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Contamination of sharks, especially tiger and bull sharks, by ciguatoxins: occurrence, analytical methods, human cases reported and ethological information

ANSES Opinion
Collective Expert Appraisal Report

August 2014 Scientific Edition
Revised edition, January 2015

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State of knowledge relating to the contamination of sharks, especially tiger and bull sharks, by ciguatoxins: occurrence, analytical methods, human cases reported and ethological information for these two shark species

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ANSES Opinion
Request No. 2013-SA-0198

The Director General

Maisons-Alfort, 06 January 2015

OPINION**
of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES)

on the health risk assessment related to the consumption of two shark species on Reunion Island, especially regarding the risk related to ciguatoxins

ANSES undertakes independent and pluralistic scientific expert assessments.
ANSES primarily ensures environmental, occupational and food safety as well as assessing the potential health risks they may entail.
It also contributes to the protection of the health and welfare of animals, the protection of plant health and the evaluation of the nutritional characteristics of food.

It provides the competent authorities with all necessary information concerning these risks as well as the requisite expertise and scientific and technical support for drafting legislative and statutory provisions and implementing risk management strategies (Article L.1313-1 of the French Public Health Code).

Its opinions are made public.
This opinion is a translation of the original French version. In the event of any discrepancy or ambiguity the French language text dated 6 August 2014 shall prevail.

1. BACKGROUND AND PURPOSE OF THE REQUEST

Since 1966, the French *département* of Reunion Island has been covered by a specific regulation (by prefectoral order) which restricts or prohibits the marketing of certain species of fish because of the risk of poisoning by marine biotoxins, especially ciguatoxins. Since 1999, the list of these species of fish has included most shark species, including those of the *Carcharhinidae* family.

The Prefect of Reunion Island requested the Directorate General for Food (DGAL) to commission a reassessment of the risk related to two shark species whose consumption is currently prohibited by the prefectoral order. These are the tiger shark (*Galeocerdo cuvier*) and the bull shark (*Carcharhinus leucas*).

In order to acquire data on the contamination of these two shark species by ciguatoxins, the services of the prefecture of Reunion Island launched a campaign in 2012 to test 12 specimens per species for ciguatoxins by means of a mouse bioassay. This sampling campaign was extended in 2013 with a goal of 45 additional specimens per species.
To date, 24 specimens have been analysed for ciguatoxins by mouse bioassay and five of them have also been analysed for heavy metals (lead, cadmium and mercury).

On 14 October 2013, ANSES received a formal request from the DGAL to answer the following questions:

Question 1: What is the current state of knowledge on contamination of shark flesh by ciguatoxins, especially tiger and bull sharks? Have any cases of food poisoning associated with the consumption of sharks already been reported?

** revised Opinion cancelling and superseding the previous Opinions of 9 July 2014 and 6 August 2014.

Question 2: What are the analytical methods currently applicable for detecting and quantifying ciguatoxins in shark flesh? Can the results from these methods be used to assess the health risks related to a possible authorisation of these species for human consumption in this area?

In the event that ANSES should identify a sufficiently reliable method for testing shark flesh for ciguatoxins, what data would be necessary to carry out this evaluation and what recommendations could be made regarding the protocol for sampling tiger and bull sharks on Reunion Island? Particular consideration shall be given to the geographical area concerned and the ethology of these two shark species in terms of the extent of their movements in the marine areas around Reunion Island.

Question 3: In 2006 and 2009, the Agency published Opinions on the consumption of pelagic predator fish at Reunion Island and the health risk related to methylmercury. Do the new analyses of shark specimens in 2013 give cause for revising the conclusions of these Opinions?

2. ORGANISATION OF THE EXPERT APPRAISAL

The expert appraisal was carried out in accordance with the French standard NF X 50-110 "Quality in Expertise – General Requirements of Competence for Expert Appraisals (May 2003)".

The assessment lies within the sphere of competence of the Expert Committee (CES) on "Assessment of the physical and chemical risks in food" (CES ERCA). ANSES entrusted the examination of this request to the Working Group on "Ciguatoxins", formed by a decision dated 19 December 2013, for Questions 1 and 2 relating to ciguatoxins. Concerning Question 3, relating to the Agency's earlier work on the health risks related to methylmercury, a study originally carried out by ANSES internally, by the Risk Assessment Department, was presented to the CES ERCA on 26 June 2014. The methodological and scientific aspects of the Group's work were submitted to the CES ERCA in plenary meetings on 19 November 2013, 17 March 2014, 14 April 2014 and 26 June 2014. All the work was adopted by the CES ERCA in a plenary meeting on 26 June 2014 and electronically on 3 July 2014. An updated version of the documents was adopted by the Chair of the CES ERCA by electronic means on 25 July 2014, following the receipt of additional information on 21 July 2014.

ANSES analyses interests declared by experts before they are appointed and throughout their work in order to prevent risks of conflicts of interest in relation to the points addressed in expert appraisals. The declarations of interest by experts are made public via the ANSES website (www.anses.fr).

3. THE CES'S ANALYSIS AND CONCLUSIONS

The Expert Committee on "Assessment of the physical and chemical risks in food" (CES ERCA) adopted the report of the collective expert appraisal produced by the "Ciguatoxins" Working Group which is annexed to this Opinion, a summary of which is presented below.

- **Question 1 concerning the current state of knowledge about contamination of shark flesh by ciguatoxins, especially tiger and bull sharks, and any cases of food poisoning associated with the consumption of shark flesh**

A review of the scientific literature and more widely a search on the internet enabled the Working Group to identify and describe in its report cases of food poisoning associated with the consumption of shark (flesh and/or liver) from the 19th century to the present day. Cases, sometimes fatal, have been reported in New Caledonia, the Cook Islands, the Gilbert Islands, French Polynesia, Madagascar (particularly in November 2013 and February 2014) and Reunion Island (in 1993). The tiger shark was the species implicated in the Gilbert Islands. This species has

been described in the literature as "ciguateric", on the basis of data collected in Samoa, Fiji and Mascarene Islands (the Mascarene Islands are an archipelago in the Indian Ocean made up of three main islands, Reunion Island, Mauritius and Rodrigues Island, as well as several small nearby islands). In Madagascar, flesh from a tiger shark has been associated with at least one case of food poisoning reported in the literature, and flesh from a bull shark with two cases. Genetic analysis of the shark involved in the food poisoning that occurred in November 2013 concluded that it was a bull shark.

The symptoms observed in these food poisoning incidents associated with the consumption of sharks correspond to the characteristic symptoms of ciguatoxins, a family of toxins produced by a micro-algae of the genus *Gambierdiscus*. However, some authors suggest that other toxins with similar properties, known as carchatoxins, might be responsible, but their structure has not yet been characterised. More than 175 different symptoms have been identified in acute and chronic phases of ciguatera (the name given to poisoning by ciguatoxins). Regional differences have been noted and can be attributed to the presence of different ciguatoxins. Ciguatoxins have thus been classified into Pacific, Caribbean or Indian Ocean groups. It cannot be ruled out that carchatoxins could be new analogues of ciguatoxins, for example highly oxidised forms.

Other data concerning the contamination of sharks (flesh or liver), in particular of tiger and bull sharks, by ciguatoxins (or similar toxins) were collected and reviewed in the WG's report. These data were found in studies carried out between the 1960s and the 1980s, based on mongoose bioassays.

➔ *On the basis of these elements, the CES ERCA believes that it is appropriate to take into account not only ciguatoxins but also another type of toxin, specific to certain shark species, currently known as carchatoxins.*

■ <u>Question 2</u> concerning the analytical methods

The analytical methods for the detection and quantification of ciguatoxins in the flesh of sharks were identified and are described in the report of the Working Group. These methods include: tests on animals, in particular the mouse bioassay, the assay using neuroblastoma cells (Neuro-2a), the radioligand receptor binding assay (RBA), immunological tests and physico-chemical methods, including liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

➔ *After considering the strengths and weaknesses of the available analytical methods in the light of the complexity and diversity of the toxins that make up the family of ciguatoxins (ciguatoxins from the Pacific, the Caribbean and the Indian Ocean), the CES ERCA recommends using a combination of the following techniques:*

- *a mouse bioassay, to find any overall toxicity in the sample;*
- *a cytotoxicity assay on Neuro-2a cells and/or a test on receptors, which both have higher specificity and greater sensitivity than the mouse bioassay;*
- *an analysis by LC-MS/MS, to try to confirm the presence of known ciguatoxins in the case of positive results by one of the above methods.*

The data thus produced could then be used in a health risk assessment for consumers.

▶ Concerning the tiger and bull sharks sampled at Reunion Island in 2012 and 2013

The results of mouse bioassays for ciguatoxins of 24 samples of shark flesh were sent to ANSES as part of the Request from the DGAL. They were all negative. However, this test is not sufficiently sensitive to detect concentrations of ciguatoxins considered to be of no risk to humans.

Considering the previous recommendations on the analytical methods, the CES ERCA believes that analysis by mouse bioassay alone does not produce sufficiently reliable results to conclude that the 24 shark specimens were safe for consumption as regards the presence of ciguatoxins.

ANSES therefore contracted a research and development agreement (RDA) with ARVAM (Agency for Research and Marine Exploitation, Reunion Island), in collaboration with IRTA (*Instituto de Investigación y Tecnología Agroalimentaria*, Spain) for these samples to be analysed by cytotoxicity assays on Neuro-2a cells. The final report was submitted to ANSES on 21 July 2014.

The results did not show ciguatoxin-like toxins to be present above the limit of detection of 0.04 µg eq. P-CTX-1 kg^{-1} of flesh. It should be noted that the detection limit is higher than the concentration considered to be of no risk to humans, which is 0.01 µg eq. P-CTX-1 kg^{-1} of fish flesh.

The RDA also included samples from the bull shark implicated in an outbreak of food poisoning that occurred in Madagascar in November 2013 (124 people intoxicated, nine of whom died) for analysis by mouse bioassay and by cytotoxicity assay on Neuro-2a cells. The sample of flesh gave a positive result by mouse bioassay, with symptoms typical of those known for carchatoxins (prostration, dyspnoea, cyanosis, convulsions and death by respiratory arrest). The analysis of a sample of flesh, a sample of stomach and three samples of dried fin by cytotoxicity assays on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated as follows:

- Flesh: 0.144 µg eq. P-CTX-1 kg^{-1} (i.e. 14 times the concentration considered to be of no risk to humans);
- Stomach: 114 µg eq. P-CTX-1 kg^{-1} (i.e. 11,400 times the concentration considered to be of no risk to humans);
- Dried fin: 0.145 µg eq. P-CTX-1 kg^{-1}; 0.158 µg eq. P-CTX-1 kg^{-1}; 0.737 µg eq. P-CTX-1 kg^{-1} (i.e. 14 to 74 times the concentration considered to be of no risk to humans).

■ **Question 2 concerning a protocol for sampling tiger and bull sharks in Reunion Island**

In order to answer this question, the Working Group collected information concerning the ethology of tiger and bull sharks in the marine areas around Reunion Island, based in particular on the preliminary results obtained by the CHARC programme (on the ecology and habitat of two species of coastal sharks on the West Coast of Reunion Island).

These sharks are apex fish-eating predators, able to feed on different species. At Reunion Island they principally show a fidelity to certain specific sites, but are able to colonise a very wide range of different habitats and to travel over long distances, even as far as Madagascar where food poisoning outbreaks associated with the consumption of shark flesh have been reported recently (November 2013 and February 2014). It would be particularly interesting to know whether these sharks also travel from Madagascar to Reunion Island. Nothing is currently known about either the origin or the dynamics of bioaccumulation of these toxins in sharks. In addition, knowledge of the lifestyle of these two shark species and their population dynamics is very fragmentary and inadequate.

➔ *In the absence of information on the size and structure of the populations of tiger and bull sharks in the marine areas around* Reunion Island *and considering the gaps in our knowledge of their feeding behaviour and their movements in the Indian Ocean, it is not possible to provide recommendations for a protocol for sampling sharks by which to assess the risks related to a possible authorisation of these species for human consumption, as regards the ciguatera risk.*

■ **Question 3 concerning the health risks related to methylmercury**

The results of the analyses of five samples of shark flesh for lead, cadmium and mercury transmitted to ANSES as part of the Request by the DGAL, are presented in Table 1. It can be seen that four out of five (i.e. 80%) of the samples of shark flesh analysed have a concentration of mercury up to 2.5 times higher than the maximum limit set by Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs.

Table 1: Results of the analyses of five samples of shark flesh for lead, cadmium and mercury (ANSES CIME method), in mg/kg fresh weight.

	Lead	Cadmium	Mercury
No. 1 - Tiger shark	< 0.040 mg/kg	0.013 mg/kg	**1.075 mg/kg**
No. 2 - Tiger shark	< 0.040 mg/kg	0.010 mg/kg	0.966 mg/kg
No. 3 - Bull shark	< 0.04 mg/kg	0.014 mg/kg	**1.598 mg/kg**
No. 4 - Bull shark	< 0.04 mg/kg	0.017 mg/kg	**2.514 mg/kg**
No. 5 - Bull shark	< 0.04 mg/kg	0.011 mg/kg	**1.132 mg/kg**
Maximum limit set by Regulation (EC) No 1881/2006	0.30 mg/kg	0.05 mg/kg	1 mg/kg

In its Opinion of 17 April 2009[1], the Agency reported the results of analyses of nine samples of shark flesh (porbeagle *Lamna nasus* and tope shark *Galeorhinus galeus*) from a control survey carried out in 2007. The mean concentration of mercury was 1.3 mg/kg of fresh weight; 44% of the samples exceeded the maximum limit of 1 mg/kg of fresh weight.

In the light of these results, the Agency updated its recommendations with regard to the consumption of pelagic fish predators, in particular swordfish, on Reunion Island, in relation to the health risk related to methylmercury (Opinion of 6 July 2006[2]) so as to include sharks in the list of fish species that should not be consumed by pregnant women or breastfeeding mothers, nor by infants (< 30 months).

➔ *The results of the analysis of five samples of shark flesh carried out in 2013 give no grounds for changing the Agency's recommendations.*

■ Conclusions of the CES

The analysis of 24 samples of the flesh of tiger or bull sharks collected on Reunion Island in 2012 and 2013, by mouse bioassay and by cytotoxicity assay on Neuro-2a cells, did not reveal the presence of ciguatoxin-like toxins above the limit of detection. It should be noted, however, that for these two tests the detection limit is higher than the concentration considered to be of no risk to humans, which is 0.01 µg eq. P-CTX-1 kg^{-1} of fish flesh.

The analysis of a sample of the flesh from the bull shark implicated in an outbreak of food poisoning that occurred in Madagascar in November 2013 (124 people intoxicated, nine of whom died) gave a positive result by mouse bioassay with symptoms typical of those known for carchatoxins. The analysis of one sample of flesh, one sample of stomach and three samples of dried fin by cytotoxicity assay on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated at 0.144 µg eq. P-CTX-1 kg^{-1} in the flesh, 114 µg eq. P-CTX-1 kg^{-1} in the stomach and 0.145 µg to 0.737 µg eq. P-CTX-1 kg^{-1} in the fin. These concentrations are 14 to 11,400 times the concentration considered to be of no risk to humans.

In addition, the preliminary results of the CHARC programme have shown that the populations of tiger and bull sharks are not limited to the marine areas around Reunion Island and that one of the specimens (a tiger shark) tagged at Reunion Island moved to Madagascar.

Accordingly, the CES ERCA considers that, in view of the data produced by the analysis of samples of flesh from 24 specimens of shark from Reunion Island and of samples from one specimen of shark from Madagascar, it is impossible to rule out the risk that sharks caught at

[1] Opinion of the French Food Safety Agency of 17 April 2009 relating to the interpretation of the results of the analyses of the monitoring campaign for chemical contaminants in 2007, particularly the search for mercury in sea lamprey and the different species of Selachii (Request No. 2008-SA-0309).

[2] Opinion of the French Food Safety Agency of 6 July 2006 on the consumption of pelagic fish predators, especially swordfish, on Reunion Island, in relation to the health risk related to methylmercury (Request No. 206-SA-0003).

Reunion Island could be contaminated by ciguatoxins (or similar toxins) in concentrations which could present a risk to the health of consumers.

Furthermore, the analyses for mercury carried out in five samples of the flesh of tiger or bull sharks showed that in 80% of cases, the concentration was higher than the maximum limit set under Regulation (EC) No 1881/2006. The CES ERCA therefore maintains the recommendation issued by the Agency in 2009 that pregnant women, breastfeeding mothers and infants (< 30 months) should avoid, as a precaution, the consumption of shark, like that of swordfish, marlin, dogfish and sea lamprey.

Finally, the CES ERCA supports the need for research identified by the Working Group (see the Report for details), focusing on:

- the development of diagnostic tools for detecting the presence of ciguatoxins (or similar toxins, such as carchatoxins) in sharks, and a plan of action in case of intoxication by consumption of shark;
- the identification of analogues of ciguatoxins or similar toxins (carchatoxins) in sharks in the Indian Ocean;
- the toxicity of these toxins, their origin and how they are transferred to sharks;
- the size and structure of shark populations in the Indian Ocean as well as their movements and the distances travelled.

4. CONCLUSIONS AND RECOMMENDATIONS OF THE AGENCY

The French Agency for Food, Environmental and Occupational Health & Safety adopts the conclusions of the Expert Committee on "Assessment of the physical and chemical risks in food".

The Agency considers that it is not possible to rule out the risk that the tiger and bull sharks caught at Reunion Island could be contaminated by ciguatoxins (or similar toxins), especially following the analyses carried out on samples of the bull shark implicated in a recent outbreak of food poisoning (with fatal cases) that occurred in Madagascar, and taking into account the movements of these shark species between the two islands.

In addition, the Agency reiterates that the consumption of shark, like that of swordfish, marlin, dogfish and sea lamprey should be avoided, as a precautionary measure, by the vulnerable populations of pregnant women, breastfeeding mothers and infants (< 30 months), because of the mercury levels found in these species.

Marc Mortureux

KEY WORDS

Ciguatoxins, sharks, Reunion Island, methylmercury

ANNEXES

Report of the collective expert appraisal by the "Ciguatoxins" Working Group, State of knowledge on the contamination of sharks, especially tiger and bull sharks, by ciguatoxins: occurrence, analytical methods, human cases reported and ethological information for these two shark species, November 2014, 82pp.

Tracking changes to the previous ANSES Opinions of 9 July 2014 and 6 August 2014.

Tracking changes to the ANSES Opinion

Date	Version	Page	Description of the change (*in blue, italic*)
24/11/2014	03	04	**Text of 6 August 2014** The analysis of a sample of flesh, a sample of oesophagus and three samples of dried fin by cytotoxicity assays on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated as follows: - Flesh: 0.144 µg eq. P-CTX-1 kg^{-1} (i.e. 14 times the concentration considered to be of no risk to humans); - Oesophagus: 114 µg eq. P-CTX-1 kg^{-1} (i.e. 11,400 times the concentration considered to be of no risk to humans); - Dried fin: 0.145 µg eq. P-CTX-1 kg^{-1}; 0.158 µg eq. P-CTX-1 kg^{-1}; 0.737 µg eq. P-CTX-1 kg^{-1} (i.e. 14 to 74 times the concentration considered to be of no risk to humans). **Revised text** The analysis of a sample of flesh, a sample of *stomach* and three samples of dried fin by cytotoxicity assays on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated as follows: - Flesh: 0.144 µg eq. P-CTX-1 kg^{-1} (i.e. 14 times the concentration considered to be of no risk to humans); - *Stomach*: 114 µg eq. P-CTX-1 kg^{-1} (i.e. 11,400 times the concentration considered to be of no risk to humans); - Dried fin: 0.145 µg eq. P-CTX-1 kg^{-1}; 0.158 µg eq. P-CTX-1 kg^{-1}; 0.737 µg eq. P-CTX-1 kg^{-1} (i.e. 14 to 74 times the concentration considered to be of no risk to humans).
24/11/2014	03	05	**Text of 6 August 2014** The analysis of one sample of flesh, one sample of oesophagus and three samples of dried fin by cytotoxicity assay on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated at 0.144 µg eq. P-CTX-1 kg^{-1} in the flesh, 114 µg eq. P-CTX-1 kg^{-1} in the oesophagus and 0.145 µg to 0.737 µg eq. P-CTX-1 kg^{-1} in the fin. **Revised text** The analysis of one sample of flesh, one sample of *stomach* and three samples of dried fin by cytotoxicity assay on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated at 0.144 µg eq. P-CTX-1 kg^{-1} in the flesh, 114 µg eq. P-CTX-1 kg^{-1} in the *stomach* and 0.145 µg to 0.737 µg eq. P-CTX-1 kg^{-1} in the fin.
25/07/2014	02	02	**Text of 9 July 2014** All the work was adopted by the CES ERCA in a plenary meeting on 26 June 2014 and by electronic means on 3 July 2014. **Revised text** All the work was adopted by the CES ERCA in a plenary meeting on 26 June 2014 and by electronic means on 3 July 2014. *An updated version of the documents was adopted by the Chair of the CES ERCA by electronic means on 25 July 2014, following the receipt of additional information on 21 July 2014.*

25/07/2014	02	03	**Text of 9 July 2014** The results of mouse bioassays for ciguatoxins of 24 samples of shark flesh were sent to ANSES as part of the Request by the DGAL. They were all negative. **Revised text** The results of mouse bioassays for ciguatoxins of 24 samples of shark flesh were sent to ANSES as part of the Request by the DGAL. They were all negative. *However, this test is not sufficiently sensitive to detect concentrations of ciguatoxins considered to be of no risk to humans.*
25/07/2014	02	04	**Text of 9 July 2014** The preliminary results obtained from the 24 samples of shark flesh from Reunion Island did not reveal any toxicity in this test at the doses tested. The RDA also included samples from the bull shark implicated in an outbreak of food poisoning that occurred in Madagascar in November 2013 (124 people intoxicated, nine of whom died) for analysis by mouse bioassay and by cytotoxicity assay on Neuro-2a cells. The sample of flesh gave a positive result by mouse bioassay; the first results obtained on Neuro-2a cells cannot yet be interpreted. The symptoms observed in mice (prostration, dyspnoea, cyanosis, convulsions and death by respiratory arrest) are typical of those known for carchatoxins. Three samples of fin and one of oesophagus were also tested on Neuro-2a cells and the first results show an activity typical of that for ciguatoxins in these samples, especially strong in the sample of oesophagus. These preliminary results are currently undergoing confirmation. **Revised text** *The final report was submitted to ANSES on 21 July 2014.* *The results did not show ciguatoxin-like toxins to be present above the limit of detection of 0.04 µg eq. P-CTX-1 kg^{-1} of flesh. It should be noted that the detection limit is higher than the concentration considered to be of no risk to humans, which is 0.01 µg eq. P-CTX-1 kg^{-1} of fish flesh.* The RDA also included samples from the bull shark implicated in an outbreak of food poisoning that occurred in Madagascar in November 2013 (124 people intoxicated, nine of whom died) for analysis by mouse bioassay and by cytotoxicity assay on Neuro-2a cells. The sample of flesh gave a positive result by mouse bioassay, with symptoms typical of those known for carchatoxins (prostration, dyspnoea, cyanosis, convulsions and death by respiratory arrest). *The analysis of a sample of flesh, a sample of oesophagus and three samples of dried fin by cytotoxicity assays on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated as follows:* - *Flesh: 0.144 µg eq. P-CTX-1 kg^{-1} (i.e. 14 times the concentration considered to be of no risk to humans);* - *Oesophagus: 114 µg eq. P-CTX-1 kg^{-1} (i.e. 11,400 times the concentration considered to be of no risk to humans);* - *Dried fin: 0.145 µg eq. P-CTX-1 kg^{-1}; 0.158 µg eq. P-CTX-1 kg^{-1}; 0.737 µg eq. P-CTX-1 kg^{-1} (i.e. 14 to 74 times the concentration considered to be of no risk to humans).*
25/07/2014	02	05	■ **Conclusions of the CES** **Text of 9 July 2014** The preliminary results obtained by the cytotoxicity assay on Neuro-2a cells of 24 samples of flesh from tiger or bull sharks collected on Reunion Island in 2012 and 2013 did not reveal the presence of ciguatoxins at a concentration causing a toxic response, at the doses tested. The results

			obtained by mouse bioassay were negative. The analysis of a sample of flesh from the bull shark implicated in an outbreak of food poisoning that occurred in Madagascar in November 2013 (124 people intoxicated, nine of whom died) gave a positive result by mouse bioassay with symptoms typical of those known for carchatoxins. Three samples of fin and one of oesophagus were also tested on Neuro-2a cells and the first results show an activity typical of that for ciguatoxins in these samples, especially strong in the sample of oesophagus. These preliminary results are currently undergoing confirmation. As the preliminary results of the CHARC programme have shown that the populations of tiger and bull sharks are not limited to the marine areas around Reunion Island and that one of the specimens (a tiger shark) moved to Madagascar, the CES ERCA considers that, as a result of the data produced by the analysis of 24 samples of shark, it is impossible to rule out the risk that sharks caught at Reunion Island could be contaminated by ciguatoxins (or similar toxins) in concentrations which could present a risk to the health of consumers. **Revised text** The *analysis* of 24 samples of the flesh of tiger or bull sharks collected on Reunion Island in 2012 and 2013, *by mouse bioassay and by cytotoxicity assay on Neuro-2a cells, did not reveal the presence of ciguatoxin-like toxins above the limit of detection. It should be noted, however, that for these two tests the detection limit is higher than the concentration considered to be of no risk to humans, which is 0.01 µg eq. P-CTX-1 kg^{-1} of fish flesh.* The analysis of a sample of the flesh from the bull shark implicated in an outbreak of food poisoning that occurred in Madagascar in November 2013 (124 people intoxicated, nine of whom died) gave a positive result by mouse bioassay with symptoms typical of those known for carchatoxins. *The analysis of one sample of flesh, one sample of oesophagus and three samples of dried fin by cytotoxicity assay on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated at 0.144 µg eq. P-CTX-1 kg^{-1} in the flesh, 114 µg eq. P-CTX-1 kg^{-1} in the oesophagus and 0.145 µg to 0.737 µg eq. P-CTX-1 kg^{-1} in the fin. These concentrations are 14 to 11,400 times the concentration considered to be of no risk to humans.* In addition, the preliminary results of the CHARC programme have shown that the populations of tiger and bull sharks are not limited to the marine areas around Reunion Island and that one of the specimens (a tiger shark) *tagged at* Reunion Island moved to Madagascar. *Accordingly,* the CES ERCA considers that, in view of the data produced by the analysis *of samples of flesh from 24 specimens of shark from* Reunion Island *and of samples from one specimen of shark from Madagascar,* it is impossible to rule out the risk that sharks caught at Reunion Island could be contaminated by ciguatoxins (or similar toxins) in concentrations which could present a risk to the health of consumers.

State of knowledge relating to the contamination of sharks, especially tiger and bull sharks, by ciguatoxins: occurrence, analytical methods, human cases reported and ethological information for these two shark species

Request 2013-SA-0198 "Request for an Opinion on the health risk assessment related to the consumption of two shark species on Reunion Island, especially regarding the risk related to ciguatoxins"

Collective expert appraisal
REPORT**

Expert Committee

“Assessment of the physical and chemical risks in food”

Working Group

"Ciguatoxins"

November 2014

** Revised report cancelling and superseding the previous reports of 26 June 2014 and 25 July 2014.

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Key words

Ciguatoxins, sharks, Reunion Island

Presentation of participants

FOREWORD: Non ANSES experts, Expert Committee and WG members, or designated rapporteurs are all appointed in their personal capacities, *intuitu personae,* and do not represent their parent organisation.

WORKING GROUP

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Members

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Mr Jean-Marc FREMY – former ANSES employee (retired) – Knowledge of ciguatoxins, ecological and toxicological aspects and analytical methods

Mr Philipp HESS – Ifremer (Nantes), French research institute for exploitation of the sea – analytical methods for ciguatoxins

Mr Marc SORIA – IRD (Reunion Island), Institute of Research for Development – ethology of sharks in the seas around Reunion Island

Mrs. Sophie TROTEREAU – ANSES/Laboratory for Food Safety (Maisons-Alfort) – analytical methods for ciguatoxins (NRL for marine biotoxins)

Mr Jean TURQUET – ARVAM (Reunion Island), Agency for Research and Marine Exploitation – Knowledge of ciguatoxins, ecological and toxicological aspects and analytical methods

EXPERT COMMITTEE

The work described in this report was monitored and adopted by the following Expert Committee (CES):

- CES ERCA "Assessment of the physical and chemical risks in food" - in plenary meetings of 19 November 2013, 17 March 2014, 14 April 2014, 26 June 2014 and by electronic means on 3 July 2014. An updated version of the results was adopted by the Chair of the CES ERCA electronically on 25 July 2014, following the receipt of additional information on 21 July, 2014.

Chair

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CONTENTS

Acronyms and abbreviations

ANSES: French Agency for Food, Environmental and Occupational Health & Safety
CTXs: ciguatoxins
C-CTXs: Caribbean ciguatoxins
Nav: voltage-gated sodium channels
DGAL: Directorate General for Food
LD_{50}: median lethal dose
EFSA: European Food Safety Authority
FBIO: Foodborne illness outbreak
I-CTXs: Indian Ocean ciguatoxins
i.p.: intraperitoneal
LC-MS/MS: liquid chromatography coupled with tandem mass spectrometry
MBA: mouse bioassay
Neuro-2a cell test: cell test on murine neuroblastoma cells
NRL: National Reference Laboratory
PbTxs: brevetoxins
b.w.: body weight
P-CTXs: Pacific ciguatoxins
RBA: Radioligand Binding Assay

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1 Background, purpose and procedure for handling the request

The content of the request is reported in Annex 1.

1.1 Background

Since 1996, the French *département* of Reunion Island has been covered by a specific regulation (by prefectoral order) which restricts or prohibits the marketing of certain species of fish because of the risk of poisoning by marine biotoxins, especially ciguatoxins. Since 1999, the list of these species of fish has included most species of sharks, including those of the *Carcharhinidae* family (Annex 2).

The Prefect of Reunion Island requested the Directorate General for Food (DGAL) to commission a reassessment of the risk related to two shark species whose consumption is currently prohibited by the prefectoral order. These are the tiger shark (*Galeocerdo cuvier*) and the bull shark (*Carcharhinus leucas*).
In order to acquire data concerning the contamination of these two shark species by ciguatoxins, the services of the Prefecture of Reunion Island launched a campaign in 2012 to test 12 specimens per species for ciguatoxins by a mouse bioassay. This sampling campaign was extended in 2013 with a goal of 45 additional specimens per species.
To date, 24 specimens have been analysed for ciguatoxins by mouse bioassay and five of them have also been analysed for heavy metals (lead, cadmium and mercury).

1.2 Purpose of the expert appraisal

The request instructs ANSES to answer the following questions:

Question 1: What is the current state of knowledge on contamination of shark flesh by ciguatoxins, especially tiger and bull sharks? Have any cases of food poisoning associated with the consumption of sharks already been reported?

Question 2: What are the analytical methods currently applicable for detecting and quantifying ciguatoxins in shark flesh? Can the results from these methods be used to assess the health risks related to a possible authorisation of these species for human consumption in this area?

In the event that ANSES should identify a sufficiently reliable method for testing shark flesh for ciguatoxins, what data would be necessary to carry out this evaluation and what recommendations could be made regarding the protocol for sampling tiger and bull sharks around Reunion Island? Particular consideration shall be given to the geographical area concerned and the ethology of these two shark species in terms of the extent of their movements in the marine areas around Reunion Island.

Question 3: In 2006 and 2009, the Agency published Opinions relating to the consumption of pelagic predator fish around Reunion Island and the health risk related to methylmercury. Do the new analyses of shark specimens in 2013 give cause for revising the conclusions of these Opinions?

1.3 Procedure for handling the request: resources used and organisation

ANSES entrusted the examination of this request to the Working Group on "Ciguatoxins", a WG of the Expert Committee on the "Assessment of the physical and chemical risk in foods" (CES ERCA), for Questions 1 and 2 relating to ciguatoxins. Concerning Question 3, relating to the Agency's earlier work on the health risks related to methylmercury, a study originally carried out internally by ANSES was presented to the CES ERCA. These latter points in response are therefore not included in the present report, but in the note "Collective Expert Appraisal: summary of the arguments and conclusions" by the CES ERCA, which was quoted in the opinion released by ANSES.

The methodological and scientific aspects of this group's work were regularly submitted to the CES. The Working Group report takes into account the additional observations and information provided by the members of the CES.

This expert appraisal was therefore performed by a group of experts with complementary skills.

The expert appraisal was carried out in accordance with the French standard NF X 50-110 "Quality in Expertise – General Requirements of Competence for Expert Appraisals (May 2003)".

1.4 Prevention of risks of conflicts of interest

ANSES analyses interests declared by experts before they are appointed and throughout their work in order to prevent risks of conflicts of interest in relation to the points addressed in expert appraisals.

The declarations of interest by experts are made public via the ANSES website (www.anses.fr).

2 Points in response to Question 1

Reminder of the content of Question 1: What is the current state of knowledge on contamination of shark flesh by ciguatoxins, especially tiger and bull sharks? Have any cases of food poisoning associated with the consumption of sharks already been reported?

2.1 Toxicity of ciguatoxins and mode of action

In introduction to the points in response to Question 1, here is a brief review of the toxicity of ciguatoxins (CTXs) and of their mode of action. A detailed review can be found in various articles or reports, some in English (EFSA, 2010), others in French (Epidemiological Bulletin no.56 of March 2013; Public Health Surveillance Bulletin for the French Antilles and French Guiana, 3 April 2013), from which the elements presented below are extracted.

Poisoning related to the consumption of fish contaminated with ciguatoxins is known as ciguatera poisoning. The source[1] of these toxins is a microalga that proliferates periodically on degraded coral substrates of the genus *Gambierdiscus*, an epiphytic benthic dinoflagellate. Herbivorous or microphageous fish ingest this microalga after which the toxins are conveyed along the food chain, up to the large fish-eating predators such as Carangidae, barracudas, moray eels and sharks. The level of contamination then depends on the feeding history of each fish, its trophic level, the geographical area concerned, and the result of the kinetics of accumulation/metabolism/excretion of toxins ingested.

The clinical syndrome can include symptoms that are gastrointestinal (nausea, vomiting, abdominal pain, diarrhoea), neurological, involving sensitivity disorders (hyperesthesia, paraesthesia, dysaesthesia), muscular, joint, dermal (pruritus, which is why ciguatera is known as "*la gratte*" or "the itch" in some regions), cardiovascular (bradycardia, low blood pressure), of varying intensity and with onset varying from 30 minutes to 48 hours after ingestion. More than 175 different symptoms have been identified in acute and chronic phases of ciguatera. Gastrointestinal symptoms usually disappear in 1 to 4 days without special treatment but some symptoms, mainly neurological, may persist for several weeks or even months.

Regional differences in the clinical syndrome have been observed and can be attributed to the presence of different ciguatoxins. Symptoms have also been found to vary widely between individuals.

In humans, according to Lehane (1999, as described in the opinion published by EFSA in 2010 at the end of Section 10), the lowest dose likely to cause symptoms in 20% of consumers would be 1 ng kg^{-1} b.w., based on a contamination of 0.1 µg P-CTX-1 kg^{-1} of fish and assuming fish consumption of 500 g and an individual with a body weight of 50 kg.

The preferred molecular targets of CTXs are the voltage-gated sodium channels (Nav), which play a fundamental role in the genesis and propagation of nerve impulses. When CTXs bind site 5 of the α subunits of these proteins of the plasma membrane of electrically excitable cells, it causes the prolonged opening of these channels even at the resting membrane voltage, which leads to a continuous influx of Na^+ ions into the cells. In order to alleviate this increase of intracellular sodium, cellular mechanisms are activated to allow an outflux of Na^+, counterbalanced by an influx of Ca^{2+}, leading to an increase in intracellular calcium. A consequence of this virtually irreversible attachment of CTXs to the VDSCs is an impairment of both nerve conduction and the morphology of the nerve cells.

[1] Some pelagic marine cyanobacteria (*Hydrocoleum spp*. and *Trichodesmium spp*.) could be another source of contamination. Studies have revealed the production of ciguatoxin-like toxins in samples of cyanobacteria populations in New Caledonia, related to cases of human poisoning following the consumption of giant clams when no *Gambierdiscus spp* had been detected during environmental monitoring of the area concerned (Laurent *et al*., 2008; Kerbrat *et al*., 2010).

This set of changes induced by CTXs (depolarisation and hyper-excitability phenomena, increase in intracellular Na^+ and Ca^{2+}, anarchic liberation of neuro-mediators, swelling due to the influx of water, etc.) explains the diversity of clinical signs observed in cases of poisoning.

- At the neurological level, the multitude of symptoms such as impaired motricity, sensitivity, cerebellum and psychiatric disorders, is a direct consequence of the alteration of the fibres of the peripheral, central and autonomous nervous system.
- At the digestive level, it is the high level of intracellular Ca^{2+} that seems to cause an increase in intestinal secretions and therefore the occurrence of profuse diarrhoea.
- At the muscular level, the increase in intracellular Ca^{2+} generates an increase in the frequency and intensity of muscle contractions, while the spontaneous and repetitive firing of action potentials induces disorganised twitching.
- At the level of the heart, in addition to the muscle effects, the CTXs affect the autonomous nervous system, with bradycardia and low blood pressure being linked to parasympathetic hyperstimulation (which explains why atropine is so effective in the symptomatic treatment of ciguatera poisoning) and to low sympathetic tone.

Figure 1 shows how the three types of symptoms develop over time. It should also be noted that the predominant symptoms are different depending on the ocean concerned. The digestive symptoms (in particular vomiting) and cardiac symptoms are dominant in the Caribbean while in the Eastern Pacific, Australia and the Indian Ocean the neurological symptoms dominate (Pottier et al., 2001).

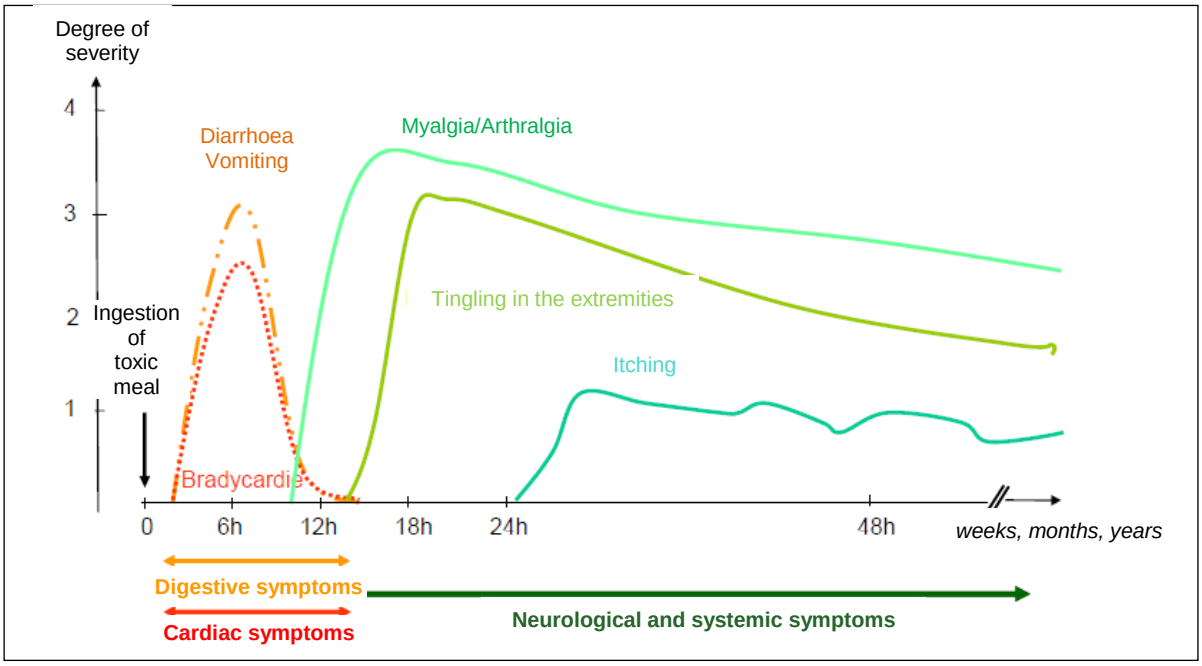


Figure 1: Chronology of the appearance of the 3 major types of symptoms observed during ciguatera poisoning (Chinain *et al.,* 2013, modified from Lawrence *et al.,* 1980)

As regards the Indian Ocean, which is the particular focus of this appraisal, ciguatera poisoning was reported historically in Mauritius (an island near Reunion Island), in the 19th century (Hamilton *et al.,* 2002). The epidemiological analysis conducted on Reunion Island shows that 484 cases of ciguatera poisoning were identified for the period 1986-1999, and another 150 cases for the period 2000-2010. Ciguatera poisoning accounts for 80% of cases of food poisoning from fish. The annual incidence rate is variable and remains low compared to other regions such as French Polynesia. The incidence rate for the period 1986-1999 has been estimated at 0.8 cases per 10,000 inhabitants, and at 0.2 for the period 2000-2010.

There have been specific local regulations since 1966, establishing a list of tropical fish species whose marketing is prohibited. This list changes periodically depending on the state of knowledge

and the local and regional epidemiological situation, thus limiting any harmful impact on health. There were two major revisions in 1982 and 1999, the latter introducing some species of shark among the list of prohibited species (the most recent update was in 2009 and can be found in Annex 2).

In clinical terms, the symptomatology typically observed on Reunion Island includes neurological signs (paraesthesia, dysaesthesia, myalgia), digestive signs (diarrhoea) and general signs (residual asthenia). The most evocative is the reversal of the hot/cold sensation or allodynia (Bagnis *et al*., 1992; Quod and Turquet, 1996). Most outbreaks of food poisoning are due to fish from the Soudan, Saya de Malha, Rodrigues or Mauritius fishing shoals. Only 10% of cases are due to fish caught in the seas around Reunion Island.

2.2 Carchatoxism, poisoning by consumption of shark

As a general rule, there is little information in the literature on poisoning by consumption of shark. The most numerous and the best described cases are recent (1990s) and mainly concern Madagascar. A few cases have nevertheless been reported in other regions of the world.

Halstead (1978) describes several cases of poisoning, the oldest of which dates from 1873 in New Caledonia, several individuals having fallen ill after consuming the liver of a shark whose species was not described. The case is reported as severe, with one fatality. Cooper (1964) reported the death of several individuals after consumption of liver from tiger shark (*Galeocerdo cuvieri*) in the Gilbert Islands, an archipelago in the Pacific Ocean (the Beru Atoll in 1957 and the Tabiteuea Atoll in 1960 and 1961). The flesh, however, was described as non-toxic.

More recently, in the Cook Islands (Pacific Ocean on the west of French Polynesia), two cases were reported (symptomatology unspecified) after consumption of whitetip reef shark (*Triaenodon obesus*) (Rongo and van Woesik, 2011).

In French Polynesia, two severe cases were described by Gatti *et al*. (2008). The intoxicated individuals had consumed the liver or viscera of sharks whose species were not specified. They were hospitalised with neurological and digestive symptoms similar to those in the cases described in Madagascar (the most severe and best documented episodes). Three other cases of poisoning by the consumption of shark viscera occurred in French Polynesia between 2002 and 2013 (Chinain, personal communication), though the species in question are not documented.

Elsewhere in the world, information is still fragmentary and lacks detail. Glaziou and Legrand (1994), in their review on the epidemiology of ciguatera poisoning, cite the *Carcharhinidae* family, some species of which are responsible for ciguatera poisoning in different regions of the world (the Americas, the central Pacific and the Asia/Indian Ocean region).

In a review article published in 1981, Bagnis provides a list of fish referred to as "ciguateric" known in the Indo-Pacific zone, based on data he collected during epidemiological investigations carried out between 1965 and 1980. Among these fish species there are several species of shark (Table 1, next page).

Table 1. List of "ciguateric" sharks identified by Bagnis (1981)

	Pacific Ocean						Indian Ocean	Caribbean
Species	French Polynesia	Samoa	Tonga	Fiji	Vanuatu	New Caledonia	Mascarene Islands *	Virgin Islands
Carcharhinus amblyrhynchos Grey Reef Shark	+	+			+			
C. melanopterus Blacktip Reef Shark		+						
C. plumbeus Sandbar Shark			+		+	+	+	
***Galeocerdo cuvieri* Tiger Shark**		+		+				
Isurus paucus Longfin Mako Shark			+		+	+		
Triaenodon obesus Whitetip Reef Shark	+	+						
Sphyrna lewini *Scalloped Hammerhead Shark*								++

* The Mascarene Islands are an archipelago in the Indian Ocean consisting of three main islands, Reunion Island, Mauritius and Rodrigues, plus several small nearby islands.

\+ indicates exceptional toxicity, very limited in time and space. ++ indicates regional toxicity, for large specimens.

In Madagascar, the first episode described occurred at Manakara (south-east coast) in 1993, which was noted for its unprecedented severity. Several hundred people (between 200 and 500 depending on the different authors) were poisoned due to the consumption of a shark which may have been a bull shark (*Carcharhinus leucas*) or a pigeye shark (*C. amboinensis*), two species that are difficult to distinguish between. This episode resulted in the deaths of between 60 and 98 people, depending on the different authors, a fatality rate of 20 to 30% (Habermehl *et al.,* 1994; Boisier *et al.,* 1995; Quod *et al.,* 2001).

Following this serious event, investigations conducted by the local authorities provided a better understanding of the situation in Madagascar. Between 1930 and 1997, more than 83 episodes of poisoning by consumption of shark were identified, affecting 1855 people. Sharks were responsible for 48% of severe cases of poisoning, the majority of them being observed on the eastern coast of Madagascar in the province of Toamasina (Champetier de Ribes *et al.,* 1999). Regarding moderate forms of poisoning, sharks account for 35% of episodes (n=71). In the order of Carcharhiniforms, two families of shark are most often cited in serious and moderate outbreaks: the *Sphyrnidae* (Hammerhead sharks) and the *Carcharhinidae* (Requiem sharks). A more detailed analysis of the investigation led to the identification of 1269 episodes of poisoning by sharks between 1993 and 1998, including 68 deaths (Champetier de Ribes *et al*., 1998). The main species implicated in eight severe episodes belong to the following three families:

- *Carcharhinidae: Carcharhinus leucas* (bull shark, two cases)*, C. amboinensis* (pigeye shark, two cases), *Galeocerdo cuvier* (tiger shark, one case)
- *Sphyrnidae: Sphyrna lewini* (scalloped hammerhead shark, one case)*, S. mokarran* (great hammerhead shark, one case)
- *Hexanchidae: Hexanchus griseus* (bluntnose sixgill shark, one case), of the order of Hexanchiformes.

A few results of analysis by mouse bioassay are presented in Table 2 (below).

The episodes occurred primarily on the east coast in the health districts of Vohipeno (twice), Manakara, Sambava, Maroantsetra and Mahanoro, but also on the south coast, at Taolagnaro, and on the southwest coast at Toliara II.
The onset of clinical signs was early, occurring less than 6 hours after the meal in question in the vast majority of cases and never exceeding 24 hours. Signs typically lasted from a few hours to 15 days. The attack rate varied between 20 and 100%. The lethality among patients varied between 0 and 32%. Neurological signs were always predominant and were present in all episodes, affecting between 50% and 100% of intoxicated subjects (80% on average):

- neurosensitive signs (100% of episodes) with paraesthesia (tingling of the extremities, burning sensation of the lips), myalgia and arthralgia, headaches
- neuro-motor impairments (60% of episodes) with balance disorders, abnormal gait
- neurosensorial signs (20% of episodes) with double or blurred vision
- impaired consciousness (40% of episodes): obfuscation, coma.

Digestive signs were also found in all episodes, affecting between 5% and 100% of intoxicated subjects (40% on average). These were essentially vomiting, diarrhoea, epigastric pain and, more rarely, stomatitis (observed in a few subjects intoxicated during one episode) and jaundice (observed in a few subjects intoxicated during one episode).
General signs were less frequent (50% of episodes), especially involving asthenia, dizziness and fever (Champetier de Ribes et al., 1998).

Table 2. A few results of analysis by mouse bioassay of sharks implicated in foodborne illness outbreaks in Madagascar

Sources: i) Workshop on the prevention and control of poisoning by consumption of marine animals in Madagascar, 21-23 April 1998, Compendium of presentations. Ministry of Health, Ministry of Fisheries and Fishery Resources and ii) Champetier de Ribes *et al*. (1998).

Date/Place	Number of consumers	Rate of illness	Symptoms	Species of shark	Toxicity
November 1993 Manakara	188	100%	Neurological	*Carcharhinus leucas* Bull Shark	30 MU per g of liver
October 1995 Tolagnaro	62	100%	Neurological	Shark not identified	< 0.1 MU per g of flesh < 1.7 MU per g of liver 5.3 < x < 10 MU per g of liver
November 1996 Sambava	80	21%	Neurological	*C. amboinenses* Balestrine Shark	0.163 ng per g of flesh
December 1996 Mahonoro	324	61%	Digestive	*Sphyrna mokarran* Great Hammerhead Shark	1.82 ng per g of flesh
December 1997 Toliara	145	82%	Neurological	*Carcharhinus leucas* Bull Shark	Flesh: 8 MU per g of flesh Liver: 0

(In this table, MU (mouse unit) corresponds to the quantity of toxins causing death in 24h to a mouse weighing between 16 and 22 g depending on the study. The amount of toxin is not calculated relative to 1 g of mouse, or the expression MUg would be used).

Since 1998, two outbreaks of foodborne illness following the consumption of shark have been reported very recently, one in the district of Fenoarivo Atsinanana in November 2013, and the other in the district of Toamasina II in February 2014 (Figure 2, below). According to the information transmitted to ANSES by the French Institute for Public Health Surveillance, dated 22 April 2014:

- In the Fenoarivo Atsinanana outbreak, 124 people, nine of whom died, were poisoned after a meal (shark meat), consumed on 10 November 2013. Leftovers were collected and

analysed in the framework of a research and development agreement contracted by ANSES and detailed later in this report; the genetic analysis concluded that the flesh was that of a bull shark;

- In the Toamasina II outbreak, four people became intoxicated following a meal (shark meat) consumed on 11 February 2014, one of whom died. Leftovers were in poor condition and were not collected (no analysis planned).

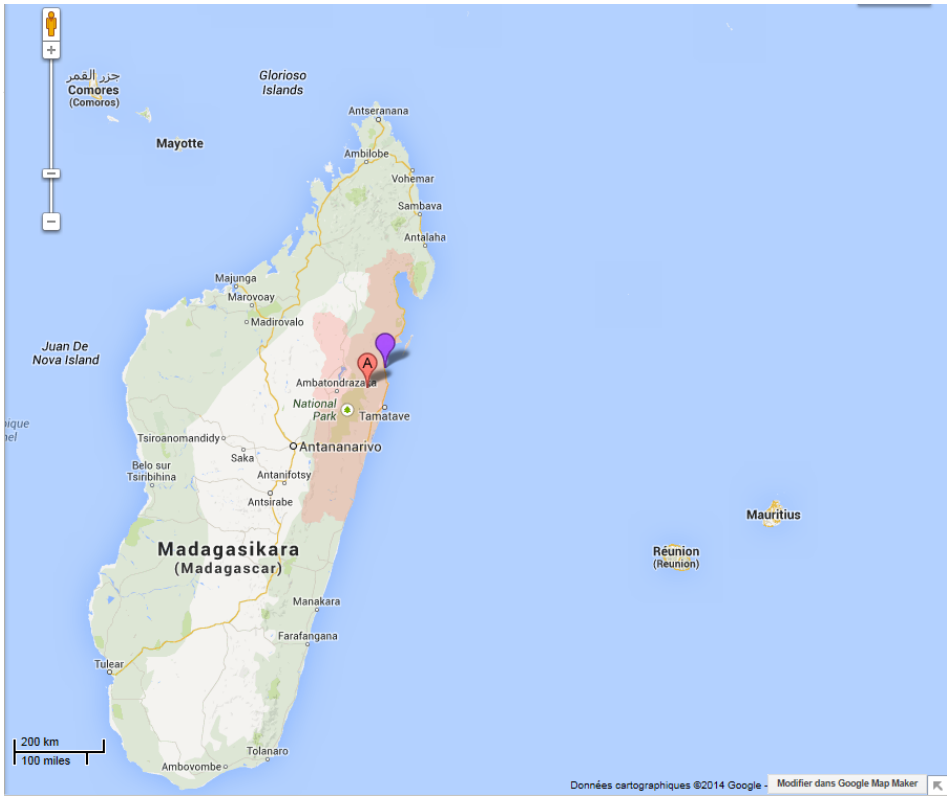


Figure 2. Map showing the location of the districts Fenoarivo Atsinanana (purple) and Toamasina II (red)

On Reunion Island, an outbreak of poisoning affecting five people was reported in 1993 following the consumption of the flesh of shark fished in the bay of Saint-Paul, although the species implicated was not formally identified. The symptoms progressively included asthenia, headache, abdominal pain, diarrhoea, vomiting, hallucinations, arthromyalgia, perioral paraesthesia and distal paraesthesia of the limbs. One of the patients presented cardiac symptoms requiring admission in intensive care, with a sinus bradycardia lasting for a month, and myalgia and asthenia lasting for three months (Le Bouquin *et al*., 1993).

Ciguatoxins or carchatoxins?

It has been suggested that poisoning due to the consumption of shark presents a clinical picture different from that of classic ciguatera poisoning and that it could be due to other toxins with similar properties, called carchatoxins (Yasumoto, 1998; Champetier de Ribes *et al*., 1998; Turquet *et al*., 2000; Gatti *et al.,* 2008).

To date, this type of poisoning by shark has mainly been found in severe forms in Madagascar. The various epidemiological investigations carried out on the spot provide a clinical picture (Table 3, next page) showing a predominance of neurological and sensorial signs (dominated by disorders of consciousness, with muscle and joint pain) and high mortality. In the case of ciguatera poisoning by consumption of reef fish from the Indian Ocean, the first symptoms are mainly gastrointestinal and are associated with neurosensitives manifestations; mortality remains exceptional (Champetier de Ribes *et al.,* 1998; Turquet *et al*., 2000).

Table 3: Differential diagnosis of poisoning by consumption of sharks and reef fish in the Indian Ocean (Turquet et al., 2000)

Type (animal implicated)	Clinical signs
Carchatoxism (sharks)	*Latency time:* 2 to 12 hours on average. *Clinical signs:* Predominance of neurological signs (paraesthesia of the extremities, superficial dysaesthesia, arthralgia, dizziness and visual disturbances) associated with mild and erratic gastroenteric symptoms. Severe forms also included ataxia, dysarthria, disorder of consciousness ranging from obfuscation to coma as well as cardiac arrhythmia and respiratory distress. *Mortality:* 7 to 30%
Ciguatera Poisoning (reef fish)	*Latency time:* 2 to 12 hours on average. *Clinical signs:* Clinical presentation of common gastro-enteritis associated with characteristic neurosensitive events (paraesthesia of the extremities and perioral paraesthesia, superficial dysaesthesia, reversal of the hot/cold sensation or allodynia). The symptomatology can also include myalgia and arthralgia, a pruritus of variable intensity and asthenia that resolves slowly. The severe forms (5%) are due to the manifestations of dehydration by persistent diarrhoea, of cardiovascular collapse by rhythm disorder and falls in blood pressure, dyspnoea by weakening of the respiratory muscles. *Mortality:* Exceptional.

In addition, leftovers of meal of the Manakara case in 1993 were tested by mouse bioassay and caused the same types of symptoms as ciguatoxins, with one slight difference: the samples of shark resulted in the death of the mice within 4 hours after injection (no mortality observed beyond 4 hours) while in the case of ciguatoxins, the mouse deaths were observed even after 24 hours (Boisier *et al.,* 1995). The toxicity was 30 MU (mouse unit, corresponding to the quantity of toxins resulting in the death of a mouse in 24h, the amount of toxin is not calculated per 1 g of mouse) per gram of shark liver (Champetier de Ribes *et al*., 1998).

These leftovers were also analysed by Prof. Yasumoto (1998), who demonstrated the presence of new toxins that he termed **carchatoxins A and B**. But the origin of this form of poisoning is still unknown.
However, the suggestion cannot be ruled out that carchatoxins could be new analogues of ciguatoxins, for example highly oxidised forms.

2.3 Other data concerning contamination of sharks by ciguatoxins (or by toxins with similar properties)

In a study published in 1980, Randall presented the results of a vast campaign conducted in the Marshall Islands (located in the Pacific Ocean north of the Gilbert Islands) concerning 807 specimens of fish, including 32 sharks. These samples were tested by bioassay on mongoose, which involved feeding the animals portions of liver representing 10% of their body weight, with or without the viscera, except for sharks for which only the muscles were used. Mongoose was used because of the similarity of its symptoms with those observed in humans, the fact that it does not regurgitate (unlike the cat) and because it was easy to acquire on the island of Hawaii where the analytical laboratory was located. Of the 32 samples of sharks tested, 30 gave negative results and two a positive result (1 grey reef shark *Carcharhinus amblyrhynchos* and 1 blacktip shark *Carcharhinus limbatus*). The three specimens of tiger shark tested all gave negative results. It should be noted that only the flesh was tested, and neither the liver nor the viscera.

In a report dated 1966, Brock *et al*. presented the results of studies conducted from 1963 to 1965 in the Johnston Atoll (located in the Pacific Ocean, to the west of Hawaii) that concerned, among others, 82 shark specimens (46 whitetip reef sharks, *Triaenodon obesus*, and 36 grey sand sharks *(Carcharhinus menisorrah)*. The mongoose bioassay, used this time with the liver of the shark,

revealed 11 positive samples (7 whitetip and 4 silky sharks). The authors consider that the toxicity of the samples was underestimated because mongooses seldom ingest the entire portion administered.

The WG therefore draws attention to the fact that, in the case of sharks, the toxins to take into account include not only ciguatoxins but also, potentially, another type of toxins currently called carchatoxins, whose structure has not yet been elucidated. However, the suggestion cannot be ruled out that carchatoxins could be new analogues of ciguatoxins, for example highly oxidised forms.

3 Points in response to Question 2

Reminder of the content of Question 2:

What are the analytical methods currently applicable for detecting and quantifying ciguatoxins in shark flesh? Can the results from these methods be used to assess the health risks related to a possible authorisation of these species for human consumption in this area?

In the event that ANSES should identify a sufficiently reliable method for testing shark flesh for ciguatoxins, what data would be necessary to carry out this evaluation and what recommendations could be made regarding the protocol for sampling tiger and bull sharks around Reunion Island? Particular consideration shall be given to the geographical area concerned and the ethology of these two shark species in terms of the extent of their movements in the marine areas around Reunion Island.

3.1 Introduction

Reviews have been published recently (Caillaud *et al*., 2010; EFSA, 2010) describing various analytical methods for screening for ciguatoxins, using different principles.

Some methods are based on the toxicological or functional properties of CTXs:

- tests on animals, in particular the mouse bioassay;
- cytotoxicity tests based on the effects of the toxins on the viability of neuroblastoma cell lines (Neuro-2a) in culture;
- receptor binding assay (RBA) functional tests, using the recognition between the ligand-CTX and the voltage-gated sodium channels (Nav) in the membranes of nerve and muscle cells.

Other methods are based on the molecular structure of CTXs:

- immunological tests, using the recognition between the CTXs and anti-CTX antibodies;
- chemical analyses, in particular the liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

Their general principles, advantages and disadvantages are presented in the following paragraphs. It is preceded by a review of the current state of knowledge on the chemical structure of CTXs.

3.2 Chemistry of ciguatoxins

The region covered by the most advanced scientific studies is the Pacific Ocean. Thus, the group led by Pr. Yasumoto has particularly contributed since 1975 to the characterisation of toxins produced by microalgae of the genus *Gambierdiscus* as well as the metabolites produced by transformations found in fish (Yasumoto, 2001; Yasumoto *et al*., 2000). Two basic structures of toxins have been described, and their metabolites identified (Figure 3, next page).

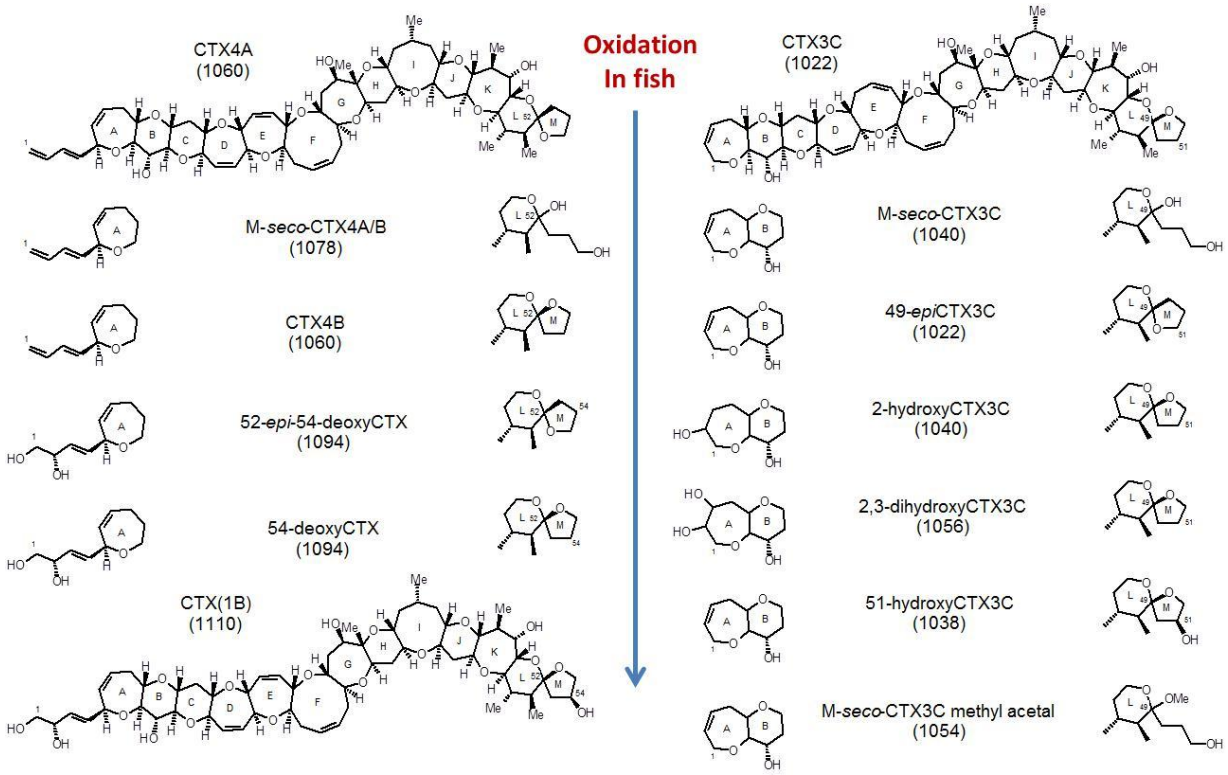


Figure 3: Metabolism by fish of algal ciguatoxins in the Pacific Ocean
(adapted from Yogi *et al*., 2014).

Some of these metabolites were also observed in the microalgae themselves, or in the extracts and fractions purified from a strain of *Gambierdiscus polynesiensis* (Chinain *et al*., 2010).

Two analogues of CTXs were described as characteristic of fish from the Caribbean area, C-CTX-1 and its epimer, C-CTX-2 (Lewis *et al.,* 1998). Although the structural differences with P-CTXs can be described as minor (for example with a stable spiroacetal – because closed – in P-CTXs compared to an unstable hemiacetal – because open – in C-CTXs; different oxidation on five quaternary carbons for the C-CTXs compared with two for the P-CTXs, Figure 4, next page), the consequences are major in terms of stability of the toxins (stable for the P-CTXs, unstable for C-CTXS) and toxicity in mice (toxicity of C-CTXs one tenth that of P-CTXs). In addition, the epitopes have the effect of preventing the anti-P-CTX antibodies from recognising C-CTXs. A point-by-point comparison can be found in the work of Pottier *et al.* (2001).

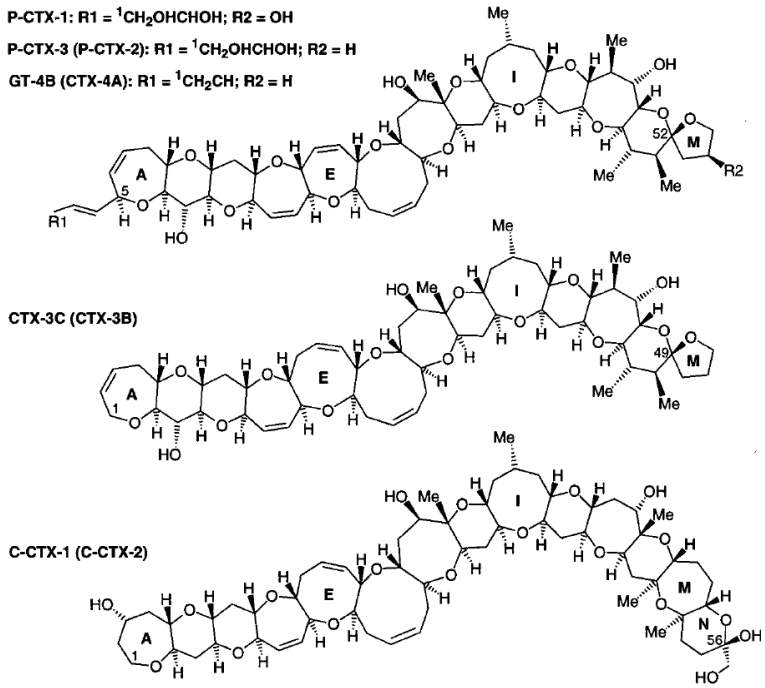


Figure 4: Comparison of structures of CTXs from the Pacific Ocean (P-CTXs) with those from the Caribbean (C-CTXs) (Lewis *et al*., 1998)

Few studies have been conducted to characterise CTXs from the Indian Ocean (I-CTXs). Hamilton *et al.* (2002) showed that I-CTXs had a chromatography elution profile different from that of Caribbean CTXs (C-CTXS) (Figure 5).

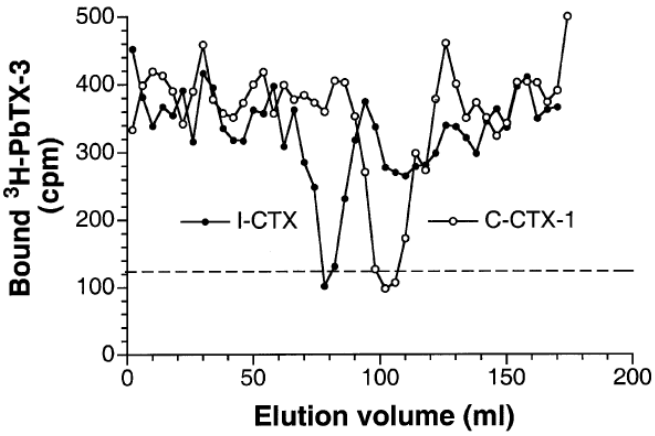


Figure 5. Elution profiles of CTXs from the Indian Ocean (I-CTX) and the Caribbean (C-CTX-1) with 2 ml min^{-1} MeOH on TSK HW-40S, a molecular-scale exclusion resin (Hamilton *et al.,* 2002)

Although the *m/z* values were measured by mass spectrometry for I-CTX-1 $[M+H]^+$ (1141.58 Da), the raw formulae have not yet been confirmed to date, and there is therefore no exact structure known for this family of I-CTXs. In addition, although the nominal mass (rounded to the nearest integer) of I-CTX-1 corresponds to that of C-CTX-1 (1141 Da), the elution spectrum by chromatography of I-CTX-1 does not correspond to that of C-CTX-1, as discussed earlier (Figure 5). There could therefore be significant differences between these analogues of CTX-1 from two different ocean regions.

Table 4 (next page) presents the state of knowledge on the diversity of ciguatoxins isolated from fish or from microalgae.

Table 4. State of knowledge on the diversity of ciguatoxins isolated from fish or from microalgae (according to Caillaud *et al*., 2010, supplemented with data from Pottier *et al*., 2002a,b and Lenoir, 2006)

Origin		Number of cycles	Number of carbon atoms	Examples of CTXs	Molecular Weight (Da)	Source
Pacific (P-)	Type I	13	60	CTX (CTX-1B, CTX-1)	1110.6	Carnivorous fish
				CTX-2-A2 (CTX-2, 52-epi-54-deoxyCTX)	1094.5	Carnivorous fish
				CTX-2-B2 (CTX-3, 54-deoxyCTX)	1094.5	Carnivorous fish
				CTX-4A	1060.8	*G. toxicus*, *G. polynesiensis*
				CTX-4B (GTX-4B, Gt 4b)	1060.8	*G. toxicus*, *G. polynesiensis*, herbivorous fish
	Type II	13	57	CTX-3C	1022.8	*G. toxicus*, *G. polynesiensis*, herbivorous fish
				CTX-2A1 (2,3-dihydroxyCTX3C)	1056.0	Carnivorous fish
Caribbean (C-)		14	62	CTX-1,-2, -1141a, -1141b, -1141c	1140.7	Carnivorous fish
				CTX-1143, -1143a	1142.7	Carnivorous fish
				CTX-1157,-1157a, -1157b	1156.7	Carnivorous fish
				CTX-1127	1126.6	Carnivorous fish
				CTX-1159	1158.6	Carnivorous fish
				CTX-1181	1180.6	Carnivorous fish
Indian (I-)		undetermined	undetermined	CTX-1	1140.6	Carnivorous fish
				CTX-2	1140.6	Carnivorous fish
				CTX-3	1156.6	Carnivorous fish
				CTX-4	1156.6	Carnivorous fish
				CTX-5	1038.7	Carnivorous fish
				CTX-6	1038.4	Carnivorous fish

From samples of shark implicated in the food poisoning episode that occurred in Madagascar in 1993 (Boisier *et al.,* 1995), Yasumoto (1998) revealed the presence of compounds of a different nature from that of the ciguatoxin of the Pacific, at least for the major analogue encountered in Pacific fish (P-CTX-1), which he named carchatoxins A and B (Figure 6, next page). Unfortunately, neither the molecular weight nor the structure of these carchatoxins are known.

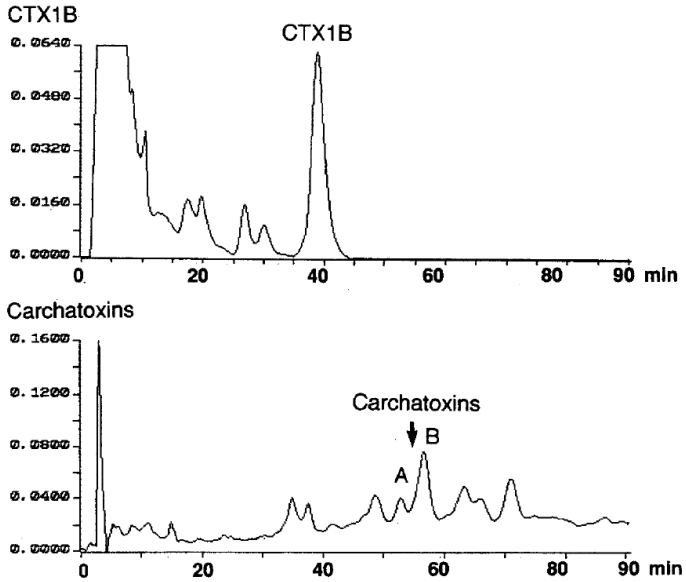


Figure 6. Carchatoxins A and B take different times to elute compared with P-CTX-1 in reversed-phase chromatography (Asahipak ODP-50, 4.6 x 150 mm, 75% MeOH, 1 ml min^{-1}, 210 nm) (Yasumoto, 1998)

3.3 Tests on animals

Tests carried out on animals involve either feeding tests on cats, mongoose or chicks or injection tests on insects or laboratory mice (Bagnis et al., 1985; Vernoux, 1991; Granade et al., 1976; Labrousse and Matile, 1996). Feeding tests are particularly interesting for studying the overall toxicity of samples of contaminated fish because the tissues are tested "as is", which therefore avoids losses due to extraction, as has been found with ciguatoxins, especially those from the Indian Ocean. Tests by injection, on the other hand, are preferred for studying semi-purified extracts (Vernoux and Lahlou, 1986; Vernoux, 1991).

The chick test is the most interesting of the feeding tests due to the presence of the crop, which can easily be filled with fish liver, and the low weight of the animal. It is well suited to the assessment of the potential toxicity of the livers of various fish (Vernoux and Lahlou, 1986; Vernoux *et al*., 1986). It was described in detail by Vernoux *et al*. (1985). The mouse bioassay is the reference injection test and is described below.

Mouse bioassay

3.3.1 General principle of the mouse bioassay

The mouse bioassay is a test widely used to detect toxins of the group of ciguatoxins in fish (EFSA, 2010; Hamilton et al., 2002; Hoffman et al., 1983; Lewis and Sellin, 1993; Vernoux, 1994). The 50% lethal dose (LD_{50}) by the intraperitoneal route in white laboratory mice is 3.6 µg kg^{-1} b.w. for C-CTX-1 and 0.25-0.35 µg kg^{-1} b.w. for P-CTX-1, which shows its good sensitivity to these toxins (Pottier et al., 2001).

The principle is to extract samples of fish tissue in acetone and then to purify them into two liquid partitions using hexane and diethyl ether. This extraction exploits the lipophilic nature of ciguatoxins which can be classified as moderate (**mean polarity** of the same order as that of the aflatoxins) because of the presence in their structures of OH polar functions against a background of hydrocarbons interspersed with regular polyether functions. The extract containing the ciguatoxins is solubilised in Tween 60 1-5% saline solution and then injected into a mouse (20 ±2 g) by the intraperitoneal (i.p.) route. Two mice are observed continuously during the first two hours, and then monitored regularly until 24 hours after injection.

The interpretation of the results is based on the symptoms observed and the time-to-death of the mice. The typical symptoms of the presence of CTXs are profuse diarrhoea, piloerection, respiratory disorders, dyspnoea and, when using male mice, transient preerectional cyanosis of the

penis (which can become priapism). With the Indian Ocean toxins, this last symptom is observed only very rarely. It therefore does not appear in the classical description for this region. The death of 1 or 2 mice within 24h is interpreted as a positive result indicating the presence of ciguatoxins (sample therefore non-edible).

The limit of quantification determined by Lewis (Lewis and Sellin, 1993) is 0.5 nmol kg^{-1} or 0.56 µg P-CTX-1 kg^{-1} of flesh. The relationship "dose *vs* time-to-death" is used for quantification and mortality is expressed in MU (mouse units). For a mixture of ciguatoxins in fish, this relationship is calculated using the equation log (MU) = 2.3 log (1+1/T), where MU is the number of mouse units, but in this definition it corresponds to the LD_{50} (1 MU = LD_{50}, the dose proving lethal to half of the population of 20 g mice exposed) and T is the time between injection and the death of the mouse. The relationship log (1+1/T) was established by injecting concentrations of pure standard of 2.5 to 70 ng P-CTX-1 (0.45 -12.5 MU) into the local species of mouse (Lewis and Holmes, 1993). One mouse unit MU (LD_{50} for a 20 g mouse) is equivalent to 5 ng P-CTX-1 (Lewis *et al.,* 1991).

These notions of a dose/time-to-death relationship were also developed previously for the ciguatoxins in the Caribbean with determination of a minimum time-to-death (Vernoux, 1986). The work is of interest because, unlike the previous studies, it determines a minimum time-to-death (significant at p<0.1%) that can be achieved experimentally by injection of high doses at 5 or 10 minimum lethal doses (MLDs), such that the determination does not seem to depend on the degree of purity of the extracts injected (Vernoux and Talha, 1989). This enables quick-acting ciguatoxins, i.e. those with a minimum time-to-death t < 10 min, with early-onset hypersalivation and violent respiratory spasms leading to death, to be distinguished from slow-acting ciguatoxins with a t < 29 min with the same symptoms but later onset.

Annex 3 presents a detailed description of different variants of the mouse bioassay.

3.3.2 Assessment of the mouse bioassay protocols described

The main extraction steps of all these methods are very similar. They include an acetone extraction followed by two liquid/liquid purification steps using hexane and diethyl ether (or vice versa). Wong *et al.* (2009) propose a further purification on Florisil. Considerable quantities of solvent are used, the acetone extraction varying from a ratio of 3/1 (Lewis, 2003) to 1/1 (NRL). Washes with hexane are practised at least twice, as are most of the partitions using diethyl ether.

It is important to note that the quantity injected into mice can vary by a factor of 2.5, in terms of fish flesh per g of mouse injected. The increase of this factor is limited by interference from the lipid extracts that it generates. Table 5 (next page) summarises these variants in the analytical protocols.

The results of bioassays depend on the partially purified ether extracts injected into the mice and the toxicity responses of the latter. Both the quantity and quality of lipid residues (presence of other toxic compounds) may vary from one species of fish to another.

Table 5. Summary of different variants of the mouse bioassay

	NRL method	Vernoux 1994	Lewis 2003	Wong 2009
Cooking		70°C	70°C	70°C
Homogenate	100 g	50 g	100 g	100 g
Extraction	Acetone (2x100 ml)	Acetone (150 ml) and then acetone 80% (30 ml)	Acetone (2x300 ml)	Acetone (2x300 ml)
Evaporation	Acetone phase	Acetone phase	Acetone phase	Acetone phase
Re-dissolution	Extract dissolved in H_2O (→40ml)	30 ml H_2O dissolved in 10ml EtOH (→40ml)	Extract dissolved in MeOH 90% (→50ml)	Extract dissolved in MeOH 90% (→50ml)
1st liquid/liquid purification	2x160 ml diethyl ether	2x40 ml diethyl ether	2x50 ml hexane	2x50 ml hexane
Evaporation	Ether phase	Ether phase	Methanol phase	Methanol phase
Re-dissolution	20 ml MeOH 80%	25 ml MeOH 80%	50 ml EtOH 25%	50 ml EtOH 25%
2nd liquid/liquid purification	2x40 ml hexane	2x50 ml hexane	3x50 ml diethyl ether	3x50 ml diethyl ether
Evaporation	Methanol phase	Methanol phase	Ether phase	Ether phase
Re-dissolution	3-5 ml EtOH		chloroform–methanol (97:3)	chloroform–methanol (97:3)
Evaporation	Methanol phase		Dry extract weighed	Dry extract weighed
SPE Florisil 500 mg/3 ml				20-40mg deposit in hexane/acetone 4:1 Elution acetone / MeOH 7:3 (8 Vm) Evaporation
Solubilisation	1 ml Tween 60 1%	2 ml Tween 60 1%/saline	1 ml Tween 60 1-5%/0.9% saline	1 ml Tween 60 1-5%/0.9% saline
Intraperitoneal bioassay	Injection 0.5 ml/ mouse 2 mice (M) 18-22 g eq. 2.5 g fish flesh/g mouse	Injection 0.04 ml g^{-1} mouse 2 mice 18-24 g eq. 1 g fish flesh/g mouse	Injection 0.1-0.5 ml/mouse (i.e. 20 mg) 2 mice (M&F) 18-22 g	Injection 0.5-0.5 ml/mouse (i.e. 20 mg) 2 mice (F) 18-22 g
Interpretation	Monitoring symptoms, loss of weight and time-to-death	Monitoring symptoms and time-to-death Limit < 0.5 MUg g^{-1} eq. flesh	Monitoring symptoms and time-to-death Toxicity in MU	Monitoring symptoms and time-to-death Toxicity in MU

One mouse unit (MU) is defined as the minimum quantity of toxin capable of killing a mouse of 20 g in 24 hours after intraperitoneal (i.p.) injection.

One MUg or mouse unit-gram is defined as the amount of toxin that can kill at the most one gram of mouse in 24h, under conditions of injection designed to determine the minimum lethal dose (MLD) or the LD_{50}.

3.3.3 Advantages and disadvantages of the mouse bioassay

The advantages and disadvantages of the mouse bioassay as implemented by the French NRL and ARVAM as part of their investigations of outbreaks of ciguatera poisoning and import controls are as follows:

Advantages:

- can be used to establish the overall toxicity taking into account the various ciguatoxins potentially present; is especially suitable for use in toxicovigilance;
- on the basis of specific symptoms, it is possible to conclude that CTXs are present, and the relationship between the dose and the time-to-death of the mice can be used to calculate the concentration of CTXs in the sample;
- does not need complex instruments.

Disadvantages:

- low specificity;
- does not provide information on the identity of each ciguatoxin analogue present;
- is not sufficiently sensitive to detect concentrations of ciguatoxins considered to be of no risk to humans. The limit of quantification is 0.56 µg P-CTX-1 kg^{-1} of flesh (Lewis and Sellin, 1993). However, EFSA (2010) stated that the concentration expected not to exert effects in humans would be 0.01 µg eq. P-CTX-1 kg^{-1} of fish flesh;
- cannot be automated; it is long and requires a large amount of solvent;
- requires a large quantity of fish samples (because of its low sensitivity); this point is critical when it is necessary to confirm the implication of small species of fish in FBOs or when only small quantities of leftovers fish are available;
- requires specific installations (an animal supply facility) and only qualified and authorised staff may conduct experiments on animals;
- results vary between laboratories, partly due to the animals (species, sex, age, weight);
- no inter-laboratory validation because of the lack of commercially available standard materials. However, inter-laboratory comparison tests are planned between ARVAM and the NRL with samples of contaminated fish;
- for ethical reasons, many countries do not want to use bioassays on animals.

3.3.4 Application of tests on animals to samples of shark

The mouse bioassay is suitable for testing shark flesh. Regarding the liver, as sharks are leading predators, the presence of various pollutants of the marine environment or natural substances (such as vitamin A or squalene) in high concentrations can make extracts toxic for mice and distort the result (Hashimoto, 1979; Quod *et al.,* 2001). Tests with chicks could be envisaged, with the reservations listed above, and after eliminating the fat from the liver extracts, which contain large amounts.

3.4 The Neuro-2a cell test

3.4.1 Principle

The Neuro-2a cell test (on murine neuroblastoma cells) is used regularly for the screening of ciguatera toxins in fish. It relies on the ability of CTXs to bind voltage-gated sodium channels (Nav), resulting in an increase in the influx of sodium ions into the cell (Manger *et al*., 1995). The combined use of ouabain (which blocks the efflux of Na+ by inhibiting the ATP-dependent Na+/K+ pump) and veratridine (which blocks the voltage-gated Na+ channel in the open position) accentuates the influx of Na+, thus leading to cell death (i.e. they act as potentiators for the action of CTXs). The limit of quantification of the test was estimated to be 0.039 µg eq. P-CTX-1 kg^{-1} by Dechraoui *et al.* (2005) and 0.0096 µg eq. P-CTX-1 kg^{-1} by Caillaud *et al.* (2012).

Other cell lines may be used, but Neuro-2a cells are highly sensitive to the action of neurotoxins (saxitoxins, brevetoxins and palytoxins).

Neuro-2a cells cultivated in 96-well plates are exposed for 24h to a standard of CTX or extracts of fish. With the dose-response curve for a standard of CTX (for example, a pure standard of P-CTX),

the measurement of cellular mortality can be used to establish the quantities of CTX-equivalent present in the fish.

Prior to the test, the fish extracts must be verified for non-toxicity at the concentrations studied, in the absence of ouabain and veratridine. This preliminary verification aims primarily to characterise the maximum concentration of extract that can be tested and for which the death of Neuro-2a cells cannot be attributed to a matrix effect. This maximum concentration of extract is likely to vary depending on the biological matrices tested.

A more detailed description of the protocol is presented in Annex 4.

3.4.2 Advantages and disadvantages of the Neuro-2a cell test

Advantages:

- high sensitivity to CTXs; it can detect levels of CTXs of the same order of magnitude as those considered without risk to humans;
- can be used to establish the overall toxicity taking into account the various ciguatoxins potentially present, and to estimate the content in CTX equivalent;
- the model uses an immortalised and therefore stable cell line, which promotes analytical repeatability;
- multiple doses and repetitions possible in 96-well plates;
- relatively widespread and used in several laboratories.

Disadvantages:

- does not provide information on the identity of each ciguatoxin analogue present;
- not inter-laboratory validated;
- possibility of interference, depending on the nature of the matrices;
- also reacts to brevetoxins or other toxins that may bind site 5 of Nav.

3.4.3 Prospects for the application of the Neuro-2a test to samples of sharks

It should be possible to apply this test to samples of sharks without difficulty. Nevertheless, it will be necessary to check the absence of any matrix effect at the concentrations tested. The test can be used to assess the toxic potential of samples and to estimate the content in CTX equivalent of the standard used, but it cannot be used to identify analogues of CTXs present.

3.5 The Radioligand Binding Assay (RBA)

3.5.1 Principle

The radioligand binding assay (RBA) is a neuro-pharmacological test which was proposed for the first time in the 1980s by Poli *et al.* (1986), when it became possible to isolate voltage-gated sodium channels (Nav) from animal tissue.
This test is based on the affinity of specific ciguatoxins (CTXs) for site 5 of the α subunits of Nav (Lombet *et al*., 1987). This site is the receptor for two families of marine polyether toxins: CTXs and brevetoxins (PbTxs), synthesised respectively by the dinoflagellates *Gambierdiscus* spp. and *Karenia brevis* (Poli *et al.,* 1986; Lombet *et al.,* 1987; Dechraoui-Bottein, 1999). In the case of the detection of CTXs, the RBA measures the binding to this receptor of a radiolabelled toxin, tritiated brevetoxin ([^{3}H]PbTx-3), in competition with the non-radiolabelled CTXs, contained in the extract to be tested (Poli *et al.,* 1986; Lombet *et al*., 1987).

The binding activity of PbTxs and CTXs has been tested on various types of tissues (brain, cardiac muscle and skeletal cells) of various origins: human, rat, marine mammals, marine turtles or neural tissue of fish, but synaptosomes – rich in Nav – prepared from the brains of rats are most commonly used (Dodd *et al.,* 1981; Dechraoui-Bottein, 1999; Bottein Dechraoui and Ramsdell, 2003; Caillaud *et al.,* 2010 for review).

A more detailed description of the protocol is presented in Annex 5.

3.5.2 Advantages and disadvantages of the RBA

Advantages:

- can provide qualitative and quantitative estimates of the ciguatoxins potentially present in a biological sample. It is particularly well suited to the detection of CTXs in complex and varied biological matrices, such as the cells of *Gambierdiscus* (Chinain *et al*., 2010a), the liver and flesh of fish (Bottein Dechraoui *et al*., 2005; Darius *et al*., 2007; Chinain *et al*., 2010b), cyanobacteria, giant clams or sea urchins (Kerbrat *et al*., 2010; Pawlowiez *et al*., 2013);
- can be used to establish the overall toxicity taking into account the various ciguatoxins potentially present;
- high sensitivity: thus, by using the P-CTX-1 as standard, the limit of detection of the RBA for the fish matrix has been estimated at 0.065 µg eq. P-CTX-1 kg^{-1} and the limit of quantification at 0.13 µg eq. P-CTX-1 kg^{-1}. The RBA has also been calibrated with different standards of ciguatoxins (P-CTX-1, P-CTX-3C);
- can be used with raw or partially purified extracts;
- can be easily automated to allow a large processing capacity, which makes it the tool of choice in large scale monitoring programmes of ciguateric risk (Bottein Dechraoui *et al.,* 2005; Darius *et al.,* 2007; Chinain *et al*., 2010a, 2010b). The RBA can be practised in tube format (Lombet *et al*., 1987; Lewis *et al*., 1991; Dechraoui *et al*., 1999; Bottein Dechraoui *et al.*, 2005; Darius *et al*., 2007; Chinain *et al*., 2010a, 2010b), but the microplate format is also successfully applied to the detection of saxitoxins (STXs) and tetrodotoxins (TTXs) which bind site 1 of Nav (Barchi and Weigele, 1979; Van Dolah *et al*., 1994; Doucette *et al.,* 1997; Doucette *et al*., 2000; Llewellyn *et al*., 2001). For example, since the end of 2011, RBA conducted in microplate format has become one of the official methods for the detection of STXs (AOAC News, 2012).

Disadvantages:

- does not provide information on the identity of each ciguatoxin analogue present;
- also reacts to brevetoxins or other toxins that may bind site 5 of Nav;
- it would probably be difficult to generalise this test across a set of laboratories because of the regulatory constraints governing the possession and handling of radioactive substances (training for Personnel Competent in Radiation Protection, costs inherent to the management, decontamination and disposal of radioactive waste, etc.) as well as the difficulties and the cost related to the synthesis of radiolabelled brevetoxin;
- not inter-laboratory validated.

3.5.3 Prospects for the application of the RBA to samples of sharks

A recent study showed that it was possible today to perform RBA analyses through brevetoxins labelled with a fluorescent element, BODIPY® conjugated to PbTx-2 (McCall *et al.,* 2012). Fluorescent RBA should thus eventually make it possible to break free of all the constraints related to radioactivity and "democratise" this test in laboratories. For the time being, it has been applied to four different brevetoxin standards (PbTx-1, PbTx-2, PbTx-3 and PbTx-9) and no significant difference between the values obtained with the radioactive vs. fluorescent RBA has been observed (McCall *et al*., 2012). Additional studies are currently under way to try to apply this technique to the CTX standards in order to determine the specificity, sensitivity, repeatability and reproducibility of these tests, and confirm that the technique is well suited to a wide range of biological matrices (including shark flesh).

3.6 Immunological tests

3.6.1 Principle

Several teams around the world (American, Japanese and French) have conducted research on the immunodetection of ciguatoxins. The general principle behind immunological tests involves attaching a specific anti-toxin antibody to the antigen for the toxin of interest extracted from the sample and then revealing this attachment using a marker, such as an enzyme (e.g. peroxidase). The base reagent is an antibody produced by a laboratory animal following the injection of a toxin-protein compound, where the toxin behaves like a hapten.

The methods developed include radio-immunoassays (the antibody is labelled radioactively and the assay measures the number of disintegrations per second) and enzyme immunoassay tests (the dosage is based on the reaction of an appropriate substrate by an enzyme marker covalently bound to the antibody, which releases a coloured component whose optical density is measured by spectroscopy) including enzyme-linked immunosorbent assay (ELISA) methods on a solid support.

A more detailed description is presented in Annex 6.

3.6.2 Advantages and disadvantages of immunological tests

Advantages:

- high specificity regarding the toxin used as a hapten;
- fast, easy to implement, inexpensive;
- the operating principle could be applied for fast-throughput screening of samples and above all it could be used directly in the field by fishing professionals and amateur fishermen (in the form of ready-to-use kits for general sale);
- promising sandwich ELISA technique.

Disadvantages:

- at present, there are no antibodies (either polyclonal or monoclonal) capable of detecting whole molecules of CTXs. The only monoclonal antibodies reported in the literature are those directed against synthetic fragments of P-CTX-1, P-CTX-3C and 51-hydroxyP-CTX-3C (right and/or left part of the molecule) (Tsumuraya *et al*., 2006, 2010; Pauillac *et al.,* 2000);
- they do not provide information on the identity of each ciguatoxin analogue present (unless research currently under way leads to the development of monoclonal antibodies specifically targeting the analogues of reference CTXs: for example P-CTX-1, P-CTX-3C, C-CTX-1, etc.);
- not inter-laboratory validated. There have been two attempts to develop such a test: the Ciguatect® and the Cigua-Check®, but these kits were finally withdrawn from sale because of the lack of conclusive results, in particular because of the high percentage of false positives and false negatives (Dickey *et al*., 1994; Bienfang *et al*., 2011).

3.6.3 Prospects for the application of immunological tests to samples of sharks

Because of the absence of antibodies (polyclonal or monoclonal) specifically targeting the ciguatoxins of the Indian Ocean, it does not seem appropriate to consider the use of immunological tests to characterise the level of contamination of sharks in the Indian Ocean by ciguatoxins.

3.7 Physico-chemical methods

3.7.1 State of the art

Methods for chemical analysis include liquid chromatography with fluorescence detection or mass spectrometry. The methods described below are based exclusively on detection by mass

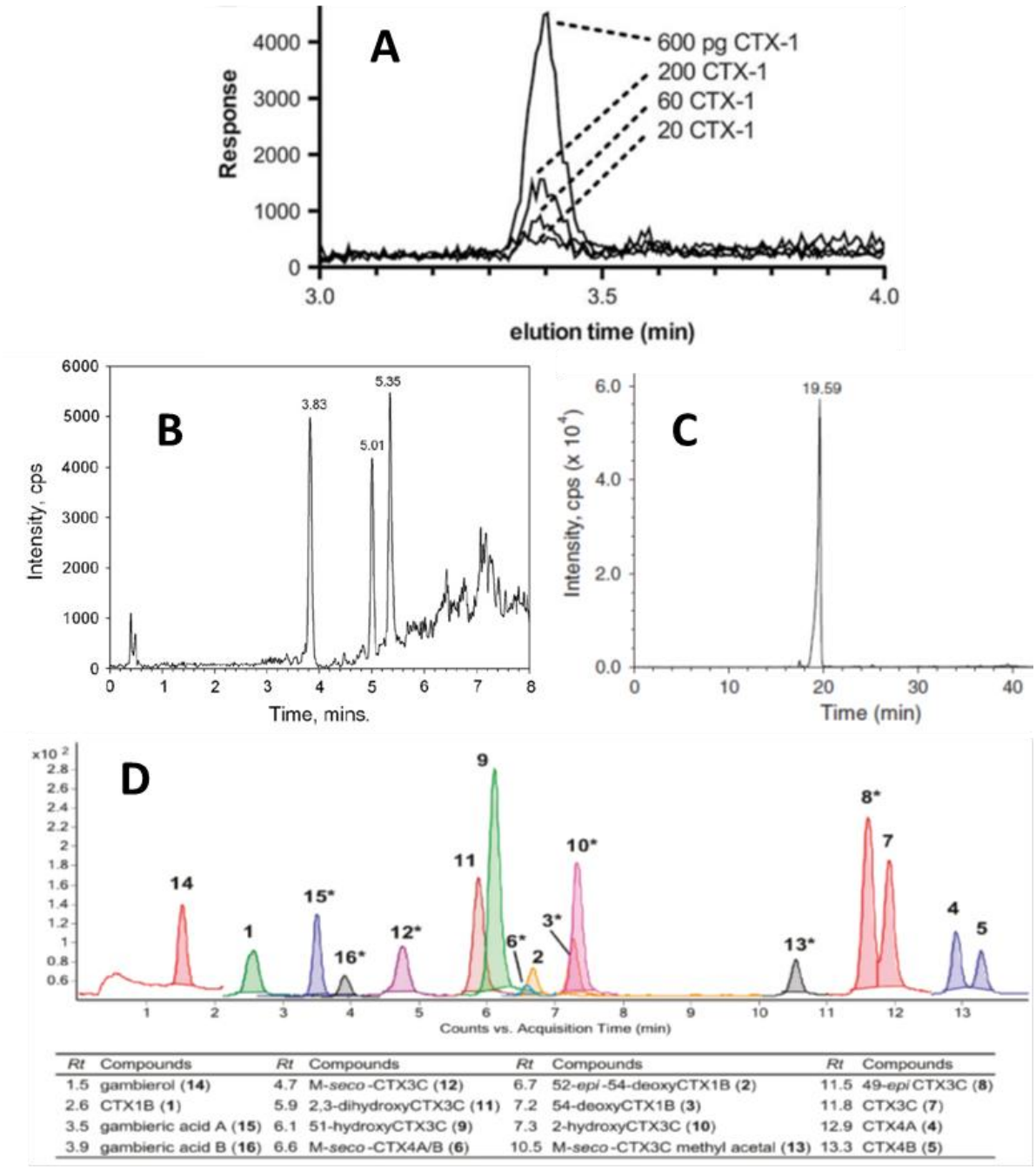
spectrometry (LC-MS/MS). Following separation by liquid chromatography, limits of detection and quantification by mass spectrometry are better than those for the method using fluorescence detection (EFSA, 2010).

To achieve acceptable limits of detection and quantification, these methods have so far focused on the detection of pseudo-molecular ion clusters, i.e. the transitions of pseudo-molecular ions to ions representing one or more losses of water.

For P-CTXs (from the Pacific), methods have been presented by Franco-Australian and Japanese teams (Lewis *et al.,* 1999, 2009; Stewart *et al.,* 2010; Yogi *et al.,* 2011), Figure 7 a), b) and d) next page.

The U.S. Food & Drug Administration has also developed a method for the detection of C-CTX-1 (from the Caribbean) (Abraham *et al*., 2012; Dickey, 2008), Figure 7c next page.

No method has been published for the quantification of I-CTXs (from the Indian Ocean) or carchatoxins.



Rt	Compounds	Rt	Compounds	Rt	Compounds	Rt	Compounds
1.5	gambierol (**14**)	4.7	M-*seco*-CTX3C (**12**)	6.7	52-*epi*-54-deoxyCTX1B (**2**)	11.5	49-*epi*CTX3C (**8**)
2.6	CTX1B (**1**)	5.9	2,3-dihydroxyCTX3C (**11**)	7.2	54-deoxyCTX1B (**3**)	11.8	CTX3C (**7**)
3.5	gambieric acid A (**15**)	6.1	51-hydroxyCTX3C (**9**)	7.3	2-hydroxyCTX3C (**10**)	12.9	CTX4A (**4**)
3.9	gambieric acid B (**16**)	6.6	M-*seco*-CTX4A/B (**6**)	10.5	M-*seco*-CTX3C methyl acetal (**13**)	13.3	CTX4B (**5**)

Figure 7. Separation and limits of quantification of recent LC-MS/MS methods for detecting CTXs of the Pacific and the Caribbean: (A) limits of detection and quantification (200 and 600 pg, respectively) by column separation according to Lewis *et al.,* 2009, (B) P-CTX-1 eluted at 3.83 min with a peak corresponding to 0.8 µg kg^{-1}, according to Stewart *et al*., 2010, (C) C-CTX-1 in fish (leftovers) peak corresponding to 1.6 µg kg^{-1} according to Abraham *et al.,* 2012, (D) separation of 16 compounds at approximately 1 ng ml^{-1} according to Yogi *et al*., 2011.

The applicability of the LC-MS/MS methods nevertheless remains very limited insofar as there is no standard readily available for the quantification of analogues found in fish. Only the Japanese distributor WAKO currently provides a non-certified standard solution of CTX-3C, the main toxin found in the Pacific strains of *Gambierdiscus*. The analogues found in fish, following the enzyme transformation of algal toxins, are only available through collaborative partnerships with expert laboratories (Richard Lewis, Australia; Takeshi Yasumoto, Japan; Mireille Chinain, Tahiti). None of these expert laboratories has been able to move on to certification of the purified analogues, mainly due to the small quantities of these toxins available, even in these expert laboratories.

A common weakness of LC-MS/MS methods is the fact that they are based on the detection of transitions corresponding to losses of water (Lewis *et al.,* 1999, 2009; Stewart *et al.,* 2010; Dickey,

2008) or the detection of transitions of sodium adducts (Yogi *et al.,* 2011). These transitions are very common for all natural compounds of the polyether or poly-hydroxy ether type. This observation has been demonstrated in many cases for lipophilic toxins such as okadaic acid (Pleasance *et al.*, 1990), azaspiracids (Rehmann *et al.*, 2008), or palytoxins (Suzuki *et al.*, 2013). Since this loss of water is not specific to CTXs, LC-MS/MS may only be used on semi-purified extracts, in order to give them a certain degree of specificity. **In the absence of a standard for defining the retention time and the molar response factors on a given mass spectrometer, LC-MS/MS cannot be used to guarantee either the identity or the quantity of ciguatoxins present.**

LC-MS/MS methods typically reach a limit of detection of approximately 0.03 µg eq. PCTX-1 kg^{-1} of fish flesh (Stewart *et al.*, 2010). Only the Japanese method recently submitted claims a limit of quantification of 0.01 µg kg^{-1}, a sufficient limit for legislation in the USA (Yogi *et al.*, 2011, US-FDA, 2011).

3.7.2 Advantages and disadvantages of physico-chemical methods

Advantages:

- high sensitivity to CTXs;
- the only technique capable of identifying toxins;
- quantification (in the absence of international standards, relative to a positive control specific to the laboratory).

Disadvantages:

- complex instrumentation;
- high cost of analysis;
- unsuited for processing multiple samples;
- not inter-laboratory validated.

3.7.3 Prospects for the application of physico-chemical methods to samples of sharks

As a result of the disadvantages presented above, it does not seem appropriate to consider the use of LC-MS/MS for analysing all samples of sharks, but this technique could be used to attempt to confirm the presence of known ciguatoxins if results are positive by other screening techniques.

3.8 Comparison of methods and proposed strategy for analysis

The objective is to propose an analysis strategy to ensure the health safety of fish for consumption. Positive reasons for choosing an analytical tool or a combination of tools capable of handling the concentrations considered to be of no risk to humans of specific CTXs in these species of fish, as well as the applicability of such tools, are presented below. This is preceded by a brief review of current data on risk assessment in these matters.

3.8.1 Current data on risk assessment, especially regarding concentrations considered to be of no risk to humans

In the Caribbean, since 1997, a concentration of 1 µg C-CTX-1 kg^{-1} of fish has been proposed as the limit for safe consumption (Vernoux and Lewis, 1997).

For the Pacific region, Lehane and Lewis (2000) estimated that a concentration of 0.01 µg P-CTX-1 kg^{-1} of fish flesh would have no effect on consumers (based on the assumption of a meal of 500 g of fish, of a factor of interindividual variability of 10 and a minimum dose causing effect of 1 ng P-CTX-1 kg^{-1} b.w.).

Dickey (2008) presented the analysis of more than 100 cases of ciguatera poisoning in the United States between 1998 and 2008 associated with Pacific and Caribbean fishing regions. According to this study, the concentration in fish likely to cause adverse effects in consumers is approximately

0.10 µg eq. P-CTX-1 kg^{-1} (Dickey, 2008) or, after conversion, 0.23 µg eq. P-CTX-3C kg^{-1} (Darius *et al.*, 2013). Dickey and Plakas (2010) proposed guideline values of 0.1 µg eq. C-CTX-1 kg^{-1} for fish caught in tropical regions of the Atlantic, the Gulf of Mexico and the Caribbean, and of 0.01 µg eq. P-CTX-1 kg^{-1} for fish from the Pacific Ocean.

In an opinion published in 2010, experts from EFSA (European Food Safety Authority) were unable to propose a toxicity reference value because of limited experimental and epidemiological data, but nevertheless concluded that a concentration of 0.01 µg eq. P-CTX-1 kg^{-1} is expected not to exert effects in sensitive individuals.

The US-FDA (2011) has also determined a guidance level of 0.01 µg eq. P-CTX-1 kg^{-1} for toxins in the Pacific and 0.1 µg eq. C-CTX-1 kg^{-1} for toxins in the Caribbean.

Although the European Regulation (EC) No 854/2004 of 29 April 2004 states that *"checks [by the competent authority] are to take place to ensure that [...] fishery products containing biotoxins such as ciguatera or other toxins dangerous to human health [...] are not placed on the market"*, to date the EU has not established a regulatory limit or defined any reference analytical method(s) applicable to CTXs.

In France, ciguatera poisoning is one of the FBOs (foodborne illness outbreaks) for which declaration is mandatory. The protocol for managing outbreaks of ciguatera poisoning in the French Antilles, updated in 2013 by the DGAL, is based on the observation of the clinical syndrome characteristic of ciguatera poisoning. This syndrome associates digestive signs (nausea, vomiting, abdominal pain, diarrhoea), neurological signs (hyperaesthesia, paraesthesia, dysaesthesia) and skin (pruritus), cardio-vascular and respiratory signs of varying intensity.

3.8.2 Comparison of the methods described

After consideration of a few points common to most of these methods, they will be compared on the basis of their advantages and disadvantages listed earlier.

3.8.2.1 Points in common

▸ *Considerations on the extraction of CTXs from the flesh of sharks (and possibly from other tissues)*

The extraction of fish tissue for subsequent examination for the presence of CTXs requires the adoption of protocols that ensure:

- a high rate of recovery of CTXs from the different tissues, minimising possible losses;
- the elimination of undesirable substances that could interfere with the execution of analyses or testing.

Several protocols for the extraction of CTXs in fish samples are described in the literature. The choice of these protocols and the need to adopt possible changes may depend on the nature of the tissues to be extracted as well as the analyses or tests to be performed. As regards CTXs in shark flesh, a first approach could be the adoption of conventional protocols taking into account the fatty nature of the shark flesh matrix. To eliminate the fat, samples could undergo extra washing with hexane, or the second extraction could be performed with acetone and 20% water. Protocols could also be adapted to treat samples that are too fatty, as is the case of shark liver. After cooking and subsequent cooling, for example, solidified supernatant fats could be eliminated as a last resort, since ciguatoxins do not accumulate in the fatty sections, unlike lipophilic contaminants such as certain pesticides (Vernoux, 1986).
However, fats are not the only sources of interference encountered in some of the tests mentioned (RBA and Neuro-2a, in particular). On the basis of the experience of the members of the WG, it is often necessary to supplement the liquid-liquid partition step with a solid phase extraction (SPE) step.

▸ *Considerations concerning CTX standards*

Another critical element is the lack of CTX standards, as noted during the most recent training session organised by the EU reference laboratory for marine biotoxins in Vigo (2-3 May 2013). A consequence of this lack of standards, for all methods, is that it is impossible to:

- calculate and verify the recovery rate;
- validate these methods by organising inter-laboratory tests.

However, partnerships should make it possible to determine qualitatively whether the P-CTX-1, P-CTX-3 or C-CTX-1 analogues are present in samples of sharks, with much work still to be done on I-CTXs.

3.8.2.2 Points specific to the different methods

The mouse bioassay provides overall toxicity, based on the animal's biological response to toxins, with the advantage of presenting a characteristic clinical picture. But it has major disadvantages, especially in terms of sensitivity and specificity: the limit of quantification is 0.56 µg P-CTX-1 kg^{-1}, whereas the concentration considered to be of no risk to humans is 0.01 µg eq. P-CTX-1 kg^{-1} of fish flesh (EFSA, 2010; US-FDA, 2011). For C-CTX-1, the guideline value is 0.1 µg eq. C-CTX-1 kg^{-1} of fish flesh (Dickey and Plakas, 2010; US-FDA, 2011) however, the limit of quantification of the mouse bioassay is about 5 µg of C-CTX-1 kg^{-1} (estimated value using the existing factor of 10 between the LD_{50} in mice of 0.35 µg eq. P-CTX-1 kg^{-1} b.w. and 3.6 µg eq. C-CTX-1 kg^{-1} b.w. in mice given by Pottier *et al.,* 2001).

The Neuro-2a cell test and the RBA also provide overall toxicity and appear ethically more acceptable than the mouse bioassay (although it remains necessary to sacrifice animals to obtain receptors for the RBA, the number of animals is limited, and the animals are not exposed to toxic effects), but these methods are more complex to implement.

The Neuro-2a test provides high sensitivity, with a limit of quantification of 0.0096 µg eq. P-CTX-1 kg^{-1} (Caillaud *et al.,* 2012). It can be used to analyse multiple samples. The US-FDA uses it in combination with LC-MS/MS in the event of positive results (Friedman *et al.,* 2008).

The RBA test can be used with raw or partially purified extracts. It is easy to automate to provide a large processing capacity, but requires the handling of radiolabelled products. Studies have shown a good match between the values of RBA and those obtained by mouse bioassay, Neuro-2a cell tests and the chemical HPLC/MS method as regards the detection of PbTxs and CTXs (Dechraoui et al., 1999; Pottier et al., 2003; Bottein et al., 2005; Bottein et al., 2007).

Analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) is increasingly used to identify CTXs in fish implicated in foodborne illness outbreaks (Caillaud *et al.*, 2011; Hamilton *et* al., 2002; Hamilton *et a*l., 2010; Lewis and Jones, 1997; Lewis *et al*., 1999, 2009; Pottier *et al*., 2002; Stewart *et al*., 2010). This method, which is both sensitive and specific, has a limit of quantification of 0.03 µg P-CTX-1 kg^{-1}. It is particularly interesting for concentrations below the limit of detection of the mouse bioassay (Wong *et al.*, 2005). However, it remains difficult to apply in large-scale monitoring programmes requiring the processing of a large number of samples and/or the participation of many laboratories.

3.8.3 Prospects and proposal of analysis strategy

Any system to determine the safety of foodstuffs in the light of their concentration in chemical contaminants must take into account, not only feasibility criteria and knowledge at time t, but also the concentration considered to be of no risk to humans and the performance of the analytical method in terms of specificity, sensitivity, robustness and repeatability.

A consideration of the advantages and disadvantages of the methods described in this report shows that while the mouse bioassy can detect overall toxicity and is simple to implement, it has a limit of quantification (approximately 0.5 µg eq. P-CTX-1 kg^{-1} and 5 µg eq. C-CTX-1 kg^{-1}) that is insufficient as regards concentrations of CTXs considered to be of no risk to humans (0.01 µg eq. P-CTX-1 kg^{-1} and 0.1 µg eq. C-CTX-1 kg^{-1}) by EFSA (2010) and US-FDA (2011).

Inversely, LC-MS/MS can achieve a degree of sensitivity close to the concentration considered to be of no risk to humans, 0.01 µg eq. P-CTX-1 kg^{-1}, but it is still a sophisticated technique requiring a high level of expertise, and is difficult to apply when a large number of samples need to be processed in a short time, and by many laboratories. This technique appears more suited for use as a method of confirmation to try to identify known toxins and characterise the toxin profile.

Between these two strategies, the cell toxicity test could eventually become an excellent candidate as a reference test for the detection of CTXs (subject to inter-laboratory validation), as Caillaud *et al*. (2012) obtained a limit of quantification of 0.0096 µg P-CTX-1 kg^{-1}. This test therefore offers greater sensitivity than the RBA (whose limit of quantification is 0.13 µg eq. P-CTX-1 kg^{-1}). Finally, because it can be adapted for the detection of a wide range of marine biotoxins such as ciguatoxins, maitotoxins, palytoxins, saxitoxins, brevetoxins, etc. (Pawlowiez *et al.,* 2013), it is being used increasingly by laboratories concerned with the issue of marine toxins. As regards the implementation of the extraction protocols for subsequent assessment of CTX-like responses by cell tests, if matrix effects are encountered, changes could be proposed.

To summarise and in conclusion, in order to determine the health status of a sample of shark in relation to ciguatera risk, it is recommended that a combination of the following techniques be used:

- a mouse bioassay, to find any overall toxicity in the sample;

- a cytotoxicity test on Neuro-2a cells and/or a test on receptors, which both have higher specificity and greater sensitivity than the mouse bioassay;

- an analysis by LC-MS/MS, to try to confirm the presence of known ciguatoxins if one of the above methods gives positive results.

The data thus produced could then be in a health risk assessment for consumers.

3.9 Comments by the WG concerning the results of analyses of tiger and bull sharks caught off Reunion Island in 2012 and 2013

In order to acquire data concerning the contamination of two species of shark (tiger shark *Galeocerdo cuvier* and bull shark *Carcharhinus leucas*) by ciguatoxins, the services of the Prefecture of Reunion Island launched a sampling campaign in 2012 and 2013 which supplied 12 specimens per species for the detection of ciguatoxins by mouse bioassay. This sampling campaign was extended in 2013 with a goal of 45 additional specimens per species.

To date, 24 specimens (12 per species) have been analysed for ciguatoxins by mouse bioassay (table 6) and five of them have also been analysed for heavy metals (lead, cadmium and mercury). The results of these analyses were transmitted to ANSES in the framework of the Request from the DGAL.

Table 6. Results of the analyses by mouse bioassay for the detection of ciguatoxins in the flesh of 24 shark specimens caught off Reunion Island in 2012 and 2013 (modified CAT 10 method, ANSES Maisons-Alfort)

N° échantillon	Espèce	Date pêche	Longueur Totale (LT, cm)	Longueur Fourche (LF, cm)	Sexe	Ref ARVAM	Résultat
001-140812	*Galeocerdo cuvier*	15-août-12	310	251	M	13/15	Négatif
001-03112012	*Carcharhinus leucas*	1-nov-12	285	234	M	13/18	Négatif
001-15112012	*Galeocerdo cuvier*	15-nov-12	362	295	M	13/16	Négatif
002-15112012	*Galeocerdo cuvier*	15-nov-12	308	261	F	13/17	Négatif
001-22112012	*Galeocerdo cuvier*	22-nov-12	321	270	M	13/19	Négatif
002-22112012	*Galeocerdo cuvier*	22-nov-12	384	312	F	13/20	Négatif
001-13122012	*Galeocerdo cuvier*	13-déc-12	323	265	F	13/21	Négatif
002-13122012	*Galeocerdo cuvier*	13-déc-12	338	290	F	13/22	Négatif
003-13122012	*Galeocerdo cuvier*	13-déc-12	285	235	F	13/23	Négatif
004-13122012	*Galeocerdo cuvier*	13-déc-12	289	241	M	13/31	Négatif
001-03042013	*Galeocerdo cuvier*	3-avr-13	390	325	F	13/32	Négatif
001-23052013	*Galeocerdo cuvier*	23-mai-13	294	239	M	13/40	Négatif
002-23052013	*Galeocerdo cuvier*	23-mai-13	325	266	F	13/41	Négatif
001-06062013	*Carcharhinus leucas*	6-juin-13	285	241	F	13/33	Négatif
002-06062013	*Carcharhinus leucas*	6-juin-13	214	178	M	13/34	Négatif
003-06062013	*Carcharhinus leucas*	6-juin-13	220	187	M	13/35	Négatif
001-10062013	*Carcharhinus leucas*	10-juin-13	232	197	M	13/36	Négatif
001-11062013	*Carcharhinus leucas*	11-juin-13	231	190	F	13/37	Négatif
001-19062013	*Carcharhinus leucas*	19-juin-13	325	275	F	13/38	Négatif
001-27062013	*Carcharhinus leucas*	27-juin-13	273	221	M	13/39	Négatif
001-03072013	*Carcharhinus leucas*	3-juil-13	223	190	M	13/42	Négatif
001-05072013	*Carcharhinus leucas*	5-juil-13	308	258	F	13/43	Négatif
001-11072013	*Carcharhinus leucas*	11-juil-13	260	220	F	13/44	Négatif
001-18072013	*Carcharhinus leucas*	18-juil-13	317	267	F	13/45	Négatif

Note contained in the analysis report: The known symptoms of carchatoxins injected into mice are: paralysis of the limbs, dyspnoea, convulsions, diarrhoea and mortality by respiratory arrest in 4h; beyond this period the animals recover (*Boisier et al.*, 1995). These symptoms were not observed in the 24 samples tested. However, certain atypical signs (brief diarrhoea) were observed for all samples of *Carcharhinus leucas* (not observed for the samples of *Galeocerdo cuvier*). This could be the result of a matrix effect. The absence of positive matrix (in-house or commercial) and the low sensitivity of the mouse bioassay makes it impossible to be more precise as to this specific point.

In the light of the evidence presented earlier in the report, although the mouse bioassay provided a negative result for the 24 samples of shark flesh analysed, it was not possible to conclude with certainty that these samples were not contaminated by toxins at levels that could present a risk to the health of consumers. This test is not sufficiently sensitive to detect concentrations of ciguatoxins considered to be of no risk to humans.

Further analyses by cytotoxicity tests on Neuro-2a cells and/or receptor tests, as well as by LC-MS/MS, would provide the necessary data.

ANSES therefore contracted a research and development agreement (RDA) with ARVAM (Agency for Research and Marine Exploitation, Reunion Island), in collaboration with IRTA (*Instituto de Investigación y Tecnología Agroalimentaria*, Spain) for these samples of shark flesh from Reunion Island to be analysed by cytotoxicity tests on Neuro-2a cells. The final report was submitted to ANSES on 21 July 2014.

The results did not show ciguatoxin-like toxins to be present above the limit of detection of 0.04 µg eq. P-CTX-1 kg^{-1} of flesh. It should be noted that the detection limit is higher than the concentration considered to be of no risk to humans of 0.01 µg eq. P-CTX-1 kg^{-1} of fish flesh (EFSA, 2010; US-

FDA, 2011). This high limit of detection is mainly due to the matrix, as this was the first time that this type of sample had been studied in the laboratory.

The RDA also included samples from the shark implicated in an outbreak of food poisoning that occurred in Madagascar in November 2013 (124 people intoxicated, nine of whom died) for analysis by mouse bioassay and by cytotoxicity tests on Neuro-2a cells. Genetic analysis of the shark involved concluded that it was a bull shark. The sample of flesh gave a positive result by mouse bioassy, with symptoms (prostration, dyspnoea, cyanosis, convulsions and death by respiratory arrest) typical of those known for carchatoxins (Boisier *et al*., 1995). The analysis of a sample of flesh, a sample of stomach and three samples of dried fin by cytotoxicity tests on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated as follows:

- Flesh: 0.144 µg eq. P-CTX-1 kg^{-1} (i.e. 14 times the concentration considered to be of no risk to humans);
- Stomach: 114 µg eq. PCTX-1 kg^{-1} (i.e. 11,400 times the concentration considered to be of no risk to humans);
- Dried fin: 0.145 µg eq. PCTX-1 kg^{-1}; 0.158 µg eq. PCTX-1 kg^{-1}; 0.737 µg eq. PCTX-1 kg^{-1} (i.e. 14 to 74 times the concentration considered to be of no risk to humans).

3.10 Points regarding the ethology of tiger and bull sharks in the seas around Reunion Island

Reminder of the content of Question 2:

In the event that ANSES should identify a sufficiently reliable method for testing shark flesh for ciguatoxins, what data would be necessary to carry out this evaluation and what recommendations could be made regarding the protocol for sampling tiger and bull sharks around Reunion Island? Particular consideration shall be given to the geographical area concerned and the ethology of these two shark speciesin terms of the extent of their movements in the seas around Reunion Island.

Summary of the preliminary results of the acoustic tagging of tiger and bull sharks, from data obtained from December 2011 to September 2013, as part of the CHARC programme

The CHARC programme (on the ecology and habitat of two species of coastal sharks along the West Coast of Reunion Island) was designed to collect information on the behaviour of tagged individuals to define the various habitats of bull and tiger sharks based on the time spent, and their movements, in this area. In collaboration with the CRESSM (Regional Committee for Studies and Sports in the Underwater Environment), three fishermen on the west coast of the island, and the association *Squal'idées* and its diving and tagging teams, the scientific team of the French Institute of Research for Development (IRD) deployed 49 listening stations and attached coded tags to 81 sharks: 39 bull sharks and 42 tiger sharks. The study area currently extends from the Port up to the city of Saint-Pierre with two additional stations at the exit points from the ports of Sainte-Marie and Sainte-Rose.

These behavioural observations were supplemented by two studies concerning the trophic ecology of the sharks studied, and the genetics of populations across the western Indian Ocean. The purpose of the study of trophic ecology is to characterise the diet by analyses of stomach contents and to identify *i*) the sources of production on which the individuals and/or species depend and *ii*) the habitat in which they feed on a regular basis. These sources are identified by a comparative analysis of the isotopes of carbon, nitrogen, and sulfur, and also of the heavy metals contained in samples taken from the environment (water, sediment and prey) and from the sharks themselves (muscle and blood). The study of the population genetics of the targeted shark species provide information on the genetic diversity of populations (speed of reproduction, inbreeding) and on the differentiation of populations (gene flows, effective dispersion within the Indian Ocean). To date, few samples have been collected.

3.10.1 The tiger shark (*Galeocerdo cuvier*)

The first results concerning tiger sharks show that little time is spent in the study area (2% of the data set). The tagged sharks were detected near three fish aggregating devices further out to sea, accounting for 26% of the total time during which these sharks were present in the network of stations. These results indicate that tiger sharks around Reunion Island occupy a habitat further from shore than the area covered by most of the listening stations.

It may be important to note the case of one female sub-adult tiger shark, 3 metres in total length, tagged at Reunion Island on 6 December 2012, where the island shelf drops steeply at the spot known as the *Sec de Saint-Paul*, 3 km off the coast, which was caught 9 months later on 28 August 2013 at Morombé on the west coast of Madagascar, a hundred kilometres north of Tuléar. In about 9 months, this individual had therefore travelled at least **1800 km** crossing the southwest part of the Indian Ocean between the two islands. **This result suggests that the tiger shark occupies a very large biogeographical area, at the scale of the Indian Ocean**.
These results are in agreement with the data from the literature on the spatial occupation of tiger sharks and their ability to travel over long distances (Heithauss *et al*., 2002; Meyer *et al.,* 2009). Thus, in a similar programme, Werry *et al*. (2014) observed the movements of 33 tagged tiger sharks in the Western Pacific zone (between New Caledonia and Australia) between 2009 and 2013. Considerable diversity was observed in the movement profiles, some (14 individuals) travelling up to 1141 km while others (especially the young, but also one adult) seemed to prefer staying within a given area.

The tiger sharks of Reunion Island therefore seem to be part of an open population at the scale of the Indian Ocean (interacting with other populations of sharks of the same species).

The study of the stomach contents of tiger sharks showed a high rate of stomachs that had either ejected their contents or were empty (58%). Those with contents showed a great diversity of prey. These tiger sharks had consumed sea birds, cephalopods and crustaceans but they were predominantly fish-eating, which corresponds to the literature. The isotope values measured show that the tiger shark essentially depends on coastal sources of production. Nitrogen values did not differ significantly from those of giant trevallies (*Caranx ignobilis*), dolphinfish (*Coryphaena hippurus*), yellowfin tuna (*Thunnus albacares*), skipjack tuna (*Katsuwonus pelamis*) and coastal dolphins (*Stellena longirostris* and *Tursiops aduncus*), which indicates that these species occupy similar trophic positions and that they consume prey at the same trophic level. These species are therefore potentially in competition with tiger sharks, although the yellowfin tuna and skipjack consume more crustaceans compared to the other species. Deep-dwelling fish have higher nitrogen values than those found in tiger sharks. Given the small size of these species as compared to sharks, it seems more likely that these species live in water masses whose base lines in nitrogen are higher than the surface waters. This result indicates that tiger sharks do not feed regularly and significantly at depth, but in surface waters (<150 m), which is corroborated by the records of the vertical profiles obtained on a few tagged sharks. It therefore seems likely that tiger sharks feed on small fish, where the island shelf drops steeply at the "*Sec de St Paul*" or *St Gilles*.

An analysis of trace elements did not suggest any clear predator-prey relationship. The values measured in the sediments show no significant difference between the various stations along the west coast of the island, and do not make it possible to identify the preferred feeding habitats of tiger sharks.

3.10.2 The bull shark (*Carcharhinus leucas*)

The first results for bull sharks show that they are not present on a permanent basis in the study area around Reunion Island. Most seem to explore the 80 km of coastline where the network of listening stations is deployed, and some explore the entire island and beyond. They usually only come close to the coast briefly, sporadically and mostly at night. They mainly approach the coast in the late afternoon (3pm to 5pm, local time) and at nightfall. They are present more frequently in winter than in summer but also, on a few sites, mainly during the transition from winter to summer or from summer to winter. They are most frequently found at three sites off the western coast: in the bay of St Paul, in the seas off St Gilles and Etang du Gol and off Sainte-Marie in the north. Their more frequent presence along the coasts in winter could be linked either to search-and-selection feeding behaviour or to reproductive behaviour. The small number of detections and the behaviour of these animals do not indicate an overabundance of individuals but occupation of the habitat that varies depending on whether they encounter favourable or unfavourable environmental conditions. More than the number of sharks present, it would seem to be the mode of occupation of the environment and the environmental constraints that lead some bull sharks to spend time along the coast. We know something of which environmental conditions are favourable to bull sharks: high turbidity, brackish water and water loaded with organic matter. Other factors, such as temperature, ocean swell or the quantity of prey, are being investigated in the framework of the CHARC programme. The data concerning these factors were collected and their analysis is under way.

Regarding the study of the movements of bull sharks, a male shark of approximately 3 metres in total length was followed in its movements over a large area for 6 months by an external satellite tag (MiniPAT, by Wildlife Computers) attached to the rear of the base of the first dorsal fin. The results of the analysis indicate that this shark left Reunion Island shortly after being tagged, to explore an area approximately 300 km to the south west of Reunion Island, not far from an area of shallows known to offshore fishermen, then returning closer to the island (still about 100 km south-southwest of Reunion Island) before setting off for the north about 300 km from the island, not far from another area of shallows also known to fishermen. Finally, it returned to the coast of the island at the end of the period of observation (Figure 8). This individual therefore seems able to travel long distances in the ocean environment and to stay away from the coast. This result had already been obtained during a similar experiment on bull sharks in the Fiji Islands in 2004

(Brunnschweiler *et al.,* 2010). This species therefore seems able to visit the pelagic zone and swim in very deep waters. The depth of the seabed is no barrier to its movements. Bull sharks are therefore perfectly capable of leaving the island and returning to it.

Studies of the stomach contents of bullsharks showed a high rate of stomachs either turned inside out (to eject the contents) or that were empty (46%). Those with contents showed little diversity of prey. The bull shark is predominantly fish-eating, consuming mostly large-sized fish (>30 cm), which concurs with the literature. The results of the isotope values and trace elements of bullsharks and of their prey are not yet available.

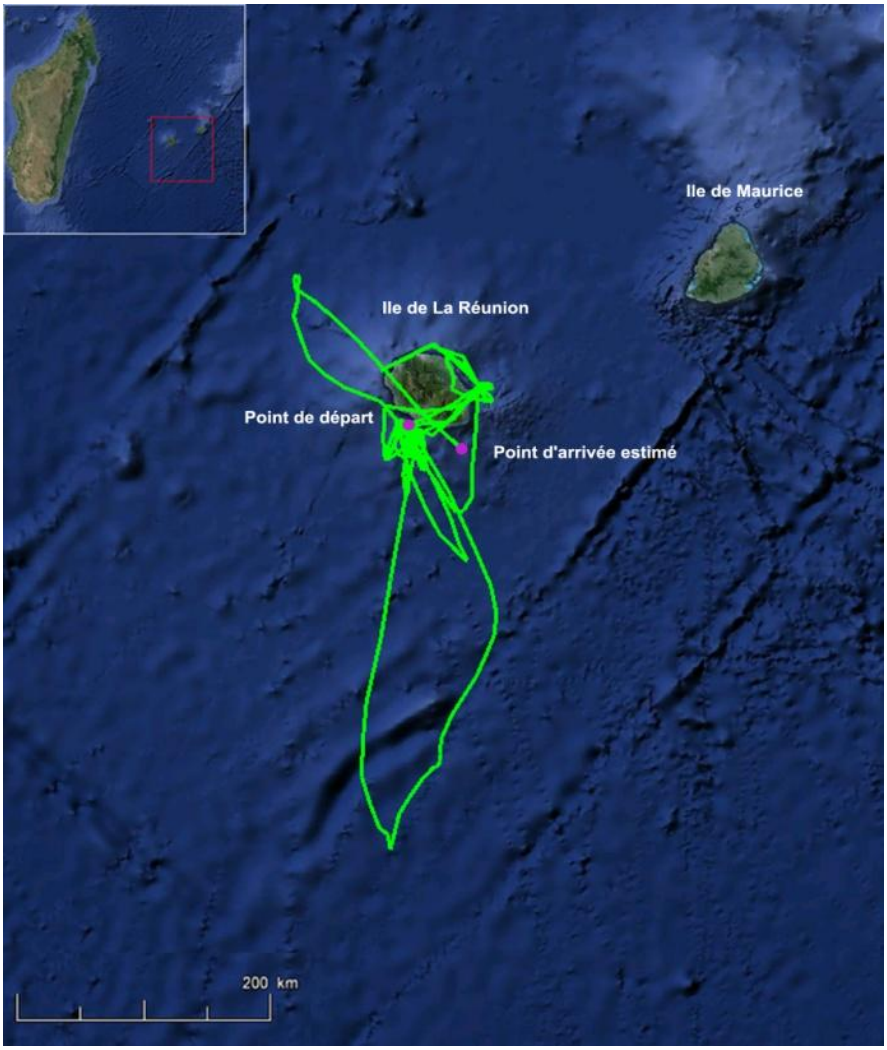


Figure 8. Estimated itinerary of a male bull shark between 15 March and 6 September from light-level readings recorded by the sensor of the MiniPAT tag attached to the fin of the shark. (NB: Journeys apparently over land are obviously impossible and only due to the imprecision of the measurement.)

3.10.3 Summary and prospects

In order to specify the ecological data that would be necessary to follow the dynamics of bioaccumulation of ciguatoxins (or carchatoxins) in the food chain, it is important to distinguish between multiple sources of variability depending on whether one is working at the scale of the individual or of the population.

At the scale of the individual, the level of contamination depends particularly on the feeding behaviour of sharks. Sharks are apex predators (predators at the top of the food chain) and can therefore become heavily contaminated through bioaccumulation (Rand *et al.,* 1995). Tiger and bull sharks are opportunistic species, capable of colonising a very wide range of habitats and of feeding on different species, but they can also feed exclusively on a single species as long as it is abundantly available (Stevens *et al.,* 1991; Snelson *et al*., 1995). More specifically, tiger and bull

sharks around Reunion Island are primarily fish-eating and, according to the initial results of the CHARC programme, they feed more on coastal fish than on pelagic or deep-sea species. These data will need to be refined, but when more is known about the origin and the location of sources of contamination, they will help to better understand the variability observed in the processes of bioaccumulation and contamination at the scale of individuals.

At the scale of the population, the level of contamination depends on the number and location of the sites of contamination, and on the size and structure of populations of the two targeted shark species(degree of connectivity between populations or sub-populations of sharks in the Indian Ocean, fragmentation of habitat of the different species, rates of exchange between these populations). To date, the initial results of the CHARC programme have shown that the tiger shark makes extensive journeys over the ocean and that the bull shark is capable of visiting the ocean waters several kilometres from Reunion Island. **The size of the populations around Reunion Island is unknown and difficult to estimate** because there is insufficient information about fishing catches. The rate of travel over long distances and the importance of this rate in the turn-over of local populations around Reunion Island and in other areas where the presence of sharks has been noted (such as Madagascar, South Africa and the Seychelles) are unknown. This rate may be very low if the events observed in the CHARC programme (a tiger shark tagged at Reunion Island caught in Madagascar, a tagged bull shark travelling several kilometres from the island) are rare and were only observed by chance. The reasons for these movements (food or reproduction) are also unknown. The tiger shark caught in Madagascar was found in a fairly large estuary, known to be a nursery area for this species. Had this female gone to give birth in this area or to find favourable feeding conditions? When the male bull shark left the island it moved towards areas of shallows known to fishermen for their greater abundance of fish. Was this shark following a migration route taking it to these feeding sites, or did it arrive there by chance?

More tagging data will be required to answer these questions. Data from other countries of the Indian Ocean rim will provide more information about the movements of these species. A study focused on the biology of these species and particularly on the genetics of populations of tiger and bull sharks at the scale of the western Indian Ocean would also be a promising area of research. The data collected principally at Reunion Island are currently insufficient but a larger programme at the scale of the Indian Ocean would provide information about the sites and periods of egg-laying and reproduction periods and about the genetic diversity of shark populations (speed of reproduction, inbreeding) and the differentiation of populations (gene flows, effective dispersion within the Indian Ocean). A kinship analysis of the samples from Reunion Island will identify the links between individuals (presence of father/mother, brother/sister, half-brother/half-sister), to identify gregarious behaviour and to study the philopatry (tendency of some individuals to remain in or return to the place where they were born). It will then be possible to deduce the extent to which these species remain faithful to some breeding sites, which will help provide crucial information for the determination of the spatial scale at which the sampling strategies must be implemented.

Concerning these sampling strategies and in particular the protocol for sampling tiger and bull sharks around Reunion Island, if we knew the percentage of individuals of the total population that were contaminated and the number of individuals making up the total population, it would be possible to estimate, from laws of probability (such as hypergeometric distribution), the number of individuals to be sampled in order to have a good or very good probability of capturing at least one individual infected by ciguatoxins (or similar toxins).

At present, in the absence of any estimate of the populations of tiger and bull sharks in the seas around Reunion Island, it is not possible to recommend a protocol for sampling sharks by which to assess the risks related to a possible authorisation of these species for human consumption, as regards the ciguatera risk.

4 Prospects - research needs

Within the strict framework of the questions raised by the Request, the WG identified research needs relating to:

1) the development of diagnostic tools for detecting the presence of ciguatoxins (or similar toxins, such as carchatoxins) in sharks, and a plan of action in case of poisoning by consumption of shark, which will involve:
   - establishing protocols for sample collection (for example, leftover of meals, fish samples taken in the same area) and any other useful information for the study of toxins in the tissues of sharks responsible for food poisoning in the area of the Indian Ocean;
   - setting up a network of laboratories and a coordinating body, in order to respond promptly in cases of ciguatera-type foodborne illness outbreaks;
   - developing reliable methods for identifying and quantifying CTX-like toxins in different shark tissues:
     - optimising extraction protocols;
     - identifying the optimum combination of methods from among mouse bioassay, the Neuro-2a test, RBA and LC-MS/MS analysis;
     - establishing the limits of quantification of the mouse bioassay, the Neuro-2a test and the RBA test;
     - in the event that toxins other than CTXs are found in sharks (carchatoxins or other toxins), developing the protocols for purification and detection specific to these toxins.
   - obtaining reference samples of I-CTXs, C-CTXs, P-CTXs and where appropriate of carchatoxins (different fish/sharks as source), tools necessary for the laboratory network and for the identification of toxins (see following points);
   - launching a programme for validating interlaboratory methods and exercises.
2) identifying I-CTXs or similar toxins (carchatoxins) in sharks in the Indian Ocean, which will involve:
   - setting up a plan for the collection of samples of sharks;
   - characterising and quantifying the I-CTXs or similar toxins (carchatoxins) in these specific populations of sharks;
   - isolating the toxic fractions (for example by bioassay-guided fraction) and purifying these fractions to identify the toxins; high-resolution mass spectrometry could contribute considerably to these studies;
   - studying the distribution of these toxins in the different tissues of sharks (flesh, liver).
3) studying the toxicity of I-CTXs or similar toxins (carchatoxins):
   - studying the acute toxicity of purified I-CTXs or similar toxins (carchatoxins) by the intraperitoneal and oral routes in mice;
   - studying their bioavailability, their tissue distribution and their metabolism in mice.
4) determining the origin and the transfer of I-CTXs or similar toxins (carchatoxins) in sharks:
   - carrying out ecological studies of the distribution and movements of sharks:
     - at the individual level, improving understanding of the feeding behaviour of sharks and the origin of the contamination by ciguatoxins or similar toxins (carchatoxins);

  - at the population level, acquiring information about the size and structure of the populations of the two target species of shark at the scale of the Indian Ocean, the rate of travel of local populations around Reunion Island and in other areas where sharks have been observed (such as Madagascar, South Africa and the Seychelles). To this end, a study focused on the genetics of populations of tiger and bull sharks at the scale of the western Indian Ocean is also a promising area of research. A kinship analysis of the samples from Reunion Island will enable identification of the links between individuals and gregarious behaviour and a study of philopatry (tendency of some individuals to remain in or return to the place where they were born);
- studying the size/weight relationship, physiological conditions and the position in the trophic chain of shark species responsible for food poisoning, but also of other fish species;
- identifying the algae/cyanobacteria at the origin of the production of I-CTXs or other toxins identified in the tissues analysed and in the areas prospected, especially by acquiring more knowledge on species of *Gambierdiscus* spp. endemic to the seas around Reunion Island and their toxin profiles.

5 Conclusions of the Working Group

An analysis of the scientific literature and more widely a search on the internet enabled the Working Group to identify and describe cases of food poisoning associated with the consumption of shark (flesh and/or liver) from the 19th century to the present day. Cases, sometimes fatal, have been reported in New Caledonia, in the Cook Islands, the Gilbert Islands, in French Polynesia, Madagascar (particularly in November 2013 and February 2014) and Reunion Island (in 1993). The tiger shark was the species implicated in the Gilbert Islands. This species was described in the literature as "ciguateric" by Bagnis (1981), on the basis of data collected in the Samoa Islands, Fiji and the Mascarene Islands (the Mascarene Islands are an archipelago in the Indian Ocean made up of three main islands, Reunion Island, Mauritius and Rodrigues Island, as well as several small nearby islands). In Madagascar, tiger shark was associated with at least one case of food poisoning reported in the literature, and bullshark with two cases (Champetier de Ribes *et al.,* 1998). Genetic analysis of the shark involved in the food poisoning that occurred in November 2013 concluded that it was a bull shark.

The symptoms observed in these food poisoning incidents associated with the consumption of sharks correspond to the characteristic symptoms of ciguatoxins. However, some authors suggest that other toxins with similar properties, known as carchatoxins, might be responsible, but whose structure has not yet been characterised. More than 175 different symptoms have been identified in acute and chronic phases of ciguatera (the name given to poisoning by ciguatoxins). Regional differences have been noted and can be attributed to the presence of different ciguatoxins. Different ciguatoxins have been identified in the Pacific (P-CTXs), the Caribbean (C-CTXs) and the Indian Ocean (I-CTXs). The suggestion cannot be ruled out that carchatoxins could be new analogues of ciguatoxins, for example highly oxidised forms.

Other data concerning the contamination of sharks (flesh or liver), in particular of tiger and bull sharks, by ciguatoxins (or similar toxins) were collected and analysed. These data were found in studies carried out between the 1960s and the 1980s, using bioassays on mongoose.

➔ On the basis of these elements, the CES ERCA believes that it is appropriate to take into account not only ciguatoxins but also another type of toxins, specific to certain species of shark, currently known as carchatoxins.

The analytical methods for the detection and quantification of ciguatoxins in the flesh of sharks were identified and are described. These methods include: tests on animals, in particular mouse bioassay, the test on neuroblastoma cells (Neuro-2a), the radioligand receptor binding assay (RBA), immunological tests and physico-chemical methods, including liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

➔After considering the strengths and weaknesses of the available analytical methods in the light of the complexity and diversity of the toxins that make up the family of ciguatoxins (P-CTXs, C-CTXs and I-CTXs), the WG recommends using a combination of the following techniques:

- **a mouse bioassay, to find any overall toxicity in the sample;**
- **a cytotoxicity test on Neuro-2a cells and/or a test on receptors, which both have higher specificity and greater sensitivity than the mouse bioassay;**
- **an analysis by LC-MS/MS, to try and confirm the presence of known ciguatoxins in the case of positive results by one of the above methods.**

The data thus produced could then be used in a health risk assessment for consumers.

Considering these recommendations (and considering that the samples all gave negative results by mouse bioassay), the analysis by mouse bioassay alone of the 24 samples of Reunion Island sharks transmitted to ANSES in the framework of this expert appraisal does not provide sufficiently reliable results to conclude that they are safe with regard to the presence of ciguatoxins.

ANSES therefore contracted a research and development agreement (RDA) with ARVAM (in collaboration with IRTA) for these samples to be analysed by cytotoxicity tests on Neuro-2a cells. The final report was submitted to ANSES on 21 July 2014.

The results did not show ciguatoxin-like toxins to be present above the limit of detection of 0.04 µg eq. P-CTX-1 kg^{-1} of flesh. It should be noted that the detection limit is higher than the concentration considered to be of no risk to humans of 0.01 µg eq. P-CTX-1 kg^{-1} of fish flesh.

The RDA also included samples from the bull shark implicated in an outbreak of food poisoning that occurred in Madagascar in November 2013 (124 people intoxicated, nine of whom died) for analysis by mouse bioassay and by cytotoxicity tests on Neuro-2a cells. The sample of flesh gave a positive result by mouse bioassay, with symptoms (prostration, dyspnoea, cyanosis, convulsions and death by respiratory arrest) typical of those known for carchatoxins (Boisier *et al*., 1995). The analysis of a sample of flesh, a sample of stomach and three samples of dried fin by cytotoxicity tests on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated as follows:

- Flesh: 0.144 µg eq. P-CTX-1 kg^{-1} (i.e. 14 times the concentration considered to be of no risk to humans);
- Stomach: 114 µg eq. P-CTX-1 kg^{-1} (i.e. 11,400 times the concentration considered to be of no risk to humans);
- Dried fin: 0.145 µg eq. P-CTX-1 kg^{-1}; 0.158 µg eq. PCTX-1 kg^{-1}; 0.737 µg eq. PCTX-1 kg^{-1} (i.e. 14 to 74 times the concentration considered to be of no risk to humans).

Ethology information concerning tiger and bull sharks in the seas around Reunion Island was collected and summarised. These sharks are apex fish-eating predators, able to feed on different species. Around Reunion Island they principally inhabit certain specific sites, but are able to colonise a very wide range of different habitats and to travel over long distances, even as far as Madagascar, where food poisoning outbreaks associated with the consumption of shark have been reported recently (November 2013 and February 2014). It would be particularly interesting to know whether these sharks also travel from Madagascar to Reunion Island. Nothing is currently known about either the origin or the dynamics of bioaccumulation of these toxins in sharks. In addition, knowledge of the lifestyle of these two species of shark and their population dynamics is very fragmentary and inadequate. Results were obtained and methods of analysis proposed (CHARC programme). However, the gaps in our knowledge concerning the ecology of these sharks need to be filled to help interpret the data acquired and to serve as a basis for a monitoring plan for assessing the risks related to a possible authorisation of these species for human consumption, as regards ciguatera risk.

Within the strict framework of the questions raised by the Request, the WG has identified research needs relating to:

- the development of diagnostic tools for detecting the presence of CTXs (or similar toxins, such as carchatoxins) in sharks, and a plan of action in case of poisoning from consumption of shark;
- the identification of ciguatoxin analogues or similar toxins (carchatoxins) in sharks in the Indian Ocean;
- the toxicity of these toxins, their origin and how they are transferred to sharks;
- the size and structure of shark populations in the Indian Ocean as well as their movements and the distances travelled.

Date of validation of the Expert Appraisal Report by the Working Group: 25 July 2014

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For elements of the ethology of tiger and bull sharks

Programme CHARC: Connaissances de l'écologie et de l'HAbitat de deux espèces de Requins Côtiers sur la côte Ouest de la Réunion,

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6.2 Standards

NF X 50-110 (May 2003) Quality in Expertise - General Requirements of Competence for Expert Appraisals. AFNOR (classified X 50-110).

6.3 Legislation and regulations

Prefectoral Order no.3621/2009/SG/DRCTCV of 24 December 2009 regulating the trade of certain species of tropical marine fish. Préfecture de Reunion Island.

Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption.

ANNEXES

Annex 1: Request letter

COURRIER ARRIVE
16 OCT. 2013
DIRECTION GENERALE

2013 -SA- 0198

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

MINISTÈRE DE L'AGRICULTURE, DE L'AGROALIMENTAIRE ET DE LA FORET

Direction Générale de l'Alimentation
Service de l'Alimentation
Sous-direction de la Sécurité Sanitaire des Aliments
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Réf. : 13-129 Saisine_Requin_974 n° [redacted]-0428

Le Directeur Général de l'Alimentation

à

Monsieur le Directeur Général de l'Agence
nationale de sécurité sanitaire de l'alimentation,
de l'environnement et du travail

27-31, avenue du Général Leclerc
94701 MAISONS-ALFORT CEDEX

Paris, le 14 OCT. 2013

Objet : Saisine relative à l'évaluation du risque lié à la consommation de deux espèces de requins.

Conformément à l'article R. 1313-1 du code de la santé publique, j'ai l'honneur de saisir l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail en vue de l'**évaluation du risque lié à la consommation de deux espèces de requins notamment vis-à-vis du risque lié aux toxines ciguatériques.**

A- Contexte

Le département de la Réunion dispose depuis plusieurs années d'une réglementation spécifique qui restreint ou interdit la commercialisation de certaines espèces de poissons au regard du risque d'intoxication par des biotoxines marines (et particulièrement la ciguatera).

A ce jour, l'arrêté préfectoral n°3621 en date du 24 décembre 2009 (ci-joint) interdit la commercialisation de la plupart des espèces de requins, et notamment les carcharinidae, ainsi qu'une vingtaine d'autres espèces de poissons susceptibles de contenir des biotoxines et notamment de la ciguatoxine.

Monsieur le Préfet de la Réunion m'a sollicité afin que soit ré-évalué le risque lié à la consommation de deux espèces de requins, aujourd'hui interdits par arrêté. Il s'agit du requin tigre (*Galeocerdo cuvier*) et du requin bouledogue (*Carcharhinus leucas*).

Afin de ré-évaluer le risque ciguatérique chez ces 2 espèces, les services de la préfecture de la Réunion envisagent un diagnostic par le biais d'une campagne de prélèvements à hauteur de 45 spécimens par espèce sur lesquels la recherche de la ciguatoxine sera réalisée par un bio-essai sur souris.

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B- Questions adressées à l'ANSES

Dans ce contexte, je vous saurais gré de bien vouloir examiner les questions suivantes :

1. **A la lueur des connaissances actuelles sur les espèces de requins tigre et bouledogue, de leur susceptibilité à accumuler des phycotoxines et métabolites de celles-ci, et de leurs implications dans des intoxications alimentaires (à Madagascar par exemple) peut-on envisager la consommation de ces espèces sans risque pour le consommateur ?**

   En raison des avis précédemment émis par l'Agence en 2006 et 2009 relatifs à la consommation des poissons prédateurs pélagiques à la Réunion vis-à-vis du risque sanitaire lié au méthylmercure, l'Anses portera une attention particulière sur ce contaminant et intègrera la question de l'impact qu'aurait la consommation de requin sur l'exposition alimentaire totale à son évaluation.

2. **Quels sont les éléments nécessaires pour acquérir des informations robustes quant à l'innocuité de consommation de ces espèces ?**

   Quelles sont les méthodes analytiques actuellement applicables à la détection et à la quantification des ciguatoxines dans la chair de requin ? Les résultats issus de ces méthodes peuvent-ils être utilisés pour mener une évaluation des risques sanitaires liés à une éventuelle autorisation de ces espèces pour la consommation humaine dans cette zone ?

   Dans le cas où l'Anses identifierait une méthode d'analyse des ciguatoxines dans la chair de requin suffisamment fiable, quelles données seraient nécessaires pour mener cette évaluation et quelles recommandations pourraient être émises concernant le protocole de prélèvement du requin tigre et du requin bouledogue à la Réunion ? Il sera notamment pris en compte la zone géographique concernée et l'éthologie de ces 2 espèces de requin en termes de capacité de déplacements dans les eaux marines réunionnaises.

Les éléments de réponse apportés seront utiles pour la révision de l'arrêté préfectoral en cours.

Mes services se tiennent à votre disposition pour vous apporter toute information complémentaire.

Je vous remercie de bien vouloir accuser réception de la présente demande et de m'apporter une réponse dans un délai de 6 mois. Si ce délai n'est pas compatible avec d'autres saisines en cours de traitement au sein de l'agence, il pourra être adapté sans remettre en cause le calendrier actuel de travail déjà établi.

Le Directeur Général Adjoint
Chef du Service de la Coordination
des Actions Sanitaires - C. V. O.

Jean-Luc ANGOT

Copies :

- DGAL/ BAST
- DPMA
- LNR des biotoxines marines (Anses Maisons-Alfort)

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Annex 2: Prefectoral Order no.3621/2009/SG/DRCTCV of 24 December 2009 regulating the trade of certain species of tropical marine fish, Prefecture of Reunion Island

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

PREFECTURE DE LA REUNION

SECRETARIAT GENERAL

DIRECTION DES RELATIONS AVEC LES COLLECTIVITES TERRITORIALES ET LE CADRE DE VIE

Saint-Denis le

ARRETE N° 3621 /2009/SG/DRCTCV

Enregistré le 24 DEC 2009

Réglementant la commercialisation de certaines espèces de poissons marins tropicaux

LE PREFET DE LA REGION ET DU DEPARTEMENT DE LA REUNION

Officier de la Légion d'Honneur
Chevalier de l'Ordre National du Mérite

Vu les Règlements 178-2008, 852-2004, 853-2004, 854-2004 , fixant notamment les règles sanitaires régissant la production et la mise sur le marché des produits de la pêche ;

Vu la directive 97/78/CEE du conseil du 18 décembre 1997 fixant les principes relatifs à l'organisation des contrôles vétérinaires pour les produits en provenance des pays tiers introduits dans la communauté ;

Vu le code de la consommation et notamment son Art. L 212.1 ;

Vu le code rural, et notamment ses articles R231-12 à 19,

Vu l'arrêté ministériel du 16 mars 1982 (ministres de la consommation, de l'agriculture, de la mer), définissant les noms français officiels et dénominations admises des poissons marins ;

Vu l'arrêté ministériel du 25 juillet 1986 (ministres de l'agriculture, de l'économie et des finances, secrétaire d'Etat à la mer), relatif à la réglementation des conditions d'importation en France des produits de la mer et eau douce destinés à la consommation humaine ;

Vu l'arrêté préfectoral N° 06-2412/SG/DRCTCV du 30 juin 2006 réglementant la commercialisation de certaines espèces de poissons marins tropicaux ;

Vu le relevé de décision de la réunion du 3 novembre 2009 qui s'est tenue en présence du Directeur de la Direction des services vétérinaires, du Directeur régional des affaires maritimes, du Président du comité régional des pêches maritimes et de l'Agence pour la recherche et la valorisation marine ;

Considérant la situation de la Région REUNION dans la zone Océan Indien où sévit le phénomène « ciguatera » de façon endémique ;

Considérant la nécessité de protéger au mieux la population réunionnaise contre ce risque, au vu de ses habitudes alimentaires et d'une affinité particulière pour ces types de poissons à risque Ciguatérique ;

Considérant qu'il y a lieu d'appliquer une réglementation sanitaire uniforme entre les poissons issus de la pêche locale et les poissons issus de la pêche en pays tiers ;

ARRETE :

Article 1

Sans préjudice de l'application des autres dispositions réglementaires visées dans les textes de référence,

1.1 Sont interdits à la commercialisation sur le territoire du département de la REUNION :

1.1.1 Les poissons vénéneux des familles suivantes : Tétraodontidae, Molidae, Diodontidae, Cantigasteridae, Balistidae, Acanthuridae.

1.1.2 Les produits de la mer contenant des biotoxines telles que ciguatoxine ou toxines paralysantes des muscles.

1.2 Tout responsable de la première mise sur le marché de poisson est tenu de vérifier que ses produits répondent aux prescriptions du point 1.1 précité.

Article 2

Pour l'application du point 1.1.2. de l'article 1er et au vu des connaissances actuelles notamment dans la zone de l'Océan Indien, la commercialisation des espèces suivantes en provenance de zones de pêche tropicales est interdite :

ESPECES DE POISSONS INTERDITES				
Famille	**Genre**	**Espèce**	**Nom commun**	**Nom local**
ACANTHURIDAE	*genus (1)*	*spp (2)*	poisson chirurgien	poisson chirurgien
BALISTIDAE	*genus*	*spp*	baliste	bourse
CARANGIDAE	*Caranx*	*ignobilis*	carangue grosse tête	carangue grosse tête
CARANGIDAE	*Caranx*	*lugubris*	carangue	carangue noire
CARANGIDAE	*Caranx*	*melampygus*	carangue aile bleue	carangue bleue
CARANGIDAE	*Carangoides*	*fulvoguttatus*	carangue amoureuse	carangue blanc
CARCHARINIDAE	*genus*	*spp*	requins gris, baleinier, tigre	requins gris, baleinier, tigre
CLUPEIDAE	*Herklotsichthys*	*quadrimaculatus*	hareng queue blanche	sardine queue blanche
CLUPEIDAE	*Amblygaster*	*sirm*	sardinelle tachetée	sardinelle tachetée
DIODONTIDAE	*genus*	*spp*	poisson porc épic	poisson porc-épic
HEXANCHIDAE	*genus*	*spp*	requin griset	requin griset
LETHRINIDAE	*Gymnocranius*	*griseus*	empereur gris	capitaine pisa
LETHRINIDAE	*Gymnocranius*	*grandoculis*	empereur tatoué	capitaine blanc
LUTJANIDAE	*Lutjanus*	*gibbus*	lutjan bossu	vivanneau pagale
LUTJANIDAE	*Lutjanus*	*sebae*	bourgeois	bourgeois
LUTJANIDAE	*Lutjanus*	*bohar*	vara vara	vara vara
SCOMBRIDAE	*Gymnosarda*	*unicolor*	thon dents de chien	thon dents de chien
SCORPAENIDAE	*Pterois et Synancea*	*spp*	poisson scorpion	poisson scorpion
SERRANIDAE	*Cephalopholis*	*argus*	vielle cuisinier	prude

SERRANIDAE	*Variola*	*louti*	croissant queue jaune	grand queue
SERRANIDAE	*Plectropomus*	*maculatus*	babonne	babonne
SPHYRAENIDAE	*Sphyraena*	*barracuda*	barracuda	békine à dents
SPHYRNIDAE	*genus*	*spp*	requin marteau	requin marteau
TETRAODONTIDAE	*genus*	*spp*	tétrodon	poisson ballon

- **(1) *genus* : tous les genres de la famille**
- **(2) *spp* : toutes les espèces du genre**

Article 3

En dérogation à l'article 2 sont autorisées les espèces suivantes :

Famille	Genre	Espèce	Nom commun	Nom local
CARCHARINIDAE	*Prionace*	*glauca*	requin à peau bleue	requin à peau bleue
CARCHARINIDAE	*Carcharinus*	*longimanus*	requin pélagique	pointe blanche du large
SPHYRNIDAE	*Isurus*	*oxyrhynchus*	requin maquereau	mako

Article 4

En dérogation à l'article 2 et en considérant la situation particulière de la REUNION par rapport au risque ciguatérique, les espèces suivantes pourront continuer à être commercialisées sous la responsabilité de leur détenteur lorsqu'elles auront été capturées dans les eaux territoriales réunionnaises.

Famille	Genre	Espèce	Nom commun	Nom local
CARANGIDAE	*Caranx*	*lugubris*	carangue	carangue noire
CARANGIDAE	*Caranx*	*ignobilis*	carangue	carangue grosse tête
CARANGIDAE	*Caranx*	*melampygus*	carangue aile bleue	carangue bleue
CARANGIDAE	*Carangoides*	*fulvoguttatus*	carangue amoureuse	carangue blanc'
CARCHARINIDAE	*Carcharinus*	*falciformis*	requin à peau soyeuse	requin à peau soyeuse
CLUPEIDAE	*Amblygaster*	*sirm*	sardinelle tachetée	sardinelle tachetée
CLUPEIDAE	*Herklotsichthys*	*quadrimaculatus*	hareng queue blanche	sardine queue blanche
LETHRINIDAE	*Gymnocranius*	*griseus*	empereur gris	capitaine pisa
LETHRINIDAE	*Gymnocranius*	*grandoculis*	empereur tatoué	capitaine blanc
LUTJANIDAE	*Lutjanus*	*gibbus*	lutjan bossu	vivanneau pagaie
SCOMBRIDAE	*Gymnosarda*	*unicolor*	thon dents de chien	thon dents de chien
SERRANIDAE	*Cephalopholis*	*argus*	vielle cuisinier	prude
SERRANIDAE	*Variola*	*louti*	croissant queue jaune	grand queue * entier < 2,5kg

Article 5

Dans le cadre des dispositions juridiques relatives à l'obligation générale de sécurité du fait des produits défectueux :

- Les pêcheurs professionnels, les entreprises de pêche, de transformation ou de commercialisation concernées par les dérogations visées aux articles 3 et 4, devront s'assurer par des analyses régulières que ces espèces ne contiennent pas de biotoxines.
- Ils seront tenus de fournir un état annuel des résultats, d'informer immédiatement les services de l'Etat concernés en cas de résultat positif et de procéder sans délais au retrait de la commercialisation des poissons concernés.

Article 6

L'importation et l'introduction en vue de leur commercialisation sur le territoire de la Réunion d'espèces de poissons de l'Océan Indien non mentionnées par l'arrêté ministériel du 16 mars 1982 ou les avis aux importateurs parus au Journal Officiel de la République Française devra faire l'objet d'une autorisation préalable.

L'autorisation pourra être sollicitée sur présentation d'un dossier d'expertise préalable réalisé à la charge de l'opérateur par le Laboratoire ARVAM.

Au titre de l'alinéa précédent, font l'objet d'une dérogation les espèces suivantes :

Famille	Genre	Espèce	Nom commun	Nom local	Origine
LUTJANIDAE	*Lutjanus*	*sebae*	bourgeois	bourgeois	Seychelles
LUTJANIDAE	*Lutjanus*	*sebae*	bourgeois	bourgeois	Seychelles
LUTJANIDAE	*Lutjanus*	*sebae*	bourgeois	bourgeois	Madagascar (1)
LETHRINIDAE	*Gymnocranius*	*griseus*	Empereur gris	Capitaine pisa	Madagascar (1)
LETHRINIDAE	*Gymnocranius*	*grandoculis*	Empereur tatoué	Capitaine blanc	Madagascar (1)

(1) Zones côtières des provinces de Diego-Suarez et Tamatave comme figurant sur la carte ci-jointe

Article 7

La commercialisation des espèces dérogatoires, visées aux articles 3, 4 et 6, n'est autorisée que pour des entreprises régulièrement déclarées qui répondent aux exigences du règlement 852-2004 ou 853-2004. Elle peut être soumise à prélèvement libératoire, à charge de l'importateur, pour ce qui est des poissons en provenance de zones de pêche hors COI.

Ces entreprises devront tenir à la disposition des services de contrôle tous les documents permettant de justifier de l'origine des produits et procéder le cas échéant et dans les meilleurs délais, au retrait de la commercialisation des produits non conformes.

Article 8

Les listes des espèces de poissons interdits ou soumis à dérogation pourront être modifiées en fonction de l'évolution des données épidémiologiques et toxicologiques.

Article 9

L'arrêté préfectoral N° 06-2412/SG/DRCTCV du 30 juin 2006 réglementant la commercialisation de certaines espèces de poissons marins tropicaux est abrogé.

Article 10

Le Secrétaire Général de la Préfecture, le Secrétaire Général pour les Affaires Economiques et Régionales, les Sous Préfets, les Maires des communes du département, le Directeur des Services Vétérinaires, le Directeur Régional des Affaires Maritimes, le Directeur Départemental de la Concurrence de la Consommation et de la Répression des Fraudes, le Directeur Régional de l'Action Sanitaire et Sociale, le Directeur Régional des Douanes, le Directeur Départemental de la Sécurité Publique, le Colonel Commandant le Groupement de Gendarmerie à La Réunion, sont chargés chacun en ce qui le concerne de l'exécution du présent arrêté qui sera publié au recueil des actes administratifs de la Préfecture.

Le Préfet,

Pour le Préfet
Le Secrétaire Général
pour les Affaires Régionales

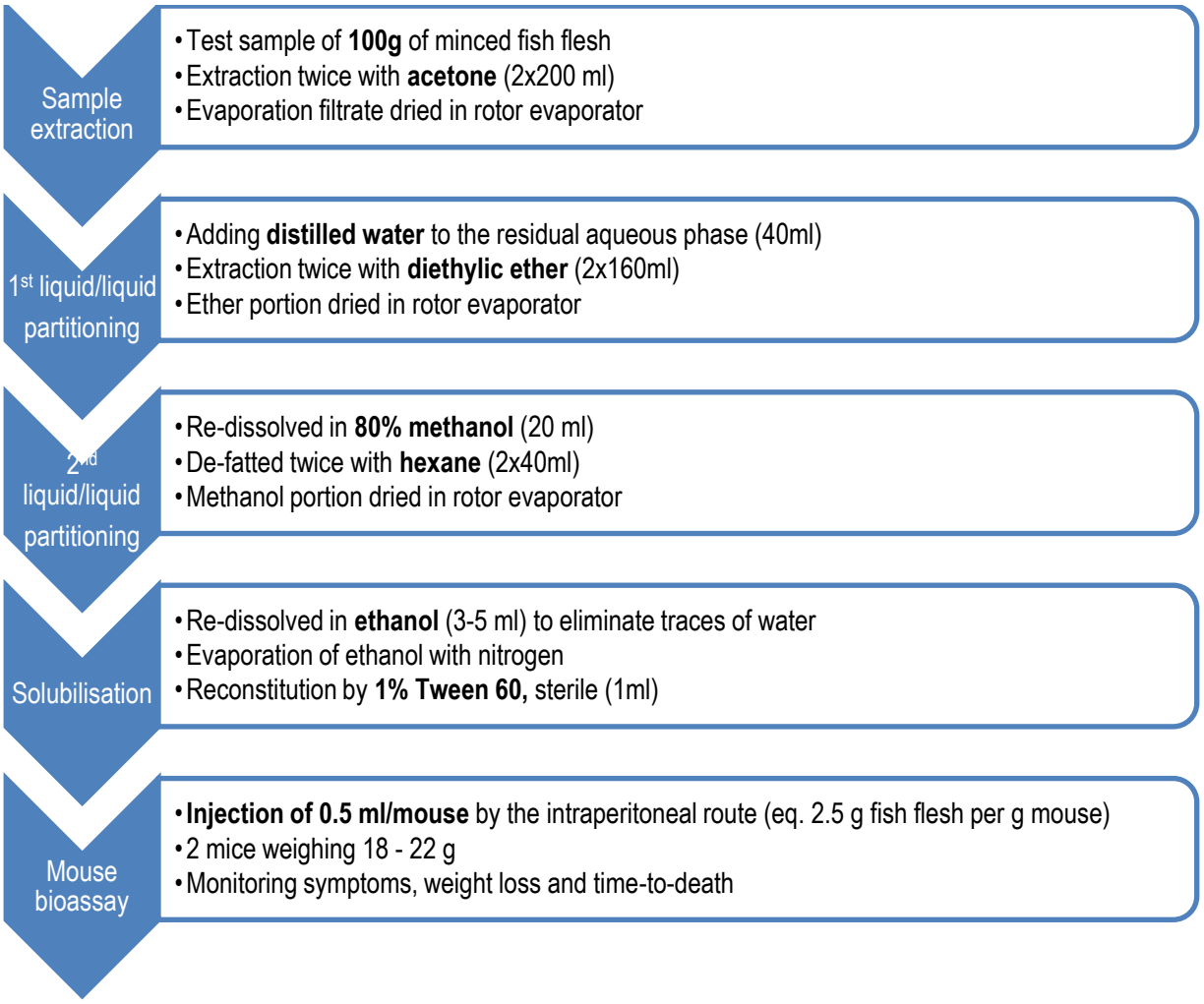
Jean BALLANDRAS

ADRESSE POSTALE : 97405 - Saint-Denis Cedex- STANDARD ☎ 0262 40 77 77)

Annex 3: Detailed description of different variants of the mouse bioassay

CAT-NAT 10 method, ANSES Maisons-Alfort

This is the mouse bioassay method for detecting ciguatoxins in fish used by laboratories accredited by the French Directorate General for Food (ARVAM and French NRL) for their investigations of suspected outbreaks of ciguatera poisoning and import controls. It can be applied to fresh, frozen or cooked fish.



Assessment of the sample's toxicity is based on the time-to-death of the mice. The death of 1 or 2 mice within 24h is interpreted as a positive result indicating the presence of ciguatoxins (sample therefore non-edible). The presence of typical symptoms and/or weight loss 24h after injection are also assumed to demonstrate that the sample is positive (limit of edibility). This method cannot be used for quantification in the absence of a standard.

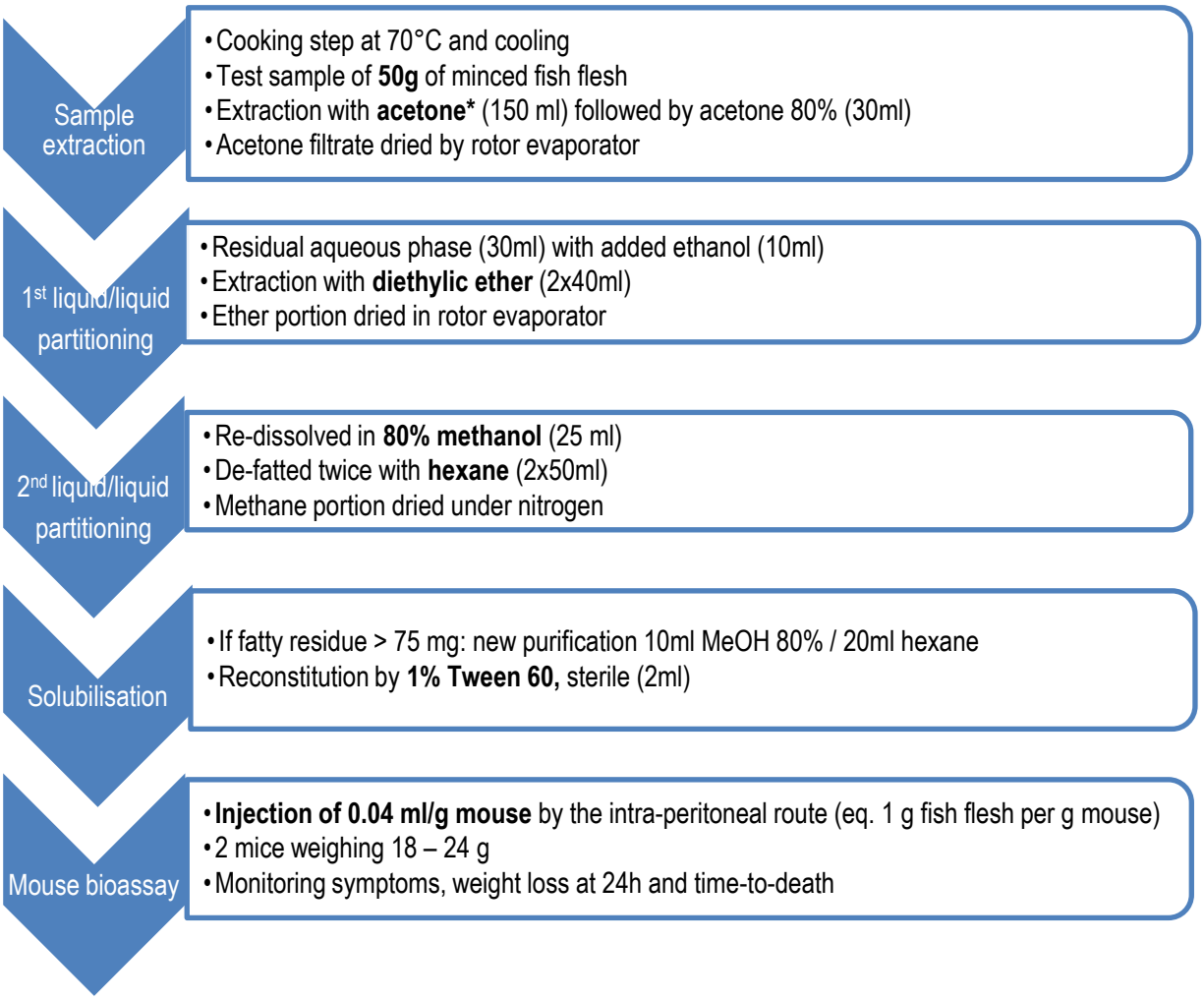
Typical symptoms of the presence of CTX are diarrhoea, piloerection, respiratory disorders, dyspnoea and sometimes cyanosis of the penis.

<table>
<tr><th>Mortality within 24h</th><th>Presence of typical symptoms</th><th>Loss of weight >5% 24 h after injection (for at least 1 mouse out of 2)</th><th>Result</th></tr>
<tr><td>2/2</td><td colspan="2" rowspan="2">Irrespective of the symptoms and/or the weight loss</td><td>POSITIVE non-edible</td></tr>
<tr><td>1/2</td><td>POSITIVE non-edible</td></tr>
<tr><td rowspan="4">0/2</td><td>Yes</td><td>Yes</td><td rowspan="3">POSITIVE limit of edibility</td></tr>
<tr><td>Yes</td><td>No</td></tr>
<tr><td>No</td><td>Yes *</td></tr>
<tr><td>No</td><td>No</td><td>NEGATIVE Edible</td></tr>
</table>

* If no symptoms are observed even though a loss of weight is recorded at the end of 24h, the observation period can be extended to 48h. If the animal regains all the lost weight at the end of 48h, the result can then be declared as "limit of edibility - negative".

Method described by Vernoux (Vernoux, 1994)

This method served as the basis for the French NRL before the previous protocol was harmonised with ARVAM in 2012.



* Extraction in methanol is possible when the sample of fish flesh does not exceed 10 g.

Assessment of the sample's toxicity is based on the time-to-death of the mice. The death of 1 or 2 mice within 24h is interpreted as a positive result indicating the presence of ciguatoxins (non-edible). Weight loss (> 5%) 24h after injection is also assumed to demonstrate that the sample is positive (limit of edibility).

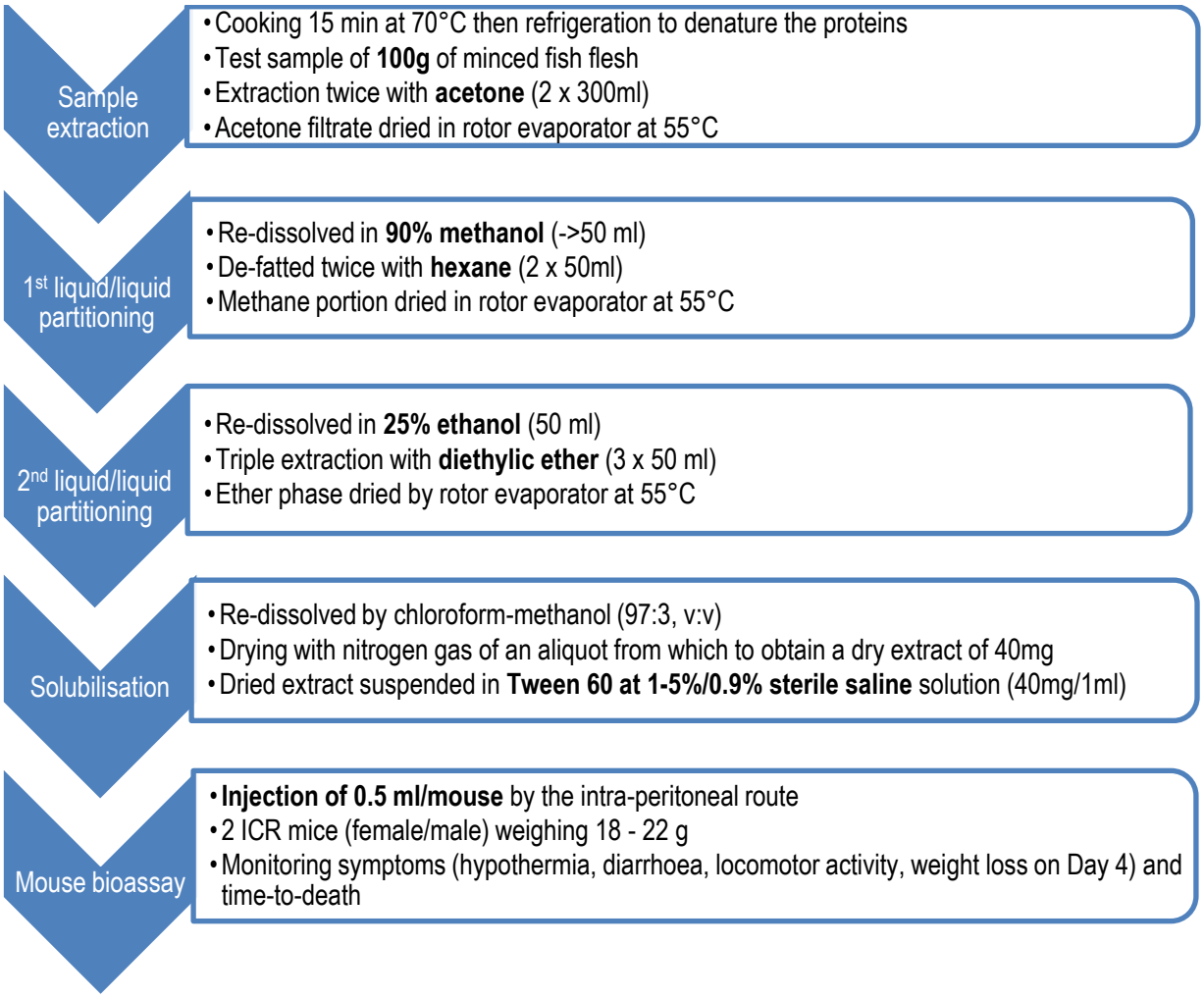
Mortality at 24 h	**Loss of weight at 24h (>5%)**	**Toxicity Class ***	**Interpretation**
2/2	-	≥ 1 MUg per g eq. flesh	Non-edible
1/2	Yes	0.5 to 1 MUg per g eq. flesh	Non-edible
0/2	Yes	< 0.5 MUg per g eq. flesh**	Limit of edibility
	No		Edible

* One MUg or mouse unit-gram is defined as the amount of toxin that can kill one gram of mouse in 24h, under conditions of injection designed to determine the minimum lethal dose (MLD, death of at least one mouse) or the LD_{50} (death of half the mice). The amount of toxin is relative to the body weight (in grams) of the mice tested.

** A study of 306 specimens of Caribbean fish whose toxicity ranged from 0.05 to 3.72 MUg per gram of fish flesh established a minimum toxin concentration of 0.5 MUg per gram of flesh (Vernoux, 1988), which is equivalent to 1.8 µg C-CTX-1 kg^{-1} of fish flesh. The level of action established by the US-FDA (2011) for C-CTXs is 0.1 µg kg^{-1} eq. C-CTX-1.

Method described by Lewis (Lewis, 2003), figure next page

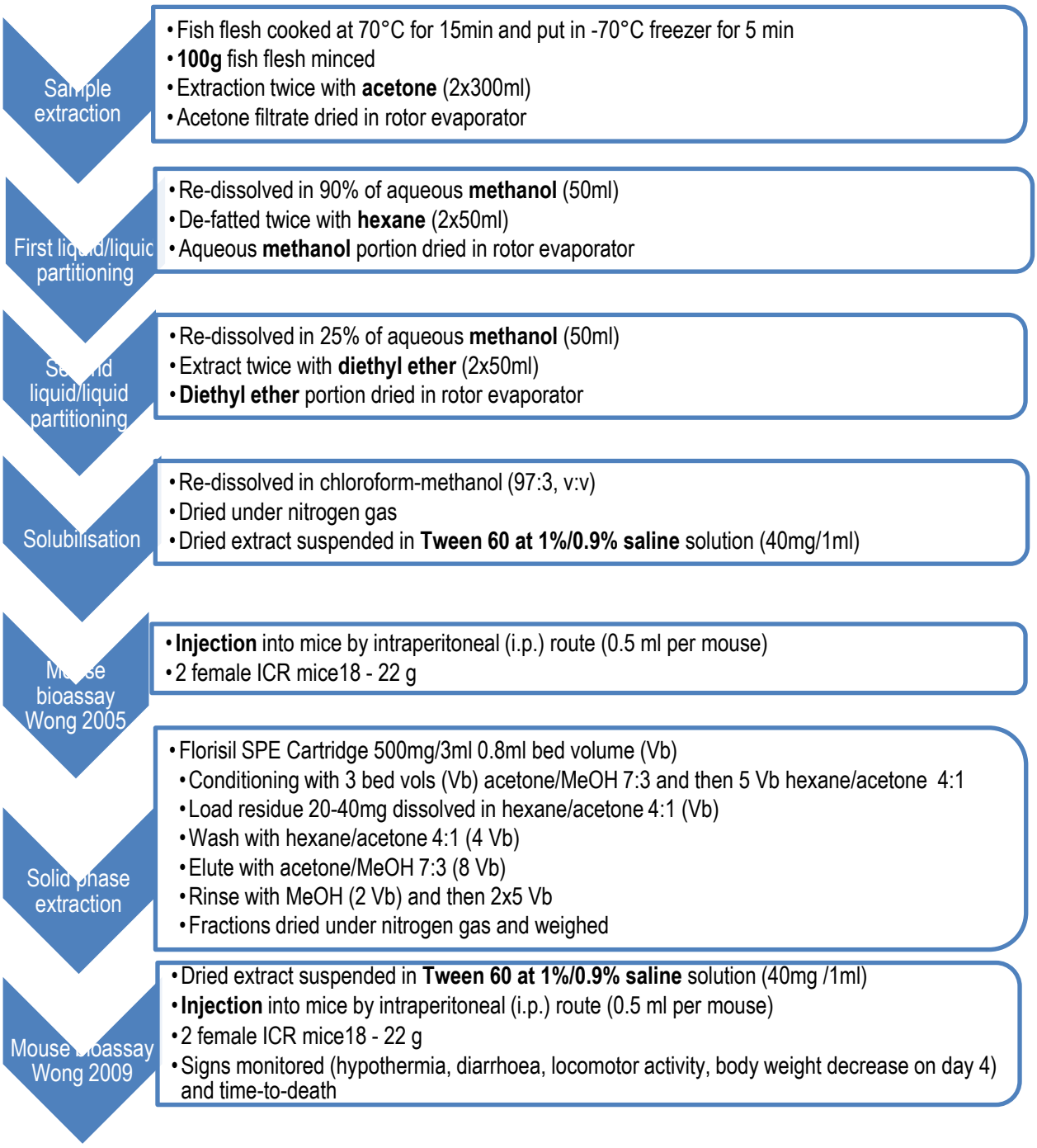
Symptoms that are not ciguateric are discarded to avoid any subjective bias in the observations (Hoffman *et al.*, 1983). The mice are observed continuously during the first five hours after injection, and then monitored regularly until the end of the 24 hours. Weight loss is measured at 24h and on Day 4. This is because only measuring loss of body weight at 24h does not sufficiently reflect the main effect of the extracts injected. The measurement of weight loss at Day 4 is more appropriate than at 24 hours to observe the actual effects of extracts. This may be due to the fact that the mice cannot have recovered completely after the i.p. injection, and the rectal measurements of bodily temperature (optional) can affect the feeding habits and behaviour of the mice.



Method described by Wong with SPE purification on Florisil (Wong *et al.,* 2009), figure next page

An improvement in the preparation of the sample with the aim of increasing the limit of detection and improving the quantification of CTXs in the flesh of fish was obtained by adding a purification step on Florisil SPE to increase the purity of the ether fractions (Wong *et al*., 2005, 2009). The purification step on a Florisil cartridge minimises potential interference due to lipids. According to the authors, this step of further purification improves the recovery rate of P-CTX-1 to 76%, as compared with 63% when using the standard protocol.

The equation is log (MU) = 2.3 log (1 +1/T), where MU is the number of mouse units (1 MU = DL_{50}) and T is the time-to-death. The log (1+1/T) relationship was established by injecting concentrations of standard between 10 and 70 ng $0.5ml^{-1}$ Tween 60 1%/0.9% saline solution into the mice. In this study the LD_{50} was established at 5.6 ng P-CTX-1.



Wong *et al*. (2005) used this protocol during a study of FBOs in Hong Kong. The sub-lethal doses were estimated at between 0.18 and 0.45 MU/20 mg extract injected. Samples found positive by bioassay were analysed by a rapid-detection Cigua-Check® kit. The kit cannot detect CTXs at a level lower than 0.05 µg kg^{-1} flesh and can also produce false positives (Lehane and Lewis, 2000). This assessment highlights the inconsistencies between the mouse bioassay and the rapid immunodetection kit. The authors therefore conclude that a further review should be carried out to check its validity and reliability as a tool for routine detection of CTXs.

Annex 4: Detailed description of the protocol for the Neuro-2a test

According to Caillaud *et al.* (2012)

The extraction procedures may be revised if the assay is to be used in combination with other analytical methods, depending on the matrix (flesh, liver, viscera).

Extraction and purification procedure of CTXs from fish samples

Fish flesh were extracted and purified according to the protocol described in Lewis (2003). Briefly, 10 g portions of fish flesh was cooked at 70°C for 10 min and mixed for 5 min in 30 ml acetone (Ultraturax, 17,500 g). Acetone soluble extract was recovered after 5-min centrifugation at 600 g at 4°C (Joan MR23i, Saint Herblain, France) and filtrated using 0.45 mm nylon filters. The extraction with acetone was repeated twice and both acetone soluble extracts were pooled and dried on a rotary evaporator (Büchi Rotavapor® R-200, Flawil, Switzerland).

The dried extract was further purified using liquid/liquid partition in order to eliminate excessive fatty acids that may interfere with the detection of CTXs. For that purpose, dried extract was dissolved in 5ml methanol:water (9:1) and partitioned twice with 5ml nhexane (1:1, v/v). The n-hexane fractions were discarded and the methanol:water fraction was dried for further purification (Büchi R-200). The dried extract was dissolved in 5ml ethanol:water (1:3) and partitioned twice with 5ml diethyl ether (1:1, v/v). The ethanol:water fraction was discarded and the diethyl ether fractions were pooled, dried, then dissolved in 4ml methanol and kept at -20ºC until analysis.

Neuroblastoma (neuro-2a) cell maintenance and cytotoxicity assay

Neuro-2a cells (ATCC, CCL131) were maintained in 10% foetal bovine serum (FBS) RPMI medium (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a 5% CO_2 humidified atmosphere (Binder, Tuttlingen, Germany) as described (Cañete and Diogène, 2008). For experiments, cells were seeded in a 96-well microplate in 5% FBS RPMI medium at an approximate density of 35,000 cells per well. Cells were incubated in the same conditions of temperature and atmosphere as described for cell maintenance.

After 24h incubation of the neuro-2a cells and just before exposure to fish extracts and CTX standard solution to be tested, one half part of the microplate received 0.1 mM ouabain (Sigma-Aldrich) and 0.01 mM veratridine (Sigma-Aldrich) allowing a reduction of 20% in cell viability (Canete and Diogène, 2008). An equivalent amount of fish extract or P-CTX-1 standard solution to be tested was first evaporated until dry under N_2 flux at 40º C (TurboVap, Caliper, Hopkinton, USA) to remove the methanol completely. Dried extracts were further dissolved in 5% FBS RPMI medium, serially diluted in the same medium and 10 ml of each concentration was directly added into the well of both halves of the microplate (with and without O/V treatment) in order to compare cell response in the presence and absence of O/V treatment. An equal volume in each well was corrected using phosphate buffer solution. All doses as well as all experiments were performed in triplicate.

The LOQ of P-CTX-1 for the method was evaluated using a non-toxic fish sample (when no toxic effects were observed with and without O/V treatment under the limit of tissue equivalent [TE] exposure set) spiked with P-CTX-1. For that purpose, neuro-2a cells were exposed to 20 mg TE ml^{-1} spiked with P-CTX-1 at concentrations ranging from 0.01 to 10 pg P-CTX-1 mg TE^{-1}. Eight repetitions of this experiment were done during the same day in order to test the repeatability of the assay (intra-assay variability) and three repetitions of the same experiment were done on different days in order to test the reproducibility of the assay (inter-assay variability).

When toxicity of fish extracts was observed only in the presence of O/V treatment and under the limit of TE exposure, this toxicity was considered as Na+ channel-activating toxicity, and for this study it was considered as CTX-like toxicity, assuming no PbTx is present. The sensitivity of the neuro-2a cells to the presence of CTX was calibrated each day of the experiment with a standard solution of P-CTX-1 at 20 ng ml^{-1}. The CTX-like content in the samples tested was quantified after analysis of cell viability, as further described below.

Cytotoxicity evaluation and results analysis

After 24h exposure of the neuro-2a cells to fish extracts and P-CTX-1 standard material, cell viability was used as an endpoint for cytotoxic effects measurement. Cell viability was assessed using the colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] MTT assay (Mosmann 1983). Absorbance values were read at 570nm using an automated multi-well scanning spectrophotometer (Biotek, Synergy HT, Winooski, VT, USA) and results were further analysed using the Prism4 software (GraphPad, San Diego, CA, USA). The viability of cells was expressed in relation to the viability of the corresponding cell control (with or without O/V treatment) and the 50% of effects ($IC_{50}^{O/V+}$) was calculated according to the dose–response curve obtained using a sigmoid regression curve with a variable Hill slope. In the present study we considered responses producing less than 20% cell mortality as a non-toxic effect; the concentration inhibiting 20% of cell viability (IC20) was set as being the limit of detection (LOD) and the LOQ of the method. For a quantitative estimation of the content in P-CTX-1 equivalents in fish extracts, the concentration of P-CTX-1 standard material that induced 50% of toxic effects ($IC_{50}^{O/V+}$ for P-CTX-1) was used to estimate the concentration of P-CTX-1 eq. in fish TE, considering the amount of TE that induced 50% of toxic effects ($IC_{50}^{O/V+}$ for fish TE). In borderline samples, the quantitative estimation of the content in P-CTX-1 equivalents was estimated at the LOQ of the method.

Significant differences between repetitions of experiments (intra- and inter-assay variability) were evaluated using ANOVA with a 95% confidence level.

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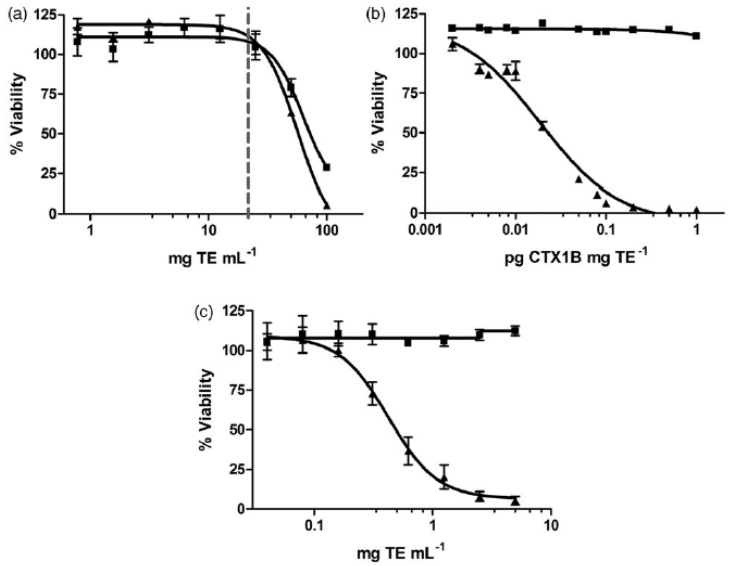


Figure 2. Dose–response curves of neuroblastoma (neuro-2a) cells exposed for 24 h to a non-toxic fish sample (*S. fasciata*, sample 4) (a), non-toxic fish sample (*S. fasciata*, sample 4) spiked with CTX1B (b) and toxic fish sample (*S. fasciata*, sample 2) (c), with (▲) and without (■) O/V pretreatment. The limit of tissue equivalent (TE) exposure for matrix interferences is represented by a dotted vertical line.

Table 3. Repeatability and reproducibility of the response of the neuroblastoma (neuro-2a) cell based-assay exposed for 24 h to a non-toxic fish sample spiked with CTX1B.

Variability	*n*	$IC_{20}^{O/V+}$ (pg CTX-1 mg TB^{-1}) ± SD	CV (%)	$IC_{50}^{O/V+}$ (pg CTX-1 mg TB^{-1}) ± SD	CV (%)
Intra-assay	8	0.008 ± 0.002	23	0.023 ± 0.004	17
Inter-assay	3	0.009 ± 0.003	31	0.029 ± 0.006	19

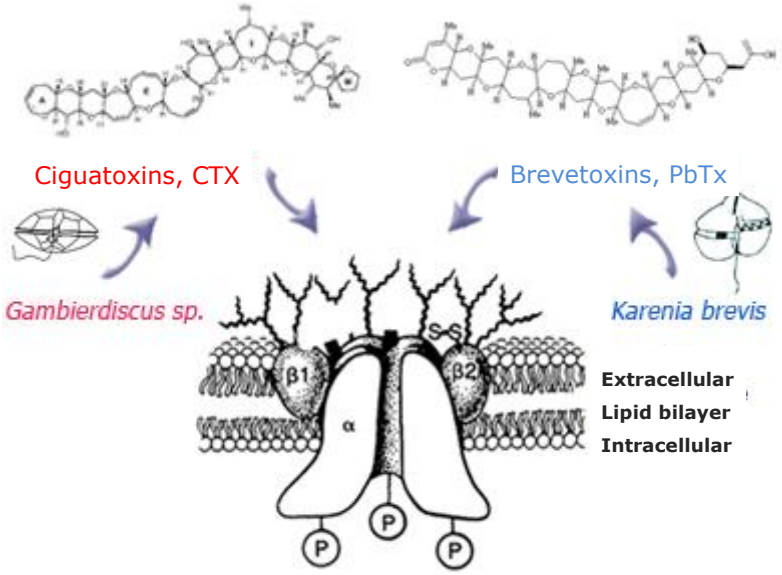
Notes: $IC_{20}^{O/V+}$ and $IC_{50}^{O/V+}$, concentration of CTX1B producing respectively 20% and 50% of toxic effects; *n*, number of replicates; TE, tissue equivalents; SD, standard deviation, CV, coefficient of variation.

Annex 5: Detailed description of the protocol for the RBA test

According to http://www.ilm.pf/testbinding

Principle behind the method

The sodium channel is a trans-membrane protein present in large quantities in the membranes of excitable cells which lets sodium ions pass through selectively. The analysis of this channel, purified from the brains of rats, showed that it comprises three sub-units. The diagram below shows the alpha sub-unit (α 260 kDa) associated with the beta 1 (β1 36 kDa) and beta 2 (β2 33 kDa) sub-units. The β1 sub-unit is linked to the α sub-unit by non-covalent bonds while the β2 sub-unit is linked by disulfide bridges (S-S). The three sub-units are heavily glycosylated on the external face of the molecule and the α sub-unit has many sites of phosphorylation (P) on its internal surface.



Specific high-affinity attachment on site 5 of the α sub-unit of the sodium channel (modified diagram from Catterall, 1986)

Attachment sites of marine toxins (STXs, TTXs, PbTx, CTXs, etc.)

Pharmacological competition studies, on ion flow measurements (^{22}Na) and mutagenesis have identified six receptor sites on the sodium channels in mammals, numbered 1 to 6. These sites are the receptors for the attachment of many neurotoxins associated with the sodium channel activated by the action potential.

Site 5 of the alpha sub-unit of the sodium channel is the receptor for two families of polyether-type marine toxins: ciguatoxins (CTXs) and brevetoxins (PbTxs), produced respectively by the dinoflagellates *Gambierdiscus* spp. and *Karenia brevis*.

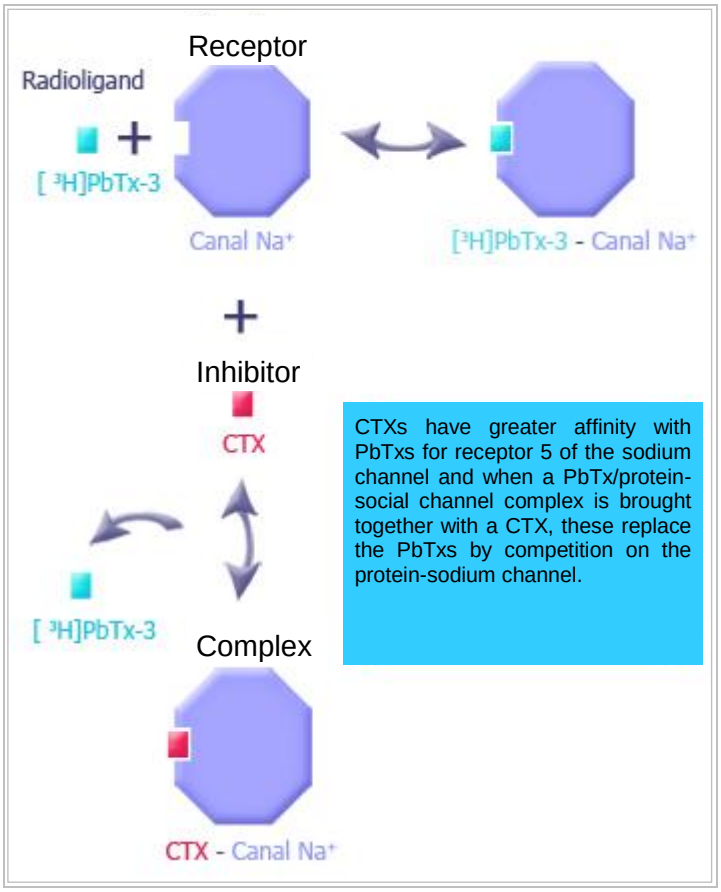
The specific property of these toxins of binding site 5 of the sodium channel made it possible to develop a detection test known as the "ligand-receptor binding assay". This test is used to detect and quantify the CTXs present in a sample of algae or fish.

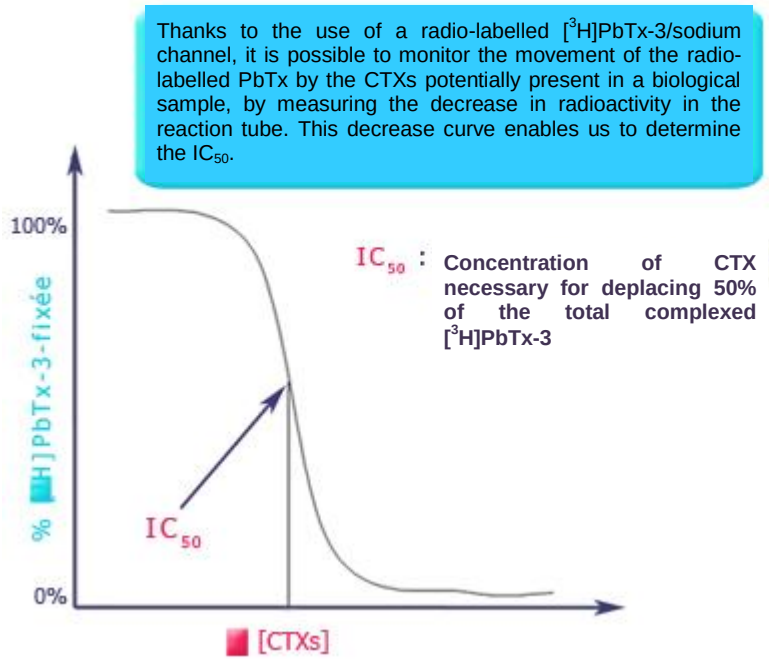
- **The theoretical principle**

If a radioligand (L*) is placed in the presence of its receptor site (R), a radioligand-receptor complex is formed. A balance is struck between these two states which can be expressed by the following equation: [L*] + [R] ⇔ [L*-R].

If increasing concentrations of non-labelled inhibitory molecules are added to this mixture and enter into competition with the radioligand, the number of receptors available will decrease, which is reflected by a decrease in the quantity of labelled complex formed. The curve representing the

percentage of complex formed as a function of the concentration of the inhibitor (I) on a semi-Log scale is a sigmoid.

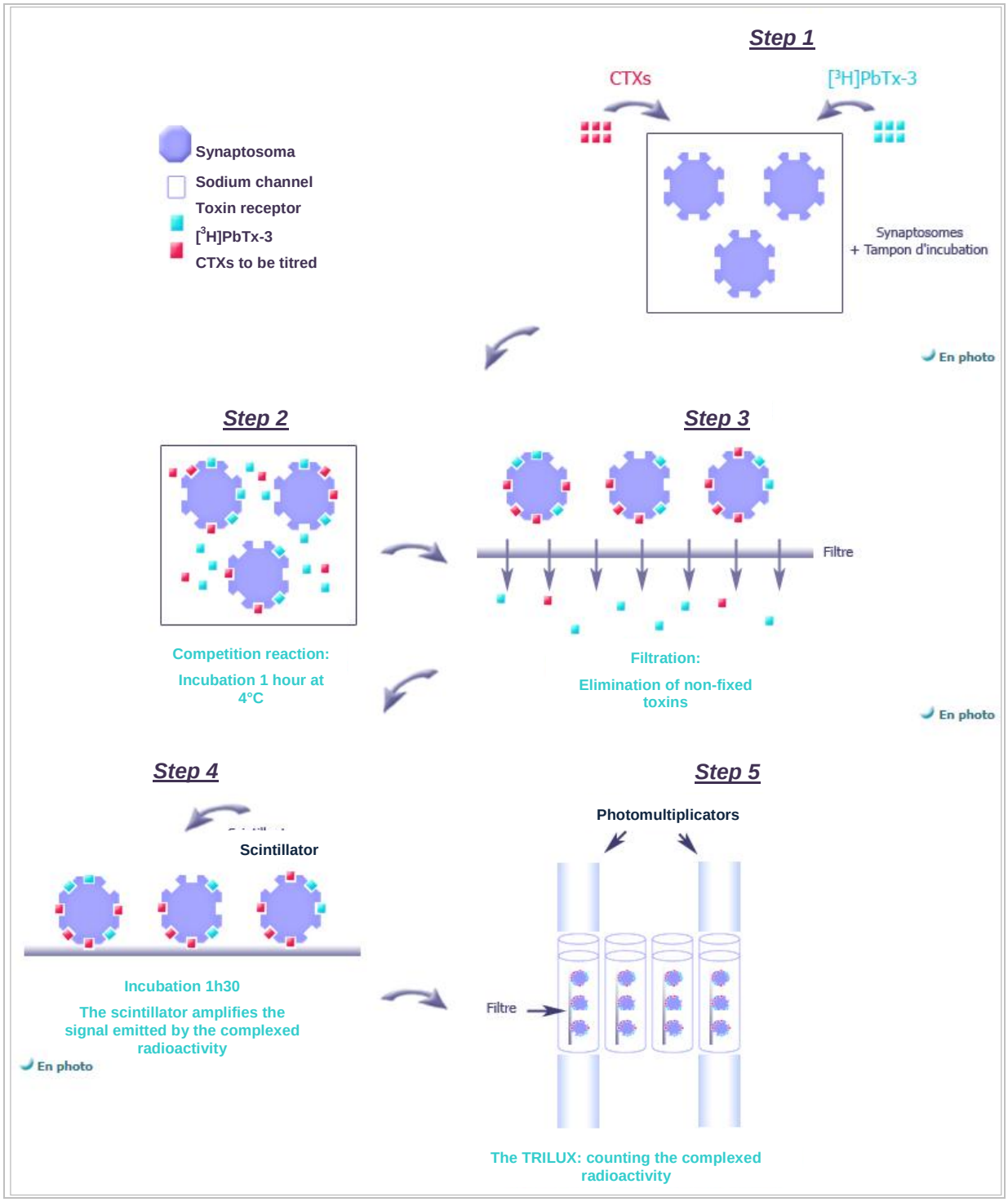




Modified diagrams from Bottein Dechraoui, 1999

The test in images

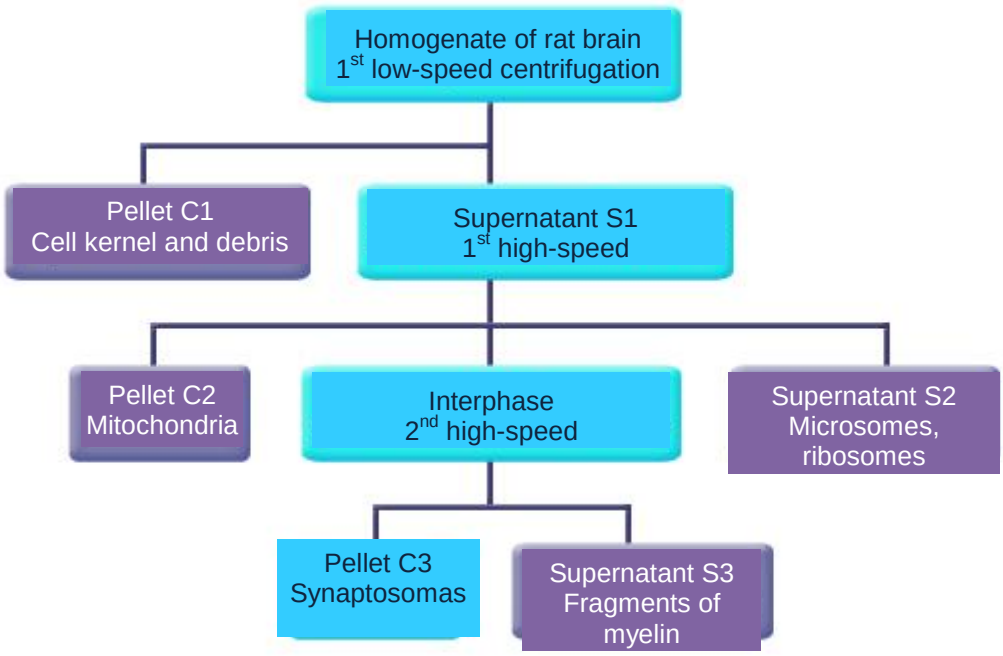
The different steps of this test follow the protocol of Dechraoui *et al*. (1999).



Obtaining and purifying sodium channels

At present, the sodium channels are obtained from membrane preparations from excitable brain cells of rats, also called synaptosomes. The protocol used is based on that of Dodd *et al*. (1981), whose major steps are listed below:

- Ordering frozen rat brain from an accredited animal supplier;
- Preparation of brain homogenates followed by three centrifugation operations as illustrated below (modified according to Bottein Dechraoui, 1999):



- The proteins of the synaptosomes are assayed according to the Bradford method, with the optimum concentration necessary for the detection test being between 60 and 80 mg ml^{-1} of protein.

The "ligand-receptor interaction" detector test requires the use of a radiolabelled toxin: tritiated brevetoxin, [^{3}H]PbTx-3.

The use of radioelements is highly specialised and is governed by the texts issued by the European Union under the name EURATOM. The French regulations and standards result from the transposition of these European texts as well as the guidelines of the International Commission on Radiological Protection (ICRP).

Annex 6: Detailed description of the various possibilities for immunological tests

Several teams around the world have carried out research on the immunodetection of ciguatoxins: mainly an American team, Hokama's team at the University of Hawaii, a Japanese team under the present leadership of Pr. Hirama and a French team under the direction of Dr S. Pauillac of the Institut Pasteur during secondment to the Louis Malarde Research Institute in Tahiti from 1992 to 1999 and, therefore, in collaboration with the Tahiti team under the present leadership of Mireille Chinain.

Historically, it was Hokama's team that launched the first studies. Thus, the first polyclonal antibodies directed against ciguatoxins were produced in sheep in 1977 by repeated injections of complexes of ciguatoxins combined with human serum albumin (HSA) obtained with carbodiimide, which is a chemical toxin coupling agent with a native or acquired acid-protein function (Hokama *et al*., 1977). A radio immuno-assay (RIA) was developed with these antibodies (Kimura *et al*., 1982a,b), and later an enzyme immuno-assay (EIA) (Hokama *et al*., 1983, 1984). A methodology by attachment to a stick of plant matter, the "Bamboo stick test", was developed for practical use by probing the flesh of fresh fish to detect the possible presence of ciguatoxins. The polyclonal antibodies were then replaced by monoclonal antibodies. Hokama thus described the use of monoclonal antibodies produced from the conjugation of okadaic acid (OA) with HSA, which had an affinity with ciguatoxins equal to or greater than that observed for OA(Hokama *et al*., 1985, 1988, 1989). Then other monoclonal antibodies directed specifically against ciguatoxins by the same method were produced, again by Hokama's team. These different antibodies were detected by labelling either with peroxydase (Solid-Enzyme ImmunoAssay or S-EIA) or with blue-coloured particles of latex (Solid-Phase Immunobead Assay or S-PIA) (Hokama *et al.* 1990, 1992, 1993, 1998a,b).

The industrialisation phase was then launched and the first immuno-test, called Ciguatect®, was marketed by Hawaii Chemtect International, Pasadena, USA. Then, after an evaluation phase and a renaming of the above company, a new test, the Cigua-Check®, based on the same analytical principle, was marketed by the Hawaiian company Oceanit Laboratories. It was Dr Park who first promoted the production and sale of these tests (Park, 1994). The principle was as follow: first any potential fish ciguatoxins are adsorbed on a bamboo stick covered with a corrective coating (to act as a non-specific adsorbant, such as silicic acid or Florisil) by simply probing the fish flesh repeatedly with this. After drying and rapid fixing in the methanol, the stick is submerged in a suspension of antibody marked with blue latex. When rinsed, the stick turns blue, darker or lighter depending on the quantity of antibodies adsorbed on the ciguatoxins held on the stick. Positive and negative controls must be performed. But this test returns up to 25% of false negatives which means that it needs standardised scientific validation before it can be used (Dickey et al., 1994). Moreover, as it is presented as being capable of detecting OA and CTXs, at the time it was never possible to know what type of antibody was used, considering that it is much easier to prepare antibodies directed against OA (which has an acid function) than against CTXs (with no acid function), as OA was readily available in significant quantities whereas ciguatoxins are available in limited quantities, often lower than 1 mg! It would also seem that the monoclonal antibodies used came from a selection of those synthesised by the population of 5C8 hybridomas obtained in 1986 (which would mean the ones targeting okadaic acid) following publication by Hokama of the data for reproducing the OA antibody method regarding the composition in antibodies of the solutions supplied with Cigua-Check® (Hokama et al., 1998a,b). Unfortunately, Legrand *et al.* (1998) and Richard Lewis (Lehane and Lewis, 2000) both confirmed the relative non-validity of this test, and even demonstrated the absence of reaction with purified C-CTX-1 (Vernoux, personal communication). At this time it was also indicated that there was little chance that antibodies prepared against P-CTXs would react fully with C-CTXs given the very different epitope terminals (Pottier et al., 2001). However, the product remained on the market both for Caribbean fish and for Pacific Ocean fish until 2005, at which time it was removed from the market (http://cigua.com/onlinestore/product_info.php?cpath=24&products_id=29; go to the last page). On the same date, the Hokama team reported having improved the specificity of the antibodies and announced that their "Bamboo stick test" was capable of detecting P-CTXs and C-CTXs at the very low levels of

0.078 to 5 ng ml^{-1} with no cross-reaction with OA, palytoxin and domoic acid (Campora et al., 2006). However, this test did not reappear on the market [moreover, this test was recently criticised and its basis questioned (Bienfang *et al*., 2011)]. Nevertheless, C.E. Campora, representing Oceanit, responded sharply (Ebesu and Campora, 2012). In reality, the path followed up to that point by Hokama's team was modified and they switched to the sandwich ELISA method (whose principle is set out below with the focus on the work of the Japanese teams because they are the ones who developed it) which shows a good correlation with the test on neuroblastoma 2A cells (Campora *et al.,* 2008). For this sandwich ELISA method, Campora *et al.* (2008) used antibodies to capture initial ABCD anti-cycles of P-CTXs produced by chickens (IgY) as well as a reactive monoclonal antibody in mice (IgG) directed against the JKLM terminal fragment already mentioned and conjugated to horseradish peroxidase (HRP). The ELISA by competitive inhibition (CIEA) detection technique leads to an LoD (limit of detection) of approximately 5 pg ml^{-1} of equivalent CTX per mg of extract (corresponding to 5×10^{-12} M), which is of the same order of magnitude as that of the test on neuroblastomas. In addition, this CIEA test provides similar results on extracts from the flesh of fish caught *in natura* or farmed by aquaculture (Campora et al., 2008, 2010).

The French and Japanese teams worked in collaboration but adopted totally different and complementary scientific approaches to try to resolve the problem of the immunodetection of ciguatoxins, which is itself a textbook case because it involves four major issues: very low quantities of usable ciguatoxins (< 1mg), very high toxicity, structural and epitopic variability, and low mass (barely more than 1000 Da), which results in haptens not directly immunogenic thus requiring a toxin-protein pairing. These quantity constraints mean that it is only possible to produce monoclonal antibodies, since these require low quantities of toxins on small laboratory animals such as rats or mice, which thus enables unlimited production of monoclonal antibodies, unlike the production of polyclonal antibodies which requires large quantities of antigen to be injected into larger animals such as sheep, goats or horses. Regarding the epitopic variability, ideally it should be possible to prepare different monoclonal antibodies and market them in a mixture, but that currently seems far off. And on the subject of toxin-protein pairs, a way must be found to miniaturise the techniques so as to permit the production of antibodies despite the very small quantities of toxins available.

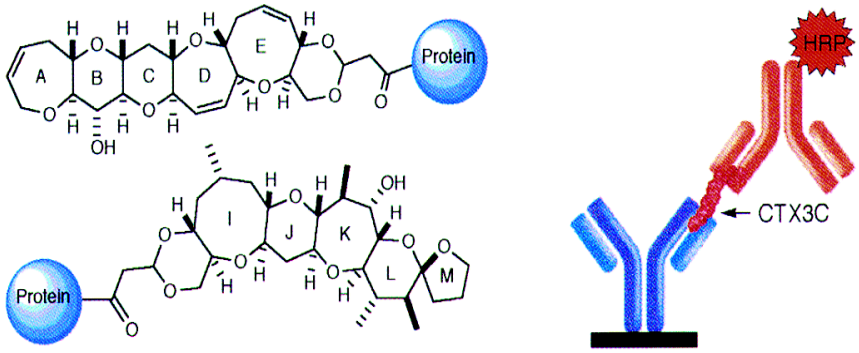
This is where Serge Pauillac's team come into the picture (Pauillac *et al.,* 1995) because at that time it had barely 1 mg of P-CTX1B to work with. In order not to waste this stock of toxin, they had to develop a whole new approach to the miniaturisation of toxin-protein pairing techniques on other model molecules that were commercially available in sufficient quantity, acetylbenzoic acid, cholesterol hemisuccinate and brevetoxin PbTx-3 type PbTx2 (which also acts on site 5 of Nav) (Pauillac *et al.,* 1998; Naar *et al*., 1998, 1999). They transformed 400 µg of PbTx-3 into its mono-succinylated derivative with a yield of 100%, which enabled them to conjugate it with HSA (human serum albumin) and to prepare specific antibodies (Naar *et al.,* 2001). The use of a synthetic fragment of the P-CTX-1, the JKLM fragment, with the help of Japanese colleagues who provided the fragment, has also been described in this context.

The Japanese teams announced that they could get round the problem of the lack of pure CTXs by preparing conjugate proteins with haptens like the JKLM terminal fragment of the CTX-1, as early as 1994 (Sasaki *et al.,* 1994). A carboxylic acid derivative with this fragment of CTX-1 was thus prepared with bovine serum albumin (BSA) and ovalbumin (OVA), for the purpose of immunisation and assessment of the responses of antibody production respectively (Pauillac *et al*., 2000). In this study, the conjugates were prepared with 3 to 5 mg of hapten using an activated ester in a semi-organic medium (bulk activated ester method) or with a microtechnique (300 µg of hapten) via the formation of a mixed anhydride in a reverse micellar medium. The two pairing methods provided densities of epitopes of the same order of magnitude: 20 and 12 for the BSA and OVA conjugates, respectively. Monitoring of the response regarding the production of monoclonal antibodies (Mabs) or of polyclonal antibodies (Pabs) in mice showed that a long-term immunisation protocol (injections 4 weeks apart) was more effective than a short-term immunisation protocol (injections two weeks apart). With detection in CIEA mode, the long-term Pabs had stronger affinity (KD = 7 x 10^{-9} M), together with narrow specificity, whereas the short-term Pabs had a weaker affinity (10 times less) but greater immunoreactivity for the JKLM fragment of CTX congeners that have it. No

cross-reaction was observed for PbTx-3, OA, monensin, or for other polyethers. In order to enhance the sensitivity of this test, a system of amplification by biotin-avidin can be used which lowers the LoD for the JKLM fragment to 1.23 × 10^{-9} M. The development and application of these immunoassays was pursued (Pauillac *et al*., 2002) and their evolution reported in 2010 in the article by Caillaud *et al.* (2010).

In Japan, Hirama's team had been working from the beginning of 1990 on the synthesis of ciguatoxins with a view to being able to produce appreciable quantities and was also attempting the successful synthesis of structures as complex as that of such polyethers as are only produced by certain dinoflagellates (Hirama *et al*., 2001; Inoue *et al.,* 2002, 2004, 2006a,b). The founding efforts of the current immunological approach, that of the direct sandwich ELISA technique, were made by Oguri *et al*. (2003), as reported in the abstract attached below.

Abstract of "Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX-3C" (Oguri et al., 2003)



Ciguatoxins are the major causative toxins of ciguatera seafood poisoning. Limited availability of ciguatoxins has hampered the development of a reliable and specific immunoassay for detecting these toxins in contaminated fish. Monoclonal antibodies (mAbs) specific against both ends of ciguatoxin CTX-3C were prepared by immunisation of mice with protein conjugates of rationally designed synthetic haptens, **3** and **4**, in place of the natural toxin. Haptenic groups that possess a surface area larger than 400 $Å^2$ were required to produce mAbs that can bind strongly to CTX-3C itself. A direct sandwich enzyme-linked immunosorbent assay (ELISA) using these mAbs was established to detect CTX-3C at the ppb level with no cross-reactivity against other related marine toxins, including brevetoxin A, brevetoxin B, okadaic acid, or maitotoxin.

These two pentacyclic fragments corresponding to the two terminal parts of CTX-3C (named ABCDE and IJKLM) were thus paired with the keyhole limpet hemocyanin (KLH) and BSA. After the mice had been immunised with the two KLH conjugates, they produced several monoclonal antibodies (Mabs). Two were selected because of their very high reactivity to their corresponding cyclic fragment as well as to CTX-3C, and also because of the lack of any cross-reaction to non-corresponding cyclic fragments or to other structurally connected marine biotoxins such as OA, PbTx congeners or maitotoxin (MTX). And therefore a direct sandwich ELISA based on the use of the selected monoclonal capture antibody i.e. MAb 10C9 (KD = 2.8 x 10^{-9} M for ABCDE) and on the detection Mab 3D11 for the other end (KD = 1.22 x 10^{-7} M for IJKLM) conjugated to horseradish peroxidase (HRP) was proposed as the reference method with an LoD of 5 Nm for the detection of CTX-3C. The authors rightly conclude that it must be possible to generalise this strategy to all CTX congeners. This assumption proved accurate, as 3 years later, the production of Mabs against the common cycles and terminals of CTX-1 and 51-hydroxy CTX-3C became possible by using a conjugate KLH protein from the synthetic HIJKLM fragment (Tsumuraya *et al*., 2006). MAb 8H4 was chosen because it is very reactive in capturing the right terminal fragment of 51-hydroxy CTX-3C (KD = 7.5 x 10^{-8} M). It also discriminates perfectly, as it distinguishes the compounds that do from those that do not contain the hydroxyl OH function in position 51 (OH-51). And as previously, a direct sandwich ELISA method was chosen, using as capture antibodies MAb 10C9 (KD = 2.8 x 10^{-9} M for the cyclic fragment of the left-hand end of CTX-3C and 51-hydroxy CTX-3C) and, as a new antibody detector, MAb 8H4 (KD = 7.5 x 10^{-8} M for the cyclic part of the

right-hand end of the 51-hydroxy CTX-3C) combined with the HRP. This gave an LoD of only 1 Nm for 51-hydroxy CTX-3C. Finally, more recently, Tsumuraya and his collaborators (Tsumuraya *et al*., 2010) reused the MAb 10C9 clone for the capture (directed against the left side of CTX-3C) with, for detection, another monoclonal antibody, MAb 3D11 (KD = 1.22 x 10^{-7} M for the right terminal of CTX-3C), and obtained an LoD of 5 Nm for CTX-3C. Furthermore, another approach described by the Hirama group involves the production of recombinant antibody fragments (rFabs) for the anti-fragment ABCD part (Nagumo *et al.,* 2004). The principle is based on the deduction that the epitope recognised by the anti-CTX MAbs encompasses more than 3 cycles, and the mouse MAbs transformed into ABC-KLH conjugates were genetically modified using a phage-display Abs technology. During the CIEA study, the three carefully selected rFabs gave KD values for the free ABCD fragments ranging from 2.4 x 10^{-5} M to 5.0 x 10^{-5} M. In addition, rFab 1C49, which has the highest reactivity for ABCD, can also react with CTX-3C, albeit less strongly. This type of experiment and other studies (Tsumoto *et al.,* 2008; Ui *et al.,* 2008; Tsumaraya *et al*., 2010) have confirmed the importance of the use of fragments with more than 4 cycles as synthetic haptens for creating hapten-protein conjugates in order to produce CTX anti-congeners with high affinity and specificity. The use of the combined monoclonal antibodies Mabs 10C9 and 3D11 (against respectively the left and right portions of CTX-3C) effectively permits a preventive neutralisation *in vivo* of poisoning in mice (Inoue et al., 2009).

There are therefore antibodies currently available with a promising sandwich ELISA technique whose principle was even used by the Hokama team, as reported above (Campora *et al*., 2008, 2010), but there remain problems regarding the diversity of ciguatoxins and the necessary versatility of the antibodies.

Annex 7: Tracking changes to the Report

Date	Version	Page	Description of the change (*in blue, italic*)
24/11/2014	03	01	**Text of 25 July 2014** July 2014 **Revised text** *November 2014*
24/11/2014	03	02	**Text of 25 July 2014** **Report**: 25 July 2014 ● version: 2 **Revised text** **Report**: *24 November 2014* ● version: *3*
24/11/2014	03	36 and 44	**Text of 25 July 2014** The analysis of a sample of flesh, a sample of oesophagus and three samples of dried fin by cytotoxicity tests on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated as follows: - Flesh: 0.144 µg eq. P-CTX-1 kg^{-1} (i.e. 14 times the concentration considered to be of no risk to humans); - Oesophagus: 114 µg eq. P-CTX-1 kg^{-1} (i.e. 11,400 times the concentration considered to be of no risk to humans); - Dried fin: 0.145 µg eq. P-CTX-1 kg^{-1}; 0.158 µg eq. PCTX-1 kg^{-1}; 0.737 µg eq. PCTX-1 kg^{-1} (i.e. 14 to 74 times the concentration considered to be of no risk to humans). **Revised text** The analysis of a sample of flesh, a sample of *stomach* and three samples of dried fin by cytotoxicity tests on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated as follows: - Flesh: 0.144 µg eq. P-CTX-1 kg^{-1} (i.e. 14 times the concentration considered to be of no risk to humans); - *Stomach*: 114 µg eq. P-CTX-1 kg^{-1} (i.e. 11,400 times the concentration considered to be of no risk to humans); - Dried fin: 0.145 µg eq. P-CTX-1 kg^{-1}; 0.158 µg eq. PCTX-1 kg^{-1}; 0.737 µg eq. PCTX-1 kg^{-1} (i.e. 14 to 74 times the concentration considered to be of no risk to humans).
25/07/2014	02	01	**Text of 26 June 2014** June 2014 **Revised text** *July 2014*
25/07/2014	02	02	**Text of 26 June 2014** **Report**: 26 June 2014 ● version: 1 **Revised text** **Report**: *25 July 2014* ● version: *2*
25/07/2014	02	03	**Text of 26 June 2014** The work described in this report was monitored and adopted by the Expert Committee (CES): CES ERCA "Assessment of physico-chemical risks in foods" - in plenary meetings of 19 November 2013, 17 March 2014, 14 April 2014, 26 June 2014 and by electronic means on 3 July 2014.

			Revised text The work described in this report was monitored and adopted by the Expert Committee (CES): CES ERCA "Assessment of physico-chemical risks in foods" - in plenary meetings of 19 November 2013, 17 March 2014, 14 April 2014, 26 June 2014 and by electronic means on 3 July 2014. *An updated version of the results was adopted by the Chair of the CES ERCA electronically on 25 July 2014, following the receipt of additional information on 21 July 2014.*
25/07/2014	02	35-36	**Text of 26 June 2014** In the light of the evidence presented earlier in the report, although the mouse bioassay provided a negative result for the 24 samples of shark flesh analysed, it was not possible to conclude with certainty that these samples were not contaminated by toxins at levels that could present a risk to the health of consumers. Further analyses by cytotoxicity tests on Neuro-2a cells and/or receptor tests, as well as by LC-MS/MS, would provide the necessary data. ANSES therefore contracted a research and development agreement (RDA) with ARVAM (Agency for Research and Marine Exploitation, Reunion Island), in collaboration with IRTA (*Instituto de Investigación y Tecnología Agroalimentaria*, Spain) for these samples of shark flesh from Reunion Island to be analysed by cytotoxicity tests on Neuro-2a cells. The preliminary results obtained from the 24 samples of shark flesh from Reunion Island did not reveal any toxicity in this test at the doses tested. The RDA also included samples from the shark implicated in an outbreak of food poisoning that occurred in Madagascar in November 2013 (124 people intoxicated, nine of whom died) for analysis by mouse bioassay and by cytotoxicity tests on Neuro-2a cells. Genetic analysis led to the conclusion that it was a bull shark. The sample of flesh gave a positive result by mouse bioassay; the first results obtained on Neuro-2a cells cannot yet be interpreted. The symptoms observed in mice (prostration, dyspnoea, cyanosis, convulsions and death by respiratory arrest) are typical of those known for carchatoxins (Boisier *et al*., 1995). Three samples of fin and one of oesophagus were also tested on Neuro-2a cells and the first results show an activity typical of that for ciguatoxins in these samples, especially strongly in the sample of oesophagus. These preliminary results are currently undergoing confirmation. **Revised text** In the light of the evidence presented earlier in the report, although the mouse bioassay provided a negative result for the 24 samples of shark flesh analysed, it was not possible to conclude with certainty that these samples were not contaminated by toxins at levels that could present a risk to the health of consumers. *This test is not sufficiently sensitive to detect concentrations of ciguatoxins considered to be of no risk to humans.* Further analyses by cytotoxicity tests on Neuro-2a cells and/or receptor tests, as well as by LC-MS/MS, would provide the necessary data. ANSES therefore contracted a research and development agreement (RDA) with ARVAM (Agency for Research and Marine Exploitation, Reunion Island), in collaboration with IRTA (*Instituto de Investigación y Tecnología Agroalimentaria*, Spain) for these samples of shark flesh from Reunion Island to be analysed by cytotoxicity tests on Neuro-2a cells. *The final report was submitted to ANSES on 21 July 2014.*

			The results did not show ciguatoxin-like toxins to be present beyond the limit of detection of 0.04 µg eq. P-CTX-1 kg^{-1} of flesh. It should be noted that the detection limit is greater than the concentration considered to be of no risk to humans of 0.01 µg eq. P-CTX-1 kg^{-1} of fish flesh (EFSA, 2010; US-FDA, 2011). This high limit of detection is mainly due to the matrix, as this was the first time that this type of sample had been studied in the laboratory. The RDA also included samples from the shark implicated in an outbreak of food poisoning that occurred in Madagascar in November 2013 (124 people intoxicated, nine of whom died) for analysis by mouse bioassay and by cytotoxicity tests on Neuro-2a cells. Genetic analysis led to the conclusion that it was a bull shark. The sample of flesh gave a positive result by mouse bioassay, with symptoms (prostration, dyspnoea, cyanosis, convulsions and death by respiratory arrest) typical of those known for carchatoxins (Boisier et al., 1995). *The analysis of a sample of flesh, a sample of oesophagus and three samples of dried fin by cytotoxicity tests on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated as follows:* - *Flesh: 0.144 µg eq. P-CTX-1 kg^{-1} (i.e. 14 times the concentration considered to be of no risk to humans);* - *Oesophagus: 114 µg eq. P-CTX-1 kg^{-1} (i.e. 11,400 times the concentration considered to be of no risk to humans);* - *Dried fin: 0.145 µg eq. P-CTX-1 kg^{-1}; 0.158 µg eq. PCTX-1 kg^{-1}; 0.737 µg eq. P-CTX-1 kg^{-1} (i.e. 14 to 74 times the concentration considered to be of no risk to humans).*
25/07/2014	02	44	**Text of 26 June 2014** ANSES therefore contracted a research and development agreement (RDA) with ARVAM (in collaboration with IRTA) for these samples to be analysed by cytotoxicity tests on Neuro-2a cells. The preliminary results obtained from the 24 samples of shark flesh from Reunion Island did not reveal any toxicity in this test at the doses tested. The RDA also included samples from the bull shark implicated in an outbreak of food poisoning that occurred in Madagascar in November 2013 (124 people intoxicated, nine of whom died) for analysis by mouse bioassay and by cytotoxicity tests on Neuro-2a cells. The sample of flesh gave a positive result by mouse bioassay; the first results obtained on Neuro-2a cells cannot yet be interpreted. The symptoms observed in mice (prostration, dyspnoea, cyanosis, convulsions and death by respiratory arrest) are typical of those known for carchatoxins (Boisier *et al.,* 1995). Three samples of fin and one of oesophagus were also tested on Neuro-2a cells and the first results show an activity typical of that for ciguatoxins in these samples, especially strongly in the sample of oesophagus. These preliminary results are currently undergoing confirmation. **Revised text** ANSES therefore contracted a research and development agreement (RDA) with ARVAM (in collaboration with IRTA) for these samples to be analysed by cytotoxicity tests on Neuro-2a cells. *The final report was submitted to ANSES on 21 July 2014.* *The results did not show ciguatoxin-like toxins to be present above the limit of detection of 0.04 µg eq. P-CTX-1 kg^{-1} of flesh. It should be noted that the detection limit is higher than the concentration considered to be of no risk to humans of 0.01 µg eq. P-CTX-1 kg^{-1} of fish flesh.* The RDA also included samples from the bull shark implicated in an outbreak of food poisoning that occurred in Madagascar in November

			2013 (124 people intoxicated, nine of whom died) for analysis by mouse bioassay and by cytotoxicity tests on Neuro-2a cells. The sample of flesh gave a positive result by mouse bioassay, with symptoms (prostration, dyspnoea, cyanosis, convulsions and death by respiratory arrest) typical of those known for carchatoxins (Boisier et al., 1995). *The analysis of a sample of flesh, a sample of oesophagus and three samples of dried fin by cytotoxicity tests on Neuro-2a cells concluded that ciguatoxin-like toxins were present, with concentrations estimated as follows:* - *Flesh: 0.144 µg eq. P-CTX-1 kg^{-1} (i.e. 14 times the concentration considered to be of no risk to humans);* - *Oesophagus: 114 µg eq. P-CTX-1 kg^{-1} (i.e. 11,400 times the concentration considered to be of no risk to humans);* - *Dried fin: 0.145 µg eq. P-CTX-1 kg^{-1}; 0.158 µg eq. PCTX-1 kg^{-1}; 0.737 µg eq. PCTX-1 kg^{-1} (i.e. 14 to 74 times the concentration considered to be of no risk to humans).*
25/07/2014	02	44	**Text of 26 June 2014** Date of validation of the Expert Appraisal by the Working Group: 25 July 2014 **Revised text** Date of validation of the Expert Appraisal by the Working Group: *25 July 2014*

Notes

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