

# Surveillance of phycotoxins in shellfish

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## Abstract

This paper presents the French national system for monitoring three groups of marine biotoxins regulated in shellfish, implemented firstly in marine production areas by the REPHY REPHYTOX network of IFREMER and secondly at the distribution level through the network of laboratories approved by the Directorate General for Food within the framework of official controls. The European regulations, the nature of the shellfish toxins, and analytical methods used are presented. The sampling procedures and strategy, as well as the results obtained by each of the two systems mentioned, are presented and discussed.

## Keywords

Phycotoxins, Shellfish, Lipophilic toxins, ASP, PSP, Surveillance

## Résumé

**Surveillance des phycotoxines dans les coquillages**  
Cet article présente le dispositif national de surveillance de trois groupes de biotoxines marines réglementées dans les coquillages mis en œuvre, d'une part au niveau de zones marines de production par le réseau RePHY-Rephytox de l'Ifremer et, d'autre part au stade de la distribution par le réseau des laboratoires agréés de la direction générale de l'Alimentation dans le cadre des plans de surveillance et des plans de contrôle mis en place chaque année. La réglementation européenne, la nature des phycotoxines recherchées et les méthodes analytiques mises en œuvre sont présentées. Les modalités et la stratégie d'échantillonnage pour chacun des deux dispositifs sont décrites. Les résultats obtenus en 2015 sont exposés et discutés.

## Mots-clés

Phycotoxines, coquillages, toxines lipophiles, ASP, PSP, surveillance

Shellfish are in direct contact with the marine environment and, due to their filtration activity (in the case of filter-feeding shellfish), concentrate contaminants found in the environment, particularly phycotoxins (algal toxins produced by toxic phytoplankton).

The following phycotoxins are regulated in shellfish under the EU hygiene package (Regulation (EC) No 854/2004 of 29 April 2004):

- lipophilic toxins including diarrhetic shellfish poison (DSP) (okadaic acid, dinophysistoxins, pectenotoxins, yessotoxins and azaspiracids), produced in particular by *Dinophysis*. These toxins are likely to cause rapid-onset gastrointestinal disorders in the consumer (30 minutes to 12 h after ingestion), mostly without severity except in people with a fragile state of health;
- amnesic shellfish poison (ASP) (domoic acid), produced in France by *Pseudo-nitzschia*. These toxins are likely to cause generally rapid-onset neurological disorders in the consumer (15 minutes to 38 h after ingestion) that can be serious, as seizures and coma may result in a fatal outcome;
- paralytic shellfish poison (PSP) (saxitoxin), produced in France by *Alexandrium*. These toxins are likely to cause rapid-onset neurological disorders in the consumer (30 minutes to 12 h after ingestion) that can be serious, as paralysis of the respiratory muscles may result in a fatal outcome.

**Table 1.** Regulatory thresholds for phycotoxins in shellfish

Name of toxin group	Regulatory threshold	
Saxitoxins (PSP-type toxins)	800 µg/kg of meat	
Domoic acid (ASP-type toxins)	20 mg/kg of meat	
Lipophilic toxins	Okadaic acid (OA) group	160 µg of okadaic acid equivalents/kg of meat (for okadaic acid, dinophysistoxins and pectenotoxins together)
	Azaspiracids	160 µg of azaspiracid equivalents/kg of meat
	Yessotoxins	3.75 mg of yessotoxin equivalents/kg of meat

The maximum regulatory levels in shellfish are established in Regulation (EC) No 854/2004 of 29 April 2004 (Annex III, Section VII, Chapter V) (Table 1).

These phycotoxins are monitored in shellfish through two complementary programmes:

- firstly, in marine production areas via the REPHY-REPHYTOX networks of Ifremer, respectively the Phytoplankton and hydrology observation and monitoring network, and the Phycotoxin monitoring network,
- and secondly at the distribution level via the surveillance and control plans (SCPs) implemented by the DGAL.

## Monitoring of phycotoxins in shellfish in marine production areas (REPHY-REPHYTOX networks)

### Materials and methods

Shellfish production areas are regularly monitored to ensure the quality of the products. The surveillance method for phycotoxins in shellfish production areas is described in Ifremer's REPHYTOX procedures dossier (Neaud-Masson & Belin)<sup>(1)</sup>.

The surveillance of phycotoxins is closely related to surveillance of toxic phytoplankton, which is managed within the framework of the REPHY network. Its procedures are currently being revised<sup>(2)</sup>.

If necessary, local REPHY-REPHYTOX procedures provide more specific information with reference to the national provisions.

The objective of REPHYTOX is the detection and monitoring of toxins that may accumulate in commercial marine products, particularly bivalve molluscs found in production areas or in natural environments farmed professionally. To meet these objectives, REPHYTOX collects shellfish samples through a network of sampling sites located

1. [http://envlit.ifremer.fr/content/download/83181/601705/version/9/file/Cahier-Procedures-REPHYTOX\\_v1.pdf](http://envlit.ifremer.fr/content/download/83181/601705/version/9/file/Cahier-Procedures-REPHYTOX_v1.pdf)

2. [http://envlit.ifremer.fr/surveillance/phytoplankton\\_phycotoxines/mise\\_en\\_oeuvre](http://envlit.ifremer.fr/surveillance/phytoplankton_phycotoxines/mise_en_oeuvre)

**Box.****Objectives**

The REPHYTOX network aims to detect and monitor regulated phycotoxins in shellfish located in marine production areas. This network is closely associated with the REPHY network, which includes in its missions the detection and monitoring of phytoplankton species producing toxins that may accumulate in shellfish.

The DGAL surveillance plans (SCP system) regarding phycotoxins in shellfish complement the REPHY-REPHYTOX monitoring programme on shellfish in the marine environment. The objective of these plans is to assess phycotoxin contamination levels of marketed shellfish and thereby, consumer exposure.

**Programming framework****Regulations**

- Regulation (EC) No 853/2004 of 29 April 2004 (Annex III, Section VII, Chapter V)
- Regulation (EC) No 854/2004 (Annex II, Chapter II, Point B)
- Regulation (EC) No 854/2004 (Annex II, Chapter II, Point D.2)

**Protocol**

- Type of contaminants detected: the three groups of regulated toxins, i.e. lipophilic toxins (okadaic acid, dinophysistoxins, pectenotoxins, yessotoxins, and azaspiracids).
- amnesic toxins in the domoic acid group, paralytic toxins of the saxitoxin group.
- Production of interest ("population"): shellfish.

- Food chain stage: shellfish sampled directly in the natural production environment (marine areas) for REPHYTOX monitoring, and shellfish placed on the market for surveillance plans.
- "Case" definition: sample contaminated by regulated phycotoxins above the thresholds determined by European regulations.
- Number of samples and sampling method: between 2500 and 3000 samples analysed each year for REPHYTOX monitoring, with at least half concerning lipophilic toxins. About 1000 samples per year for the surveillance plan.
- Sampling strategy: targeted for REPHYTOX monitoring, and random for surveillance plans, with the number of samples to collect per region being proportional to the human population.
- Analytical methods, types of samples:
  - > Testing (detection and quantification) for lipophilic toxins<sup>(1)</sup> by chemical analysis (liquid chromatography coupled with tandem mass spectrometry).
  - > Testing for saxitoxin group toxins by bioassay, with quantification based on the survival time of mice injected with shellfish extracts.
- Testing for domoic acid group toxins (domoic acid and its epimer epi-domoic acid) by chemical analysis (liquid chromatography coupled with ultraviolet detection).

1. Testing is performed for regulated toxins (okadaic acid, dinophysistoxins and pectenotoxins - OA+DTXs+PTXs, yessotoxins - YTXs and azaspiracids - AZAs) and also certain non-regulated toxins (spirolids - SPXs, gymnodimines - GYM, and pectenotoxin-2-seco acid - PTX2sa).

along the entire coast, with spatial coverage that must fulfil two requirements: scientific relevance and optimisation of the cost/effectiveness ratio. There may be overlap between the REPHYTOX sampling sites and those of the REPHY network. In any event, there is a close relationship between REPHYTOX and a certain number of REPHY sites since the phytoplankton results at REPHY sites in a given area determine when detection of toxins at the REPHYTOX sites in the area is triggered. If toxic phytoplankton are found<sup>(3)</sup> (above the thresholds defined for each toxic species in the REPHYTOX procedures), toxin analyses are triggered in shellfish with a weekly interval.

In some cases, monitoring of toxic phytoplankton is not sufficiently reliable to guarantee the food safety of shellfish in an area, and analyses of toxins are then systematically carried out in shellfish. This is the case:

- in areas at risk for lipophilic toxins during predefined risk periods. These areas are considered more sensitive on the basis of historical toxin contamination data and may be subject to shellfish contamination even if there are only very low quantities of toxic phytoplankton that are difficult to detect, which justifies systematic analysis in shellfish,
- in offshore sources, which are systematically monitored for the three types of toxins every fifteen days (1 month before and then during the farming period). The depth of the water column in this case makes it impossible to clearly determine all the phytoplankton species present.

In the case of lipophilic toxins, mussels are considered a sentinel species because historical data have shown that they always become contaminated more quickly than all other shellfish. When there are mussels available for a production zone, they are therefore analysed on a first-line basis, while other shellfish are analysed as soon as mussels are found to contain toxins. There is no sentinel species for ASP or PSP.

There are about 250 potential sampling points for shellfish all along the coast of mainland France. The samples can be for various types of shellfish, from offshore sources or farmed using different methods

(stake, rope, tray culture, etc.).

Changes to the system for monitoring shellfish production areas (specifically sampling conditions) are defined within the framework of a national steering committee (COFIL) that brings together the various government bodies concerned: the Directorate General for Food, the Directorate for Marine Fisheries and Aquaculture, the Directorate General for Health, Ifremer, ANSES, and the French Public Health Agency. The committee meets at least once a year.

The analytical methods used in the REPHY and REPHYTOX systems are as follows:

- > Quantitative analysis of domoic acid (ASP toxin) in shellfish by high-performance liquid chromatography with ultraviolet detection (HPLC-UV): ANSES method PBM BM LSA-INS-0140.

*Principle:* domoic acid and its epimer epi-domoic acid (if present) are extracted from a homogenised tissue sample using 50% aqueous methanol. The extract is then filtered and analysed by isocratic high-performance liquid chromatography (HPLC) with ultraviolet detection.

- > Determination of lipophilic marine biotoxins in molluscs by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS): ANSES method PBM BM LSA-INS-0147.

*Principle:* toxins in groups OA, PTX, AZA and YTX are extracted using methanol from a homogenised tissue. An aliquot of the methanol extract is treated by alkaline hydrolysis to convert possible acyl esters of OA and/or DTX into free toxins. The extracts are then purified by SPE (optional step) and analysed by gradient elution liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Non-hydrolysed extracts are used to test for the presence of free OA, free DTX1 and free DTX2, PTX1, PTX2, AZA1, AZA2, AZA3, YTX, homo YTX, 45 OH YTX, and 45 OH homo YTX. Hydrolysed extracts are used to determine the total quantity of toxins of the OA group.

- > Bioassay in mice for the determination of saxitoxin group toxins (paralytic phycotoxins - PSP) in shellfish - ANSES method PBM BM LSA-INS-0143.

*Principle:* the bioassay method in mice includes a toxin extraction step from meat by hot acid hydrolysis, followed by intraperitoneal (IP) injection of 1 ml of extract in at least three mice. The survival

3. The following toxic species are tested for: *Alexandrium*, *Dinophysis*, *Pseudo-nitzschia*, *Ostreopsis*, *Gonyaulax spinifera*, *Lingulodinium polyedrum*, *Protoceratium reticulatum*, and *Prorocentrum lima*.



**Figure 1.** Geographic distribution of episodes of documented toxicity in shellfish on the coast: lipophilic toxins (left), paralytic toxins – PSP (centre), and amnesic toxins – ASP (right)

time (interval between injection and death) is recorded and the toxicity determined in mouse units (MU) from Sommer's table.

The bioassay is quantitative when the mice die between five and seven minutes after injection. Several dilutions may be needed to obtain a survival time between five and seven minutes. The MU measurement is then converted into  $\mu\text{g}$  STX diHCl equivalents (eq)/kg.

The National Reference Laboratory (NRL) for marine biotoxins coordinates three networks of accredited laboratories, one for each type of phycotoxin analysed: ASP network, PSP network, and lipophilic toxin network. These networks include Ifremer laboratories for the monitoring of production areas.

## Results for 2015

### Lipophilic toxins

Of the 1300 analyses performed for these toxins, 140 results (about 11%) were non-compliant (i.e. above the regulatory threshold of 160  $\mu\text{g}/\text{kg}$ ) for the okadaic acid + dinophysistoxins + pectenotoxin group. This percentage is higher if only mussels are considered (15%).

The maximum concentrations detected at the national level for the various shellfish species were as follows: 3003  $\mu\text{g}/\text{kg}$  in mussels from Etang de Salses-Leucate (western Mediterranean) in January, 615  $\mu\text{g}/\text{kg}$  in oysters from the Bay of Arcachon in May, 322  $\mu\text{g}/\text{kg}$  in great scallops from Pays de Caux in January and 1315  $\mu\text{g}/\text{kg}$  in *Donax* from the coast of Gironde in May (Figure 1). For the azaspiracid and yessotoxin groups, no non-compliant results were observed in 2015.

### Paralytic toxins (PSP)

Of the 529 bioassays performed for these toxins, 19 results (i.e. 4% [95CI: 2-5]) were non-compliant (i.e. above the

regulatory threshold of 800  $\mu\text{g}/\text{kg}$ ). This percentage is much higher if only mussels are considered (19%), bearing in mind that only mussels and oysters were contaminated in 2015.

The maximum concentrations detected at the national level for these two shellfish species were as follows: 3136  $\mu\text{g}/\text{kg}$  in mussels from Etang de Thau (western Mediterranean) in October and 1622  $\mu\text{g}/\text{kg}$  in oysters from the Penzé river (north-west Brittany) in July (Figure 1).

### Amnesic toxins (ASP)

Of the 661 analyses performed for these toxins, 40 results (about 6%) were non-compliant (i.e. above the regulatory threshold of 20 mg/kg). This percentage is higher if only great scallops are considered (10%), with these shellfish showing the highest contamination. The maximum concentrations detected at the national level for the two most affected shellfish species were as follows: 284 mg/kg in great scallops from the Roadstead of Brest in January and 33 mg/kg in *Donax* from the coast of Gironde in May (Figure 1).

## Discussion

### Lipophilic toxins

Concerning lipophilic toxins, the configuration of toxic episodes in 2015 is quite similar to what is observed each year. Firstly, from a geographical point of view: i) rare episodes in the Channel, primarily around the Seine estuary, ii) multiple episodes on the Atlantic coast, in particular in western and southern Brittany and in the Bay of Arcachon, areas where lipophilic toxins have been observed repeatedly for over 30 years, and iii) mostly localised episodes in lagoons in the Mediterranean. Secondly, in terms of distribution through the year: i) for coastal shellfish, toxicity was observed from the spring in the Atlantic areas, more commonly in summer in the Channel, and more during the winter in Mediterranean lagoons, ii) for pectinids (primarily great scallops), contamination can be observed during fishing periods, i.e. in the winter. In line with other years, mussels are the most highly affected shellfish, bearing in mind that many other shellfish species can be affected if the episodes continue for an extended period. Considering the results obtained since 2010 (first year when chemical analyses were used to detect these toxins), the 2015 results are rather high for certain types of shellfish in view of the national median value (340  $\mu\text{g}/\text{kg}$ ) calculated based on values above the food safety thresholds. These results are, however, well below the maximum levels reached in certain years, in particular for specific shellfish: for example, 37,296  $\mu\text{g}/\text{kg}$  and 11,755  $\mu\text{g}/\text{kg}$  in mussels and cockles, respectively, from the Bay of Arcachon in April 2012. Concerning the azaspiracid and yessotoxin groups, the lack of non-compliant results in 2015 confirms the results obtained for these toxin groups since their detection was implemented along the coastline of France.

### Paralytic toxins (PSP)

For paralytic toxins, the three areas most affected in 2015 (Abers in Brittany, the Roadstead of Brest, and the Etang de Thau in the Mediterranean) were among the four zones most commonly affected by episodes of contamination by PSP phycotoxins (adding to these the Penzé River in north-west Brittany) since 1988<sup>(4)</sup>. These episodes, which thus remain limited from a geographic point of view, are still of concern given how dangerous these toxins are. In terms of occurrence through the year, the results for 2015 confirm trends observed to date: contamination is always observed between June and September in the Channel-Atlantic zone and always between September and December for the Etang de Thau. Until now, non-compliances have only been observed in mussels, oysters, cockles, or clams. Shellfish from offshore sources (including great scallops) have never been affected by a PSP episode. Taking into account the results obtained since 1990, the results for 2015 are rather high for mussels in view

4. Year of first detection of PSP toxins in France.

of the national median (1622 µg/kg) calculated on the basis of values higher than the food safety threshold for all shellfish. Importantly, however, results are well below the maximum values reached in some years, in particular for certain shellfish, such as: 11,664 µg/kg in mussels from the Roadstead of Brest in July 2012, and 7,360 µg/kg in oysters from the Abers (north-west Brittany) in August 2001.

### Amnesic toxins (ASP)

For amnesic toxins, the areas affected in 2015 (western and southern Brittany, Pertuis Charentais) are among the zones that have regularly been affected by ASP episodes since the year 2000, when the first ASP toxins were identified in France. The Seine estuary, and less often the western Mediterranean, are also zones that have been affected since 2000. In terms of occurrence through the year, the results for 2015 confirm trends observed to date: all year for great scallops, and generally between March and June for the other shellfish, irrespective of the region. As a general rule, episodes of ASP affect mainly, if not exclusively in certain years, great scallops. This type of shellfish also shows the highest concentrations with a particularly protracted decontamination period that can reach several months. Other shellfish may also be affected, including mussels, oysters, *Donax*, and clams, but at concentrations rarely exceeding 100 mg/kg, and above all with decontamination periods that are often very short. Taking into account the results for the period 2000-2015, the results for 2015 for great scallops are rather high in view of the national median (41 mg/kg) calculated on the basis of values higher than the food safety threshold for these shellfish. The values are nonetheless lower than the maximum levels reached in certain years, the record being 861 mg/kg in the Roadstead of Brest in April 2014.

Contamination records for the three toxin groups are available at: <http://envlit.ifremer.fr/var/envlit/storage/documents/parammaps/toxines/index.html#>

## Monitoring of phycotoxins in shellfish at the time they are placed on the market (SCP system)

### Materials and methods

Surveillance plans for contamination of shellfish by phycotoxins at the distribution level, implemented by the DGAL, complement the REPHY-REPHYTOX monitoring programme.

These plans are part of the general framework for assessing compliance of foodstuffs, which falls under the responsibility of the competent authorities. The regulatory criteria for phycotoxins in shellfish at the distribution level are described in Annex II, Chapter II, Point D.2 of Regulation (EC) No 854/2004.

The objective of these plans is also to assess the level of phycotoxin contamination of marketed shellfish. As a result, the data help to estimate consumer exposure. In 2015, 918 samples were planned by the DGAL for the full year, with regional distribution determined proportionally to the human population, i.e. 306 samples for detection of ASP, PSP and lipophilic toxins, respectively.

Samples were taken randomly at the distribution level in hyper- and supermarkets or in retail stores (fishmongers): this involved samples of live farmed (shellfish aquaculture) or fished bivalve molluscs,

preferably sourced in France or in another Member State of the European Union.

The collected samples were forwarded to the accredited laboratory networks according to the types of phycotoxins to detect. The analytical methods used were the same as those implemented for the REPHY-REPHYTOX system.

### Results

Of the 918 collected shellfish samples, 897 yielded an analytical result. The analysis completion rate was 97%. Among the 897 analytical results, three values exceeding the regulatory thresholds were observed, i.e. a non-compliance rate of 0.33% (95CI-[0.11-0.98])<sup>5</sup> for the three groups of regulated toxins. Table 2 presents the overall results.

#### Amnesic toxins (ASP)

Of the 301 samples collected, 297 were analysed. No values exceeding the threshold for domoic acid were found, corresponding to a compliance rate of 100% (95CI-[98.7-100]) for samples in this toxin group.

#### Paralytic toxins (PSP)

Of the 303 samples collected, 300 were analysed. No values exceeding the threshold for saxitoxin were found, corresponding to a compliance rate of 100% (95CI-[98.7-100]) for samples in this toxin group.

#### Lipophilic toxins

Of the 309 samples, 300 were analysed. Three values exceeding the threshold for lipophilic toxins of the okadaic acid group (OA+DTXs+PTXs) were detected, corresponding to a non-compliance rate of 1% (95CI-[0.34-2.90]) for samples in this toxin group.

The first case involved live bulk mussels sourced from Spain that showed levels above the regulatory threshold (170.3 µg of okadaic acid equivalents/kg). Following this non-compliance, the affected mussels were withdrawn and recalled, with information provided to consumers.

The second case involved live mussels sourced from Spain that showed levels above the regulatory threshold (204.1 µg of okadaic acid equivalents/kg). Following this non-compliance, the affected mussels were withdrawn and recalled, with information provided to consumers. In view of this non-compliant result and the closure of the corresponding production area a short time after the harvest, an alert report was forwarded to the Spanish authorities via the RASFF<sup>6</sup>.

The third case involved living mussels sourced from Ireland that showed levels above the regulatory threshold (230.1 µg of okadaic acid equivalents/kg). It was not possible to implement management measures directly in France on the product batches affected by this non-compliance. The mussels had been distributed and consumed in full. An alert report was forwarded to the Irish authorities via the RASFF.

Furthermore, on the basis of the full results, it can be observed that 87.6% (263/300) of the samples did not have quantifiable lipophilic toxin levels.

5. 95CI: 95% confidence interval  
6. Rapid alert system for Food and Feed

**Table 2.** Breakdown of the samples and results by type of matrix and by analyte

	Number of samples					Number of samples analysed	Number of non-compliant samples	Compliance rate (%)
	Mussels	Oysters	Great scallops	Others*	Total			
ASP toxins	162	56	7	76	301	297	0	100
PSP toxins	179	66	3	55	303	300	0	100
Lipophilic toxins	199	55	7	48	309	300	3 (mussels)	99
<b>Total</b>	<b>540</b>	<b>177</b>	<b>17</b>	<b>179</b>	<b>913</b>	<b>897</b>	<b>3</b>	<b>99.6</b>

\* European bittersweet clam, queen scallop, cockles, clams, or no species indicated.

For okadaic acid, dinophysistoxins and pectenotoxins taken together, 28 samples showed a quantifiable toxin level below the regulatory threshold of 160 µg of okadaic acid equivalents/kg of meat:

- 15 samples had toxin levels between the quantification limit and 45 µg of okadaic acid equivalents/kg of meat,
- 13 samples had toxin levels between 45 µg and 160 µg of okadaic acid equivalents/kg of meat.

For the azaspiracids, only one sample had a quantifiable toxin level lower than the regulatory threshold of 160 µg of azaspiracid equivalents/kg of meat. This was a sample of mussels sourced from the Netherlands with a level of 80 µg of azaspiracid equivalents/kg.

For the yessotoxins, five samples had toxin levels between the quantification limit and 1711 µg of yessotoxin equivalents/kg of meat. This involved three samples of mussels from Italy, one sample of mussels from Denmark, and one sample of mussels from France (Etang de Diana in Corsica).

### **Discussion**

The results of the 2015 surveillance plan for contamination of shellfish by phycotoxins at the distribution level indicate that, like in previous years, the contamination rate for bivalve molluscs by phycotoxins is low, with an overall non-compliance rate of 0.33% (95CI-[0.11-0.98]). The findings from this surveillance plan indicate that monitoring of marine production areas by Ifremer, associated with management measures, ensures a good food safety status for national products placed on the market. The three cases of non-compliance detected as part of the surveillance plan involved shellfish from other Member States of the European Union, which were therefore not produced and monitored in marine areas of France.

In addition, the surveillance plan ensures verification of compliance for products placed on the market in France, whether they

are produced locally or imported. The combination of the two surveillance programmes makes it possible to ensure a high level of consumer protection.

Only one sample of French shellfish (mussels from Île de Groix) was involved in a case of collective foodborne illness in 2015, confirming the effectiveness of the national surveillance programme, and in particular upstream surveillance in the production areas.

In 2016, the DGAL decided to monitor only contamination of mussels by lipophilic phycotoxins at the distribution stage. This decision is based on the results of the REPHYTOX monitoring programmes implemented, which show that mussels are the bivalve molluscs that are most frequently contaminated by phycotoxins, and in particular lipophilic phycotoxins. The objective of this plan is to assess contamination levels of mussels on the market by lipophilic phycotoxins and thereby, consumer exposure.

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