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First report on amnesic and diarrhetic toxins detection in French scallops during 2004–05 monitoring surveys

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Abstract In the context of the French phytoplankton and phycotoxins monitoring network (REPHY), shellfish scallops were harvested systematically during the authorised fishing season for the purpose of investigating paralytic toxins (PSP-mouse test), amnesic toxins (ASP-chemical analysis), and lipophilic toxins (DSP-mouse test). For all shellfish samples that tested positive in the DSP mouse bioassay used for lipophilic toxins detection, liquid chromatography coupled with mass spectrometry (LC-MS/MS) was used to search for the following lipophilic toxins: okadaic acid, dinophysistoxins, pectenotoxins, azaspiracids, yessotoxins, spirolides, and gymnodimine. In order to investigate the presence of okadaic acid esters (DTX3), alkaline hydrolysis was performed on all samples, with LC-MS/MS analyses being applied to the samples before and after hydrolysis. During 2004–05, the results revealed two consecutive contamination periods of French scallops (the Bay of Seine, Normandy): firstly by domoic acid for the duration of the 8 months from November 2004 until to June 2005, and secondly by okadaic acid and DTX3 associated with *Dinophysis* up until the month of December 2005. This paper reports on the first occurrence of domoic acid, okadaic acid, and DTX3 in French scallops. Paralytic toxins, however, were not detected.

Keywords scallops; domoic acid; okadaic acid and derivatives; *Dinophysis*

INTRODUCTION

In France, among the toxic microalgae found along French coasts, three general types—*Dinophysis*, *Alexandrium and Pseudo-nitzschia*—are specifically targeted for ongoing monitoring (Lassus et al. 1988; Masselin et al. 1992, 2001; Amzil et al. 2001; Amzil & Mathias 2006). When these algae are detected, the phytoplankton and phycotoxin monitoring network (REPHY), in charge of the French shellfish monitoring program, performs toxicity analysis on exposed bivalves harvested along the coastline. As shellfish sites out in open sea (like scallop sites) are some distance from the coast, the depths involved do not allow exhaustive phytoplankton sampling. This is why scallop samples were harvested only weekly during the autumn fishing season for the purpose of investigating paralytic, amnesic, and lipophilic phycotoxins according to European regulatory methods.

In November 2004, domoic acid (DA) concentrations in scallops from Normandy (Bay of Seine, English Channel) were over the sanitary threshold (20 µg DA/g whole tissue) for the first time. The ban imposed on fishing had significant economic consequences (300 fishing boats and more than 1000 fishermen). In addition, in the same area, the scallops were contaminated with *Dinophysis*. This second crisis from September to December 2005 was the first occurrence of DSP contamination of scallops in France.

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In this study, we present the analysis results for lipophilic and amnesic toxins detected using the DSP mouse bioassay and chemical analysis, respectively. For scallop samples tested using mouse bioassay, liquid chromatography coupled with mass spectrometry in tandem (LC-MC/MS) was used to search for the following lipophilic toxins: okadaic acid (OA), dinophysistoxins (DTXs), pectenotoxins (PTXs), azaspiracids (AZAs), yessotoxins (YTXs), spirolides (SPXs), and gymnodimine (GYM). In addition, amnesic and lipophilic toxin distribution was evaluated in various scallop organs.

MATERIALS AND METHODS

Reference materials and samples

- Certified reference materials provided by IMB-Halifax, NS, Canada: (1) standard solutions of paralytic, amnesic, and lipophilic phycotoxins; (2) mussel homogenates: one containing OA and DTX-1 toxins, and another containing amnesic toxins.
- Irish mussel samples containing azaspiracids (AZA1,2,3) and Italian mussel samples containing yessotoxins (YTX, Homo-YTX, 45-YTX, 45-homo-YTX) were used to investigate these toxins without quantification.
- Scallop samples (*Pecten maximus*) were collected on a weekly basis during the fall fishing period from different locations.

ANALYSIS METHODS

300 g of total scallop meat was ground in an Ultra-turrax (8000 rpm) in order to obtain a homogenate sample to investigate amnesic, paralytic, and lipophilic phycotoxins in accordance with European official methods. The remaining homogenate was later used for chemical analyses in order to evaluate which paralytic toxins and lipophilic toxins were involved. As the toxins found to be present included both lipophilic and amnesic toxins, we will limit ourselves to a rapid description of the methods of detection used for these toxins.

Mouse bioassay for lipophilic phycotoxin detection

Out of 300 g of total scallops meat homogenate, 100 g was used for the mouse bioassay according to the Hannah method (Hannah et al. 1995). The bioassay is regarded as positive if at least two out of three mice die within 24 h.

Chemical analysis by liquid chromatography/diode array detection (LC/DAD) for amnesic toxin detection

The DA assay was performed by LC/DAD according to the Quilliam method (Quilliam et al. 1995): C_{18} reverse-phase (Vydac, 4.6 × 250 mm) at 40°C with CH_3CN/H_2O 0.1% TFA (10:90) at 1 mL/min. DA detection was performed at a wavelength of $\lambda = 242$ nm.

LC-MS-MS analyses of lipophilic toxins

Out of 300 g of total scallops meat homogenate, 2 g were used for extraction of lipophilic toxins according to the DSP mouse-assay protocol optimised with MUS-2 reference material (Fig. 1). This procedure was applied to shellfish extracts previously "mouse-assayed" in order to: (1) identify and quantify the lipophilic toxins; and (2) attempt to find a correlation between chemical analyses and mouse bioassays.

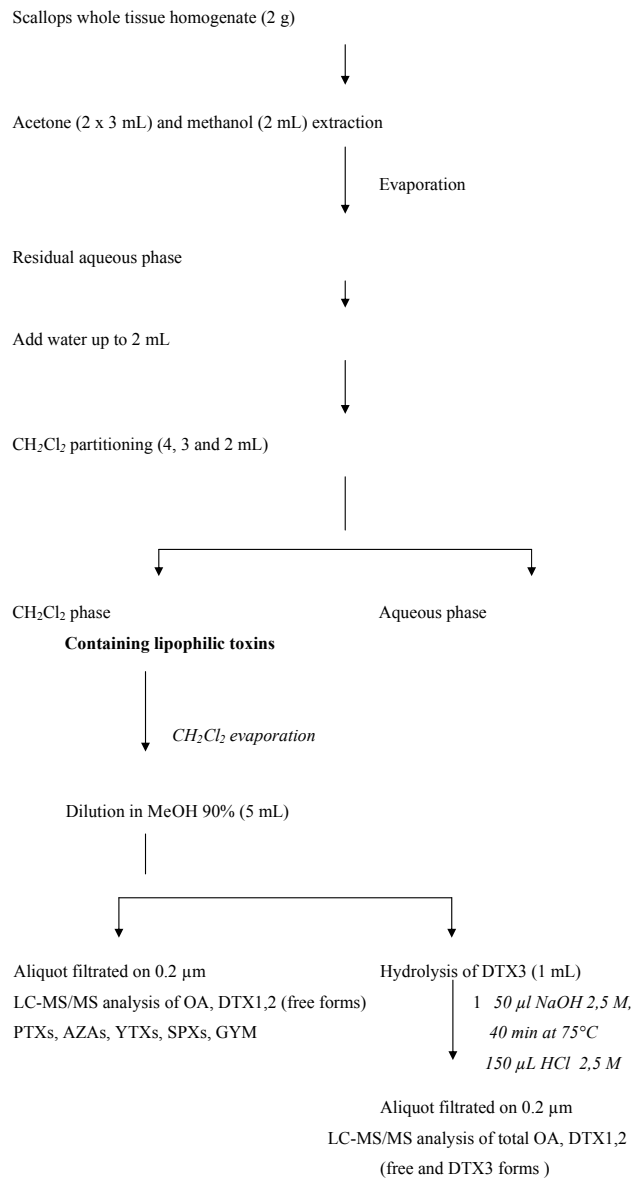

Fig. 1 Extraction procedure for lipophilic toxin analysis by liquid chromatography mass spectrometry (LC-MS/MS) applied to shellfish extracts that had been previously "mouse-assayed".

Mass spectral experiments were performed using an API-2000 triple quadruple mass spectrometer equipped with a turbo-ionspray source, coupled to an Agilent model 1100 LC. LC-MS-MS analyses for lipophilic toxins were performed by modifying the Quilliam method (Quilliam et al. 2001): 3 µm Hypersil C8 DBS column (50 × 2 mm) at 20°C; eluent A was H_2O and B was 95% acetonitrile/H_2O solution, both eluents containing 2 mM ammonium acetate and 50 mM acetic acid. A gradient elution of 10–100% B in 10 min followed by 100% B in 15 min was used. The flow rate was 200 µL/min and a 5 µL sample injection volume was used.

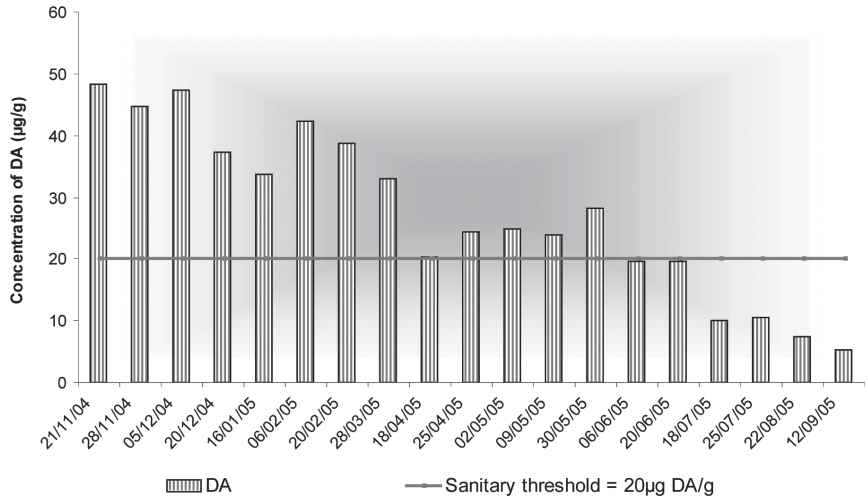

Fig. 2 Domoic acid (DA) accumulation in scallop whole tissue from samples taken on the Normandy coast from November 2004 to September 2005.

RESULTS AND DISCUSSION

Amnesic shellfish toxins (ASP)

Figure 2 shows the concentration evolution of DA accumulated in scallop whole tissue from the authorised start of the fishing season in November 2004 through to September 2005. In contrast with other shellfish species, the de-contamination period for DA was very long (around 8 months). The Blanco work on Spanish scallops initially contaminated at 250 µg/g whole tissue indicated that approximately 1 yr is needed for full decontamination (Blanco et al. 2002). By way of comparison with other shellfish, the decontamination period for mussels (*Mytilus edulis* or *M .galloprovincialis*) initially contaminated at 50 µg/g is much faster, with 50% of DA being eliminated in 24 h (Novaczek et al. 1992).

During the ASP episode in Normandy, no *Pseudo-nitzschia* sp. bloom was observed, and DA was not detected in regular phytoplankton sampling. As the phycotoxin monitoring program started with the fall fishing period, it is likely that the toxicity peak associated with *Pseudo-nitzschia* cells occurred earlier, and that the fishing period coincided with the decontamination period.

From 2004, other incidences of ASP scallop contamination were observed every year in the spring in the west and south of Brittany. In addition, we observed a geographical extension to other scallop sites around Atlantic coast islands (Belle-Isle, Ile d'Yeu) in the autumn of 2006.

Domoic acid distribution in different scallop organs

The analysis results for DA distribution in the different organs (digestive gland, gonad, muscle) of contaminated scallop samples at different levels (28.3–15.2 and 4.3 µg DA/g whole tissue) presented in Table 1 indicate that DA was mainly accumulated in the digestive gland (>95%), <5% was found in the gonad tissue (due to the intestinal loop within the gonad), and no toxin was detected in muscle. These results confirm data obtained previously on domoic acid distribution in scallops (Arévalo et al. 1998). This is why the European regulation allows

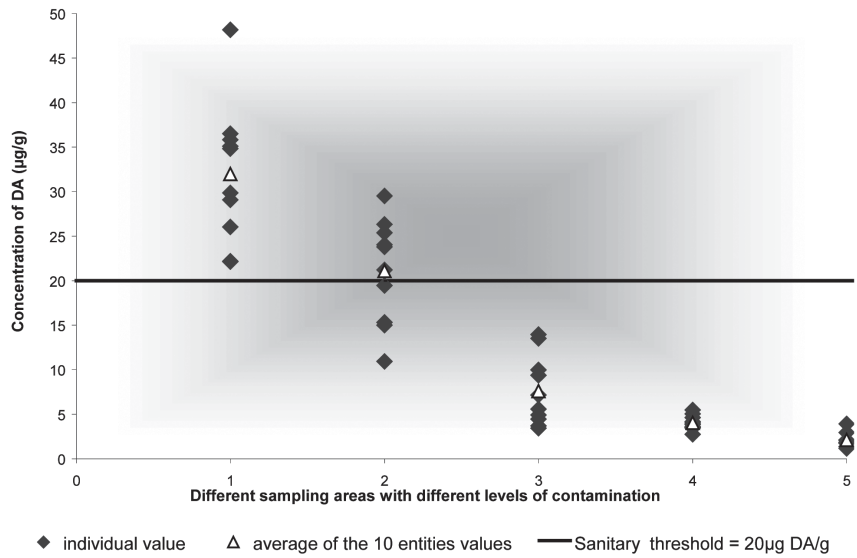

Fig. 3 Inter-individual variability of domoic acid (DA) accumulation in scallop whole tissue at different level of contamination.

Table 1 Results of domoic acid (DA) distribution in different scallop organs.

µg of DA/g	Whole tissue	28.3	15.2	4.3
DA proportion in different scallop organs	muscle	<DL*	<DL	<DL
	gonad	3.4%	<DL	<DL
	digestive gland	96.6%	100%	100%

*Detection limit: 0.15 µg DA/g.

the marketing of muscle from scallops with between 20 and 250 µg DA/g whole tissue contamination. Some countries, like Spain, adhere to this rule (Salgado et al. 2003).

Inter-individual variability of domoic acid accumulation

An inter-individual variability study of DA accumulation was carried out on five sets of scallop whole tissue (10 individuals per set) from different areas at different contamination levels. Statistical results for DA concentration (Fig. 3) show that variance and standard deviation increase with contamination level: inter-individual variability is greater for higher contamination levels. For scallops harvested in same area, DA concentration for a 10-scallop ground homogenate is close to the mean of each individual concentration value, and this is the basis for carrying out the amnesic toxin analysis on a minimum quantity of a 10-scallop individual homogenate in the framework of any monitoring program.

Lipophilic toxins

The overall findings from this comparative study between total OA amount (OA + DTX3) in scallop extracts and DSP mouse bioassay results are illustrated using two types of graph:

(1) the graph in Fig. 4 shows scallop extracts mouse-activity. Usually, the mouse-toxicity of a substance is a function of survival time (ST). For convenience purposes, we decided to represent the mouse-activity (A) on the graph (i.e., inversely proportional to survival

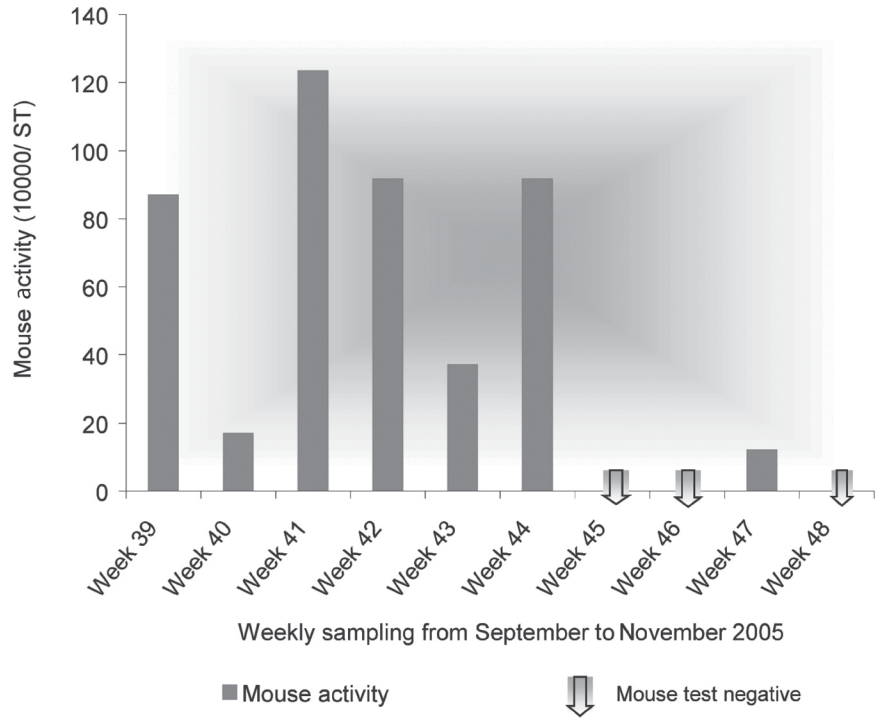

Fig. 4 Mouse activity (DSP mouse test) for scallop whole tissue extracts.

time) (A = 1/ST × 10000). For extracts with a negative mouse bioassay result, the extract activity is deemed to be null;

(2) the graph in Fig. 5 represents the OA concentration (free OA + DTX3 form) measured by LC-MS/MS in scallop whole tissue samples. For the purpose of comparison with the mouse-assay results, the DSP sanitary threshold (eq. 160 µg OA/kg of whole tissue) is indicated in the graph.

Chemical analysis (Fig. 5) indicates that all scallop samples contained OA both in free form (the minority) and in DTX3 acyl-ester forms (the majority). However, DTX1, YTXs, AZAs, SPXs, and GYMs toxins were not detected. The proportion of DTX3 in all samples is <80% of total OA-equivalent concentration, hence making DTX3 detection essential to properly assess actual shellfish toxicity. Comparison of chemical analysis results and mouse tests reveals an overall concordance. In contrast to the de-contamination period for DA, lipophilic toxin de-contamination lasted only 5 weeks. In fact, 55,400 *Dinophysis* cells/L were observed in mid September (week 37) and quickly decreased over 3 weeks down to 1000 cells/L.

Canadian work on another scallop species, *Argopecten irradians*, showed that for those scallops contaminated initially with concentrations exceeding the sanitary threshold, the de-contamination period was 2 weeks, and was shorter for gonad tissue than for digestive glands (Bauder et al. 1996).

There is already some data available on scallop diarrhetic toxin contamination associated with *Dinophysis*. Japanese research in the past has shown an accumulation of dinophysistoxine-1 (DTX1) in scallops with the presence of *D. fortii* (DTX1 producer) in sea water on the one hand, and a rapid transformation of DTX1 into acyl-ester derivative DTX3 in scallops on the other (Suzuki et al. 1997, 1999; Suzuki & Mitsuya 2001).

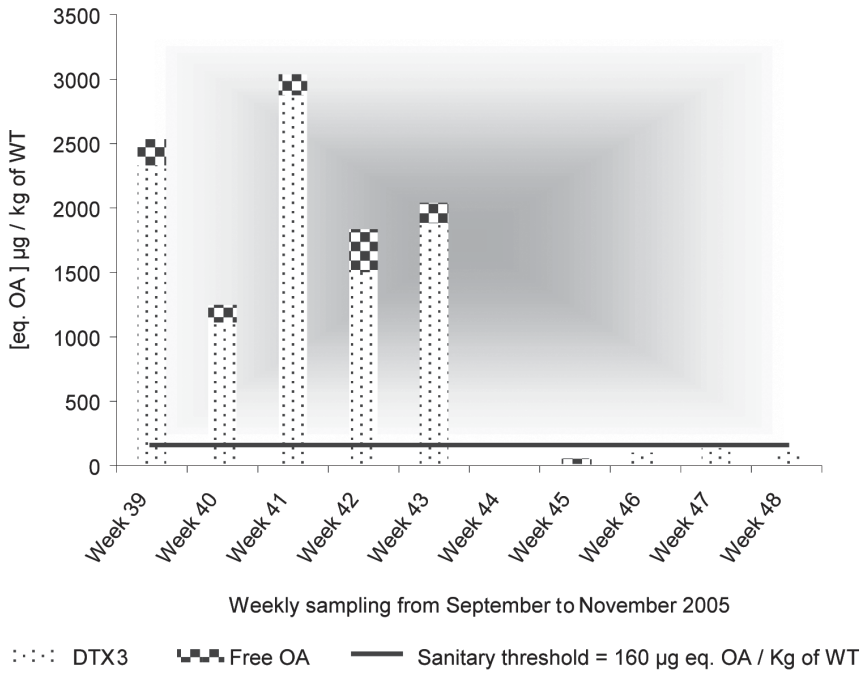

Fig. 5 Equivalent okadaic acid (OA) concentration (free OA + DTX3 form) in scallop whole tissue (WT).

OA/DTX3 distribution in different scallop organs

The study of toxin distribution in different organs (digestive gland, gonad, muscle), of contaminated scallops samples at different levels, harvested at five different areas over a 6 week period, indicated that almost all OA/DTX3 toxins were accumulated in the digestive gland, and were not detected in scallops muscle.

Toxin distribution in different organs varies according to scallop species and therefore according to organ sizes. The work carried out by Bauder (Bauder et al. 1996) on *Argopecten irradians* (Canadian scallops) contaminated with *Prorocentrum lima* (an AO and DTX1 producer) indicated that the majority of the toxin accumulated in the digestive gland (76%) rather than in the gonad (12%), muscle (4%), mantle (4%), or gill (4%) tissue. The specific weight of toxin per organ indicated that the gonad and digestives gland accumulated equivalent toxicity.

CONCLUSION

During the 2004–05 monitoring periods, our results revealed two consecutive contamination events in French scallops (the Bay of Seine in the English Channel): firstly by DA from November 2004 to June 2005, and secondly by OA and its acyl ester derivatives (DTX3) from September to December 2005. Study of toxin distribution in different scallop organs indicated that: (1) almost all of these toxins were accumulated in the digestive glands; and (2) the decontamination period for DA was very long (around 8 months), whereas in the case of OA and DTX3, it lasted only 5 weeks. This paper reports on the first occurrence of DA, OA, and DTX3 in French scallops. Paralytic toxins, however, were not detected.

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