Anses Opinion
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The Director General

Maisons-Alfort, 22 December 2021

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

on the state of knowledge on brevetoxins in shellfish, data on toxicity, occurrence and brevetoxin-producing microalgae

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 published on its website. This opinion is a translation of the original French version. In the event of any discrepancy or ambiguity the French language text dated 2 March 2021 shall prevail.

On 14 January 2020, ANSES received a request from the Directorate General for Food (DGAL) and the Directorate General for Health (DGS) to undertake an expert appraisal on the acute and chronic toxicity of brevetoxins likely to expose consumers to a risk by ingestion, inhalation or contact.

1. BACKGROUND AND PURPOSE OF THE REQUEST

In France, many unregulated toxins are studied by the network for monitoring the emergence of marine biotoxins in shellfish (EMERGTOX), whose steering committee is chaired by the DGAL and includes the DGS, the Directorate for Maritime Fisheries and Aquaculture, *Santé publique France*, ANSES, Ifremer and the Directorate for Water and Biodiversity (DEB).

Brevetoxins have been included in this network's work programme since January 2018.

They were first detected in France in November 2018 in mussels in Corsica (117 µg/kg digestive gland). Associated water samples collected for phytoplankton monitoring showed the presence of *Karenia* spp. (microalgal genus including some species that produce brevetoxins).

There is no maximum limit for these toxins in Regulation (EC) No 853/2004. The United States, Australia/New Zealand and Mexico apply a threshold of 800 µg BTX-2 equivalents/kg shellfish flesh. In Standard CODEXSTAN 292–2008 (rev. 2015) of the *Codex Alimentarius*, the

* revised Opinion cancelling and superseding the previous Opinion of 2 March 2021

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maximum limit for brevetoxins is 200 mouse units (MU)[1] or equivalent per kg of live mollusc flesh.

In this context, ANSES was asked to answer the following questions:

Question 1. What toxicological data are available for brevetoxins? Do these data enable ANSES to propose health-based guidance values for acute and chronic oral exposure?

Question 2. Based on the toxicological data identified by ANSES, is it possible to propose a guidance level in shellfish above which further investigations would be required as part of the EMERGTOX network?

Question 3. What methods could be recommended for monitoring brevetoxins in marine environments as part of the EMERGTOX network? What further investigations would be required if the guidance level was exceeded?

Question 4. Given the toxicological data and in view of the context, is there a public health concern associated with shellfish consumption based on the levels of contamination identified in certain marine areas of France?

Question 5. Is there a health concern related to exposure to brevetoxins via direct contact with contaminated water, associated in particular with swimming, water activities or inhalation of sea spray?

2. ORGANISATION OF THE EXPERT APPRAISAL

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

In the expert appraisal contract of 31 March 2020, ANSES informed the DGAL and DGS that questions 1 to 4 fall within the scope of the Expert Committee on "Assessment of physico-chemical risks in food" (CES ERCA). Question 5 falls within the scope of the CES on “Water risk assessment” (CES EAUX) and will be addressed later on. The amendment of 27 January 2021 states that the expert appraisal work will be validated by the CES EAUX in the first half of 2021. A final report containing the answers to all of the questions will be submitted in December 2021 at the latest.

ANSES entrusted examination of the first four questions to the Working Group (WG) on “Brevetoxins”, set up by a decision of 14 May 2020 following a public call for applications.

The methodological and scientific aspects of the work of the “Brevetoxins” WG were regularly submitted to the CES ERCA at plenary sessions on 5 March 2020, 7 October 2020, 4 November 2020, 6 January 2021 and 10 February 2021. The report produced by the Working Group takes account of the observations and additional information provided by the CES members and the reviewers. The expert work was adopted by the CES ERCA on 10 February 2021, unanimously by the experts present.

This work was therefore conducted by a group of experts with complementary skills.

[1] The mouse bioassay is an analytical method used to detect marine biotoxins in shellfish. A mouse unit (MU) is the amount of raw extract required to kill 50% of mice.

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 experts' declarations of interests are made public via the website: https://dpi.sante.gouv.fr/.

3. ANALYSIS AND CONCLUSIONS OF THE "BREVETOXINS" WG AND THE CES ERCA

The Expert Committee on "Assessment of physico-chemical risks in food" (CES ERCA) endorsed the collective expert appraisal report prepared by the "Brevetoxins" WG, which is summarised below.

The expert appraisal work was based on a scoping review. Two databases (Scopus and PubMed) were consulted on 14 May 2020 which led to the identification of 868 references, some of which were then selected using various criteria (detailed in the report, Section 1.5.1). The literature search was updated in October 2020, identifying four additional publications which, together with an article published online in December 2020, were added to the body of references.

3.1. Routes of exposure for humans

Humans can be exposed to brevetoxins through food, by inhalation and by mucocutaneous contact.

3.2. Chemical characterisation

Brevetoxins are a group of lipophilic marine biotoxins having around 30 metabolites whose chemical structure is known. There are also around 30 other metabolites whose empirical and structural formulas have not been chemically characterised; these metabolites are mainly products of metabolism by shellfish.

3.2.1. List of brevetoxins for the expert appraisal

Based on data on toxicity, on occurrence in shellfish and the availability of standards, the WG proposed considering the following brevetoxins (BTXs) as priorities: BTX-1, BTX-2, BTX-3, BTX-B1, BTX-B2, S-desoxy-BTX-B2, BTX-B3, and BTX-B4*(a et b).*

3.2.2. Analytical methods

Three main types of methods can be used to analyse brevetoxins in microalgal cells, algal bloom water samples, marine organisms (molluscs, fish) and sea spray/aerosol samples:

1. physico-chemical methods (such as liquid chromatography-mass spectrometry (LC-MS)) enable either the targeted identification and quantification of brevetoxins for which standards are available or the non-targeted high-resolution detection of potential new brevetoxin analogues;
2. biochemical methods, such as specific binding tests (receptor binding assay (RBA)) or immunological tests (radioimmunoassay (RIA), ELISA), enable the overall quantification of brevetoxins;

3. biological methods (*in vivo* and *in vitro*), in particular the mouse bioassay, fish bioassay and neuroblastoma cell-based assay (Neuro-2a), are able to determine the overall biological activity of brevetoxins.

Several of these methods have been validated through intra- and inter-laboratory studies. However, none have been validated through inter-laboratory studies in accordance with the guidelines of standards such as ISO 5725 and AOAC Appendix D. Therefore, to date, there are no standardised methods for the detection of brevetoxins.

3.3. Brevetoxin-producing microalgae

Brevetoxins are produced by marine microalgae, especially *Karenia brevis. K. brevis* has not been detected on the coast of metropolitan France to date. Other microalgae are suspected of producing brevetoxins. These include *K. mikimotoi, K. bicuneiformis, Chattonella marina, C. antiqua, Heterosigma akashiwo* and *Fibrocapsa japonica*. These species are found on the coasts of France, but their ability to produce brevetoxins has not been confirmed, except for *K. papilionacea*, whose production of BTX-2 has been confirmed in laboratory conditions.

Moreover, these microalgae also produce other toxins and secondary metabolites that may pose a risk to human health but are not covered by this request.

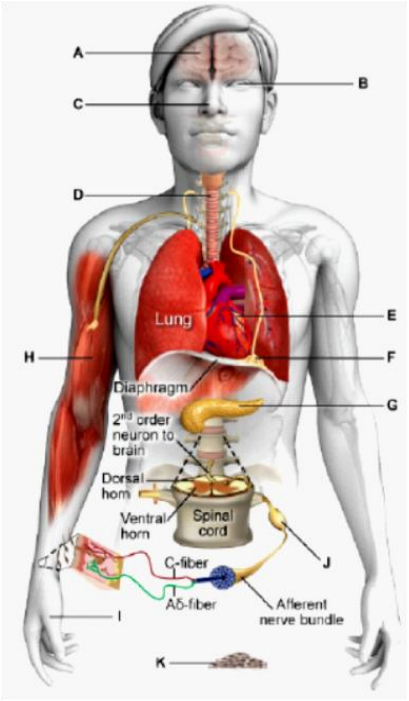
3.4. Effects of brevetoxins on organisms

3.4.1. Molecular targets and mode of action

Brevetoxins are neurotoxins whose primary targets are voltage-gated Na^+ channels (Na_v). Na_v channels are a class of transmembrane proteins that allow the passive diffusion of Na^+ ions on the surface of the membranes of excitable cells in particular but also of non-excitable cells. Na_v channels have three states: closed, open and inactivated. The binding of brevetoxins to site-5 of the Na_v channels activates them by slowing the inactivation process and shifting their activation to more negative potentials than the normal activation threshold of these channels. Brevetoxins can therefore be considered as Na_v channel activators, like ciguatoxins. Thus, they depolarise the membranes of nerve and muscle cells and promote their excitability as well as $Ca2^+$-dependent intracellular mechanisms.

This interaction between brevetoxins and Na_v channels as well as the tissue distribution of Na_v channels (Figure 1) explain the primarily neurological nature of the symptoms observed in humans and animals, which involve the central and peripheral nervous, but also gastrointestinal and cardiovascular systems.

Brevenal, produced by *K. brevis*, acts as an antagonist of brevetoxins by binding to Na_v channels and disrupting the binding of brevetoxins.



.	Tissue	Nav Subtype
A	Central nervous system	1.1, 1.2, 1.3, 1.6
B	Retina	1.8, 1.9
C	Olfactory sensory neurons	1.7
D	Sensory neurons and vagal sensory neurons innervating airways	1.7, 1.8, 1.9
E	Heart muscle	1.5, 1.8
F	Nerves, musculature involved in ventilation	1.1, 1.2, 1.3, 1.4, 1.6, 1.7
G	Pancreatic β-cells	1.7
H	Skeletal muscle	1.4
I	Skin	1.7, 1.8
J	DRG neurons	1.1, 1.3, 1.6, 1.7, 1.8, 1.9
K	Metastatic cancer cells	1.1, 1.9

Figure 1: Tissue expression of Na_v channels (according to the article by Lera Ruiz and Kraus, 2015, in ACS Publications)

3.4.2. *In vivo* toxicokinetics and toxicity

As liposoluble polyethers, brevetoxins are able to cross most biological barriers (gastrointestinal, blood-brain, placental). Studies in rodents show that once ingested, they are rapidly absorbed (within a few minutes) and distributed in most organs (liver, intestines, skeletal muscle) where they can persist for several days. They are metabolised in the liver and excreted mainly via the hepatobiliary route and to a lesser extent through urine.

Very few human toxicokinetic data are available. The only available information comes from measurements of brevetoxins in urine within a few hours of ingesting brevetoxin-contaminated shellfish. This method can be used to confirm a case of poisoning, provided that it is performed rapidly after ingestion.

In laboratory animals, following acute exposure to brevetoxins, a variety of effects are observed due to impairment of the central and peripheral nervous systems. The neurovegetative effects include hypersalivation, watery eyes, urination and defecation, and sometimes rhinorrhoea and compulsive chewing motions. The neuromuscular symptoms include tremors, muscle fasciculations and Straub tail reaction. The cardiorespiratory symptoms can include dyspnoea, respiratory discomfort and cardiac (contractility and heart rate) and haemodynamic disorders. Lastly, the central signs include ataxia, seizures and a decrease in body temperature.

In sheep exposed by intranasal instillation, respiratory difficulties (bronchoconstriction) were observed; simultaneous administration of brevenal, a *K. brevis* metabolite, reduced the respiratory signs caused by the brevetoxins.

The occurrence of delayed effects or sequelae following acute exposure was discussed by the WG, as was the possibility of long-term effects following repeated exposure to low doses (chronic toxicity). However, the WG did not identify any studies in the literature addressing

these aspects. They should be considered, due to the similarity between brevetoxins and ciguatoxins, which are the neurotoxins responsible for ciguatera whose effects last for several months or even years following acute exposure.

The effects of brevetoxins on reproduction and development in mammals are not known.

3.4.3. Toxicity in humans

The symptoms – and their duration – associated with exposure to brevetoxins depend on the route of exposure (food, inhalation, mucocutaneous contact). Knowledge on exposure by ingestion is briefly summarised below.

Neurotoxic shellfish poisoning (NSP) is a syndrome caused by the ingestion of shellfish contaminated by brevetoxins. The symptoms generally start one to 24 hours after exposure and can last up to three days. NSP is primarily characterised by the occurrence of digestive and neurological symptoms. The digestive signs include abdominal pain, nausea, vomiting and diarrhoea. The neurological signs mainly consist of paraesthesia (lips and extremities), dizziness, asthenia, partial paralysis of the limbs, speech impairment, loss of coordination and coma in the most serious cases. Reversal of temperature sensation, mydriasis and cardiovascular disorders (bradycardia, arterial hypotension) have also been reported. There is no antidote for brevetoxins; treatment is symptomatic. No deaths have been reported to date.

A few hundred cases of human poisoning associated with the consumption of brevetoxin-contaminated shellfish have been described in international journals. The small number of such events may be due to the introduction of regulations on the marketing of shellfish based on the monitoring of *K. brevis* cells along American coasts in the Gulf of Mexico and in Australia/New Zealand. Nonetheless, the number of cases of human poisoning remains underestimated, including in Florida where the reporting of this poisoning is mandatory.

The symptom most commonly observed is paraesthesia of the extremities and peri-oral area, noted in the most benign cases: tingling felt after consuming only two or three oysters (McFarren *et al.*, 1965). In severe cases (observed in young children), loss of consciousness, seizures, tachycardia and tachypnoea have been described.

More than 10 symptoms – mainly gastrointestinal, neurological and cardiovascular – can be associated with seafood poisoning caused by brevetoxins.

3.4.4. Genotoxicity

Studies dealing with the mutagenicity and genotoxicity of certain brevetoxins have found significant effects on various parameters. However, the experimental conditions of these studies have many major methodological limitations and/or show major deviations preventing their results from being validated or their interpretation from being endorsed. No conclusion can therefore be drawn regarding the mutagenic and genotoxic potential of brevetoxins. The observed effects on these biological parameters are “warnings” at the very most.

To obtain reliable and consolidated results, the strategy implemented should be based on a step-wise approach as recommended by the European Food Safety Authority (EFSA) (2011) for the production and assessment of data on the genotoxic and mutagenic potential of a substance (see Section 3.8 on research requirements).

3.4.5. Ecotoxicity

In addition to affecting human health, blooms of *K. brevis* and other species of the genus *Karenia* have caused mass mortality in fish, sea birds, turtles and marine mammals. The effects of brevetoxins on marine and coastal organisms have been described following experimental or environmental exposure. Brevetoxins cause episodes of high mortality during severe blooms reaching several million cells per litre.

Several routes of exposure have been identified: (1) direct exposure via the ingestion of microalgal cells and/or the filtration of water containing brevetoxins released following cell lysis, (2) ingestion via the food chain, and/or (3) inhalation, when brevetoxins are aerosolised during *Karenia* blooms.

The survival of marine invertebrates is not heavily affected, but sublethal effects (changes in development, growth, and eating and swimming behaviour) have been observed. In fish, exposure to sublethal concentrations of brevetoxins and/or *K. brevis* cells causes signs mainly involving the gills and respiratory function as well as the central nervous system and swimming behaviour. In marine mammals (manatees, dolphins), terrestrial mammals in coastal areas (coyotes, dogs) and sea turtles, neurological signs (primarily disorientation, ataxia and seizures) have been observed in parallel with the quantification of brevetoxins in biological fluids and tissue during/following *K. brevis* blooms.

3.5. Hazard characterisation

Data on acute toxicity in animals are very limited and do not enable a no observed adverse effect level (NOAEL) or a lowest observed adverse effect level (LOAEL) to be identified. The only study by oral administration aimed to determine median lethal doses (LD_{50}) for BTX-2 and BTX-3. This measure cannot be used as the point of departure for an acute reference dose (ARfD) because it would not be protective enough.

In humans, the data are also too limited to enable establishment of an ARfD.

Nevertheless, on the basis of data associated with human NSP cases after consumption of brevetoxin-contaminated shellfish, the WG identified lowest levels (ingested quantities of brevetoxins) associated with symptoms (“acute LOAELs”) and minimum concentrations in shellfish associated with symptoms (Table 1).

Table 1: Lowest levels of brevetoxins associated with symptoms ("acute LOAELs") and minimum concentrations in shellfish associated with symptoms

Studies	Lowest levels with symptoms ("acute LOAELs")	Minimum concentrations in shellfish flesh associated with symptoms
McFarren *et al.* (1965)	405-540 MU/person for moderate symptoms. A level of 54-81 MU/person induced minor symptoms (paraesthesia) in one case. Symptoms were associated with a level of 91 MU/person (the authors used a flesh weight of 20 g per oyster). *Note from the WG:* Using a consumed flesh weight of 10 g* instead of 20 g, the WG considers that the lowest level with symptoms would be **27-40.5 MU/person** (two or three oysters with 10 g of flesh, contaminated at a rate of 135 MU/100 g flesh)	135 MU/100 g for moderate symptoms. > 65 MU/100 g
Hemmert, 1975	**0.3-0.4 MU/kg bw**	75-118 MU/100 g
Morris, 1991		35 and 60 MU/100 g
Watkins, 2008; Terzagian, 2006		24 and 42.9 mg/kg
For comparison, the maximum level used by the *Codex Alimentarius*, the US FDA, Australia/New Zealand and Mexico		20 MU/100 g 800 µg BTX-2/kg

MU: mouse unit; *The WG considered a flesh weight of 10 g/oyster taking into account a condition index of 10% (this ranges from 6.5 to 10.5%) and the assumption of 100 g oysters (calibre 2, 86-110 g), shell included.

A mouse unit (MU) is the amount of raw extract that kills 50% of mice (20 g) within 930 minutes (15.5 hours). 1 MU = 3.4 µg BTX-3 or 4 µg BTX-2, according to Baden and Mende (1982).

Based on the data reported by Morris (1991), Gessner (2000) estimated the level causing minor symptoms at 42 to 72 MU/person. This value is often used in reviews but the WG considers it to be highly uncertain. It assumes that the consumption of 12 oysters (120 g considering a flesh weight of 10 g per oyster) is the threshold causing symptoms in a low proportion of individuals (two cases/15 in the group of low consumers) out of a total of 84 consumers. However, it is based on oyster contamination levels measured in leftovers from two meals (35 and 60 MU/100 g) that only caused four of the 48 quantified cases. The article by Morris (1991) does not state how many oysters contaminated at 35 and 60 MU/100 g were actually consumed by these four cases.

The studies by McFarren *et al.* (1965) and Hemmert (1975) are not mentioned in the EFSA Opinion (2010).

The WG found these studies to be particularly relevant for the expert appraisal since they contain detailed information about symptoms, portion sizes, body weights (for one study) and brevetoxin quantification in leftovers.

The WG identified two "acute LOAELs": 27-40.5 MU/person based on McFarren *et al.* (1965) and 0.3-0.4 MU/kg bw reported by Hemmert (1975).

The WG selected BTX-3 (and not BTX-2) as the reference brevetoxin in shellfish. BTX-3 is the reference BTX for the ELISA test; it has a lower LD_{50} value than BTX-2. Moreover, BTX-2 occurs in smaller quantities in shellfish compared to BTX-3. The limit of 20 MU/100 g or 800 µg BTX-2/kg corresponds to 680 BTX-3/kg shellfish flesh.

In BTX-3 equivalents (1 MU = 3.4 µg BTX-3), the "acute LOAELs" would be 92-138 µg BTX-3 eq./person based on McFarren *et al.* (1965) and 1.02-1.36 µg BTX-3 eq./kg bw based on Hemmert (1975).

3.6. Recommended guidance level in shellfish

To assess the degree of protection provided by the maximum level of 20 MU/100 g (800 µg BTX-2/kg shellfish flesh), the WG compared exposure associated with the consumption of shellfish contaminated at this level using the two selected "acute LOAELs", based on several consumption assumptions (Table 2).

Table 2: Assessment of the protective nature of the maximum level of 20 MU/100 g (800 µg BTX-2/kg shellfish flesh or 680 µg BTX-3/kg shellfish flesh)

Consumption assumptions	Exposure corresponding to the consumption of shellfish contaminated at the ML = 20 MU/100 g shellfish flesh	Lowest dose with symptoms ("acute LOAEL")	
400 g of shellfish	**80 MU/person**	0.3-0.4 MU/kg bw (Hemmert, 1975), assumption of a 70 kg body weight = **21-28 MU/person** i.e. 1.02-1.36 µg BTX-3 eq./kg bw	**27-40.5 MU/person** (calculated by the WG based on McFarren *et al.*, 1965) i.e. 92-138 µg BTX-3 eq./person
Consomer study (oysters) **P95 = 182.4 g** **P97.5 = 255 g**	36.5 MU/person 51 MU/person		
Consomer study (mussels) **P95 = 200 g** **P97.5 = 300 g**	40 MU/person 60 MU/person		
Consomer study (clams) **P95 = 50 g** **P97.5 = 60 g**	10 MU/person 12 MU/person		

The estimates are based on calculation assumptions made both by the authors and by the WG, each associated with a margin of error that is difficult to estimate. The values in Table 2 should therefore be considered with a moderate level of uncertainty for the study by Hemmert (1975) and with a high level of uncertainty for that by McFarren et al. (1965).

Based on the acute LOAEL of 0.3-0.4 MU/kg bw reported by Hemmert (1975), with an assumed body weight of 70 kg and protective default consumption of 400 g of shellfish flesh, the WG calculated a level of 52.5-70 MU/kg shellfish flesh. According to Baden and Menden (1982), 1 MU = 3.4 µg BTX-3, which would correspond to a concentration of **179-238 µg BTX-3 eq./kg shellfish flesh** (three to four times lower than the limit of 800 µg BTX-2/kg corresponding to 680 BTX-3/kg shellfish flesh).

Based on the acute LOAEL of **27-40.5 MU/person** calculated by the WG from the study by McFarren *et al*. (1965), assuming protective default consumption of 400 g of shellfish flesh, the WG calculated a level of 67.5-101.25 MU/kg shellfish flesh, i.e. a concentration of **230-344 µg BTX-3 eq./kg shellfish flesh** (two to three times lower than the limit of 800 µg BTX-2/kg corresponding to 680 BTX-3/kg shellfish flesh).

In conclusion, the maximum level of 20 MU/100 g shellfish flesh (equivalent to 800 BTX-2/kg shellfish flesh) does not appear protective enough. **The WG therefore recommends a**

guidance level of 180 µg BTX-3 eq./kg shellfish flesh (corresponding to the sum of all tested BTX metabolites). It was not deemed necessary to apply an additional safety factor due to the protective assumptions on which the calculations were based (default consumption of 400 g of shellfish flesh and a 70 kg body weight).

3.7. Monitoring

In the United States, monitoring systems have been put into place, in particular in Florida, Texas, Delaware and Alabama. The risks associated with blooms of the *Karenia* genus are monitored and controlled via the regular monitoring of *K. brevis* in water. Brevetoxins are monitored in shellfish and air, or in response to an episode of mass mortality in fish or when respiratory symptoms have been reported in humans. Citizen science networks are also involved in this monitoring.

In the United States, Mexico, Australia and New Zealand, the health authorities have established thresholds: a) for the number of microalgal cells in water that can lead to preventive management measures; b) for brevetoxins in shellfish flesh requiring the closing of production areas.

In France, REPHY[2] is in charge of observing all phytoplankton species in coastal waters, through regular sampling from 177 sites spread out across the metropolitan coast. Brevetoxins are among the emerging toxins monitored by the EMERGTOX network.

Brevetoxins (BTX-2 and/or BTX-3) were first detected in France in November 2018, with a maximum level, observed in November 2020, of 57.4 µg/kg total mussel flesh for the sum of BTX-2 and BTX-3. They have only been detected in mussels from the Diana lagoon in Corsica. The occurrence of brevetoxins in shellfish may be due to the presence of *Karenia sp.* and/or other organisms potentially producing these toxins, as was found in the water samples collected in parallel with the shellfish samples. It is therefore essential to identify the species of microalgae producing these toxins in the Diana lagoon in Corsica.

3.8. Research requirements

The WG identified a lack of knowledge and recommended the following lines of research.

Concerning toxicity data

- The effects of brevetoxins should be covered by an acute oral toxicity study in rodents conducted according to OECD Guideline 424 for neurotoxicity testing (single administration, 14-day observation period); it should describe all of the visible clinical symptoms (in particular behavioural changes such as hyper-reactivity) and evaluate biological parameters (respiratory and cardiovascular parameters, monitoring of internal temperature, gastrointestinal effects) as thoroughly as possible, to determine the dose-effect relationship. Such a study would help define a critical effect of acute poisoning and identify a no observed effect level as the point of departure for deriving a health-based guidance value. This recommendation should apply firstly to BTX-3 and then to other major brevetoxins in shellfish.
- To obtain reliable and consolidated results concerning the mutagenic and genotoxic potential of brevetoxins, the strategy implemented should use the step-wise approach

[2] Observation and Monitoring Network for Phytoplankton and Hydrology in coastal waters (REPHY) implemented by the French Research Institute for Exploitation of the Sea (Ifremer)

recommended by EFSA (2011), starting with the following basic battery of *in vitro* tests: 1) an Ames test[3] (bacterial reverse mutation test) according to OECD Guideline 471, 2) a chromosome aberration test, preferably the *in vitro* micronucleus test according to OECD Guideline 487. The first brevetoxin to be studied should be BTX-3. The potential of the other major metabolites found in shellfish should also be investigated.

- Toxicokinetic data should be acquired for BTX-3 following oral exposure in rodents according to OECD Guideline 417.
- Subacute/subchronic toxicity studies should be considered. They should be conducted by repeated oral administration for 28 or 90 days in rodents according to OECD Guidelines 424, 407 and 408. This recommendation should apply firstly to BTX-3 and then to other major BTX in shellfish. The chronic toxicity of brevetoxins should also be considered in studies in rodents.
- The developmental and neurodevelopmental toxicity of brevetoxins should be explored in a study aiming to identify the consequences for offspring of oral exposure to brevetoxins in pregnant females (OECD Guidelines 414 and 426).
- Metabolites in shellfish for which there are no data on toxic potential should be isolated and identified (clarify their chemical structure). Sufficient quantities should be produced to be able to study their toxic potential *in vitro* (RBA and/or Neuro-2a).
- Studies should be undertaken on the relative toxicity of BTX-3 compared with major brevetoxins in shellfish to determine toxic equivalency factors (TEFs).
- The effects of mixtures of metabolites should also be investigated.

Concerning analytical methods, it is necessary to:

- produce and make available a larger number of standards and reference materials for brevetoxins and their metabolites;
- develop physico-chemical analytical methods based on mass spectrometry, capable of analysing both native brevetoxins and their most polar metabolites so that these may be taken into account when present in shellfish;
- undertake rigorous intra-and inter-laboratory validation studies (AOAC or ISO) to assess the reliability of physico-chemical, biochemical and biological methods;
- develop analytical methods for the detection of brevetoxins in urine or blood samples to confirm cases of human poisoning.

Concerning monitoring

- The kinetics of shellfish brevetoxin contamination and decontamination should be studied (using strains of brevetoxin-producing microalgae identified in Corsica).
- An ecophysiological evaluation of the brevetoxin-producing microalgae identified in Corsica is recommended with a view to determining the environmental factors that promote cell proliferation and toxin production.

[3] Special attention should be paid in the event of a negative test with a maximum analysable level < 250 µg/dish, which is the limit of sensitivity estimated for most relevant mutagens in the Ames test. In this case, the Ames test should be considered as insufficiently relevant and gene mutation induction potential should be studied in mammalian cells for example, using the MLA/TK test (OECD, 2016b).

- Studying dissolved brevetoxins with passive samplers (providing an integrative view of the situation) would help better identify the areas impacted.
- A study in overseas territories (particularly in the Caribbean Sea) could investigate the presence of brevetoxin-producing microalgae and brevetoxins in shellfish.
- The WG recommends studying whether there is associated fish mortality in nearby geographical areas. It would be worthwhile to analyse fish samples collected in the area impacted by brevetoxins (muscle and liver).
- If cases of human food poisoning with brevetoxins occur, the patients should be monitored to determine whether there are any persistent effects (sequelae).

4. CONCLUSIONS AND RECOMMENDATIONS OF THE WG ON "BREVETOXINS" AND THE CES ERCA

Question 1. What toxicity data are available for brevetoxins? Do these data enable ANSES to propose health-based guidance values for acute and chronic oral exposure?

The data in the literature from oral acute toxicity studies in animals are very limited and do not enable a no observed adverse effect level (NOAEL) or a lowest observed adverse effect level (LOAEL) to be identified. The WG identified a single study conducted in mice to determine the median lethal dose (LD_{50}) for BTX-2 and BTX-3. Acute toxicity has been studied following intraperitoneal and intravenous administration for BTX-1, BTX-2 and BTX-3. Brevetoxins cause neurological (central and peripheral), cardiovascular and respiratory symptoms: these mainly take the form of muscle fasciculations, ataxia, a decreased respiratory rate, cardiac activity disorders and a decrease in body temperature. Neurological symptoms can also be neurovegetative and include salivation, watery eyes, urination and defecation.

In humans, the ingestion of brevetoxin-contaminated shellfish can cause a syndrome called Neurotoxic Shellfish Poisoning (NSP). The symptoms generally start one to 24 hours after ingestion and can last up to three days. NSP is primarily characterised by the occurrence of neurological and digestive symptoms. More specifically, the neurological signs mainly consist of paraesthesia (of the lips and extremities), dizziness, asthenia, partial paralysis of the limbs, speech impairment, loss of coordination and coma in the most serious cases. Reversal of temperature sensation, mydriasis and cardiovascular disorders (bradycardia, arterial hypotension) have also been reported. The digestive signs include abdominal pain, nausea, vomiting and diarrhoea. No deaths associated with brevetoxins have been reported to date.

A few hundred cases of food poisoning have been described and published in international scientific journals. They have occurred in Florida, North Carolina, New Zealand and Mexico. No cases caused by the consumption of fish have been reported to date.

The small number of such events may be due to the introduction of regulations on the marketing of shellfish based on the monitoring of *K. brevis* cells along the East Coast of the United States, in particular in the Gulf of Mexico, and in Australia/New Zealand.

Based on data on cases of human poisoning associated with the consumption of brevetoxin-contaminated shellfish, the WG identified two lowest levels associated with symptoms ("acute LOAELs"): 27-40.5 MU/person based on McFarren *et al.* (1965) and 0.3-0.4 MU/kg bw reported by Hemmert (1975). In BTX-3 equivalents, the LOAELs would be 92-138 µg BTX3 eq./person based on McFarren *et al.* (1965) and 1.02-1.36 µg BTX-3 eq./kg bw based on

Hemmert (1975). However, these data are too limited to be able to establish an acute reference dose (ARfD).

The quality of the available genotoxicity data was considered insufficient to draw any conclusion.

Given the similarity between brevetoxins and ciguatoxins, which are the neurotoxins responsible for ciguatera, whose effects can last several months or even years following acute exposure, the WG considers that long-term effects following acute exposure to brevetoxins cannot be ruled out.

To the WG's knowledge, no repeated-dose oral toxicity studies are available, meaning that it is not possible to propose a chronic health-based guidance value.

Information is also lacking regarding the potential reproductive and developmental toxicity of brevetoxins.

Question 2. Based on the toxicological data identified by ANSES, is it possible to propose a guidance level in shellfish above which further investigations would be required as part of the EMERGTOX network?

To assess the degree of protection provided by the maximum level of 20 MU/100 g (800 µg BTX-2/kg shellfish flesh), the WG compared exposure associated with the consumption of shellfish contaminated at this level using the two identified “acute LOAELs”, based on several consumption assumptions.

The WG selected BTX-3 as the reference BTX in shellfish (and not BTX-2). BTX-3 is the reference brevetoxin for the ELISA test; it has a lower LD_{50} value than BTX-2. Moreover, BTX-2 occurs in small quantities in shellfish compared to BTX-3.

Based on the “acute LOAEL” of **0.3-0.4 MU/kg bw** reported by Hemmert (1975), with an assumed body weight of 70 kg and protective default consumption of 400 g of shellfish flesh, the WG calculated a level of 52.5-70 MU/kg shellfish flesh. According to Baden and Menden (1982), 1 MU = 3.4 µg BTX-3, which would correspond to a concentration of **179-238 µg BTX-3 eq./kg shellfish flesh** (three to four times lower than the limit of 800 µg BTX-2/kg corresponding to 680 BTX-3/kg shellfish flesh).

Based on the “acute LOAEL” of **27-40.5 MU/person** calculated by the WG from the study by McFarren *et al*. (1965), assuming protective default consumption of 400 g of shellfish flesh, the WG calculated a level of 67.5-101.25 MU/kg shellfish flesh, i.e. **230-344 µg BTX-3 eq./kg shellfish flesh** (two to three times lower than the limit of 800 µg BTX-2/kg corresponding to 680 BTX-3/kg shellfish flesh).

The WG considers that the maximum level of 800 µg BTX-2/kg shellfish flesh does not appear protective enough and recommends a **guidance level of 180 µg BTX-3 eq./kg shellfish flesh (corresponding to the sum of all tested BTX metabolites).**

Question 3. What methods could be recommended for monitoring brevetoxins in marine environments as part of the EMERGTOX network? What further investigations would be required if the guidance level was exceeded?

Monitoring of brevetoxin-producing microalgae

Since 2008, the majority of the water samples collected for REPHY have been from subsurface water (one metre below the surface). The WG recommends developing a sampling procedure that would include the water column. This recommendation is not specific to this formal request dealing with brevetoxins.

The frequency applied by REPHY (once a fortnight in routine conditions, once a week if the alert threshold is exceeded for microalgae producing regulated phycotoxins) seems appropriate: since shellfish sampling as part of EMERGTOX takes place on a monthly basis, there are at least two water samples preceding the shellfish samples.

The data currently available are not sufficient to propose a threshold concentration of microalgae (number of cells per litre) applicable in France (more particularly in Corsica, which is the only area of metropolitan France affected by brevetoxins to date) as part of EMERGTOX.

To compensate for the lack of an alert threshold for *Karenia* spp. in France, the WG recommends collecting samples of water on a weekly basis in the event that brevetoxins have been quantified in shellfish at a given location.

Concerning water analyses, it is first necessary to have an unfixed raw-water sample to be able to observe living cells. Then, if target species are detected, an organisation specialising in microalgal taxonomy should be called upon.

If brevetoxins are detected in shellfish, given the difficulty of identifying brevetoxin-producing microalgae using optical microscopy (in particular to distinguish between *K. brevis* and *K. papilionacea*), the WG recommends using molecular biology (PCR amplification of several molecular markers, in the D1-D2 region of 28S rDNA, the V4 region of 18S rDNA and the ITS2 intergenic region).

Furthermore, regarding the situation in Corsica, the WG recommends:

- isolating and culturing potential brevetoxin-producing species of microalgae;
- testing for the potential presence of brevetoxin-producing microalgae on any other shellfish production sites, including in the open sea;
- determining an alert threshold for brevetoxin-producing microalgae (that would trigger brevetoxin analyses in shellfish).

Monitoring of brevetoxins in shellfish

The WG recommends a guidance level of 180 µg BTX-3 eq./kg shellfish flesh for the sum of all tested BTX metabolites.

In the event that this guidance level is exceeded in mussels or oysters in Corsica (species monitored by EMERGTOX at the Diana site, the only area of metropolitan France affected by brevetoxins to date), the WG recommends:

- actively providing information to healthcare professionals to improve the detection of potential poisoning cases;
- testing for brevetoxins in the other shellfish species produced in the affected area;

- testing for the potential presence of brevetoxins in shellfish on any other shellfish production sites, including in the open sea.

Moreover, the WG recommends identifying high-risk periods in Corsica during which shellfish could be sampled more frequently.

In the event of recreational shellfish harvesting in the nearby geographical area, the WG recommends that these results be taken into account by the risk manager.

These recommendations also apply in the event that this guidance level is exceeded in shellfish in production areas other than Corsica.

Currently, as part of the EMERGTOX network, only BTX-2 and BTX-3 are analysed using the LC-MS/MS multi-toxin method that targets several groups of regulated and unregulated lipophilic toxins at European level. The detection of brevetoxins in France since 2018 should lead testing to be extended to other brevetoxins.

The WG recommends developing complementary approaches:

- an ELISA test to screen for type-B brevetoxins produced by microalgae as well as metabolites formed in shellfish flesh. This approach is able to take into account brevetoxins that cannot be analysed by LC-MS/MS since the corresponding reference substances are not commercially available;
- a targeted physico-chemical analytical method specific to brevetoxins for which standards are available using LC-MS/MS. The WG also recommends developing an analytical method that can detect less lipophilic metabolites of brevetoxins in shellfish, to be able to take them into account if necessary;
- a non-targeted analysis using LC-HRMS to obtain spectral information for identifying possible new brevetoxins and/or new metabolites or degradation products.

The list of metabolites that the WG considers as priorities is as follows: BTX-1, BTX-2, BTX-3, BTX-B1, BTX-B2, S-desoxy-BTX-B2, BTX-B3, and BTX-B4(a and b).

The WG recommends undertaking analyses in total flesh instead of the digestive gland (to be more representative for risk assessment purposes).

Epidemiological monitoring

In early 2021, the WG on Natural toxins of ANSES's Health Alerts & Vigilance Department (DAVS) developed a reporting form for Poison Control and Monitoring Centres (CAPTVs) in order to identify cases of poisoning with marine biotoxins. Brevetoxins are included on this form, which will later be supplemented for aspects involving exposure to brevetoxins by inhalation and mucocutaneous contact.

Question 4. Given the toxicological data and in view of the context, is there a public health concern associated with shellfish consumption based on the levels of contamination identified in certain marine areas of France?

Since the EMERGTOX network started testing for brevetoxins at the beginning of 2018, quantified concentrations have only been reported in mussels from the Diana lagoon (Corsica). The maximum concentration reported to date is 345 µg/kg digestive gland for the sum of BTX-

2 and BTX-3 (November 2020), which corresponds[4] to an estimated value of 57 µg/kg total flesh for the sum of BTX-2 and BTX-3.

The WG notes that the concentrations of the two brevetoxins in shellfish increased from 20 µg/kg total flesh in November 2018 to 44 µg/kg in January 2019 and then to 57 µg/kg in November 2020.

These data are too limited to serve as a basis for estimating the dietary exposure of consumers.

The concentrations are below the new guidance level proposed by the WG (180 µg BTX-3 eq./kg shellfish flesh), but the WG underlines that the total concentration of brevetoxins may be underestimated due to the fact that only two brevetoxins out all of the potentially toxic metabolites have been analysed by EMERGTOX.

That is why the WG recommends expanding the study of the following BTXs for multi-dimensional or high-resolution LC-MS analyses as soon as possible: BTX-1, BTX-2, BTX-3, BTX-B1, BTX-B2, S-desoxy-BTX-B2, BTX-B3, and BTX-B4(a and b).

Therefore, the WG considers that a health concern following acute exposure cannot be ruled out.

Lastly, the WG cannot rule on potential effects associated with repeated exposure to low concentrations.

5. AGENCY CONCLUSIONS AND RECOMMENDATIONS

The French Agency for Food, Environmental and Occupational Health & Safety endorses the conclusions of the Working Group on “Brevetoxins” and the Expert Committee on “Assessment of physico-chemical risks in food” (CES ERCA).

This expert work is a step forward in taking into account brevetoxins in shellfish in France. Although these marine biotoxins pose a proven risk in Florida in particular, as well as in Australia/New Zealand and Mexico, they constitute an emerging risk in France and more broadly in Europe, where they are not regulated. It was thanks to the network for monitoring the emergence of marine biotoxins in shellfish (EMERGTOX), set up by the DGAL, that brevetoxins were first detected in French shellfish in November 2018.

The analysis of the literature data enabled a guidance level in shellfish to be proposed, expressed in BTX-3 equivalents for the sum of all of the tested brevetoxins, by combining two complementary analytical approaches, ELISA and LC-MS/MS.

To date, brevetoxins have been detected in French shellfish exclusively in Corsica, in the Diana lagoon. The Agency therefore recommends prioritising research work to better understand this situation in order to identify the species of producing microalgae, propose an alert threshold (in number of cells in water) and characterise the toxic profile.

To be able to establish an acute health-based guidance value, the Agency has also identified the implementation of an acute oral toxicity study in rodents, according to OECD Guideline 424 for neurotoxicity testing, as a priority action.

[4] Assuming that BTX are concentrated exclusively in the digestive gland and based on preliminary results from 2018 and 2019 in total flesh.

In early 2021, thanks to the work carried out by the Working Group on Natural toxins of ANSES's Health Alerts & Vigilance Department (DAVS), a reporting form was developed for Poison Control and Monitoring Centres (CAPTVs) in order to identify cases of poisoning associated with the consumption of shellfish contaminated by marine biotoxins. Brevetoxins are included on this form, which should facilitate the identification of cases and help more precisely estimate the level of health concern related to brevetoxins in France.

Dr Roger Genet

KEYWORDS

Brevetoxins, emerging toxins, marine biotoxins, shellfish, Karenia sp., Raphidophytes, toxicity, occurrence

REFERENCES

Refer to collective expert appraisal report

SUGGESTED CITATION

ANSES (2021). Opinion of the French Agency for Food, Environmental and Occupational Health & Safety of 22 December 2021 on the state of knowledge on brevetoxins in shellfish, data on toxicity, occurrence and brevetoxin-producing microalgae (Request No 2020-SA-0020). Maisons-Alfort: ANSES, 18 p. The opinion is accompanied by a collective expert appraisal report (in French).

ANNEXE

Tracking changes to the ANSES Opinion.

Date	Page	Description of the change
March 2021		First version of the Opinion
December 2021	3	**3.2.1. List of brevetoxins for the expert appraisal** Based on data on toxicity, on occurrence in shellfish and the availability of standards, the WG proposed considering the following brevetoxins (BTX) as priorities: BTX-1, BTX-2, BTX-3, BTX-B1, BTX-B2, ~~desoxy-BTX-B2~~, S-desoxy-BTX-B2, ~~sulfoxide-BTX-B2~~, BTX-B3, and BTX-B4*(*a *and b).*
	15	The list of metabolites that the WG considers as priorities is as follows: BTX-1, BTX-2, BTX-3, BTX-B1, BTX-B2, ~~desoxy-BTX-B2~~, S-desoxy-BTX-B2, ~~sulfoxide-BTX-B2~~, BTX-B3, and BTX-B4(a *and b)*.
	16	That is why the WG recommends expanding the study of the following BTX for multi-dimensional or high-resolution LC-MS analyses as soon as possible: BTX-1, BTX-2, BTX-3, BTX-B1, BTX-B2, ~~desoxy-BTX-B2~~, S-desoxy-BTX-B2, ~~sulfoxide-BTX-B2~~, BTX-B3, and BTX-B4(a *and b)*.