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Two decades of marine biotoxin monitoring in bivalves from Portugal (1986–2006): A review of exposure assessment

Paulo Vale *, Maria João Botelho, Susana Margarida Rodrigues, Susana Sousa Gomes, Maria Antónia de M. Sampayo

Instituto Nacional de Investigação Agrária e das Pescas (IPIMAR), Av. Brasília, s/n, 1449-006 Lisboa, Portugal

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Abstract

Foodborne outbreaks attributed to marine biotoxins were first reported in Portugal in 1946. A regular monitoring programme was implemented in 1986 for PSP, in 1987 for DSP and in 1996 for ASP. The gradual introduction of HPLC methodologies for DSP and PSP allowed a better understanding of toxin biotransformation by bivalves, supplying more selective and sensitive data than mouse bioassays. A comprehensive exposure assessment from DSP toxins in bivalves from the whole coast was only obtained more recently with the introduction of LC–MS methodology. Data on maximum toxin levels found, geographic distribution, seasonality of toxin families, and frequency of samples above current regulatory limits is presented in order to review the data available on exposure assessment after two decades of monitoring. Contamination with DSP toxins was more severe in estuarine and offshore bivalves from the NW and in offshore *Donax* spp. from the SW and south coasts. DSP toxins were recurrent every year mainly between late spring/early autumn. PSP toxins appeared intermittently in some years between 1986 and 2006, predominantly in autumn. Bivalves from the entire coast were severely contaminated, although bivalves from the NW coast were affected more often. ASP toxins appeared between spring and autumn around the entire coast, but toxin levels rarely exceeded the regulatory limit. Azaspiracids occurred in trace levels below the regulatory limit. Yessotoxins and pectenotoxins occurred in bivalves but have no known effects on the consumers. Several intoxication outbreaks attributed to PSP and DSP occurred during the two decades of the monitoring programme.

Keywords: Bivalves; Foodborne illness; Marine biotoxins; Monitoring program; Portugal; Risk assessment

1. Introduction

Human poisonings after consumption of bivalves contaminated with microalgae toxins have been recorded in Europe for several decades. Paralytic shellfish poisoning (PSP) contamination events in Norway are among the earliest recorded in Europe (1901, 1939 and others). For all the seven Norwegian outbreaks recorded between 1901 and 1992 a total of 32 victims including two fatalities were reported (FAO, 2004). In 1946 Portugal became one of the first European countries reporting a poisoning with 100 victims, including 6 fatalities of children, attributed to consumption of bivalves picked at Óbidos lagoon (Correia, 1946). In 1955 another poisoning involving 21 victims and one child fatality was described from the same lagunar area (Pinto and Silva, 1956). This phenomenon was peculiar to this lagoon alone, and occurred only when the connection to the sea was obstructed for a long time. In the 1960s a gastro-intestinal illness outbreak in Holland that was initially

* Corresponding author. Tel.: +351 213027000; fax: +351 213015948.
E-mail address: pvale@ipimar.pt (P. Vale).

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attributed to pathogenic microorganisms, was later attributed to diarrhetic shellfish poisoning (DSP) (Kat, 1979).

Reports of marine biotoxins from other European countries appeared in the 1970s and 1980s when aquaculture farming began developing. In 1976 mussels from the Galician Rias were exported to other countries and a large PSP outbreak of 120 people occurred in Western Europe following consumption of mussels. No deaths were recorded during this event (IPCS, 1984).

To prevent intoxication events such as this and allow a safe trade of shellfish products, Portugal introduced a monitoring program for toxic phytoplankton and PSP toxins in 1986 and for DSP toxins in 1987, as only PSP and DSP biotoxins were known in temperate waters at this time. In 1989 the first reports of amnesic shellfish poisoning (ASP) in North America (Quilliam and Wright, 1989) appeared in scientific literature, and in 1996 a new gastrointestinal illness—azaspiracid poisoning (AZP) was described in Europe (McMahon and Silke, 1996).

This paper reviews data from the Portuguese biotoxin monitoring programme for the two decades of its existence, including the more recent detected toxins, particularly—domoic acid (DA), azaspiracids (AZAs), pectenotoxins (PTXs) and yessotoxins (YTXs). Maximum concentrations found, geographic distribution of toxin families, seasonality and frequency of samples with levels above the current regulatory limits—Regulation EC 853/2004 (Anon., 2004a) will be presented in order to review the data available so far on exposure assessment. Residual levels found will also be presented in order to discuss the risks of prolonged exposure.

The monitoring program covers lagunar and estuarine areas, as well as offshore areas from all around the coast (Fig. 1). A production area from the NW coast (Aveiro lagoon) suffering from recurrent biotoxin contamination will be used throughout the presentation as a typical example of high contamination and worst-case scenario. In 2002, following recommendations of a European working group on sampling plans (CRL, 2001) the programme was updated in order to improve the study of biotoxin contamination using the concept of indicator species—the species that has the highest rate of toxin accumulation. For lagunar/estuarine areas *Mytilus galloprovincialis* and *Cerastoderma edule* (exceptionally *Tapes decussatus* in some areas) were chosen as indicators, while in offshore areas *Donax trunculus* and *Spisula solida* are now used. The recommendation of weekly testing, now also incorporated into Regulation EC 854/2004 (Anon., 2004b), was

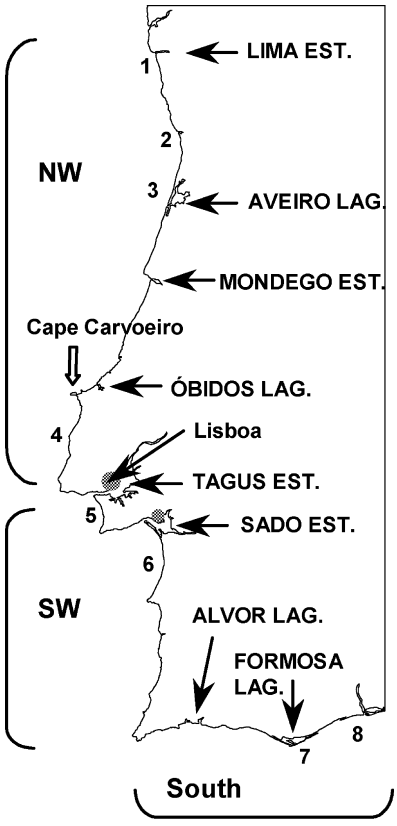

Fig. 1. Localisation of main inshore and offshore bivalve production areas. Est. = estuary; Lag. = lagoon; NW = northwest offshore coast; SW = southwest offshore coast. Offshore areas are numbered from north to south.

introduced in 2002, except during winter when the monitoring switches to fortnightly/monthly sampling if no PSP is detected during autumn/early winter. The study of time-series previous to 2002 is hampered due to the lack, in some production areas, of weekly testing of the indicator species, which might result in missing toxin peaks, and thus maximal exposure levels.

2. Diarrhetic shellfish poisoning

The first set of data on DSP toxins was obtained in mid 1987 by analysing the digestive glands of bivalves using a mouse bioassay method. Although several approaches were used throughout the years (Yasumoto et al., 1978, 1984; Le Baut et al., 1991), none of them provided results free from overestimation or underestimation of DSP toxins. Today it is known that a large variety of compounds can give false positive results in the mouse bioassay, such as yessotoxins. One of the approaches to avoid free fatty acid's interference (Le Baut et al., 1991) was later demonstrated to underestimate DSP toxicity

Table 1
Maximum DSP concentrations in estuarine/lagunar production areas between 2003 and 2006 (levels in μg OA equiv./100 g shellfish meat)

Coastline	Estuary/lagoon	Indicator species	2003	2004	2005	2006
NW	Lima estuary	*Mytilus* spp.	140	23	87	99
	Aveiro lagoon	*Mytilus* spp.	418	156	659	77
	Mondego estuary	*Mytilus* spp.	103	85	310	110
	Óbidos lagoon	*Mytilus* spp.	34	90	55	213
SW	Tagus estuary	*Mytilus* spp.	na	1	1	1
	Albufeira lagoon	*Mytilus* spp.	7	na	43	53
	Sado estuary	*Scrobicularia plana*/ *Cerastoderma edule*	nd	nd	6	nd
South	Alvor lagoon	*Tapes decussatus*	7	17	5	na
	Formosa lagoon	*Mytilus* spp.	10	18	25	14

na: data not available; nd: not detected.

(Vale and Sampayo, 1999). The use of whole flesh in the assays can also lead to false negatives, in particular when toxin concentrations are close to the regulatory limit (Vale et al., 2006). A major disadvantage of the method is that it provides only qualitative data on DSP toxins, and exposure assessment cannot be performed with this type of data. The first set of quantitative data was obtained in routine testing in 1994 using a fluorometric HPLC method (Lee et al., 1987). This method is not sensitive enough to detect routinely low levels of toxins in whole flesh assays, and is thus more suited to analysis of digestive glands only. A major improvement in quantitation of DSP toxins started in 2000 with the introduction of analysis by LC–MS (Suzuki and Yasumoto, 2000).

Two types of toxin profiles have been found in Portuguese shellfish: contamination with okadaic acid (OA) only, or simultaneous contamination with OA and dinophysistoxin-2 (DTX2). The first profile occurred when bivalves predated on *Dinophysis acuminata*, the second one when *Dinophysis acuta* was the main toxic *Dinophysis* spp. present in the plankton (Vale and Sampayo, 2000). Besides these parent toxins, it was demonstrated that bivalves also contained a complex mixture of esterified OA/DTX2 (Vale and Sampayo, 1999). Most commercial bivalves contain DSP toxins almost completely esterified with several fatty acids, with the exception of *M. galloprovincialis* and *D. trunculus*, where free and esterified DSP toxins can be found in variable proportions (Vale, 2006, 2007).

Since 2003, all monitoring samples were tested by LC–MS using whole flesh after alkaline hydrolysis to release esters and quantify the total amount of parent toxins present. Maximal DSP toxin levels found between 2003 and 2006 are presented in Tables 1 and 2. The distribution of DSP toxins along the coast follows a recurrent pattern: in the northwest coastline bivalve species from both offshore and estuarine/ lagunar areas might be strongly contaminated, while in the southwest and south coasts, high DSP levels are found only in bivalves from offshore areas, almost exclusively in *D. trunculus*. Oceanographic conditions show that the highest concentrations of toxic *Dinophysis* spp. are associated with the regular upwelling off the NW coast of Portugal, in particular north of Cape Carvoeiro (Fig. 1) (Palma et al., 1998). Also higher

Table 2
Maximum DSP concentrations in offshore production areas (see Fig. 1) between 2003 and 2006 (levels in μg OA equiv./100 g shellfish meat)

Coastline	Offshore area (no.)	Indicator species	2003	2004	2005	2006
NW	Viana (1)	*Mytilus* spp.	238	49	386	103
	Matosinhos (2)	*Spisula solida*	131	20	154	14
	Aveiro (3)	*S. solida*	35	20	60	14
	Ericeira (4)	*Mytilus* spp.	11	146	46	104
SW	Lisbon (5)	*Donax* spp.	na	na	276	516
	Setúbal/Sines (6)	*Donax* spp.	123	32	173	260
South	Faro/Olhão (7)	*Donax* spp.	144	199	71	96
	Tavira/VRSA (8)	*Donax* spp.	56	137	18	57

na: data not available; VRSA: Vila Real de Santo António.

Table 3
Average DSP levels in selected production areas from the NW and south coasts (μg OA equiv./100 g shellfish meat)

	2003	2004	2005	2006
Aveiro offshore				
S. solida	7.7	3.2	8.0	4.2
Aveiro lagoon				
Mytilus spp.	56.3	23.6	93.6	19.5
C. edule	25.5	13.3	65.8	11.0
Óbidos lagoon				
Mytilus spp.	7.8	9.2	10.4	29.0
C. edule	2.7	3.5	5.6	13.7
Formosa offshore				
Donax trunculus	16.5	42.2	10.2	21.3
S. solida	2.4	7.6	2.7	1.3[a]
Formosa lagoon				
Mytilus spp.	3.2	5.7	2.6	3.0
T. decussatus	1.0	1.4	1.8	0.7

[a] Average underestimated due to prolonged ban for stock reposition.

concentrations of cells are found inshore thus favouring bivalve contamination. At Aveiro lagoon, for instance, five bivalves species might be strongly contaminated (*M. galloprovincialis*, *C. edule*, *T. decussatus*, *Venerupis pullastra*, *Solen marginatus*), but oysters rarely exceeded the RL for DSP toxins.

Bivalves from Tagus and Sado estuaries and Formosa lagoon usually contain very low concentrations of toxins in comparison with their counterparts in Lima/Mondego estuaries and Aveiro/Óbidos lagoons. As maximal levels presented in Tables 1 and 2 do not show the prevalence of toxins throughout the year, we tried to compare some of the main production areas by using the average level found with year round sampling. Table 3 exemplifies a high-risk area from the NW coast (Aveiro), a moderate risk area from the NW (Óbidos) and a low-risk area from the south coast (Formosa). The severity of DSP contamination between 2003/2006 in Aveiro lagoon was so great that the annual average DSP concentration in mussel (*Mytilus* spp.) was always above the regulatory limit of 16 μg OA equiv./100 g. The average DSP levels in cockle (*C. edule*) were above the EU regulatory limit (RL) in 2003 and 2005, but during 2004 and 2006 the toxicity was not so severe and these levels were below the RL. In the Óbidos lagoon average DSP levels were always below the RL in both indicator species with the exception of 2006 when a localised high contamination confined to this region occurred. In the Formosa lagoon both indicator species presented average DSP levels below the RL. In offshore areas, from the two indicator species used in the programme, *S. solida* is the only one commercially exploited in the NW coast. In the NW coast average DSP levels were always below the RL. In the south coast *S. solida* was always below the RL, while *D. trunculus* DSP average was always close or above the RL.

The differences observed between *Mytilus* spp./*D. trunculus*, and *C. edule*/*R. decussatus*/*S. solida*, respectively, are attributable to species-specific metabolism. It was found esterified toxins are cleared faster than free toxins, either in wild populations (Fig. 2a and b) or in laboratory tanks (Vale, 2004a, 2006). In *Mytilus* spp. and *D. trunculus* OA and DTX2 are not completely esterified, thus remaining a large proportion of free toxins that in turn depurate slowly. The biotransformation in these bivalves is also toxin-specific: OA is commonly more esterified than DTX2. After the decline of a *D. acuta* bloom, mussels and donax clams will have a high percentage of DTX2 due to the slow elimination of unesterified DTX2. In remaining commercial bivalves, either OA or DTX2 are completely esterified,

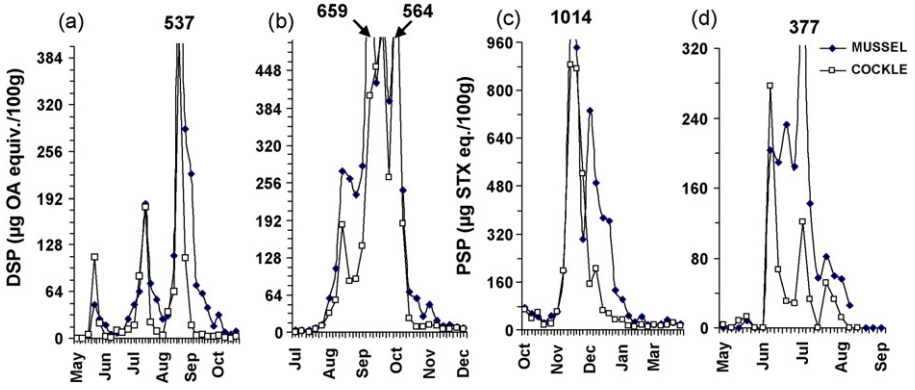

Fig. 2. Temporal evolution of toxicity in mussel and cockle: (a) DSP in Aveiro lagoon in 2002; (b) DSP in Aveiro lagoon in 2005; (c) PSP in Aveiro lagoon in 2005/2006; (d) PSP in Óbidos lagoon in 2006. Maximum toxin levels in mussels are indicated.

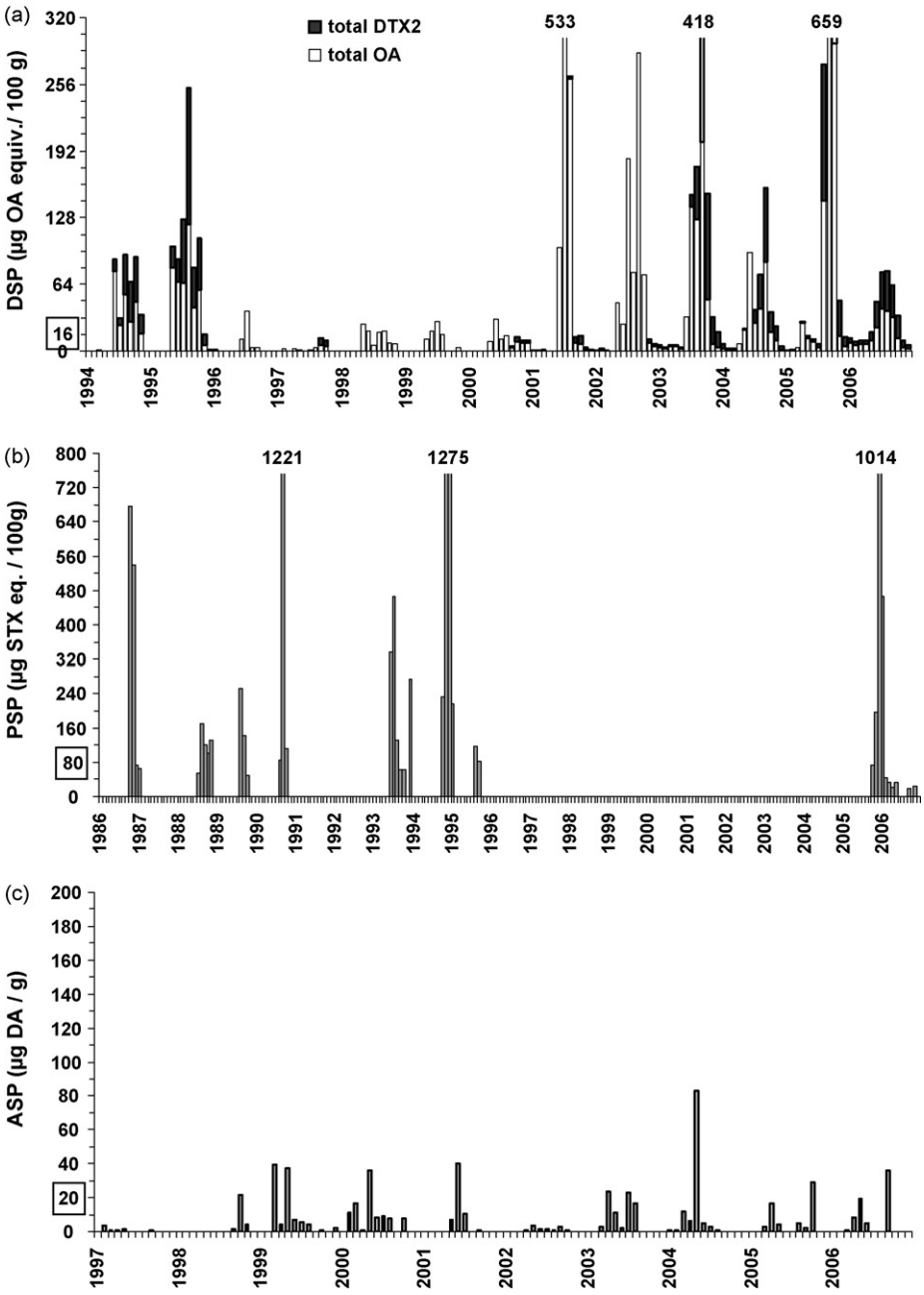

Fig. 3. Maximum monthly biotoxin levels in bivalves from Aveiro lagoon: (a) DSP in mussel; (b) PSP in mussel; (c) ASP in cockle. Analyses for DSP were performed in digestive glands between 1994 and 2000 and in whole flesh from 2001 on. Analyses for PSP were performed by mouse bioassay between 1986 and 2002 and HPLC from 2003 on. Scales were set at 10 times the EU regulatory limit for PSP and ASP, and at 20 times the RL for DSP toxins (boxes).

leading to a fast detoxification from both toxins. The behaviour of bivalves towards DTX1, another common DSP toxin worldwide, is not known due to its absence in the Portuguese coast. A recent review of data from other countries showed DTX1 esters can occur in several bivalves, additionally to the Japanese scallops where these were discovered for the first time (Vale, 2007).

Contamination with DSP toxins might present different patterns from year to year. Several peaks might occur a few weeks apart, or a large peak comprising several successive weeks or months with DSP toxins at high levels might occur (Fig. 2a and b). The DSP season usually starts around May/June (Fig. 2a). In some years the DSP season can start later

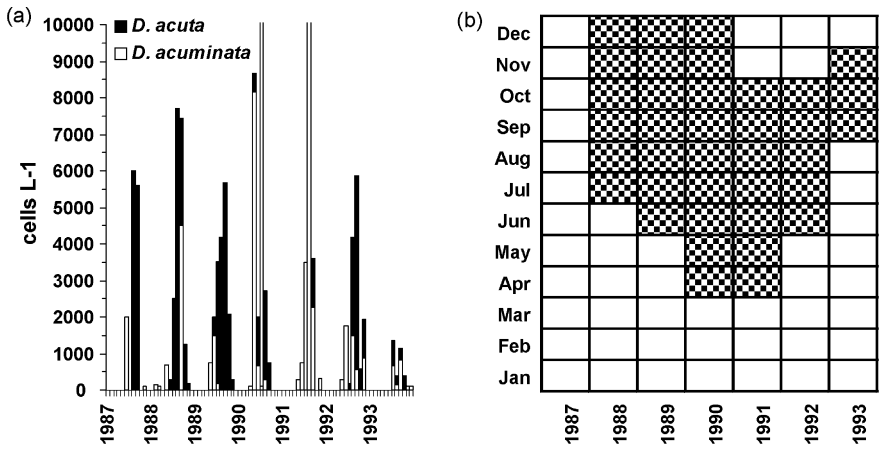

Fig. 4. (a) Maximum monthly cellular concentrations of *Dinophysis* spp. and (b) months where mussel harvest was closed at Aveiro lagoon during the period 1987–1993 (dark squares).

in August (Fig. 2b). Exceptional DSP contamination in *D. trunculus* has been recorded once in February in the south coast. The monthly maximal DSP concentrations in mussel from Aveiro lagoon between 1994 and 2006 are presented in Fig. 3a. Low toxic levels were observed between 1996 and 2000. In the remaining years the RL was always exceeded during at least 3 or more months, in some instances by 10–40 times.

The DSP contamination observed at Aveiro lagoon closely followed a cyclic pattern for these two decades. Although no quantitative data on DSP toxins is available for 1987–1993, the concentrations of toxic *Dinophysis* spp. above 5000 cells/l were abundantly found in this period (Fig. 4a) and would favour a high contamination with DSP toxins as suggested by the toxicity found with mouse bioassays (Fig. 4b) (Vale and Sampayo, 2003). In the north of Portugal, rainfall is more abundant than in the south and river drainage plays a very important role in disrupting optimal conditions for toxic *Dinophysis* growth. The average maximum levels (ML) of DSP toxins were plotted together with the average rainfall during 1994–2006 in Fig. 5a. The onset of the DSP season follows closely the reduction in rainfall. The high average DSP concentrations observed in October after the restart of the rainfall season in late September, reflects the slow detoxification of the indicator species used and also the low impacts on river drainage of early rain derived from soil absorption (Vale and Sampayo, 2003). The average ML was above the RL between May and November, reflecting the important role DSP toxins play in bivalve toxicity in Portugal.

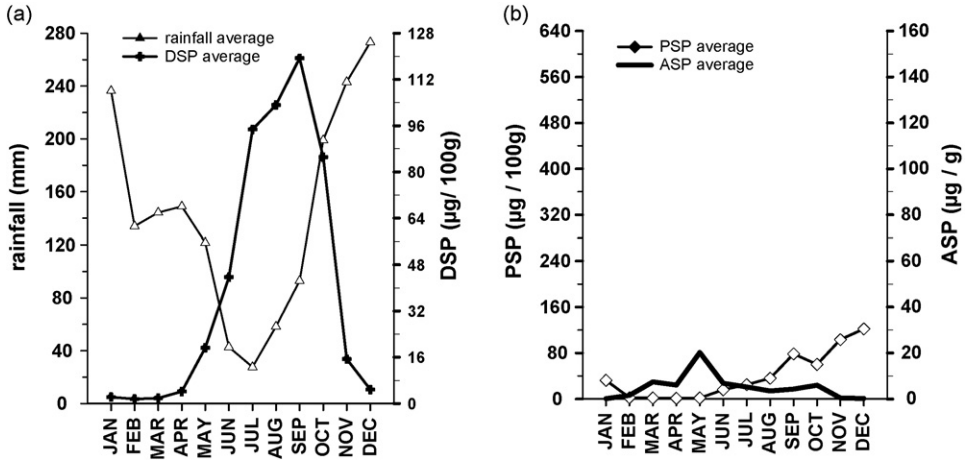

Fig. 5. Average monthly maximum levels of biotoxins in bivalves from Aveiro lagoon (as presented in Fig. 3): (a) DSP in mussel; (b) PSP in mussel and ASP in cockle. All scales were set at 8 times the respective EU regulatory limits. In (a), DSP is overlaid with average rainfall for the period 1994–2006.

Table 4
Maximum PSP concentrations in estuarine/lagunar production areas in 1994/1995 and 2005/2006 (levels in μg STX equiv./100 g shellfish meat)

Coastline	Estuary/lagoon	Indicator species	1994[a]	1995[a]	2005	2006
NW	Lima estuary	*C. edule*[b]/*Mytilus* spp.	na	150	590	97
	Aveiro lagoon	*Mytilus* spp.	1275	217	1014	466
	Mondego estuary	*Mytilus* spp.	523	83	309	244
	Óbidos lagoon	*Mytilus* spp.	2900	247	144	377
SW	Tagus estuary	*Venerupis* spp.[b,c]/ *Mytilus* spp.	2600	55	nd	15
	Albufeira lagoon	*Mytilus* spp.	685	66	nd	nd
	Sado estuary	*S. plana*[b]/*C. edule*	nd	66	nd	nd
South	Alvor lagoon	*T. decussatus*	215	3360	nd	na
	Formosa lagoon	*C. edule*[b]/*Mytilus* spp.	317	460	nd	15

na: data not available; nd: not detected.
[a] Data obtained by mouse bioassay.
[b] Indicator species available for the years 1994/1995.
[c] Species harvested near the offshore coast.

3. Paralytic shellfish poisoning

Although the Óbidos lagoon events in the 1940s and 1950s were attributed to the microalgae *Alexandrium minutum*, other PSP events caused by this microorganism were not found outside this lagoon. However, blooms of this organism in adjacent areas of the Iberian coast, such as the north of Galicia or Cataluna (Franco et al., 1994; Garcés et al., 2004) pose a threat to bivalve safety. Another microalgae bloom, *Gymnodinium catenatum* (Franca and Almeida, 1989), has also often affected the Portuguese coast since 1986. Its distribution between 1986 and 1990 was restricted to the NW coast, but in 1992 it was detected in the south and southwest coasts (Sampayo et al., 1997). In the autumn of 1994 the entire coast was simultaneously affected, and in 1995 the south coast was mainly affected. From 1996 no new blooms were detected in the entire coast until the autumn 2005 bloom, which affected only bivalves from the NW coast. A bloom in 2006 was restricted to between offshore Lisbon and the Óbidos lagoon.

The monitoring programme for PSP toxins started in 1986 by analysing the whole flesh of bivalves with a mouse bioassay method standardised in the 1950s (McFarren, 1959), adopted until now as an official method throughout the world (AOAC, 1990; Anon., 2005). The method provides useful information on toxin levels but not on toxin profiles. The monitoring programme introduced in 1996 an HPLC method with pre-column derivatisation based on Lawrence et al. (1995).

Tables 4 and 5 show the maximal levels found in 2005/2006 and in the two previous blooms from 1994/1995. In 1994 bivalves exceeding the regulatory limit of 80 μg STX equiv./100 g occurred along the entire coast, although the highest levels were observed on the NW and SW coasts (Fig. 1). The PSP toxin level of

Table 5
Maximum PSP concentrations in offshore production areas (see Fig. 1) in 1994/1995 and 2005/2006 (levels in μg STX equiv./100 g shellfish meat)

Coastline	Offshore area (no.)	Indicator species	1994[a]	1995[a]	2005	2006
NW	Viana (1)	*Mytilus* spp.	na	na	1590	264
	Matosinhos (2)	*S. solida*	122	na	331	170
	Aveiro (3)	*S. solida*	90	na	770	56
	Ericeira (4)	*Mytilus* spp.	3600	227	nd	148
SW	Lisbon (5)	*Ensis* spp.[b]/*Donax* spp.	350	na	nd	149
	Setúbal/Sines (6)	*Ensis* spp.[b]/*Donax*	1600	120	nd	nd
South	Faro/Olhão (7)	*S. solida*[b]/*Donax* spp.	65	510	nd	11
	Tavira/VRSA (8)	*S. solida*[b]/*Donax* spp.	54	99	nd	nd

na: data not available; VRSA: Vila Real de Santo António.
[a] Data obtained by mouse bioassay.
[b] Indicator species available for the years 1994/1995.

3.6 mg/100 g at Ericeira coast in 1994 is the highest concentration registered in Portuguese bivalves so far. In 1995 the highest levels were found during autumn in the south coast. Although concentrations above the RL were also observed in the NW and SW coasts, these were restricted to the first trimester and coincided with the detoxification from the autumn 1994 bloom. In 2005 only bivalves harvested between Óbidos lagoon and Lima estuary showed PSP toxins above the RL. The positive levels found in these areas in 2006 represented the winter 2005/2006 detoxification period. New contamination exceeding the RL was found in summer in Óbidos lagoon.

The PSP season commonly starts in autumn (Fig. 2c). High contamination levels may be restricted to summer (Fig. 2d). Long-term trends are exemplified in Fig. 3b. Contamination with PSP toxins did not present a regular cyclic pattern as found for DSP. High monthly levels rarely exceeded 15 times the RL, and occurred for a reduced number of consecutive weeks/months. The average ML peaked in November and December, the only 2 months where the average exceeded the RL (Fig. 5b). The higher abundance of *G. catenatum* may be found at midshelf related to the offshore displacement of blooms under upwelling conditions (Moita et al., 1998). The upwelling displacement might contribute to bivalves not being contaminated by *G. catenatum* toxins, as often as it occurs with contamination from the more coastal *Dinophysis* species.

Toxicity differences among bivalves have been observed with the mouse bioassay (Franca and Almeida, 1989), and some differences are now better understood thanks to toxin profile analysis by HPLC. When PSP toxins occurred in the Figueira estuary in 1994, the clam *Scrobicularia plana* presented contamination above the RL throughout 1995 and trace levels were still observed by mouse bioassay during 1996. HPLC analysis confirmed the presence of PSP toxins (Vale and Sampayo, 2001a). When naturally contaminated specimens were available again in 2005, artificial detoxification experiments demonstrated this clam presented a detoxification rate lower than mussels or cockles (Artigas et al., in press).

The oceanic clam *S. solida* presented a profile dominated by decarbamoyl analogues, quite different from all other bivalve species when contaminated by *G. catenatum*, which are rich in *N*-sulfocarbamoyl analogues. This profile has been attributed to a strong carbamoylase activity, which converts in a few hours all carbamate or *N*-sulfocarbamoyl toxins, rendering them into the corresponding decarbamoyl analogues. This activity was lost upon thermal denaturation (Artigas et al., 2007). This enzymatic activity also occurred in *S. plana*, but due to its slow nature was only possible to be observed *in vivo*: it took at least 1 week to observe a conversion of half the toxins ingested in a toxification experiment (Artigas et al., 2007). This activity might increase PSP toxicity by one order of magnitude, as decarbamoyl analogues are much more potent than *N*-sulfocarbamoyl analogues.

The remaining commercial bivalve species studied so far presented a profile that reflected more clearly contamination by *G. catenatum*. In estuarine bivalves toxicity has been observed in the following decreasing order: *M. galloprovincialis* > *C. edule* ≫ *T. decussatus* ≈ *V. pullastra* ≈ *S. marginatus* ≈ *Crassostrea* spp. (Fig. 2c and d). Artificial experiments also demonstrated mussel detoxified more slowly than cockle (Artigas et al., in press). Toxin profiles between these species do not show major differences that help to explain this data.

The quality of oysters from Sado estuary is poor due to their growth in an area contaminated by metals, which requires their relocation to relaying waters. Since 1986 these oysters have been tested for PSP, presenting recurrently a mouse survival time characteristic for the presence of PSP trace levels. When analysed by HPLC, the absence of toxins was confirmed, leaving the justification of the short mouse survival times to the presence of zinc and cadmium (Vale and Sampayo, 2001a).

4. Amnesic shellfish poisoning

Although the ASP toxin domoic acid (DA) is a water-soluble compound, it possesses low toxic potency in rodents and the mouse bioassay for PSP can only be used to detect high DA concentrations which cause illness in humans. For that reason HPLC methodology is used to detect DA. The Portuguese programme introduced regular testing in late 1996, although the presence of this toxin in bivalves had already been confirmed in 1995. The methodology used followed the same acid extraction employed for PSP, followed by ultraviolet detection (Lawrence et al., 1991). In-house studies showed rapid decomposition of the toxin, and a methanolic extraction was adopted to address this in 1999 (Vale and Sampayo, 2001b).

LC–MS was used to confirm the profiles previously determined by HPLC-UV—in plankton samples DA and isodomoic-A were present and in bivalves isodomoic-D was also found. Specific metabolisation of DA by bivalves does not play an important role in altering profiles, as the molecule is known to suffer

Table 6
Maximum ASP concentrations in estuarine/lagunar production areas in 2003/2006 (levels in μg DA/g shellfish meat)

Coastline	Estuary/lagoon	Indicator species	2003	2004	2005	2006
NW	Lima estuary	*Mytilus* spp.	12	4	3	25
	Aveiro lagoon	*Mytilus* spp.	18	79	25	14
	Mondego estuary	*Mytilus* spp.	14	14	2	2
	Óbidos lagoon	*Mytilus* spp.	11	8	4	5
SW	Tagus estuary	*Mytilus* spp.	na	1	nd	nd
	Albufeira lagoon	*Mytilus* spp.	nd	na	nd	1
	Sado estuary	*S. plana*/*C. edule*	1	3	nd	nd
South	Alvor lagoon	*T. decussatus*	nd	nd	nd	na
	Formosa lagoon	*Mytilus* spp.	1	1	nd	2

na: data not available; nd: not detected.

spontaneous isomerisation. Isomers represented a minor contribution to the toxin profile and routinely were not quantified for monitoring purposes (Vale and Sampayo, 2001b). The production of DA has been associated with the occurrence of *Pseudo-nitzschia australis* (Palma, 2003).

Tables 6 and 7 illustrate the maximum levels of ASP toxins found between 2003/2006. Concentrations exceeding the RL of 20 μg DA/g were rarely found. The duration of ASP toxins in bivalve tissues was usually short when compared with DSP or PSP toxins. Levels exceeding the RL usually were found only during 1 week, and rarely occurred in consecutive weeks. In Tables 6 and 7 mussel was presented as indicator species in order to compare with the maximal levels for DSP and PSP found in the same production areas in the same year. Other species such as cockle or clams may contain levels above mussels. The explanation derives on the behaviour of the diatoms that produce ASP. Unlike dinoflagellates that produce DSP and PSP toxins, diatoms do not migrate so quickly on the water column and tend to sink. The mussel used as indicator species is picked in the intertidal zone all around the coast (except at Albufeira lagoon, where a few artisanal rafts exist), becoming less contaminated with ASP. The highest concentration of DA registered so far, on two occasions, was 160 μg/g, in *T. decussatus* from Alvor lagoon in 2001 and later in *V. pulastra* from Aveiro lagoon in 2005. Monitoring of DA in the scallop *Pecten maximus*, a species particularly slow in depurating this toxin, was rarely done due to the low abundance of this species. High toxic levels were occasionally found up to 266 μg DA/g.

Bivalve contamination was generally observed between spring and autumn. Fig. 3c illustrates the highest monthly concentrations observed in cockles from Aveiro lagoon between 1987 and 2006. Concentrations never exceeded twice the regulatory limit, with the exception of May 2004. The average monthly maximum levels peaked in May, but not exceeding the current RL (Fig. 5b).

5. Azaspiracid shellfish poisoning

The specific analysis for AZP toxins was set-up for the first time for the digestive glands of mussels harvested during 2002 at Aveiro lagoon using LC–MS methodology- so far the only methodology available for

Table 7
Maximum ASP concentrations in offshore production areas (see Fig. 1) in 2003/2006 (levels in μg DA/g shellfish meat)

Coastline	Offshore area (no.)	Indicator species	2003	2004	2005	2006
NW	Viana (1)	*Mytilus* spp.	4	5	2	26
	Matosinhos (2)	*S. solida*	6	4	7	64
	Aveiro (3)	*S. solida*	21	18	28	10
	Ericeira (4)	*Mytilus* spp.	1	3	36	148
SW	Lisbon (5)	*Donax* spp.	na	na	3	5
	Setúbal/Sines (6)	*Donax* spp.	18	3	4	10
South	Faro/Olhão (7)	*Donax* spp.	2	2	3	6
	Tavira/VRSA (8)	*Donax* spp.	3	1	8	1

na: data not available; VRSA: Vila Real de Santo António.

this family of toxins (Vale, 2004b). The azaspiracids AZA1 through AZA-5 were not detected. From 2003 forward, the specific analysis for AZP was carried out in mussel and cockle from Aveiro lagoon and *D. trunculus* from the Setúbal and Olhão offshore coasts. Again, no toxins were detected above the detection limit of the method (0.5 μg/g). In the summer of 2006 traces of AZA2 and AZA1 were detected in mussels from Aveiro. Screening of other mussel samples showed traces were also present between the Óbidos lagoon and Lima estuary (NW coast). The concentrations found did not approach the RL of 16 μg AZA1 equiv./100 g shellfish meat. A screen of the digestive glands of all commercial bivalve species from the Aveiro lagoon (*M. galloprovincialis*, *C. edule*, *T. decussatus*, *V. pullastra*, *S. marginatus*, and *Crassostrea* spp.) and offshore Aveiro (*S. solida*) also showed the presence of traces of AZA2 and AZA1.

6. Other lipophilic toxins

The current EU legislation also contains compounds from the pectenotoxin and the yessotoxin groups (Anon., 2005). Regarding pectenotoxins, only PTX1 and PTX2 are included. The microalgae *D. acuta* has been correlated with PTX2 contamination of Portuguese bivalves. The contamination is coincidental in time with presence of DTX2, and has been found all around the coast. The bivalves quickly metabolise PTX2 to its seco acid (PTX2sa), resulting in the continuous presence of only trace levels of PTX2 (Vale and Sampayo, 2002a).

Analyses for four compounds from the yessotoxin group must also be conducted (Anon., 2005). Due to the poor sensitivity of our LC–MS system, the screening for these compounds was carried out only when a commercial immunoassay kit became available. From the samples collected in 2005, specimens from Aveiro lagoon, Lisbon offshore, Formosa lagoon and Olhão offshore were selected for testing. In lagunar areas, YTX analogues were detected in the following decreasing concentrations: *M. galloprovincialis* ≫ *C. edule* > *T. decussatus* ≈ *V. pullastra* ≈ *S. marginatus*. In offshore areas the following decreasing concentrations were found: *S. solida* > *Donax* spp. ≈ *Chamelea gallina* (Gomes et al., in press). Levels around the EU regulatory limit of 1 mg/kg shellfish meat were found in mussels and cockles harvested during 2005 and 2006 at the Aveiro lagoon, and in mussels from the Formosa lagoon in 2005. However, these levels might be overestimated due to the antibody broad specificity. At the Aveiro lagoon, contamination with YTXs might have a broader seasonality than contamination with DSP, starting in early spring and lasting until late autumn. The presence of YTX analogues in Aveiro lagoon has been related to the presence of *Protoceratium* spp. and *Gonyaulax spinifera* in the plankton (Gomes et al., in press), and in offshore Lisbon with *Lingulodinium polyedrum* (Gomes et al., 2007).

7. Risk assessment

The regulatory levels currently in use in Europe were based mainly on data derived from poisoning incidents from around the world. Taking into account the first confirmed outbreak of DSP in Japan, the lowest level causing gastroenteritis in humans was estimated to be equivalent to the ingestion of 48 μg OA (Yasumoto et al., 1985). A recent outbreak in Norway with mussels occurred with levels about 55–65 μg OA equiv./100 g (Aune, 2001). If the consumption of shellfish meat is estimated to be around 100 g/meal, there is a safety margin of 3 with the current European RL of 16 μg OA equiv./100 g. In Portugal several reports of DSP outbreaks are known, but in no instance was shellfish found contaminated with levels below 50 μg OA equiv./100 g, confirming the adequacy of the RL in force. The first diagnosed report of 18 cases was attributed to ingestion of *D. trunculus* contaminated at 130 μg DSP/100 g, which would have provided 130 μg of OA equivalents in case of a 100 g meal, the highest meat intake recorded (Vale and Sampayo, 1999). In the SW and south coast of Portugal this bivalve is consumed a lot. Despite being the bivalve most contaminated in these areas, other reports of poisonings were rare and restricted to periods where DSP toxin levels were above 130 μg/100 g (Table 8). The small size of this bivalve reduces the risk of poisoning, as ingestion of a large number of individuals is necessary. In the NW coast contamination with DSP toxins is much more severe and all bivalves might pose more serious threats to consumer safety. In particular, razor clams possess a large body size and a family case was studied in which a DSP toxin concentration of only 50 μg OA equiv./100 g was apparently capable to cause gastroenteritis (Table 8). The maximal record of DSP was registered in intertidal mussels picked at Póvoa do Varzim beach (north of Oporto): 1800 μg OA equiv./100 g. Despite their small size (0.6–0.7 g edible tissue/individual), these were implicated in a human outbreak (Table 8).

The exposure assessment known so far for DSP led us to believe that many more poisonings go unreported, in particular in high risk areas such as Aveiro lagoon,

Table 8
Outbreaks of gastroenteritis reported to IPIMAR and attributed to bivalves contaminated with DSP biotoxins (1998–2006)

Date	Outbreak place	No. of cases	Reporter	Vector	Production area (no.)	DSP (μg/100 g)	Reference
February 1998	Loulé	18	LHA	*Donax* spp.	Faro/Olhão (7)	130	Vale and Sampayo (1999)
July 2001	Aveiro	4	VET	*Solen* spp.	Aveiro lagoon	50	Vale and Sampayo (2002b)
August 2002	Ericeira	Unknown	LHA	*Mytilus* spp.	Ericeira (4)	260	Unpublished
September 2002	Póvoa Varzim	13	LHA	*Mytilus* spp.	Matosinhos (2)	1800	Vale et al. (2003)
August 2004	Faro/Olhão	Unknown	other	*Donax* spp.	Faro/Olhão (7)	200	Unpublished
June 2005	Sesimbra	Unknown	VET	*Donax* spp.	Lisboa (5)	270	Unpublished

Toxin levels are from uncooked shellfish meat. LHA: Local Health Authority; VET: Veterinarian inspector at local fish market.

where mussels, cockles, razor clams and two species of clams are available for consumption. We studied, in the period 2001–2003, the weekly frequency of gastroenteritis cases admitted to hospital emergency units (Burri and Vale, 2006). The weekly number of gastroenteritis cases in hospitals closest to the lagoon increased when DSP levels were above 150 μg OA equiv./100 g (Fig. 6). This study led us to conclude that the scientific information generated in the monitoring programme was not yet adequate to protect the local population. The prohibition on commercial harvest did not prevent recreational harvest due to the inadequate dissemination of information. In 2003, the regional centre of IPIMAR (CRIP-Centro) started promoting pamphlet distribution, outdoor signposts, and information sessions to fisherman associations and schools in the area to alert the populations of the health dangers posed by bivalves (biotoxins and pathogenic microorganisms) (Burri et al., 2006).

In the beginning of this monitoring programme no reports of PSP outbreaks were registered (Franca and Almeida, 1989). The severity of the PSP contamination in 1994 caused at least one outbreak after the consumption of rocky mussels picked at Ericeira coast contaminated with 3.6 mg STX equiv./100 g uncooked whole flesh (Table 5). The detailed neurological characterization of the symptoms was performed on nine hospitalised patients (Carvalho et al., 1998). Other family members were also poisoned, but only those with the severest symptoms were hospitalised. Unfortunately, ingestion data is not available for that incident. In the 1955 outbreak at Óbidos lagoon cockles were contaminated at a level of 16,600 mouse units, which is circa 3 mg STX equiv./100 g (Pinto and Silva, 1956). No other confirmed outbreaks were reported to us until now, despite concentrations of PSP toxins that could cause at least mild symptoms being recorded on other occasions. From a review of the PSP poisonings in eastern Canada between 1944 and 1971, Prakash et al. (1971) reported that mild, severe, and extreme poisonings resulted from ingestion of an average of 1, 1.9 and 2 mg PSP, respectively. However the person-to-person variation in sensitivity to PSP was so great that average doses had little significance. Compared with residents, non-residents of shore communities had lower tolerance to PSP, young children were more sensitive than adults, and women were more sensitive than men. Intakes up to 2 mg PSP were reported within residents without any symptoms.

Risk assessment for ASP estimated the minimal amount to cause mild symptoms circa 60–110 mg DA, and more serious neurological incidents 135–295 mg DA

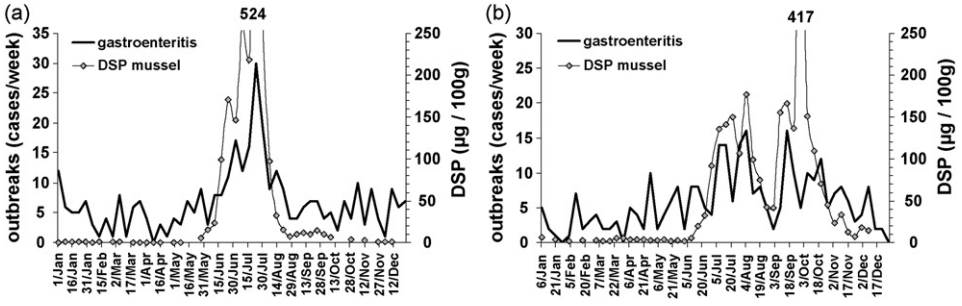

Fig. 6. Relationship between the number of patients admitted at the emergency service of Estarreja Hospital (north of Aveiro lagoon) and DSP concentration during: (a) 2001 in mussel; (b) 2003 in razor clams (reprinted from Burri and Vale, 2006).

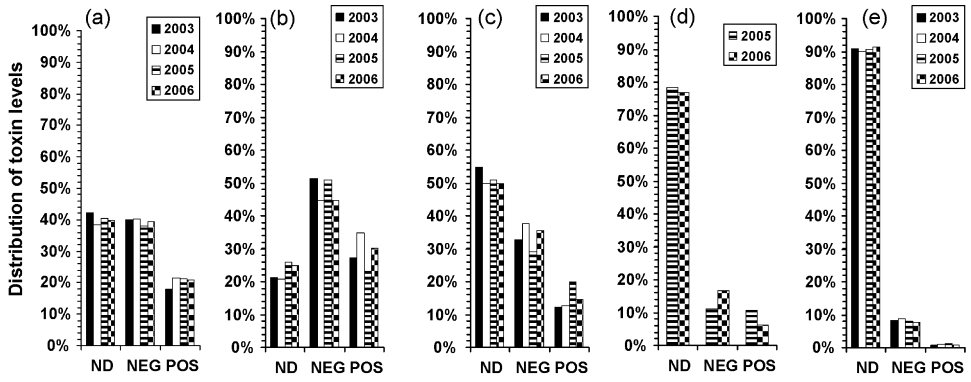

Fig. 7. Relative distribution of toxin levels found in all the samples analysed between 2003 and 2006 by three categories: above regulatory level (POS), between 0.1 and 1.0 times the regulatory level (NEG), and residual levels lower than 0.1 times the regulatory level (ND). (a) DSP toxins; (b) DSP toxins only in *Mytilus* spp. and *Donax* spp.; (c) DSP toxins in the remaining species; (d) PSP toxins; (e) ASP toxins.

(Todd, 1993). The maximal ASP levels, observed on rare occasions in Portuguese shellfish, would cause an intake of just 16 mg DA with a 100 g meal. It is not surprising that ASP outbreaks are rare among the Portuguese population. For ASP, the current RL possess a safety margin much larger than for DSP or PSP.

Toxins from the PTX and YTX family have no proven effects on human health. Despite PTX2 being acutely toxic to mice by intraperitoneal (i.p.) injection, no adverse effects have been seen with PTX2sa. Neither PTX2 nor PTX2sa are overtly toxic to mice by the oral route and no diarrhoea is observable in mice (Miles et al., 2004). Yessotoxins are also acutely toxic to mice by i.p. injection, however, no mortality, signs of toxicity or cumulative effects are induced by the repeated oral exposure to YTXs, which may reflect the poor absorption of this substance by animals (Tubaro et al., 2004). An EU Working Group on Toxicology recommended deregulation of YTXs (CRL, 2005), and no YTXs or PTXs have been included in step 5 (out of 8) of the Codex Committee on Fish and Fishery Products (Anon., 2006).

Passive surveillance systems collect a relatively small amount of information on outbreaks as this depends on the public's willingness to report illnesses as well as the willingness of the public health authority to investigate and submit reports (Todd, 2006). More difficult to assess are chronic effects attributable to prolonged exposure to marine biotoxins. For the particular case of okadaic acid, its tumour promotion properties have caused serious concern for the establishment of regulatory limits (Fujiki and Suganuma, 1999). An assessment of that data by an EU panel on toxicology concluded the tumour promotion by OA was observed in long term repeated dose studies at high dose levels, and as such these long-term studies were not considered representative of true consumption patterns (CRL, 1999). The Codex Committee on Fish and Fishery Products promoted an extensive joint FAO/IOC/WHO ad hoc Expert Consultation panel to provide scientific advice to enable the establishment of maximum levels in shellfish for shellfish toxins (Anon., 2004c). This panel also considered that there is insufficient data available to establish a tolerable daily intake (TDI) for OA or any other known marine biotoxins A review of bivalves contaminated with low levels of OA is shown in Fig. 7. Although circa 20% of all samples analysed by the monitoring programme by chemical methods between 2003/2006 contained DSP toxins in excess of 16 μg/100 g, circa 40% of samples contained residual DSP levels between 1.6 and 16 μg/100 g (Fig. 7a). These levels were not distributed uniformly by the species tested. Due to the slow detoxification mechanisms existent in *Mytilus* spp. and *Donax* spp., circa 45–51% of samples contained residual DSP levels between 1.6 and 16 μg/100 g (Fig. 7b), while in the remaining species residues were comprised only between 29 and 38% (Fig. 7c). For PSP, levels between 8 and 80 μg STX equiv./100 g were always below 17% of the samples tested (Fig. 7d), and for ASP residual levels between 2 and 20 μg DA/g were always below 9% (Fig. 7e).

8. Final considerations

Between 1986 and 2006 a total of 15,175 samples were officially analysed, comprising 11,250 for PSP toxins, 9944 for DSP toxins, 8240 for ASP toxins and 936 for AZAs. From 1993 forward at least 700–800 samples were received annually. During the prolonged absence of *G. catenatum* blooms, the testing of PSP toxins was reduced between 1999 and 2002, then

increased in 2003 due to non-animal assays being used. Not all samples were tested for DSP until 1998 when the first outbreak report alerted to the real risks posed by this biotoxin.

The data summarised here reported bivalve species, geographic areas, seasons and specific biotoxins that were particularly dangerous for human consumption. In general, DSP toxins are an annual threat to consumer safety, while the risk posed by PSP toxins is intermittent. The northwest coast is more prone to biotoxin events due to the oceanographic particularities of the region. In addition to the estuarine and offshore areas reported in Tables 1, 2 and 4–7, the Tagus and Sado estuaries contain bivalves typically from estuarine habitats (*V. pullastra*, *Venus verrucosa*, respectively) that, when harvested from the outer boundaries of these estuaries, were found contaminated by high biotoxin levels at the same periods as offshore species.

The large impact of DSP in Portuguese bivalves is similar to many other European countries, and causes more closures of shellfish beds than PSP contamination (FAO, 2004). Although mussels are widely used as indicators for different types of marine environmental contamination, their use alone as indicator species for marine biotoxins is not adequate, as harvest of other commercial species might be negatively discriminated due to the highest and longest PSP and DSP contamination events often observed in mussels. The use of cockles or clams (except *Donax* spp.) was preferred in order to decide whether harvest of the remaining species needed to be banned or not.

The risk assessment performed to date, allows for a better management of the monitoring programme in order to minimise the hazardous to public health derived from marine biotoxins. Much of this knowledge was obtained only recently when exposure assessment to DSP toxins was obtained by HPLC methods. This information is useful for managing end product testing more resourcefully. Regulation EC 854/2004 requires, in addition to the monitoring of relaying and production zones, a laboratory control system to verify food business operators' compliance with the requirements for the end product at all stages of production, processing and distribution in order to verify that the levels of marine biotoxins do not exceed safety limits (Anon., 2004b).

Despite two decades of marine biotoxin monitoring, several cases of human poisonings were still recorded. Mussels and donax clams are the most important vectors for DSP (as well as PSP) and also the bivalve species most easily picked at low tide in beaches by locals or tourists. Prohibition of commercial harvesting traditionally has been posted at the headquarters of the maritime authorities for the regions affected and faxed to purification and dispatch centres around the country. Since 2003 it has also been available on the IPIMAR web page, but the general public is not alerted. The negative effects on bivalve trade by publicising the risks associated with shellfish consumption creates a difficulty on how dissemination of the information to the general public should be conducted in the most effective way.

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