Approaches able to deal with these problems would require a reduction of the actual complexity, perhaps by using well known toxins as indicators or by quantifying the overall toxicity of the samples or their effect on some key (indicator) processes involved in the cellular/molecular mechanisms of toxicity. An effort to identify these indicator toxins or processes and to evaluate “in vivo” assays, if any, –such as the mouse bioassay- which can be reliably used to quantify the overall toxicity, should be made in the near future.

Mitigating the consequences of toxic episodes for shellfish cultivators or harvesters is another important research area that needs to be developed. Our understanding of the processes of toxin retention by shellfish is very rudimentary and, as a result, it is difficult to devise effective mechanisms to accelerate toxin depuration or elimination from live or processed shellfish. Understanding the retention mechanism involved at any level (organ-cellular-molecular) would open up a number of different possibilities, very likely making use of different disciplines, to achieve safe products in less time, substantially reducing the producers’ economic losses. Controlling toxic phytoplankton populations or their availability to shellfish may also be a way to minimise the incidence but, in the former case, the possible ecological impacts should be carefully evaluated and, in the latter, the systems implemented such as, for example, the automatic relocation of shellfish in the water column, might be difficult to harmonise with the requirements of a reliable monitoring system.

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