Phycotoxin monitoring in France : risk-based strategy and main results (2006-2008)

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Toxin monitoring is carried out along the coasts of France by a national network, "REPHY", which is the National Monitoring Network for Phytoplankton and Phycotoxins. Sampling strategy differs according to the type of zone coastal or offshore and to the family of toxins. For PSP and ASP toxins, monitoring is based on the detection of toxic phytoplankton species. For lipophilic toxins, a systematic analysis of toxins is performed in risk areas during high risk periods. Experimental monitoring for palytoxins has also been conducted along the Mediterranean coast since 2007. The total number of toxin analyses is in the order of 2500 to 3000 per year. The detailed results for each family of toxins showed that the most frequent toxic events for the period from 2006 to 2008 were lipophilic, with many types of shellfish and many regions concerned. Many of these episodes, detected on the basis of bio-assay, were not associated with the presence of OA+DTX+PTX above the sanitary threshold. During the same period, PSP toxic events were very scarce and ASP toxic events concerned mainly scallops in Brittany. The current system, with a risk-based strategy, takes into account high risks based on past problems in France (such as lipophilic, PSP or ASP toxic events), but it also attempts to anticipate the emergent problems, such as palytoxins.

Keywords :Monitoring, Phytoplankton, Toxins, Sampling, Shellfish, French coasts

Introduction

The purpose of this paper is to present phycotoxin monitoring in France, and its strategy. After a brief description of the monitoring strategy, the main results are presented.

Toxin monitoring is carried out along French coasts by the National Monitoring Network for Phytoplankton and Phycotoxins, known as REPHY. This national network, set up in 1984, covers the coast of mainland France (Channel, Atlantic and Mediterranean, including the Mediterranean saltwater lagoons). The two main objectives of this monitoring are: (i) environmental, with the knowledge of marine phytoplankton species distribution and inventory of toxic, harmful or exceptional events, and (ii) sanitary, with the detection of toxic species in water and quantification of toxins in shellfish (Belin, 2009). Although the network covers these two objectives, the current paper is mainly concerned with the sanitary aspects associated with harmful algae.

1. Materials and Methods

Sampling strategies were specified for water sampling (phytoplankton and physico-chemical parameters) and shellfish sampling (phycotoxins). The whole monitoring process was carried out by several Ifremer coastal laboratories, located all along the coast of France; these performed sampling, analysis and data acquisition. In addition, toxin chemical analysis was also carried out centrally, in the Ifremer Phycotoxin Laboratory, Nantes.

1.1. Sampling methods

In the context of water sampling, phytoplankton monitoring was carried out on three levels: (i) the first concerned 29 sampling stations at which all phytoplankton species, including toxic species, were identified and counted, (ii) the second concerned 103 stations at which only blooming and toxic species were counted. At all these stations, sampling was carried out regularly throughout the year, at intervals between once a month and once a fortnight. Besides phytoplankton, all the following parameters were measured: chlorophyll-a, temperature, salinity, turbidity, dissolved oxygen and nutrients. With these two levels of monitoring, it was possible to answer different types of environmental and sanitary questions. The third level of sampling (iii) concerned 108 stations that were sampled episodically, counting only the toxic species. A few physico-chemical parameters were also measured with the toxic phytoplankton: temperature, salinity and turbidity. Shellfish sampling concerned between 200 and 300 stations, which were situated in coastal production areas or natural banks, offshore natural or fishing banks. At these stations there were many types of shellfish (oysters, mussels, clams, cockles, scallops, queen scallops, etc), with a great diversity of culture methods.

1.2. Analysis methods

Phytoplankton was observed by inverted microscopy. Species were identified according a taxonomic reference list, and counted. Chlorophyll-a was analysed by spectrophotometry or fluorimetry. Other physico-chemical parameters were measured with *in situ* probes.

The methods used for toxin detection were the European regulatory methods: (i) mouse bio-assay for lipophilic toxins, based on Yasumoto *et al.* (1984) or Hannah *et al.* (1995), (ii) mouse bio-assay for PSP toxins, based on AOAC (1995), and (iii) HPLC/UV analysis for ASP toxins, based on Quilliam (1995).

LC-MS/MS analysis was also carried out on about 30% of the shellfish samples tested by lipophilic bio-assay, to improve knowledge of the different groups of these toxins. The detection of palytoxins was performed with LC-MS/MS analysis.

1.3. Strategies

The detailed strategies differed according to the type of zone coastal or offshore and to the family of toxins. For coastal zones, this was different for PSP and ASP on one hand, and for lipophilic toxins on the other.

For PSP and ASP toxins in coastal zones, detection of toxic species above an alert threshold acted as a trigger to then make toxin detection tests in shellfish, since there have

never been situations in coastal areas where PSP or ASP toxins have been detected without a strong warning signal in the phytoplankton. The phytoplankton species and alert thresholds for PSP and ASP toxins were, respectively: (i) *Alexandrium* species, at counts above 1000 to 10 000 cells per liter according to the region, and (ii) *Pseudo-nitzschia* species, at counts above 100 000 to 300 000 cells per liter, according to the species present in the sample.

For lipophilic toxins in coastal zones, the phytoplankton warning signal was considered too weak with toxin-producing species like *Dinophysis.* Therefore, two complementary systems were set up: (i) systematic toxin research in high-risk areas during risk periods, (ii) 10 "reference" stations sampled all year round, with both bio-assays and LC-MS/MS analysis. For all these cases, sampling and analysis were performed once a week. Table 1 describes the risk areas and risk periods defined for the year 2009. For each area, the risk period was based on the historical toxic events of the six previous years (2003 to 2008). Each area was then defined by the months during which systematic monitoring had to be made in 2009. Along the Channel coast (Normandy), the risk period began in summer, but along the Atlantic Ocean (Brittany), it began in spring. In some Mediterranean lagoons, the risk period was very long and not dependant on the season. An additional component was the existence of reference stations for lipophilic toxins: 10 stations, distributed all along the coast, were sampled once a month throughout the year, using both bio-assays and LC-MS/MS analysis, with the objective of detecting unknown or atypical toxins. These reference stations were chosen to represent different types of area: non-risk or risk ones, with presence or absence of unexplained or suspicious results. This general strategy for the monitoring of lipophilic toxins in coastal zones was proposed several years ago, to compensate for the fact that it is practically impossible to monitor all shellfish, everywhere, all the time in France, due to the very high number of shellfish areas, coupled with their diversity and scattered distribution. This strategy as a whole has been accepted by the European Food and Veterinary Office (FVO).

For offshore zones (natural and fishing banks), the strategy was identical for the three families of toxins (lipophilic, PSP and ASP) with systematic toxin research carried out during fishing periods. Sampling was performed once a week, with or without phytoplankton monitoring.

Finally, experimental monitoring for palytoxins has been set up along the Eastern part of the French Mediterranean coast since 2007. LC-MS/MS analysis were carried out on marine animals (mainly sea-urchins) during two periods: (i) during *Ostreopsis* bloom periods in summer, (ii) during sea-urchin fishing periods, in autumn – winter.

2. Results

The overall number of toxin analyses was of the order of 2500 to 3000 analyses per year. In 2007 for instance, more than 1200 bio-assays were performed for lipophilic toxins, added to which there were about 300 LC-MS/MS analyses. There were also about 400 PSP bio-assays and about 500 HPLC analyses for ASP.

Table 1. Risk areas (from North to South in Channel and Atlantic and from West to East in Mediterranean) and risk periods described for French monitoring in 2009, based on the historical events in coastal zones of the six last years

		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Channel and Atlantic	Antifer							■	■	■	■		
	Courseulles - Port Bessin								■	■			
	Iroise				■	■	■	■	■	■			
	Baie de Douarnenez					■	■	■	■	■		■	
	Baie d'Audierne						■	■					
	Concarneau						■						
	Aven, Belon et Laïta					■	■						
	Rade de Lorient					■	■	■			■		
	Baie d'Etel					■	■						
	Rivière d'Etel					■	■						
	Baie de Quiberon					■	■						
	Rivière de Crach						■						
	St Philibert-Le Breneguy						■						
	Rivière de Pénerf						■						
	Baie de Vilaine					■	■	■					
	Traicts du Croisic						■						
	Estuaire de la Loire						■	■					
	Bassin d'Arcachon				■	■	■	■	■				
Med.	Etang de Salses-Leucate	■	■	■	■	■	■	■			■	■	■
	Etangs Palavasiens					■	■	■	■	■			
	Etangs de Diana - Urbino	■	■										

2.1. Lipophilic toxins

All the toxins of the lipophilic family were observed during the period from 2006 to 2008, except for the Gymnodimines. Apart from Okadaic Acid (OA) and Dinophysistoxins (DTX), which had been observed from the beginning of French monitoring, the first observations of other toxins were: (i) Pectenotoxins (PTX) in the Eastern Mediterranean in 2004, (ii) Spirolides (SPX) on the Atlantic coast in 2005, (iii) Azaspiracids (AZA) in the Channel in 2006, (iv) Yessotoxins (YTX) in Western Mediterranean in 2007. The bio-assays with results above the sanitary threshold are summarized for each of the years from 2006 to 2008 (Fig. 1, 2 and 3). In 2006, several types of shellfish were concerned: oysters and mussels, but also clams, cockles and scallops. The most affected region was Brittany in the west of France. In 2007 and 2008, the affected areas and shellfish were about the same as the year before. However, in 2008, there was a slight decrease in the number of areas and number of types of shellfish.

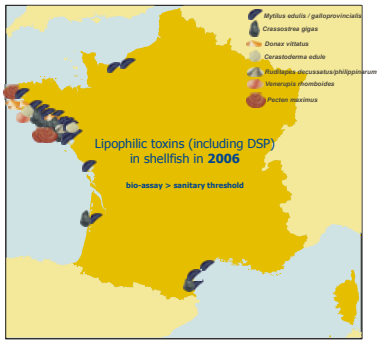

Fig. 1. Lipophilic toxins. Bio-assay results above the sanitary threshold in 2006

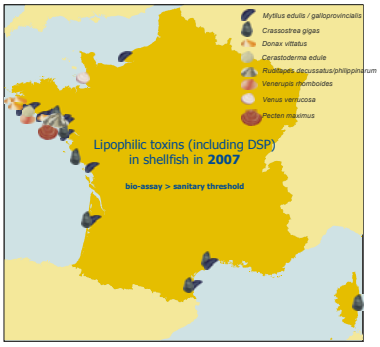

Fig. 2. Lipophilic toxins. Bio-assay results above the sanitary threshold in 2007

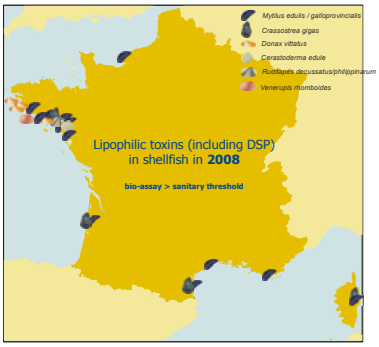

Fig. 3. Lipophilic toxins. Bio-assay results above the sanitary threshold in 2008

A comparison of bio-assays and LC-MS/MS results was made for the year 2007 for which about 300 shellfish samples were analyzed by both methods. Table 2 summarizes the results for the zones where positive bio-assays were observed. Two different cases could be described. The first case concerned the positive bio-assays (two or three dead mice out of three) which were associated with the presence of OA+DTX above the European threshold *i.e.* 160 µg.kg^{-1} (these results are noted "concordance = yes"). Three types of shellfish were concerned: mussels, oysters and wedge shells (*Donax*). The maximum concentrations per zone and per year show that some toxic events led to rather elevated concentrations in shellfish (up to 885 µg.kg^{-1} in the west of Brittany). All of these toxic events were associated with presence of *Dinophysis*, even though this organism sometimes had very low counts. The second case concerned the positive bio-assays that were not associated with the presence of OA+DTX+PTX above the sanitary threshold (these results are noted "concordance = "no"). Six types of shellfish were concerned by these problems, including mussels, oysters, cockles, clams and scallops. In several cases, chemical analysis showed complete absence of known toxins, and these discrepancies still remain unexplained. In a few cases of negative chemical results coupled with positive bio-assays, the presence of PTX or YTX was observed, though under the sanitary threshold.

These conclusions prompted a statistical study on the results obtained on the same samples analyzed with both detection methods (bio-assay and LC-MS/MS analysis) during the period from 2003 to 2008 (Belin *et al.,* 2009). The degree of concordance between the results was estimated with the Kappa coefficient (Cohen, 1960), which quantifies the intensity or the quality of the agreement between qualitative judgments.

Then a qualitative assessment of this concordance was described with the scale proposed by Landis & Koch (1977): very good, moderate or poor agreement. From an overall point of view (*i.e.* all zones and shellfish taken together), the statistical test concluded that the concordance was poor between the two methods. The overall agreement percentage between the results obtained on 1034 samples was 70%, but there were great differences between the different shellfish.

2.2. PSP toxins

There were no reported PSP toxins above the sanitary threshold (*i.e.* 800 µg.kg^{-1} equ.STX) in 2006 and 2008 on the French coasts. In 2007, there were only two toxic events in the western Mediterranean lagoons, with the following concentrations (in µg.kg^{-1} equ.STX): 830 in Salses-Leucate lagoon and 1270 in Thau lagoon. For both sites, the shellfish concerned were mussels. These events were associated with the presence of *Alexandrium minutum* in the first case and *A. tamarense* in the second case

2.2. ASP toxins

The results for each of the years from 2006 to 2008 are summarized in Figs. 4, 5 and 6. In 2006, the affected shellfish were scallops, wedge shells, or clams. The maximum concentration was observed in clams was 88 mg.kg^{-1} equ. DA. In 2007, toxic events were mainly associated with scallops, and were observed only in southern Brittany, with the maximum concentration of 183 mg.kg^{-1} equ. DA in Brest Bay (this was the maximum concentration ever observed in France for ASP). In 2008, the pattern was the same as in 2007: scallops in Brittany, with a maximum concentration of 83 mg.kg^{-1} equ. DA. Toxic events during these three years were generally associated with blooms of different species of *Pseudo-nitzschia*, but information for offshore areas is lacking.

2.3. Palytoxins

As the *Ostreopsis* concentrations had increased from year to year since 2006 along the Mediterranean coast, chemical analyses by LC-MS/MS were performed in different marine animals in 2008. Palytoxins were detected in sea urchins in two zones of Eastern Mediterranean : 175 µg.kg^{-1} of digestive gland in Marseille, and more than 400 µg.kg^{-1} in Villefranche. As a precaution, given the high concentrations of *Ostreopsis*, two beaches were closed for several days in these regions, even though there were no observed respiratory difficulties or cases of intoxication.

Table 2. Comparison between results obtained on samples analyzed by two methods (bio-assay and LC-MS/MS) during the year 2007. Bio-assay "+" means positive bio-assay. "< dl" means under the LC-MS/MS detection limit

		Shellfish	Bio-assay	AO+DTXs µg/kg	PTXs µg/kg	YTXs µg/kg	Concordance between bio-assay and LC-MS/MS analysis
Channel and Atlantic	Antifer	*Mytilus edulis / galloprovincialis*	+	670	< dl	< dl	yes
	Iroise	*Donax vittatus*	+	885	27	< dl	yes
	Baie de Douarnenez	*Donax vittatus*	+	586	< dl	< dl	yes
	Baie d'Audierne	*Donax vittatus*	+	314	315	< dl	yes
	Iles de Glénan	*Glycymeris glycymeris*	+	58	43	< dl	**no**
	Bénodet	*Mytilus edulis / galloprovincialis*	+	10	< dl	< dl	**no**
	Concarneau	*Cerastoderma edule*	+	< dl	< dl	< dl	**no**
		Mytilus edulis / galloprovincialis	+	518	11	< dl	yes
	Rade de Lorient	*Mytilus edulis / galloprovincialis*	+	109	< dl	< dl	**no**
	Baie d'Etel	*Donax vittatus*	+	429	24	< dl	yes
	Rivière d'Etel	*Mytilus edulis / galloprovincialis*	+	116	< dl	< dl	**no**
	Courreaux de Belle île	*Pecten maximus*	+	< dl	< dl	< dl	**no**
	Le Pô	*Crassostrea gigas*	+	67	< dl	< dl	**no**
		Ruditapes decussatus / philippinarum	+	130	< dl	< dl	**no**
	Rivière de Crach	*Ruditapes decussatus / philippinarum*	+	142	< dl	< dl	**no**
	St Philibert-Le Breneguy	*Ruditapes decussatus / philippinarum*	+	42	< dl	< dl	**no**
	Rivière de Pénerf	*Mytilus edulis / galloprovincialis*	+	181	< dl	< dl	yes
	Baie de Vilaine	*Mytilus edulis / galloprovincialis*	+	540	< dl	< dl	yes
	Traicts du Croisic	*Crassostrea gigas*	+	105	< dl	< dl	**no**
	Estuaire de la Loire	*Mytilus edulis / galloprovincialis*	+	78	< dl	< dl	**no**
	Olonne	*Crassostrea gigas*	+	230	< dl	< dl	yes
	Baie de l'Aiguillon	*Mytilus edulis / galloprovincialis*	+	74	< dl	< dl	**no**
	Bassin d'Arcachon	*Mytilus edulis / galloprovincialis*	+	25	12	< dl	**no**
		Crassostrea gigas	+	11	5	< dl	**no**
Mediterr.	Etang de Salses-Leucate	*Mytilus edulis / galloprovincialis*	+	181	391	22	yes
		Crassostrea gigas	+	28	108	< dl	**no**
	Etangs Palavasiens	*Mytilus edulis / galloprovincialis*	+	34	71	115	**no**
		Crassostrea gigas	+	10	47	< dl	**no**

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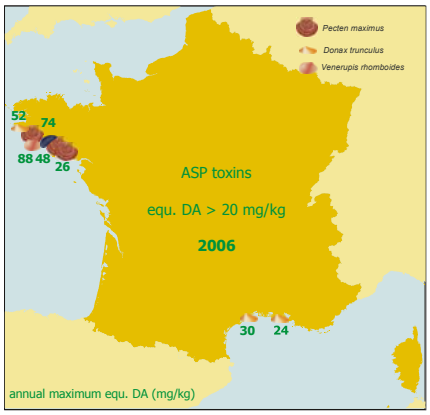

Fig. 4. ASP toxins. HPLC/UV results above the sanitary threshold (i.e. 20 mg.kg^{-1} equ. DA) in 2006

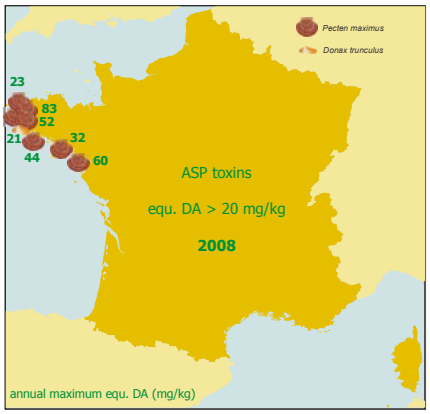

Fig. 5. ASP toxins. HPLC/UV results above the sanitary threshold (i.e. 20 mg.kg^{-1} equ. DA in 2007

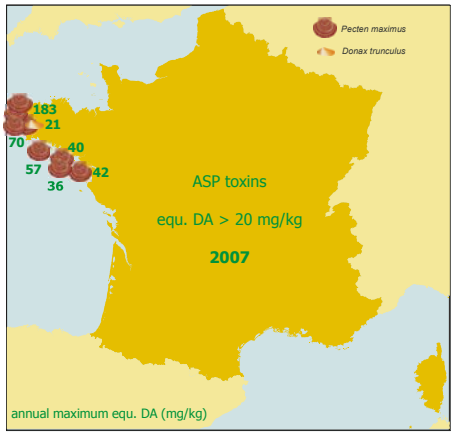

Fig. 6. ASP toxins. HPLC/UV results above the sanitary threshold (i.e. 20 mg.kg^{-1} equ. DA) in 2008

Discussion and Conclusion

Before 2005, sampling strategy for phycotoxin monitoring was primarily based on toxic species detection, which triggered the research for DSP, PSP and ASP toxins in shellfish. Important changes have been made in French toxin monitoring strategy since 2005. The main reasons for these changes were: (i) acquired experience from previous toxic episodes, and from time-series data, (ii) new knowledge on toxins and toxin-producing species. The current system with a risk-based strategy takes into account the "high risk" posed by traditionally identified problems, such as lipophilic toxins, which can be

observed in shellfish before the detection of *Dinophysis* in water samples. This new strategy also contributes to the understanding of emerging problems, such as *Ostreopsis* and palytoxins. Toxin monitoring mainly focus on bivalve molluscs, since the observed concentrations of the three groups of toxins (lipophilic, PSP and ASP) should not lead, at the present time, to a risk for other marine animals, such as gastropods for instance. However, the example of palytoxins with contaminations observed in sea urchins is a proof of a possible evolution of the surveillance to also include other marine organisms. The French system is not a perfect one, but it is adequate for the particular structure of shellfish production areas in France and the cost/benefit ratio, and it appears to be appropriate in scale and pragmatic in application.

Acknowledgements

We particularly wish to thank all staff at the Ifremer coastal laboratories who perform sampling, phytoplankton identification and counts, toxin analysis, and data storage. The authors also would like to acknowledge the financial contribution provided by the French Ministry for Food, Agriculture and Fisheries, General Directorate for Food, programme 206, as well as the contribution provided by the French Ministry for Research, programme 187, to the French national surveillance network for phytoplankton and phycotoxins (REPHY), run by IFREMER.

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