AGENCE FRANÇAISE
DE SÉCURITÉ SANITAIRE
DES ALIMENTS

Maisons-Alfort, 16 September 2008

OPINION

of the French Food Safety Agency on the relevance of tools for detecting lipophilic phycotoxins in shellfish

THE DIRECTOR-GENERAL

1. SUMMARY OF THE REQUEST

The French Food Safety Agency (Afssa) was requested by the French Directorate General for Food (DGAL) on 09 September 2008 to provide scientific and technical support to the proposal of the French regional shellfish farming association (SRC) for Arcachon Aquitaine, to revise the tests for detecting lipophilic phycotoxins in shellfish.

In response to this request Afssa has summarised economic, regulatory and scientific data on detection tools.

2. METHOD OF EXPERT ASSESSMENT

An emergency collective expert assessment group (GECU) on phycotoxins, which was created at the instigation of the Director-General of the French Food Safety Agency in consultation with the Chairman of the scientific panel "Chemical and physical residues and contaminants" was asked to perform this expert assessment.

The “Phycotoxins” GECU comprises:

- experts from the scientific panel "Chemical and physical residues and contaminants", including the Chairman of the panel;
- an expert from Ifremer[1], Environment, Microbiology and Phycotoxins Department;
- experts from the Toxins, organic pollutants and pesticides unit (TOP) of the laboratory for studies and research on the quality of foods and food processes (LERQAP), French National Reference Laboratory (NRL) for marine biotoxins;
- an expert from the Department for the Evaluation of Nutritional and Health Risks (DERNS).

Following consultation of the “Phytotoxins” GECU on 10 September 2008, Afssa is issuing the following opinion.

3. CONTEXT: SHELLFISH MARKET DATA IN FRANCE AND WORLDWIDE

3.1 Oyster production

According to data published by Ifremer[2], in the year 2000 French oyster production (in the region of 130,000 tons per year) accounted for **91% of production in the Europe of 15 Member States**, making France the fourth largest producer in the world behind China, Japan and Korea. French trade with European Union countries is limited to 3,000 tons of imports per year into France and 6,000 tons per year of exports from France (details in annex 1).

[1] Ifremer: French Research Institute for Exploitation of the Sea
[2] http://www.ifremer.fr/reper/pagesthemes/Socio-economie/ostreiculture.htm).

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Seven regions are involved in national production: Brittany (30%), Poitou-Charente (22.5%), Lower Normandy (22.5%), the Mediterranean (9%), Arcachon-Aquitaine (8.3%) and Pays de la Loire (7.5%)[3].

3.2 Mussel production

Spain is the largest mussel producer with 46% of European production[4]. France is the third largest producer in Europe with 12% of production, i.e. approximately 75,000 tons per year, behind Spain and Italy, and the fifth largest worldwide (with 5% of global production) behind China, Spain, Italy and New Zealand.
Mussel production is divided between a greater number of countries than oyster production.

According to data from the year 2000, French trade is mainly conducted with European countries and New Zealand. France imports 46,250 tons of mussels per year and exports 5,384 tons.

3.3 Oyster consumption

Unlike mussels, which are eaten throughout the year, 70% of oyster production is consumed in winter (between November and January), mainly during the Christmas and New Year celebrations.

According to the CALIPSO study[5] (2006) on high consumers of seafood, the average consumption of oysters in France is 41 g/week (grams per week) and 144 g/week at the P95 (95th percentile) for men; for women the average is 28 g/week and P95 is 90 g/week; for the elderly (men and women aged 65 and over) the average is 51.3 g/week and P95 is 144 g/week.

4. HISTORY

Shellfish contamination, which has been evidenced since the 1970s, has spread along the coastlines of the whole world. In recent years not only have more and more geographical regions been affected, but new phytoplankton species and toxin families have also appeared. Analysing and characterising constantly evolving toxicity episodes has therefore been made difficult.

4.1 Toxic planktonic algae

Worldwide approximately 4,000 species of planktonic algae have been identified, 250 of which can proliferate in a bloom and approximately 70 are currently known to be toxic for fauna, flora and occasionally for the shellfish consumer (source: Ifremer).

Periodical poisonings linked to the consumption of shellfish have been detailed for a long time, but a correlation between the presence of certain planktonic algae and shellfish toxicity for consumers was only highlighted in the 1970s. At that time Japanese teams established a link between shellfish contamination, the presence of *Dinophysis fortii* in sea water and human poisoning (Frémy and Lassus, 2001).

4.2 Phycotoxins

Up to 2004 four groups of toxins were defined, each according to the clinical symptoms of poisoning after consuming shellfish:

- Liposoluble diarrhetic toxins: Diarrhetic Shellfish Poisoning (DSP);

[3] http://www.ifremer.fr/littoralbasnormand/page.php?numpage=119
[4] http://www.ifremer.fr/reper/pagesthemes/Socio-economie/ValerieBarbierUniPoitiers/mytiliculture.htm
[5] CALIPSO: seafood consumption study and biomakers of exposure to trace elements, pollutants and Omega-3, AFSSA/DGAl/INRA, 2006, AFSSA/DGAL/INRA, 2006.

- Hydrosoluble paralytic toxins: Paralytic Shellfish Poisoning (PSP);
- Hydrosoluble amnesic toxins: Amnesic Shellfish Poisoning (ASP); and
- Liposoluble neurotoxins: Neurotoxic Shellfish Poisoning (NSP).

Amongst these four groups, liposoluble diarrhetic toxins have proven to be more complex as science advanced. Originally it was believed that this group consisted mostly of the okadaic acid and dinophysistoxins produced by the genus *Dinophysis.* Because of the toxins' liposolubility, their toxicity was researched by giving fractions of contaminated shellfish extracted with organic solvents to rats or mice. Progressively the monitoring operations using this rodent bioassay and further parallel research highlighted several families of liposoluble toxins produced by different species and genera of phytoplankton with varying modes of action or biological effects. Therefore a distinction was made between toxins causing diarrhetic effects in humans (okadaic acid, dinophysistoxins and azaspiracids) and those for which no symptoms have ever been reported in humans despite hepatotoxic (pectenotoxins) or cardiotoxic (yessotoxins) effects in animals.

Therefore the designation changed to "lipophilic toxins", which includes four regulated families:

- okadaic acid and dinophysistoxins (DTXs), produced in particular by the genus *Dinophysis*;
- Pectenotoxins (PTXs), produced mainly by *Dinophysis spp.*;
- Yessotoxins (YTXs), produced mainly by *Lingulodinium polyedrum*;
- Azaspiracids (AZAs), whose producing genus has not yet been identified.

This group also includes emerging toxins such as gymnodimines and spirolides. Their risk for consumer health has not been analysed.

The group of **paralytic toxins**, which are hydrosoluble and produced especially by the genus *Alexandrium,* has at least 24 identified analogues, including the most famous, saxitoxin (STX).

The group of **amnesic toxins**, which are hydrosoluble and produced by diatoms of the genus *Pseudo-nitzschia,* consists mainly of domoic acid (DA) and its isomers.

The group of **neurotoxins** consists of brevetoxins and congeners. Because of their toxicity for fish, which makes it possible to detect their presence easily, and geographical distribution, which remains limited, no European regulation exists. These are lipophilic toxins causing very particular symptoms and can therefore be distinguished from diarrhetic lipophilic toxins (by mouse bioassay).

Growing knowledge and evidence of a larger number of families of phytoplankton/phycotoxins, including some emerging toxins, have led to updating of the regulations.

At its 25th session, the Codex Committee on Fish and Fishery Products (CCFFP) asked FAO and WHO to provide scientific advice on biotoxins in conjunction with its work on Proposed Draft Standards for Live and Processed Bivalve Molluscs. The following specific requests were made: Provide scientific advice to the CCFFP to enable the establishment of maximum levels in shellfish for shellfish toxins (PSP-, DSP-, ASP-, AZP- and NSP-toxins, and YTXs and PTXs);

- Provide guidance on methods of analysis for each toxin group;
- Provide guidance on monitoring of biotoxin-forming phytoplankton and bivalve molluscs (including sampling methodology);
- Provide information on geographical distribution of biotoxin-forming marine phytoplankton.

In 2004, the Joint FAO-IOC-WHO[6] *ad hoc* Expert Consultation on Biotoxins categorized the biotoxins into 8 distinct groups based on chemical structure:

- **the okadaic acid and dinophysistoxins group;**
- **the saxitoxins group;**
- **the domoic acid group;**

[6] Food and Agriculture Organization of the United Nations/Intergovernmental Oceanographic Commission/World Health Organization

- **the pectenotoxins group;**
- **the yessotoxins group;**
- **the brevetoxins group;**
- **the azaspiracids group; and**
- **the cyclic imines group (including spirolides).**

Since 2003, dinoflagellates of the genus *Ostreopsis* have appeared along Mediterranean coastlines (Greece, Spain, Italy and France), making it necessary to consider a ninth group: palytoxins. Palytoxins and an analogue, ovatoxin, were detected for the first time in France in sea urchins in the Mediterranean Sea in 2008 and were linked to the development of *Ostreopsis cf. ovata* (Ifremer/ Phycotoxins laboratory data).

4.3 History of the situation in France

In France planktonic algae were considered to be responsible for thousands of diarrhetic poisonings in Brittany and Normandy in 1983; the phytoplankton species belonged to the genus *Dinophysis*, which is capable of producing diarrhetic toxins.

In order to guarantee the health of consumers of shellfish produced in France, Ifremer established the REPHY network[7] in 1984 and began to monitor production areas with surveillance of the environment (detection of potentially toxic phytoplankton) combined with health controls of the bivalves (measurement of the global toxicity level). These measures made it possible to:

- highlight the presence of a toxin (okadaic acid) in shellfish in contaminated areas;
- identify the geographical expansion of the areas contaminated by diarrhetic phycotoxins;
- detect the first cases of shellfish contaminated with paralytic toxins in North Brittany in 1988, and again ten years later in the Mediterranean Sea (Thau Lake), (Masselin *et al*., 2001);
- detect the contamination of shellfish with amnesic toxins in Brittany for the first time in 2000 (Amzil *et al*., 2001).

In recent years the improvement of knowledge at European and international level and in the methods of analysing toxins monitored by the REPHY network have made it possible to identify toxicity episodes linked to a larger variety of regulated and emerging (spirolides) toxins.
The following were identified:

- dinophysistoxin-2 and dinophysistoxin-3 linked to the presence of *Dinophysis acuta,* in South Brittany in 2002 (Amzil and Mathias, 2006);
- pectenotoxin-2 and a derivative in the lakes of Salses, Leucate and West Corsica in 2004 linked to the development of *Dinophysis spp.* (Amzil *et al*., 2007);
- spirolides linked to the presence of *Alexandrium ostenfeldii* in the Arcachon Basin in 2005 (Amzil *et al*., 2007);
- compounds from the palytoxin group: i) in samples of *Ostreopsis cf. ovata* from French Mediterranean coasts in 2006; ii) in sea urchins from the Mediterranean Sea in 2008 (Ifremer/Phycotoxin laboratory data)
- azaspiracids off the coast of Roscoff in 2006 (Amzil *et al*., 2008);
- yessotoxins linked to the presence of *Lingulodinium polyedrum* and *Gonyaulax spinifera* in Thau Lake in 2007 (Amzil et al., 2008), and in the Arcachon Basin linked to the presence of *Gonyaulax spinifera* in 2008.

No human death linked to the consumption of shellfish contaminated with phycotoxins has been reported in France.

[7] Réphy: *Réseau de surveillance du phytoplancton et des phytotoxines* (Phytoplankton and phycotoxin monitoring network)

5. CHANGES TO THE REGULATIONS

The implementation of European regulations on live bivalve molluscs began in 1991 and has progressively been supplemented by various texts (list in the bibliographic references and details in annex 2). These regulations were later included in the Hygiene Package.

5.1 Group of saxitoxins (formerly "PSP toxins")

This family of toxins (more than twenty analogues) has, since 1991, been regulated by Directive 91/492/EC, which fixed a maximum level of 800 µg/kg of shellfish flesh and defined a biological analysis method (mouse bioassay). The directive did, nevertheless, offer the possibility to include a chemical method. In the event of challenged results the biological method was to be considered as the reference method.

The maximum level has not been changed by the various amendments to the regulation. Since 2006 it has, however, been possible to use a validated chemical method as an alternative to the bioassay.

Mouse bioassay for paralytic toxins has been validated by the AOAC (Association of Official Analytical Chemists). It allows the global toxicity of shellfish to be detected and quantified by comparing it to saxitoxin toxicity, the only molecule for which a standard could be fixed.
This relatively specific bioassay (especially because of the extraction) does not present any particular problems when interpreting the results as the targeted toxins all have the same toxic effect (immediate neurotoxicity). It is not currently being reviewed except for ethical aspects.

5.2 Group of domoic acid (formerly ASP toxins)

In 1997 the regulations were extended to include the domoic acid group in Directive 97/61/EC, which sets a maximum level of 20 mg/kg of shellfish flesh using a HPLC/UV (high-performance liquid chromatography with UV detection) validated analysis method. This method can be applied as this group of toxins consists of one main analogue, domoic acid, which is the most toxic and accounts for at least 95% of the quantity of toxins present.

The maximum level and the reference method have not been amended by the various changes to the regulation.

5.3 Family of lipophilic toxins (formerly DSP toxins)

At the implementation of the initial regulation in 1991 the term "diarrhetic toxins" included only okadaic acid and dinophysistoxins, and no quantitative limit value had been fixed. The directive indicated that the control was to be undertaken using the "customary" biological methods with rodents without giving any further details.
In the years following this directive other groups of toxins were detected by these biological methods, showing that the family of lipophilic toxins was less homogenous than originally believed in 1991.
In this context an official discussion started in 2001 to improve the regulations for lipophilic toxins and led to Decision 2002/225/EC establishing specific regulatory limit values for four groups of toxins and stating their reference methods: mouse or rat bioassay (for the okadaic acid and azaspiracids groups). This decision presents basic guidelines for the analytical protocol and states that the period of observation must last 24 hours to enable all toxins to be detected.

The maximum level for shellfish (whole body or each edible part separately) is:

- 160 micrograms in equivalent-okadaic acid per kilogram of okadaic acid, dinophysistoxins and pectenotoxins;
- 1 milligram in equivalent-yessotoxin per kilogram; and
- 160 micrograms in equivalent-azaspiracids1 per kilogram for azaspiracids.

Decision 2002/225/EC introduced the option to apply alternative methods, provided that either alone or combined they can detect at least the following analogues, that they are not less effective than the biological methods and that their implementation provides an equivalent level of public health protection: okadaic acid and dinophysistoxins (DTX1, DTX2 and DTX3): a hydrolysis phase can be necessary to detect the presence of DTX3;

- pectenotoxins: PTX1 and PTX2;
- yessotoxins: YTX, 45 OH YTX, Homo YTX, and 45 OH Homo YTX;
- azaspiracids: AZA1, AZA2 and AZA3.

The total toxicity shall be calculated using toxic equivalency factors (TEF) based on the toxicity data available for each toxin.

The decision also states that if new analogues of public health significance are discovered, they should be included in the analysis.

Finally it is indicated that the performance characteristics of these alternative methods should be defined after validation following an internationally agreed protocol.

Article 5 of the decision details that "When the results of the analyses performed demonstrate discrepancies between the different methods, the mouse bioassay should be considered as the reference method."

In 2004 the entry into force of Regulation 853/2004 (Hygiene Package) did not result in any modifications of the maximum level or applicable methods for these toxins.

In 2005 Regulation (EC) No. 2074/2005 reiterated the detection methods for all of the phycotoxin groups. It stated that the biological methods could be replaced by other methods as soon as the reference material for the detection of toxins listed in annex III, section VI, chapter V of Regulation (EC) No. 853/2004 becomes readilyavailable, these methods have been validated and the regulation has been amended accordingly.

5.4 International work

As early as 2002 the *Codex Alimentarius* focused on biotoxins, through its committee on fish and fishery products (CCFFP), in the aim of establishing a proposed draft standard for live and processed bivalve molluscs. A joint scientific FAO/WHO/IOC expert group was therefore created. The conclusions of this group, which were presented in 2005, resulted in contradictory opinions and discussions between the States. It was therefore decided to create a working group to supplement the expert assessment in the report in the framework of devising these standards and codes of use.

Parallel to the international works led by the Codex, a working group consisting of international experts and called the "WG on Toxicology" was created by the CRL (Community Reference Laboratory) in 2005 in order to conduct **a risk assessment for lipophilic toxins** and to establish safety limit values for shellfish for each sub-family of this family of toxins.

The works led by the Codex and CRL show a general lack of toxicological data and the complexity of the biotoxins domain, leading to differences for some families in fixing the acute reference dose for humans (ARfD), the portion consumed to take into account and the regulatory limits for shellfish.

It can reasonably be considered that the works of the Codex will not be completed for several years.

5.5 Work of EFSA

Given the development of scientific knowledge, the works of the Codex and the CRL, the detection of emerging toxins and the questioning of the mouse bioassay by professionals and

some scientists, the DG SANCO requested that the European Food Safety Authority (EFSA) respond to these issues in late 2006.

The Commission requested that EFSA evaluate the current European regulatory limit for public health and the methods for analysing various marine toxins as established in European regulations, including new emerging toxins and based on the available prior studies (Codex, CRL, ECVAM).

An EFSA working group comprising scientists specialising in biotoxins was created. An initial report on the okadaic acid family and dinophysistoxins was published in early 2008. The report states that the ARfD for these toxins is similar to the values proposed by the international working groups of the Codex and the CRL (0.3 µg/kg p.c.).

Based on recent data the working group's experts concluded that mouse bioassay only has a 40 to 50% chance of detecting a positive response for a sample containing okadaic acid at the current regulatory limit of 160 µg/kg. .

The experts reiterate that current European legislation permits the replacement of the bioassays, provided that alternative methods have been validated according to an internationally recognised protocol. They also state that at the time of the expertise, no detection method for the okadaic acid group and dinophysistoxins has been validated (see 6.1.2). Based on currently available data inhibition tests for protein-phosphatases and liquid chromatography coupled with mass spectrometry have the greatest potential to replace the mammalian assays, and to detect levels of OA-group toxins below the current EU regulatory limit., but a large number of obstacles must be overcome before these methods can be used routinely.

Finally, the sensitivity of the mouse bioassay for okadaic acid content at the regulatory limit of 160 µg eq OA/kg is limited, even with an observation period of 24 hours, but no other method of detection is currently available.

In order to protect against the acute effects of OA-group toxins, the working group considered that it is important to use a high portion size rather than a long-term average consumption in the health risk assessment of shellfish consumption.
Based on a daily amount of 400 g the experts concluded that shellfish should not contain more than 45 µg equivalent OA/kg in order to stay below the acute reference dose.

The other toxin groups (saxitoxins, domoic acid, pectenotoxins, yessotoxins, brevetoxins, azaspiracids and cyclic imines [incl. spirolides]) are currently under evaluation, and the works will not be finished before mid-2009.

6. CHARACTERISATION OF TOXIC EPISODES

The characterisation of a toxic episode involving lipophilic toxins is based on:

- the identification of a bloom of a potentially toxic phytoplanktonic genus or species
- a positive result for the mouse bioassay, and
- an identification of lipophilic toxins in shellfish by chemical analysis in a sufficient quantity to explain the toxicity effects observed in mice.

Although the presence of a potentially toxic phytoplankton is one aspect of characterising a toxic episode and an indicator of possible shellfish contamination, it does not make it possible to rule on the safety of the shellfish. This is determined by analysing the contamination level of the shellfish themselves.

6.1 Methods of detecting toxins in shellfish

For several years consumer health safety has been ensured by regulatory measures (maximum levels in foodstuffs) based on the toxicological profile and the safety of a substance detected in

rodents. These tests evaluate the acute (LD50) mid (90 days) and long term (24 months) toxicity and have been standardised (Organisation for Economic Cooperation and Development, OECD). Therefore the mouse test has historically been applied for the monitoring of phycotoxins, and the modifications of the protocol enabled an estimate of the quantity of certain toxins present in shellfish flesh. This bioassay makes it possible to detect a known or unknown danger.

6.1.1 Mouse bioassay

Explanation of the safety limit value of 160 µg/kg for OA and DTXs

The European regulatory limit for diarrhetic phycotoxins (okadaic acid and dinophysistoxins) results from epidemiological data from Japan. Contaminated shellfish were analysed and toxicological studies with mice showed that a mouse unit (MU), corresponding to the minimum dose injected by intraperitoneal route causing the death of half of the mice in 24 hours (LD50[8] at 192 µg of OA/kg of mice), is approximately 4 µg of OA.

The data from Japan showed that the limit value to cause effects on humans corresponds to 12 MU, i.e. 48 µg of OA ingested, or 0.8 µg/kg b.w. (average body weight of 60 kg).

To take into account the variable sensitivity of the individuals exposed (age and health of shellfish consumers), a safety factor of 3 has been chosen because of the great number of available epidemiological data, leading to an acute reference dose of 0.27 µg of OA/kg b.w. For an average individual weighing 60 kg, the portion of shellfish should not contain more than 16.2 µg toxin (0.27 µg x 60 kg).

The average amount of shellfish consumed for the first risk assessment in the context of Directive 91/492 was 100 g. The resulting regulatory limit is 160 µg eq. OA/kg.

The injection of the equivalent of 25 g of shellfish flesh into each mouse (i.e. the equivalent of 5 g of hepatopancreas, the organ concentrating almost all the lipophilic toxins) makes it possible to test the safety limit of 160 µg eq OA/kg for shellfish. Each mouse weighing 20 g responds to 4 µg eq OA (corresponding to the LD50 of 192 µg per OA/kg of mice). The test is considered to be positive if two in three mice die and negative if only one mouse dies.

Justification of the 24-hour observation period

The observation period of 24 hours is necessary to check:

- the level of regulated lipophilic phycotoxins (except yessotoxins), meaning 160 µg equivalent OA/kg of shellfish flesh linked to mouse mortality in 24 hours,
- the level of DTX3 derivatives, and
- the azaspiracids level.

a. <u>Concerning the need to check the level of regulated lipophilic phycotoxins (except yessotoxins), meaning 160 µg equivalent OA/kg of shellfish flesh linked to mouse mortality in 24 hours</u>

Works published between 1985 and 1991 proposed dose-response curves for mouse mortality following administration by intraperitoneal route of toxins from mussels naturally contaminated by a *Dinophysis* bloom and a standard okadaic acid. They also proposed a comparative study of the period of death of mice in bioassay by IP route and young mice intoxicated by oral route (Marcaillou-Le Baut et al., 1985; Marcaillou-Le Baut and Masselin 1990; Marcaillou et al., 1991). The authors concluded that there is no significant difference between the bioassay at 5 hours and 24 hours.

These results were not confirmed by the work of Vale and Sampayo (1996) and Vieytes *et al*. (1997) which showed that mouse mortality at 5 hours is only observed at concentrations equal to or higher than 400 µg/kg of flesh for okadaic acid (i.e. over twice the maximum level of 160 µg/kg).

[8] Lethal dose 50 (dose causing the death of half of the animals following administration of a single dose of a substance in 24 hours (OECD guideline 401).

In 1998, based on these studies the CRL reaffirmed the necessity to maintain the observation period at 24 and not 5 hours to cover the risk for the consumer concerning OA and its analogues (Miguez *et al*., 1998).

b. Concerning the need to check the level of DTX3 derivatives

DTX3 are a group of metabolites from esterification of the native toxin (OA or DTX1 or DTX2) by a series of fatty acids. They were identified by Yasumoto *et al*. (1984) in the early 1980s. According to Holland *et al*. (2007), the esters are two to three times less toxic by IP route than the native molecule. However, their toxicity for mice by oral route is similar to the native molecule. Finally, epidemiological data show that DTX3 are poisonous for humans (Vale and Sampayo, 1999).
Again according to Holland *et al.* (2007) these esters are present in different types of shellfish at varying but significant levels: 10 to 50% in mussels (*Mytilus edulis*), 10 to 85% in scallops and >90% in oysters (*Crassostrea gigas*).

In France, systematic research by chemical analysis of shellfish samples giving a positive mouse bioassay result has identified the presence of DTX3 in proportions between 50% to 100% of the total concentration in OA equivalent (Amzil and Mathias, 2006; studies of emerging toxins in the context of the DPMA/Ifremer agreement since 2002).

It appears, therefore, absolutely necessary to respect the 24-hour period to take DTX3 into account, whose toxicity reaction is slower than OA because of their chemical structure (higher molecular weight and non-polar).

c. Concerning the need to check the level of azaspiracids

Azaspiracids were detected following several cases of human poisoning (Netherlands, France, Ireland and Italy) by Irish mussels in the late 1990s. At the time of the poisoning Ireland applied a bioassay with an observation period limited to five hours, which did not make it possible to detect this toxicity. On this occasion Ireland reintroduced the 24-hour test in accordance with the recommendations of the CRL/NRL network.

d. Advantages and disadvantages of mouse bioassay

- It presents good performances for the detection of overall toxicity in all families of lipophilic toxins;
- It is the only test available allowing routine detection of a new or emerging toxicity or new analogues of an already known toxin;
- It makes it possible to obtain a qualitative response to the safety limit value and gives indications on the nature of the involved toxins through the symptoms of the mice;
- It causes specificity and sensitivity problems, which are partly linked to the variability of the biological material (mouse);
- It has logistical requirements (network transfer, trained staff, mouse supply, costs); and
- It is performed by intraperitoneal (IP) route whereas humans are exposed by oral route.

Cellular toxicity tests

Compared to the LD50 test based on mouse mortality, *in vitro* cellular toxicity tests are based on cell death (IC50) and observation of the mechanisms causing the death (e.g. cytotoxicity, cellular viability, apoptosis, etc.). These non-specific tests make it possible to detect any substance that is toxic for the cell. They are recognised in assessment protocols for chemical substances (especially REACH) and validated by ECVAM. More specific tests are based on the alteration of a biological process (enzymatic inhibition, connection to a receptor, macromolecular damage, etc.). These tests are currently under development in a research programme led by Afssa to achieve a better understanding of atypical toxic episodes, but are not suitable for a monitoring system.

Conclusions concerning the mouse bioassay

The mouse or rat bioassays used for the official detection of lipophilic toxins are not valid according to international standards (unlike the bioassay for paralytic phycotoxins), but have been practiced for more than thirty years at international level.

In a mouse bioassay aiming to detect lipophilic toxins, an observation period of five hours would only make it possible to detect high amounts, i.e. at least twice the safety limit. A 24-hour observation period is absolutely necessary to detect the limit value of 160 µg eq OA/kg, DTX3 and azaspiracids.
Finally, mouse bioassay makes it possible to detect emerging toxins presenting a potential danger to humans.

6.1.2 Other detection methods

To compensate for certain problems posed by mouse bioassay and following the division of lipophilic phycotoxins into four distinct groups each with different safety limits, Decision 2002/225/EC included the option of using "alternative" methods for detecting regulated lipophilic phycotoxins on the condition that the chosen method or combination of methods is no less effective than the official mouse bioassay in consumer protection, makes it possible to detect at least four groups of phycotoxins (okadaic acid/dinophysistoxins, pectenotoxins, yessotoxins, and azaspiracids) and has been validated by inter-laboratory analysis at international level.

In regulatory texts the alternative methods are intended to replace mouse bioassay in the context of monitoring phycotoxins in shellfish for the purposes of managing production areas (opening and closure). However, these methods do not provide a safeguard against new emerging toxins or new analogues.

In this context the Commission financed three projects as part of the sixth FP to develop methods to replace the bioassay:

- BIOTOX: Development of cost-effective tools for risk management and traceability systems for marine biotoxins in seafood. Ended in March 2008.
- DetecTox: Development of an SPR-based biosensor for the detection of lipophilic phycotoxins in shellfish residues.
- BIOTOXMARIN: Development of novel analytical tools for the detection of marine biotoxins.

The works to develop replacement methods for mouse bioassays are based on three approaches:

- **physico-chemical:** these methods make it possible to separate and quantify individual toxins, but only target those toxins for which a standard is available. The detection and quantification of new analogues or toxins is not possible. They give no indication as to the toxicity level.
- **immuno-chemical:** these methods make it possible to detect and quantify toxins (antigens) with a specific structure recognised by the antibody(ies) used in the test. They give a global result without any information on the toxicity levels of the detected analogues. They can detect new analogues if the antibody recognises them, but do not allow the detection of new families of toxins.
- **functional:** these methods make it possible to detect and quantify all toxins with the same mechanism of action. They give a global result. They allow the detection of new analogues but not of new families of toxins with a different mechanism of action.

a. Physico-chemical analysis by LC-MS/MS
The methods developed are based on the combination of liquid chromatography and a mass spectrometer in tandem (LC-MS/MS). They aim to detect and quantify all lipophilic phycotoxins in one single analysis (multi-toxin method). Thanks to the mass spectrometer they allow the formal identification of different phycotoxins with great sensitivity and specificity.

The development of these methods is currently confronting the problem of unavailability of standards for the analogues of the basic lipophilic toxins. They are not all available and come mostly from one single supplier (National Research Council, Canada). The available standards are as follows:

- OA and DTX1
- PTX2 and PTX2-seco acid

- AZA1
- YTX
- SPX1 (13 desmethyl spirolide C), and
- GYM (gymnodimine)

The methods developed make it possible to detect and quantify the basic toxins for which a standard is available whereas analogues of toxins can be identified but not quantified. Pending standards, scientists undertake quantification by extrapolation using the available standard that is representative of the family, e.g. DTX2 is quantified based on OA. Such an approach is based on the premise that all the molecules from the same family have the same instrumental response factor. At national level, the chemical analysis method in LC-MS/MS is currently used as a supplement to the mouse bioasay to gather data on the concordance of the results from the mouse bioassay and the chemical analysis (PHYC-Ifremer and NRL-Afssa).
In order to be able to compare the mouse bioassay giving a toxicological response to physico-chemical methods giving a molecular response, it is necessary to have toxic equivalent factors (TEFs).

Few TEF values have been determined and/or approved at international level to date. In its opinion from 2008, EFSA proposed TEFs for DTX1 (1) and for DTX2 (0.6) as compared to OA.
One of the objectives of the BIOTOX project conducted in the framework of the sixth FP was to develop and validate one or more LC-MS methods for the analysis of lipophilic toxins. Due to unsatisfactory results from inter-laboratory trials this project did not make it possible to approve a single method of LC-MS analysis. The difficulties arise from the complexity of using the MS tool, the variety of commercially available analysers and the nature of the shellfish matrix. The studies extending this research programme mention the option of taking an approach based on performance criteria, which would offer a larger choice for the method, along the same lines as the conclusions of EFSA.

Advantages and disadvantages of chemical analysis

- It is specific and sensitive to the known toxins;
- It allows the determination of the toxic profile (individual nature and quantity) of known toxins;
- It provides a quantitative and/or qualitative response with a quantification limit well below the safety limit and variability lower than the bioassay (<10%);
- It does not allow routine identification of a new toxin or new analogues of an already known toxin;
- It requires less time to implement than the bioassay;
- It is not currently approved at international level to allow the transfer to the monitoring network; and
- It needs highly trained staff.

b. Immuno-chemical methods

An ELISA test is specific to a family of toxins (structure). Several ELISA kits have been developed for okadaic acid, and a test for yessotoxins is currently under validation. These methods are seldom applied and can result in cross-reactions and therefore misleading positive results. In addition, it is not possible to cover all groups of lipophilic toxins with one test.

c. Functional methods

A functional test is specific to a mechanism of action. Works based on the inhibition of phosphatase proteins have been developed and resulted in two types of tests with different detection techniques (colorimetric and fluorometric). Only the okadaic acid family from the lipophilic toxins group can be quantified with such tests.

Advantages and disadvantages of immuno-chemical and functional methods

- They are quick and sensitive with well-defined matrixes but their reliability is unknown with shellfish of different species and origins;

- They have a specificity ranging from limited (immuno-chemical) to high (functional);
- They make it possible to detect the presence of a family of toxins but not to determine the toxic profile within this family;
- They do not make it possible to identify new toxins;
- They should make it possible to provide at least a semi-quantitative or quantitative response (depending on the kit);
- The logistical requirements are generally favourable to network transfer;
- They have not been the subject of inter-laboratory trials for international validation; and
- Once validated at Community level they could be used for self-testing.

Conclusions on other detection methods

The most advanced methods to detect all lipophilic phycotoxins referred to in the regulations are based on liquid chromatography in combination with a mass spectrometer (LC-MS/MS), but the results of inter-laboratory trials have shown the difficulty of quantifying results reproducibly when applying this one method alone. Therefore it could be proposed that each laboratory adapts the method to their instrument and an inter-laboratory validation could be based on performance criteria.

Work must be continued in:

- evaluating the relevance and implementing performance criteria;
- continuing the work to produce standards;
- continuing TEF determination to weight the results from chemical analysis; and
- conducting a national study to adapt the shellfish monitoring system, particularly to introduce the technique to new laboratories, which will require significant investment in equipment and staff training.

6.2 Review of the situation in Arcachon

Tables 1 and 2 show the monitoring of the tests conducted in Arcachon in 2007 and 2008 from mouse bioassay and chemical analysis (Ifremer results).

Information on the interpretation of the results:

– the interpretation concerns only tests for which bioassay and chemical results are available;

– the data have to be interpreted with caution as the bioassay expresses overall toxicity activity taking into account all the active molecules, with possible synergistic or antagonistic effects between them and with the matrix, whereas chemical analysis measures the quantities of known toxins. For a better interpretation of the two methods, the TEF for each toxin has to be known and the results from chemical analysis weighted. EFSA only established TEFs for DTX1 and DTX2 in 2008. To simplify, the chemical data are compared to the regulatory limit with all TEFs equalling 1, corresponding to the highest percentage of non-concordance. In certain circumstances a bioassay can be positive with quantities of toxins under 160 µg/kg.

Other aspects are likely to influence the concordance of the results of the bioassay and chemical analysis, especially:

- the variability of inter-individual response of mice;
- a "matrix effect";
- the presence of components that could cause an interference in the mouse bioassay, which is toxic for mice by intraperitoneal route but not toxic for humans by oral route (e.g. fatty acids);
- presence of new toxin(s) detected by bioassay.

Table 1: Year 2007 – comparison of the results of testing of shellfish from the Arcachon Basin (Arguin Sud and Grand Banc) by mouse bioassay (MB) and chemical analysis (C) to the regulatory limit

		Period	No. of tests	MB + C + (%)	MB – C – (%)	MB + C – (%)	MB – C + (%)	Concordance (%)	Non concordance (%)	Comments
ARGUIN	Oysters	05/02 to 24/09	14	0	93	7	0	93	7	One unexplained non-concordant toxicity result late March
	Mussels	05/02 to 27/08	11	0	73	27	0	73	27	Three unexplained non-concordant toxicity results (late March; mid-April; mid-June)
GD BANC	Oysters	12/03 to 03/09	10	0	100	0	0	100	0	No toxicity
	Mussels	18/06 to 03/09	5	0	80	20	0	80	20	One single unexplained non-concordant toxicity result mid-June

Table 2: Year 2008 – comparison of the results of testing of shellfish from the Arcachon Basin (Arguin Sud and Grand Banc) by mouse bioassay (MB) and chemical analysis (C) to the regulatory limit

		Period	No. of tests	MB + C + (%)	MB - C – (%)	ME + C – (%)	B – C + (%)	Concor-dance	Non-concor-dance	Comments
ARGUIN	Oysters	11/02 to 15/07	16	0	100	0	0	100	0	No toxicity
		21/07 to 13/08	5	0	40	60	0	40	60	Three non-concordant results **Toxicity not linked to a known toxin**
		18/08 to 08/09	5	Negative bioassays, no chemical						No toxicity
	Mussels	11/02 to 14/04	5	0	100	0	0	100	0	No toxicity
		28/04 to 12/05	3	67	0	33	0	67	33	**Toxicity episode explained by OA and DTXs** (one result of 87µg/kg at the end of the episode)
		19/04 to 26/05	2	0	50	50	0	50	50	Transition period between OA/DTXs and YTXs episode
		02/06 to 21/07	8	0	13	88	0	13	88	**Toxicity episode explained by YTXs** 126 to 680 µg/kg (regulatory limit value 1000 µg/kg)
		28/07 to 18/08	4	0	25	75	0	25	75	Three non-concordant results **Toxicity not linked to a known toxin**
		25/08 to 08/09	3	Negative bioassays , no chemical						No toxicity
GD BANC	Oysters	28/04 to 28/07	15	0	100	0	0	100	0	No toxicity
		04/08	1	0	0	100	0	0	100	One result only **Toxicity not linked to a known toxin**
		11/08 to 08/09		Negative bioassays, no chemical						No toxicity
	Mussels	28/04 to 12/05	3	67	0	33	0	67	33	**Toxicity episode explained by OA and DTXs** (one result of 91µg/kg at the end of the episode)

		19/04 to 02/06	3	0	100	0	0	100	0	No toxicity one concordant result with 127 µg/kg
		09/06 to 15/07	6	0	67	33	0	67	33	**Toxicity episode explained by YTXs** 81 to 388 µg/kg (official limit value 1000 µg/kg)
		21/07 to 18/08	5	0	100	0	0	100	0	No toxicity
		25/08 to 08/09	Negative bioassays, no chemical							No toxicity

2008 assessment:

- One toxicity episode explained by OA/DTXs toxins affecting only mussels from Arguin and Grand Banc; late April to mid-May;
- One toxicity episode explained by Yessotoxins (YTXs) affecting only mussels from Arguin and Grand Banc; early June to mid-July;
- One unexplained toxicity episode affecting mussels (Arguin and Grand Banc) and oysters (Arguin); late July to mid-August;
- Arguin is more affected than Grand Banc.

Concerning concordance between detection tools and the notion of atypical toxicity

According to data extracted by Ifremer from the Quadrige database[9] on 8 September 2008, 3,198 shellfish samples were processed over the 2006–2008 period to detect lipophilic toxins across all French coastlines: mouse bioassay and/or chemical analysis by LC-MS/MS.

From the 3,189 samples analysed using both methods:

- 2,618 samples tested negative with mouse bioassay (2,440 with three surviving mice and 178 with two surviving mice), and
- 571 samples tested positive with mouse bioassay (393 with three dead mice and 178 with two dead mice).

Negative samples from mouse bioassay

2,618 samples tested negative with mouse bioassay.
321 were also analysed by LC-MS/MS.
From these 321 samples, 14 tested chemically positive (value and geographical distribution in table 3). The observed discrepancies with the highest chemical values should be studied further in light of data from ongoing research programmes.

Table 3: Geographical distribution of the fourteen samples leading to negative results by mouse bioassay and positive results by chemical analysis (OA+DTXs+PTXs ≥ 160 µg eq. OA/kg or AZAs ≥ 160µg eq. AZA/kg)

Quadrige Zone	Number of samples concerned	Max. value found in OA+DTXs+PTXs	Max. value found in AZAs
01			
035 Les Abers	2		274
040 Baie d'Audierne	1	228	
060 Estuaire de la Loire	1	406	
077 Bassin d'Arcachon	1	167	
083 Etang de Salses Leucate	3	988	
088 Côte languedocienne	1	403	
114 Etangs de Diana-Urbino	3	330	

It should be noted that from the 321 analysed (LC-MS/MS) samples, 81 were positive for SPXs and 17 for YTXs, at concentrations lower than the health safety threshold (when it exists, i.e. only for YTXs).

[9]For this period not all of the Quadrige data have been validated as yet. The results may therefore be modified at a later time.

Positive samples from mouse bioassay

From the 571 samples giving a positive mouse bioassay result, 368 have been analysed by LC-MS/MS. Among these:

- 171 samples led to positive results with LC-MS/MS,
- 197 samples led to negative results with LC-MS/MS (OA+DTXs+PTXs < 160 µg eq. OA/kg)

The geographical distribution of these samples is shown in table 4.

Table 4: geographical distribution of the 197 samples leading to positive results by bioassay and negative results by chemical analysis

Quadrige Zone	Concerned shellfish	Year	Number of samples concerned
010 Antifer	mussels	2007	2
036 Iroise	donax	2007	1
039 Baie de Douarnenez	donax	2007	1
040 Baie d'Audierne	donax	2007	1
041 Iles de Glénan	pink clams	2006	2
		2007	2
042 Bénodet	mussels	2007	1
043 Concarneau	mussels and cockles	2007	5
044 Aven, Belon and Laïta	oysters	2006	1
045 Rade de Lorient	mussels	2006	5
		2007	6
		2008	1
046 Baie d'Etel	donax	2006	1
		2007	10
		2008	1
047 Rivière d'Etel	oysters and mussels	2006	4
		2007	3
		2008	1
048 Courreaux de Belle île	scallops	2007	2
049 Baie de Quiberon	oysters	2006	1
050 Le Pô	oysters and clams	2007	3
051 Rivière de Crach	clams	2007	4
052 St Philibert – Le Brénéguy	mussels and clams	2006	1
		2007	2
056 Rivière de Pénerf	mussels	2007	3
057 Baie de Vilaine	mussels and oysters	2006	3
		2007	9
		2008	3
059 Traicts du Croisic	oysters and cockles	2007	1
		2008	1
060 Estuaire de la Loire	mussels	2007	1
		2008	1
065 Pertuis Breton	mussels	2006	2
066 Baie de l'Äiguillon	mussels	2007	1
077 Arcachon Basin	mussels and oysters	2006	30
		2007	5
		2008	20 (4 of them with YTX content levels between 388 and 680)
083 Etang de Salses Leucate	mussels and oysters	2006	8
		2007	14
		2008	11
089 Etangs Palavasiens	mussels and oysters	2006	1
		2007	16
		2008	4
114 Etangs de Diana -Urbino	mussels	2008	2
		Period total	197
		2006 total	59
		2007 total	93
		2008 total	45

In part of these samples, the presence of SPXs (mostly in low quantity) and/or AZAs (in very low quantity), and/or YTXs (in varying quantities but lower than the health safety threshold) was observed.

Conclusion

– The comparison between chemical analysis and mouse bioassay must be cautious but it corroborates the fact that the toxicity detected by mouse bioassay is much more global than the results of chemical analysis by LC-MS/MS on known targeted toxins.

– The occurrence of positive bioassay without a known toxin being detected has been observed in numerous zones along the coastline; 25% of these situations have been observed in Arcachon and were more frequent in 2007 than 2006 and 2008.

7. THE SITUATION IN OTHER EU MEMBER STATES

The inspection reports of the Food and Veterinary Office (FVO) make it possible to obtain information on the practices applied in other countries concerning mouse bioassay for lipophilic toxins.

Mouse bioassay was applied in accordance with the prescriptions of Decision 2002/225 by:

- Belgium (2001 and 2005 inspections),
- Italy (2004 inspection),
- Spain (2004 inspection),
- Denmark (2004 inspection), and
- Ireland (2001 inspection).

It should be noted that Italy conducts a mouse bioassay with an observation period of five hours in the sole aim of detecting yessotoxins. This measure is linked to the specific limit value of these toxins of 1 mg/kg of shellfish (compared to other lipophilic toxins with a limit value of 160 µg/kg).

However the FVO noted that mouse bioassay was not applied in compliance with the prescriptions of Decision 2002/225 by:

- Germany (2002 and 2005 inspections),
- The United Kingdom (2004 inspection),
- Portugal (2004 inspection), and
- The Netherlands (2001 inspection).

In the inspection reports the Office reiterates that the Community prescriptions currently in force concerning marine biotoxins should be respected and ask the competent authorities to take action to correct failings in the methods used. The inspection report on Portugal concludes that the alternative methods will only be internationally recognised if reference materials become available.

8. CONCLUSIONS AND RECOMMENDATIONS

Concerning the observation period of the mouse bioassay

Based on the arguments presented, Afssa concludes that the 24-hour period of observation in the mouse bioassay is absolutely necessary in order to detect a limit of 160 µg eq OA/kg, DTX3 and azaspiracids and therefore ensure consumer safety as all these toxins have been linked to diarrhetic shellfish poisoning in humans.

Concerning alternative detection methods to mouse bioassay

In the framework of monitoring phycotoxins in shellfish for the purposes of managing production areas (opening/closure), the chemical multi-toxin methods of the CL-MS/MS type are the most advanced, but additional research is necessary to apply the methods on a large scale. Several immuno-chemical or functional methods allow the detection of toxin families but must be used in combination to cover the four regulated groups concerned.

None of these alternative methods makes it possible to monitor and detect emerging toxins that are potentially toxic for humans.

None of the methods for detecting toxins of the okadaic acid and dinophysistoxins group has been validated by inter-laboratory trials. Pending an internationally recognised chemical multi-toxin method, plans could be made, together with national and Community regulatory bodies, to:

- recognise an intra-laboratory validation based on performance criteria;
- analyse toxin families separately (e.g. target one or two of the four lipophilic toxin families);
- consider all of the toxic equivalence factors (TEF) as being equal to 1 as validated TEF do not exist for all of the toxins; and
- consider that all active related molecules respond in the same way, in the absence of certain standards.

Using this chemical method would also make it necessary to adapt the national shellfish monitoring system.

9. LITERATURE

List of regulatory texts

Council Directive 91/492/EEC of 15 July 1991 laying down the health conditions for the production and placing on the market of live bivalve molluscs.

Council Directive 97/61/EC of 20 October 1997 amending the Annex to Directive 91/492/EEC laying down the health conditions for the production and placing on the market of live bivalve molluscs.

Commission Decision 2002/225/EC of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve molluscs, echinoderms, tunicates and marine gastropods.

Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.

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10. KEYWORDS

Marine biotoxins, mouse bioassay, lipophilic toxins.

Pascale BRIAND

ANNEX 1
Economic data on oyster production in France

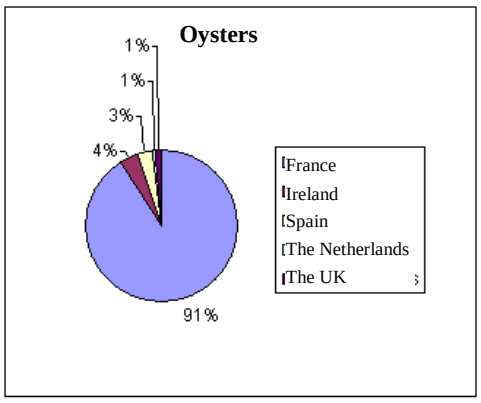

Figure 1: Distribution of oyster production in EU countries in 2000
(source: LEN-CORRAIL, calculated with FAO data).

Table 1: Distribution of French oyster production in EU countries in 2000

Oysters	Import to France		Export from France	
	Origin	**Weight (t)**	**Destinations**	**Weight (t)**
Flat oysters (less than 40g)	The UK Ireland Spain	72 44 13	Spain Italy Belgium Germany Switzerland	180 151 43 9 14
	Total	44	Total	407
Rock oysters	Ireland The UK Spain Netherlands Portugal Denmark	1,456 620 316 315 253 9	Italy Belgium Germany Spain Switzerland Ireland Luxembourg Netherlands	3,458 718 481 203 236 83 73 62
	Total	2,995	Total	5,671
Product total		3,139		6,077
			Balance	2,938

Source: annual foreign trade report 2001-OFIMER

ANNEX 2
About the regulations

1. Directive 91/492

Council Directive 91/492/EEC of 15 July 1991 laying down the health conditions for the production and the placing on the market of live bivalve molluscs.

In chapter VI the directive makes provision to check the possible presence of toxin-producing plankton in production and relaying waters and biotoxins in live bivalve molluscs.
The total Paralytic Shellfish Poison (PSP) content in the edible parts of molluscs (the whole body or any part edible separately) must not exceed 80 microgrammes per 100 g of mollusc flesh in accordance with the biological testing method — in association if necessary with a chemical method for detection of Saxitoxin — or any other method recognised by the Standing Veterinary Committee (European Committee). If the results are challenged, the reference method shall be the biological method.

Concerning lipophilic toxins the directive fixed no limit value but stated that "the **customary biological testing methods must not give a positive result** to the presence of Diarrhetic Shellfish Poison (DSP) in the edible parts of molluscs (the whole body or any part edible separately)."

2. Decision 2002/225/EC

Commission Decision 2002/225/EC of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve molluscs, echinoderms, tunicates and marine gastropods.

Directive 2002/225 is the first regulatory text to differentiate between the different families of lipophilic toxins with specific regulatory limits and to mention a bioassay (mouse or rat) as a reference method. These limits will also be adopted in the Hygiene Package (853/2004).

The maximum level in the animals referred to (the whole body or any part edible separately) is:

- 160 micrograms of okadaic acid equivalents per kilogram for okadaic acid, dinophysistoxins and pectenotoxins.
- 1 milligram of yessotoxin equivalents per kilogram, and
- 160 micrograms of azaspiracids1 equivalents per kilogram for azaspiracids.

This decision states in Article 5: "When the results of the analyses performed demonstrate discrepancies between the different methods, the mouse bioassay should be considered as the reference method."

The detection methods are described in the annex of the decision:
A single mouse bioassay involving acetone extraction can be used to detect okadaic acid, dinophysistoxins, pectenotoxins and yessotoxins.
Azaspiracids detection at the regulatory levels by means of this procedure requires the use of the whole body as the test portion.
Three mice should be used for each test. The death of two out of three mice within 24 hours after inoculation into each of them of an extract equivalent to 5 g of hepatopancreas or 25 g whole body should be considered as a positive result for the presence of one or more of the toxins mentioned in Article 1 at levels above those established in Article 2, 3 and 4.

Alternative detection methods
A series of methods such as high performance liquid chromatography (HPLC) with fluorimetric detection, liquid chromatography (LC)-mass spectrometry (MS), inmunoassays and functional assays such as the phosphatase inhibition assay can be used as alternative or complementary

methods to the biological testing methods, **provided that either alone or combined** they can detect at least the following analogues, that they are not less effective than the biological methods and that their implementation provides an equivalent level of public health protection:
— okadaic acid and dinophysistoxins (DTX1, DTX2 and DTX3): an hydrolysis step may be required in order to detect the presence of DTX3,
— pectenotoxins: PTX1 and PTX2,
— yessotoxins: YTX, 45 OH YTX, homo YTX, and 45 OH homo YTX,
— azaspiracids: AZA1, AZA2 and AZA3.
If new analogues of public health significance are discovered they should be included in the analysis. Standards will have to be available before chemical analysis will be possible. Total toxicity will be calculated using conversion factors based on the toxicity data available for each toxin.
The performance characteristics of these methods should be defined after **validation following an internationally agreed protocol.**

3. Regulation (EC) No. 853/2004

Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.

The regulation states in section VII: live bivalve molluscs must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the following limits:

(a) for Paralytic Shellfish Poison (PSP), 800 micrograms per kilogram;
(b) for Amnesic Shellfish Poison (ASP), 20 milligrams of domoic acid per kilogram;
(c) for okadaic acid, dinophysistoxins and pectenotoxins together, 160 micrograms of okadaic acid equivalents per kilogram;
(d) for yessotoxins, 1 milligram of yessotoxin equivalent per kilogram; and
(e) for azaspiracids, 160 micrograms of azaspiracid equivalents per kilogram.

4. Regulation (EC) No. 2074/2005

Commission Regulation (EC) No. 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No. 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No. 854/2004 of the European Parliament and of the Council and Regulation (EC) No. 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No. 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No. 853/2004 and (EC) No. 854/2004

In Annex II, **this regulation specifies the methods for detecting each family** of phycotoxins:

- **Paralytic toxins** (PSP): **biological testing** method or any other internationally recognised method. The biological testing method may be carried out in association, if necessary, with another method for detecting Saxitoxin and any of its analogues for which standards are available. If the results are challenged, the reference method shall be the biological method.
- **Amnesic toxins** (ASP): **high-performance liquid chromatography (HPLC)** or any other recognised method. If the results are challenged, the reference method shall be the HPLC method.
- **Lipophilic toxins** (DSP):
  - **Biological methods**, a series of mouse bioassay procedures, differing in the test portion (hepatopancreas or whole body) and in the solvents used for extraction and purification.
    Three mice shall be used for each test. Where two out of three mice die within 24 hours of inoculation with an extract equivalent to 5 g hepatopancreas or 25 g whole body, this shall be considered a positive result for the presence of one or

more toxins as referred to in Chapter V(2)(c), (d) and (e) of Section VII of Annex III to Regulation (EC) No. 853/2004 at levels above those laid down.
A rat bioassay may be used to detect okadaic acid, dinophysistoxins and azaspiracids. Three rats shall be used for each test. A diarrhetic response in any of the three rats shall be considered a positive result for the presence of okadaic acid, dinophysistoxins and azaspiracids at levels above those laid down in Chapter V (2)(c) and (e) of Section VII of Annex III to Regulation (EC) No. 853/2004.

- **Concerning the other detection methods**, the regulation repeats the same requirements as Decision 2002/225/EC.
  It states, however, that the biological methods shall be replaced by alternative detection methods as soon as reference materials for detecting the toxins prescribed in Chapter V of Section VI of Annex III to Regulation (EC) No. 853/2004 are readily available, the methods have been validated and this Chapter has been amended accordingly.

ANNEX 3
Review of international work

At the 25th session of the Codex Committee on Fish and Fishery Products (CCFFP) in 2002, the Committee members asked the FAO and WHO to provide scientific opinions on biotoxins and to present their works on the Proposed Draft Standard for Live and "Processed" Bivalve Molluscs.

At the 26th session in 2003, the CCFFP made the following specific requests to be covered through this advice:

- Provide scientific advice to enable the establishment of maximum levels of toxins in shellfish for shellfish toxins (PSP-, DPS-, ASP-, AZP- and NSP-toxins, and YTXs and PTXs);
- Provide guidance on the methods of analysis for each toxin group;
- Provide guidance on monitoring biotoxin-forming phytoplankton and bivalve molluscs (including sampling methodology); and
- Provide information on the geographical distribution of biotoxin-forming marine phytoplankton.

In 2004 the Joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs led to the production of a report addressing the aforementioned requests.

At its 27th session in 2005, the CCFFP presented the conclusions of this report, which caused contradictory opinions and lively discussions between the States, leading to the creation of a new working group chaired by Canada. The objective of this working group was to prepare for the CCFFP a document presenting an evaluation of the report of the joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs and stating how the experts' report can help to advance the works on the Codex Proposed Draft Standard and the Proposed Draft Code of Practice for Bivalve Molluscs. The report of the working group had to present how the CCFFP could use the experts' advice and recommendations in the approaches that the Committee could envisage in order to incorporate advice into future standards and codes of practice.

Table 1 below summarises the proposed toxin limits for which the Joint *ad hoc* Expert Consultation was able to establish a provisional acute reference dose (ARfD) based on available data. The table shows the proposed limits based on the ARfD and on a consumption of 250g of shellfish (consumption corresponding to the 97.5th percentile of the population, according to the experts). Finally, the current EU limit values are shown in grey.

Table 1: Provisory ARfD and regulatory limits proposed by the Joint FAO/IOC/WHO ad hoc Expert Consultation per biotoxin and reminder of the current EU regulatory limits

Biotoxins	Provisional ARfD (for an adult weighing 60 kg)	Regulatory limit based on the ARfD and on consumption of 250g	Current regulatory limit value in EU Member States
AZA	0.04 µg/kg bodyweight (bw)	0.0096 mg/kg meat	0.16 mg eq. AZA/ kg meat
Brevetoxins	- *	- *	-
Cyclic immines	- *	- *	-
DA	0.1 mg/kg bw	24 mg/kg meat	20 mg/kg meat
OA and DTXs	0.33 µg/kg bw	0.08 mg/kg meat	0.16 mg/kg meat
PTXs	- *	- *	0.16 mg eq. OA/ kg meat
STXs	0.7 µg/kg bw	0.17 mg/kg meat	0.8 mg/kg meat
YTXs	50 µg/kg bw	12 mg/kg meat	1 mg/kg meat

AZA: azaspiracids – DA: domoic acid – OA and DTXs: okadaic acid and dinophysistoxins - PTX: pectenotoxins - STX: saxitoxins – YTX: yessotoxins - *toxin family examined but not deemed to have priority

Parallel to this international work led by the Codex, another working group called the “WG on Toxicology”, created by the CRL (Community Reference Laboratory) and comprised of international experts, held a meeting in 2005.

The objective of the "WG on Toxicology" was to perform **a risk assessment for lipophilic toxins** in order to establish safety limits for shellfish for each lipophilic toxin family.

The conclusions of the working group are shown in ***table 2*** below:

Table 2: Provisory ARfD and regulatory limits proposed by the Toxicology working group (CRL) and reminder of the current regulatory EU limit values

Biotoxins	Provisional ARfD (for an adult weighing 60 kg)	Regulatory limit based on the ARfD and on consumption of 250g	Current regulatory limit value in EU Member States
OA and DTXs	0.33 µg/kg bw	0.08 mg/kg meat	0.16 mg/kg meat
PTXs	3 µg/kg bw	0.72 mg/kg meat	0.16 mg eq. OA/ kg meat
YTXs	50 µg/kg	-	1 mg/kg meat
AZA	0.127 µg/kg bw	0.032 mg/kg meat	0.16 mg eq. AZA/ kg meat
Gymnodimines	75 µg/kg bw	-	-
Spirolides	1.67 µg/kg bw	0.4 mg/kg meat	-
Palytoxins	1.07 µg/kg bw	0.25 mg/kg meat	-
Ciguatoxins	1.75 ng/kg bw	0.00004 mg/kg meat	-

ANNEX 4
Description of the mouse bioassay for lipophilic toxins

The detection methods are described in the annex of Decision 2002/225/EC:
A single mouse bioassay involving acetone extraction can be used to detect okadaic acid, dinophysistoxins, pectenotoxins and yessotoxins.
Azaspiracids detection at the regulatory levels by means of this procedure requires the use of the whole body as the test portion.
Three mice should be used for each test. The death of two out of three mice within 24 hours after inoculation into each of them of an extract equivalent to 5 g of hepatopancreas or 25 g whole body should be considered as a positive result for the presence of one or more regulated toxins.

In its current state the Community regulation accepts only the mouse bioassay as the reference method to detect the presence of lipophilic toxins in shellfish, as its efficiency has been proven during several years of application in many countries. The bioassay has not been validated[10] according to standard criteria but by long experience that made it possible to improve the conditions of its application and the acquisition of data from inter-laboratory analysis of the efficiency performed at national and Community level. There is no officially recognised alternative method at present.

The mouse bioassay for lipophilic phycotoxins combines the preparation of a liposoluble semi-purified extract with organic solvents and the intraperitoneal (IP) injection of this extract in mice according to a defined chemical and biological protocol to control a single dose corresponding to the safety limit value. Therefore in the meaning of the regulation:

- A mouse bioassay is negative if the result shows zero or one in three dead mice within 24 hours of the inoculation. This indicates that the shellfish either do not contain lipophilic toxins or in quantities below the safety limit. They comply with the regulatory limit and can therefore be consumed.
- A mouse bioassay is positive if the result shows two or three in three dead mice within 24 hours of the inoculation. This indicates that the shellfish contain quantities of lipophilic toxins above the safety limit. They do not comply with the regulatory limit and are therefore not fit for consumption.

[10] On the 1999 proposal of the Community Reference Laboratory (CRL) to validate and standardise the mouse bioassay, the European Committee for Standardisation Committee issued a refusal in order not to contradict European regulations aiming to replace animal testing.

ANNEX 5
History of the methods combined with mouse bioassay for lipophilic toxins by Ifremer since the creation of REPHY (1984).

In 1983 and 1984

The limit value was apparently variable. The following information is an extract from the report:
Berthomé J.P. & Lassus P., 1985. – Manifestation et suivi de l'algue toxique *Dinophysis acuminata* sur les côtes françaises, en 1984. – IFREMER / DRV-85.01-SR-NTES internal report: 28 p.
"*… the limit of 48 hours, the period ending in the test result, then appeared to be unrealistic. Therefore we limited the observation time of mice to 24 hours and estimated that after four hours we can give an opinion… The last results obtained appear to show that the period of time to be chosen is five hours…*"

From 1985 to 1992

Acetone extraction
Limit: five hours
1st available reference: Note Berthomé & Belin (322/89) from 21 March 1989

1992

Introduction of a methanol extraction method followed by a hexane bath to remove fatty acids that could result in false positives:
– acetone extraction
– methanol extraction - water
– hexane bath
Limit: five hours
Reference: CR Meeting REPHY 1992

1994

Introduction of a separation dichloromethane / methanol - water that makes it possible to collect the diarrhetic toxins in the dichloromethane to be collected and to eliminate the atypical toxins in methanol:
– acetone extraction
– methanol extraction - water
– hexane bath
– separation dichloromethane/methanol - water
Limit 5 hours
Reference: CR Meeting REPHY 1994

1996

– acetone extraction is replaced by methanol - water extraction
– followed by hexane
– followed by dichloromethane
Limit: five hours
Reference:
Belin C., Marcaillou-Le Baut C., Amzil Z. & Le Doux M., 1996. REPHY (Réseau de Surveillance du Phytoplancton et des Phycotoxines). Méthodes de détection des phycotoxines diarrhéïques (DSP) et paralysantes (PSP). Méthodes biologiques sur souris. IFREMER / DEL / 96.17 / Nantes internal report: 28 p.

1998

First analysis of data to evaluate the consequences of a change of the observation time from 5 to 24 hours. Examination of the mouse-test DSP problem in CSTS [scientific and technical committee for monitoring] → recommendations: apply the test described in 1996.
Reference: reports of the REPHY meeting in 1998

1999

Experimental protocol to compare acetone and dichloromethane/methanol-water procedures.
Discontinuation of the hexane bath (a part of the DTX3 thought to be removed in the hexane phase)

Reference:
Amzil Z., 1999. Procédure analytique pour déterminer à la fois la toxicité globale des coquillages sur souris et le type de toxines impliquées. 1999 experimental protocol. Report RST.DEL/99 04/Nantes, 10 p.
Amzil Z. & Belin C., 2000. Bilan du protocole expérimental Ifremer sur le dépistage des toxines diarrhéiques. Document de travail. Report DEL/MP/RST/00/10/Nantes, 89 pages.
Amzil Z., 2001. Addendum au rapport DEL/MP/RST/00/10/Nantes: synthèse et interprétation des résultats des tests-souris et analyses physico-chimiques. 6 pages.

2000
– extraction methanol / water
– separation dichloromethane/methanol - water

2001
Same protocol as in 2000

2002
Yasumoto method 1984 modified:
– acetone extraction
– separation dichloromethane / water
Reference: Amzil Z., 2001. Toxines diarrhéiques (DSP) et associées. Guide et Manuel. Document de prescription REPHY. 21 pages.
Change of observation time: 24 hours instead of 5 hours.

2003–2006
Yasumoto method 1984 modified
Reference: Guides et Manuels 2003 (addition to Manuels 2001), and later 2004 and 2006.