The Director General

Maisons-Alfort, 12 March 2019

# OPINION

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

### on the assessment of the health risks associated with pinnatoxins in shellfish

*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 12 March 2019 shall prevail.*

On 11 January 2016, ANSES received a formal request from the Directorate General for Food (DGAL) and the Directorate General for Health (DGS) to undertake the following expert appraisal: Request for an opinion on the acute and chronic toxicity of pinnatoxins.

## 1. BACKGROUND AND PURPOSE OF THE REQUEST

In a 2012 report, the French Research Institute for Exploitation of the Sea (Ifremer) revealed the presence of pinnatoxins (PnTXs) produced by the dinoflagellate *Vulcanodinium rugosum* in mussels from the Ingril lagoon (Hérault). Concentrations varied greatly depending on the years (2010, 2011 and 2012), with a maximum of 1244 µg of PnTX G per kg of shellfish (wet weight) in 2010 (Ifremer report, September 2012, "Pinnatoxines en lien avec l'espèce *Vulcanodinium rugosum*" ["Pinnatoxins related to the species *Vulcanodinium rugosum*"]). In July 2015, a concentration of 1143 µg of PnTX G per kg of shellfish was reported by Ifremer.

The presence of these toxins could explain cases of discrepancies between the results obtained by mouse bioassay and those obtained by chemical analysis (LC-MS/MS) in mussels from the Ingril lagoon, in the context of the vigilance scheme for lipophilic marine biotoxins in shellfish led by the DGAL.

In addition, research by the University of Trieste, in collaboration with CNRS (Gif-sur-Yvette) and ANSES (Fougères), has established a median lethal dose (LD$_{50}$) of around 200 µg/kg of body weight (bw) in mice by gavage (ANSES-University of Trieste-CNRS report, 2014).

In this context, on 11 January 2016, ANSES received a formal request from the DGAL and the DGS to respond to the following questions:

French Agency for Food, Environmental and Occupational Health & Safety,
14 rue Pierre et Marie Curie, 94701 Maisons-Alfort Cedex
Telephone: +33 (0)1 49 77 13 50 - Fax: + 33 (0)1 49 77 26 26 - www.anses.fr

1) Are there any toxicological data for pinnatoxins and is there a recognised $LD_{50}$ for acute effects in particular? Are there any data for the chronic risk in the form of a toxicity reference value (TRV)?

2) Given these toxicological data and in view of the context, is there a public health concern regarding the levels of contamination identified by Ifremer in certain French shellfish production areas?

3) What methods could be recommended for monitoring pinnatoxins in the marine environment, with a view to including these toxins in the vigilance scheme for lipophilic marine biotoxins in shellfish, led by the DGAL?

## 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)".

It falls within the sphere of competence of the Expert Committee on "Assessment of physico-chemical risks in food" (CES ERCA). ANSES entrusted examination of this request to the "Pinnatoxins" Working Group, set up by a decision of 14 November 2017 following a call for applications.

The methodological and scientific aspects of the work of the "Pinnatoxins" WG were regularly submitted to the CES ERCA at plenary sessions on 15 September, 18 October and 14 December 2018, and 25 January 2019. 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 25 January 2019 by all the experts present, with the exception of one who abstained[1].

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 ANSES website (www.anses.fr).
Due to the ownership of a patent concerning the detection of pinnatoxins, Mr Jordi Molgó was not selected to be a member of the Working Group. In view of his specific skills, however, he was appointed rapporteur to contribute to the work on hazard identification and characterisation. He did not participate in the discussions or in the drafting of the conclusions regarding surveillance of pinnatoxins and other related toxins.
Ms Valérie Fessard (ANSES, Fougères laboratory), a member of the "Pinnatoxins" Working Group, was not involved in the *in vivo* study described in the ANSES-University of Trieste-CNRS report, 2014.

## 3. ANALYSIS AND CONCLUSIONS OF THE "PINNATOXINS" 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 "Pinnatoxins" Working Group, a summary of which is presented below.

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[1] Considering that his personal schedule did not allow him to attend several of the CES ERCA meetings during which this matter was presented and discussed, and that in his view simply reading the documents communicated to CES members did not constitute a sufficient basis for forming a fully reasoned judgement on this matter, the expert considered it advisable to abstain.

### 3.1. Hazard identification

#### 3.1.1. Chemical characterisation of toxins produced by *Vulcanodinium rugosum*

In the framework of this expert appraisal, hazard identification took into account the toxins produced by the dinoflagellate *V. rugosum*.

Pinnatoxins (PnTXs) belong to the group of cyclic and macrocyclic imines which, to date, includes 40 compounds, without considering acyl esters, which are products of shellfish metabolism. This group includes different families determined by their structural characteristics: prorocentrolides, spiroprorocentrimine, gymnodimines (GYMs), spirolides (SPXs), pinnatoxins (PnTXs), pteriatoxins (PtTXs) and portimine.

PnTXs are soluble in solvents such as acetone, isopropanol and methanol (Zendong *et al.* 2014). They are amphoteric compounds, i.e. both acidic and basic, and are therefore ionised, which also explains their relative water solubility. In addition, they have lipophilic properties, hence their detection during the mouse bioassay used to screen for lipophilic toxins.

Once absorbed by molluscs, certain marine toxins can undergo metabolic reactions leading to a modification of their chemical structure, particularly when in the form of acyl derivatives. As with dinophysistoxin-3 (DTX3), the WG assumes that alkaline hydrolysis of their ester bond releases the parent compound. PtTXs are most likely the metabolites of certain PnTXs. The toxicity of the metabolites may vary from that of their precursors.

Based on the shellfish consumed and the toxins produced by *Vulcanodinium rugosum*, the "Pinnatoxins" WG selected PnTXs, PtTXs and portimine as the hazards to be taken into account in this expert appraisal.

#### 3.1.2. Analytical methods

Most of the different methods used to analyse toxins produced by *V. rugosum* concern PnTXs. They are mainly physico-chemical methods using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Biological (mouse bioassays) or biochemical (functional tests) methods can also be used to analyse these toxins.

None of the methods have yet undergone inter-laboratory validation or standardisation. In addition, there are very few standards (reference substances) available for these toxins, which limits and complicates their detection and quantification. Indeed, to date, only PnTX A and G are marketed as calibration solutions and only PnTX G has been certified (CRM-PnTX G). Table 1 shows the different analytical methods used to detect toxins produced by *V. rugosum*, indicating their performance and advantages/disadvantages.

**Table 1: Summary table of analytical methods for the detection of *V. rugosum* toxins**

| Method type | Sensitivity and extraction performance | Advantages | Disadvantages |
|---|---|---|---|
| Chemical methods: LC-MS/MS and LC-HRMS | Limit of detection: 0.09 to 0.5 µg/kg of shellfish meat<br><br>Limit of quantification: 0.9 to 3 µg/kg of shellfish meat<br><br>Extraction performance (PnTX G):<br>mussels: 78% to 94%<br>oysters: 67% | - High specificity of chromatographic separation of toxins and detection by mass spectrometry<br>- Very good sensitivity | - Cost of equipment and qualified personnel<br>- No inter-laboratory validation for PnTXs<br>- Limited availability of standards (only PnTX A and G are available as calibration solutions)<br>- Specificity in mass spectrometry can be a critical issue for some toxins: confusion is possible between PnTX G and spirolide B or 13-desmethyl spirolide D under certain conditions (co-elution of these toxins and lack of transition or confirmation methods) |

| Method type | Sensitivity and extraction performance | Advantages | Disadvantages |
|---|---|---|---|
| Biological method: mouse bioassay | Limit of detection: 13 to 40 µg/kg of shellfish meat | - Measurement of overall toxicity based on the animal's biological response<br>- Easy to implement<br>- Inexpensive equipment | - Qualitative method<br>- Lack of specificity: this method cannot be used to identify PnTXs<br>- Ethical reasons (3Rs rule)<br>- No inter-laboratory validation for PnTXs |
| Biochemical method: nicotinic acetylcholine receptor (nAChR) binding assay | Limit of quantification: 2.2 to 7.2 µg/kg of shellfish meat<br><br>Extraction performance (PnTX A):<br>shellfish: 82% | - Easy to implement<br>- Inexpensive equipment<br>- Rapid method | - No inter-laboratory validation for PnTXs<br>- Lack of specificity: this method does not provide information on the PnTX analogues present, nor does it distinguish PnTXs from other compounds that also act on nAChRs |

### 3.2. *Vulcanodinium rugosum*

*Vulcanodinium rugosum* is the PnTX-producing dinoflagellate that was identified by Ifremer in 2009, based on water samples from Ingril. It is a new species belonging to a new genus (Nézan and Chomérat, 2011).

The identification of this dinoflagellate in France originates from an atypical situation that occurred in 2006, as part of official surveillance of shellfish production areas (http://envlit.ifremer.fr/infos/rephy_info_toxines). The mouse bioassay used to screen for lipophilic toxins had revealed neurotoxic effects after the injection of extracts from mussels from the Ingril lagoon (Mediterranean Sea, east of the Thau lagoon, Hérault). This is unusual because the effects observed for lipophilic toxins are diarrhoeal, not neurotoxic. This neurotoxicity could not be explained by the presence of the regulated toxins (ASP[2], PSP[3], DSP[4]) screened for by chemical analysis. In addition, observations of water samples were unable to identify any microalgae species known to produce neurotoxins.

Since *V. rugosum* was first identified in 2009, high concentrations of PnTXs have been measured in mussels from the Ingril lagoon for several months each year. However, *V. rugosum* is rarely detected in water samples taken as part of surveillance (in the water column), which can be explained by the benthic nature of this dinoflagellate. It is important to note that the Ingril lagoon is physically connected to the Thau lagoon (fourth largest oyster farming area in France) via the Rhône-to-Sète canal.

Located at the interface between sea and river basins, coastal lagoons have been described as reservoirs of remarkable biodiversity (Cognetti and Maltagliati, 2000; Costanza *et al.,* 1998). These ecosystems act as filters and buffers by trapping a large proportion of the natural or anthropogenic inputs from the river basins.
Lagoons are often presented as being major phytoplankton-producing ecosystems, from 200 to 400 g of carbon per m[2] per year according to Nixon (1981), and subject to great environmental fluctuations. Due to their particular way of functioning, they are regularly faced with episodes of phytoplankton blooms, which can include toxic dinoflagellate species that affect how these environments are used. This global phenomenon has an impact on other European coasts besides Mediterranean coastal areas (Penna *et al.,* 2005; Sournia *et al.,* 1992).

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[2] ASP: Amnesic shellfish poisoning, amnesic toxins, domoic acid

[3] PSP: Paralytic shellfish poisoning, paralytic toxins of the saxitoxin family

[4] DSP: Diarrhoetic shellfish poisoning, diarrhoeal toxins from the okadaic acid family

- *V. rugosum* lifecycle

As with almost all species belonging to the dinoflagellate group, *V. rugosum* is assumed to have two distinct phases: a vegetative propagation phase (asexual reproduction) and a sexual phase. The asexual phase seems to correspond to the pelagic phase, when the cells are present in the water column. The cycle also includes a benthic phase. The sexual reproduction phase results in the formation of a planozygote that produces a resistance cyst. The pelagic and benthic phases are closely related, but the environmental and/or physiological factors controlling the transition from one to the other are not yet known.

The formation of resistance cysts, which can occur at the end of sexual reproduction, is an important step for survival in adverse environmental conditions due to the chromosomal mixing, which increases intra-species genetic diversity.

Cysts are also one of the ways in which the species spreads, through the transfer of sediments or shellfish from one area to another (cysts are present in the gastrointestinal tract and in intervalvular liquid), as well as via ship ballast water (Garrett *et al.,* 2014). Identifying resistance forms and understanding their distribution area are therefore important for preventing the risks of spread and contamination. Similarly, determining the key factors for growth and toxin production by *V. rugosum* is essential for better understanding the risk associated with this new species.

- Factors influencing *V. rugosum* growth and toxin production

Temperature is the most important factor for the growth of *V. rugosum* (Abadie *et al.,* 2016). The data suggest that *V. rugosum* is a thermophilic species, which would explain its development in the Ingril lagoon from June to September and the highest concentrations of PnTX G being found in mussels during this period.

Lastly, it should be noted that no other phytoplankton species have been reported to produce PnTXs, according to the literature available to the "Pinnatoxins" WG (last update of the literature review: September 2018).

### 3.3. Hazard characterisation of the toxins produced by *V. rugosum*

For characterising the hazard, the Working Group used all the published data for *in vitro, ex vivo* (mouse hemidiaphragm) and *in vivo* toxicity, by the intraperitoneal and oral (gavage and feed) routes.

#### 3.3.1. Acute *in vivo* toxicity

- *V. rugosum* extracts

Extracts from cultures of *V. rugosum* strains isolated from the port of Rangaunu (New Zealand), the port of Franklin (Australia) or the Ingril lagoon (France), were tested orally or intraperitoneally in mice (Rhodes *et al.,* 2010, 2011; ANSES-University of Trieste-CNRS Report, 2014). The results of these studies showed that the compounds contained in the methanolic extracts of *V. rugosum* culture were acutely toxic to mice by the oral and intraperitoneal routes, leading to their death by respiratory arrest. The main signs of toxicity before death in mice observed in the ANSES-University of Trieste-CNRS report study (2014) were: piloerection, prostration, hypothermia, hind leg paralysis, abdominal breathing and cyanosis.

The "Pinnatoxins" WG stressed the limited value of this type of study with *V. rugosum* extracts (whose composition is not known) for hazard characterisation, and stated that it is more appropriate to consider studies using purified toxins.

- Purified toxins: pinnatoxins, pteriatoxins and portimine

*In vivo* acute toxicity studies with purified toxins were reviewed using an analysis grid and assessed for quality using ToxRTool, a tool that ranks them according to the Klimisch rating.

Data on the acute toxicity of purified PnTXs are very limited. Indeed, the available studies were carried out in only one species (mice), one sex (females, which are considered to be more sensitive than males) and with very few animals tested per dose.

Orally, only three studies are available and focused on PnTX E, F, G and H (Munday *et al.*, 2012; ANSES-University of Trieste-CNRS Report, 2014; Selwood *et al.*, 2014). The WG did not find any information regarding PtTXs or portimine by the oral route. The studies by Munday *et al.* (2012) and the one described in the ANSES-University of Trieste-CNRS Report (2014), which are of major importance for hazard characterisation, were reviewed in detail collectively by the "Pinnatoxins" WG.

Regarding the intraperitoneal (IP) route, four studies have been published on PnTX E, F, G and H and portimine (Munday *et al.*, 2012; Selwood *et al.*, 2010, 2013, 2014). The WG identified additional unpublished information regarding PnTX A (personal communication from J. Molgó), but did not identify any robust information regarding PtTXs.

Despite the limited number of studies and mice tested per dose for each of the PnTXs, there is a consistent set of information enabling the main characteristics of the acute toxicity of this toxin family to be outlined.

Firstly, the toxicity of the PnTXs is rapid, with symptoms appearing within minutes of administration (whether oral or IP). This fact is already known, since PnTXs (like other cyclic imines) belong to the fast-acting toxins group.

The second characteristic point is the appearance of neurotoxic symptoms, quickly leading to the mouse's death by respiratory arrest (see 3.3.4). Clinical signs of toxicity, regardless of the route of administration and the PnTX analogue, include decreased mobility (sometimes preceded by an initial phase of hyperactivity immediately following administration), paralysis of the hind legs and breathing difficulties (Munday *et al.*, 2012), with tremors and jumps also being reported (ANSES-University of Trieste-CNRS Report, 2014). In the study by Munday *et al.* (2012), the authors reported that at sublethal doses (without specifying which ones), some mice recovered completely after exhibiting symptoms.

$LD_{50}$ values vary according to the PnTX analogue and route of administration (Table 2). Orally, $LD_{50}$ values range from 25 to 2800 µg/kg bw (for PnTX F and PnTX E respectively). The analogues tested can be ranked as follows in decreasing order of toxicity: PnTX F > PnTX G ~ PnTX H >> PnTX E. By IP, they range from 13 to 115 µg/kg bw (for PnTX F and PnTX A respectively) and the analogues can be ranked as follows: PnTX F > PnTX G > PnTX E > PnTX H > PnTX A.

$LD_{99}$ values for some PnTXs and PtTXs have been reported in the literature, but the WG considered these data unreliable due to a lack of information on the protocol (Uemura *et al.*, 1995; Chou *et al.*, 1996; McCauley *et al.*, 1998; Takada *et al.*, 2001).

Portimine has lower acute IP toxicity than that of PnTXs, with an estimated $LD_{50}$ of 1570 µg/kg bw. No effect was observed at 500 and 700 µg/kg bw (Selwood *et al.*, 2013). The authors indicate that the signs of toxicity prior to death in mice appeared less rapidly after IP administration compared to PnTXs (without however mentioning which signs of toxicity were observed). Toxicity by oral administration is unknown.

**Table 2: Acute *in vivo* toxicity in mice of PnTXs and portimine**

**Oral administration**

| Toxin (purity) | Route of administration and number of mice | LD$_{50}$ (µg/kg bw) | MTD (µg/kg bw) | References | Study quality |
|---|---|---|---|---|---|
| PnTX E* | Gavage<br>Number of mice not specified (OECD GL 425)<br>Fed (non-fasted) mice | 2800<br>CI95: 2380-3000 | 600 | Munday *et al.,* 2012 | ToxRTool: 13<br>Klimisch: 2 |
| PnTX F* | Gavage<br>Number of mice not specified (OECD GL 425)<br>Fed (non-fasted) mice | 25<br>CI95: 19.1-35.1 | 9.9 | Munday *et al.*, 2012 | ToxRTool: 13<br>Klimisch: 2 |
|  | 16h fasted mice | 29.9<br>CI95: 25-32 | Not determined |  |  |
| PnTX F* | In cream cheese<br>Number of animals not specified (OECD GL 425)<br>Fed (non-fasted) mice | 50<br>CI95: 39.4-62.8 | 16.0 | Munday *et al.,* 2012 | ToxRTool: 13<br>Klimisch: 2 |
|  | In peanut butter<br>Number of mice not specified (OECD GL 425)<br>Fed (non-fasted) mice | 50<br>CI95: 37.9-71.5 | Not determined |  |  |
|  | In mouse food<br>Number of mice not specified (OECD GL 425)<br>16h fasted mice | 50<br>CI95: 37.9-71.5 | Not determined |  |  |
|  | In cream cheese, 16h fasted mice | 77<br>CI95: not calculated | Not determined |  |  |
|  | In peanut butter, 16h fasted mice | 50<br>CI95: 39.4-62.8 | Not determined |  |  |
| PnTX G* | Gavage<br>Number of mice not specified (OECD GL 425)<br>Fed (non-fasted) mice | 150<br>CI95: 105-100 | 75 | Munday *et al.,* 2012 | ToxRTool: 13<br>Klimisch: 2 |
| PnTX G (declared purity 100%) | Gavage<br>Groups of 3 to 8 mice (8 for the control group; 3 for the doses 8, 20, 50, 120 µg/kg bw; 5 for the doses 220, 300, 370, 400 µg/kg bw)<br>Mice fasted for 3 hours before administration | 208<br>CI95: 155-281 | 120 | ANSES-University of Trieste-CNRS report, 2014 | ToxRTool: 17<br>Klimisch: 2 |
| PnTX G* | In cream cheese<br>Number of mice not specified (OECD GL 425)<br>Fed (non-fasted) mice | 400<br>CI95: 380-470 | 153 | Munday *et al.,* 2012 | ToxRTool: 13<br>Klimisch: 2 |
| PnTX H* | Gavage<br>Number of animals not specified<br>Fasting not specified | 163<br>CI95: 139-175 |  | Selwood *et al.,* 2014 | ToxRTool: 4, because there were few details in the article, but this team has already described the protocol for other PnTXs |

\* Purity verified by NMR according to the authors but percentage not mentioned in the publication
CI95: 95% confidence interval
MTD (Maximum Tolerated Dose): dose at which no mortality or clinical signs are observed

**Intraperitoneal (IP) administration**

| Toxin (purity) | Route of administration and number of mice | LD$_{50}$ (µg/kg bw) | MTD (µg/kg bw) | References | Study quality |
|---|---|---|---|---|---|
| Synthetic PnTX A (purity > 97%) | IP n=18 mice, 9 doses tested (1 to 3 mice/dose) Fed (non-fasted) mice | 114.8 | | J. Molgó (personal communication) | ToxRTool: 16 |
| PnTX E* | IP Number of animals not specified (OECD GL 425) | 57 (fed) CI95: 39.7-75.3  48 (16h fasted) CI95: 33.5-63.5 | 22  Not determined | Munday *et al.,* 2012 | ToxRTool: 13 |
| | IP (OECD GL 425) n=2 at 36 µg/kg, n=3 at 45 µg/kg, n=1 at 54 µg/kg, n=1 at 60 µg/kg. | 45 (fed) CI95: 32-58 | | Selwood *et al.*, 2010 | ToxRTool: 15 |
| PnTX F* | IP Number of animals not specified (OECD GL 425) | 12.7 (fed) CI95: 9.5-14.6  14.9 (16h fasted) CI95: 12.6-15.8 | 3.2  Not determined | Munday *et al.,* 2012 | ToxRTool: 13 |
| | IP (OECD GL 425) n=1 at 10.1 µg/kg, n=1 at 12.7 µg/kg, n=3 at 16.0 µg/kg, n=2 at 20.1 µg/kg. | 16 (fed) CI95: 12-23 | | Selwood *et al.*, 2010 | ToxRTool: 15 |
| PnTX G* | IP Number of animals not specified (OECD GL 425) | 48 (fed) CI95: 36.3-68.1  42.7 (16h fasted) CI95: 40-50 | 18.8  Not determined | Munday *et al.,* 2012 | ToxRTool: 13 |
| PnTX G* | IP (OECD GL 425) n=2 at 40 µg/kg, n=3 at 50 µg/kg, n=1 at 60 µg/kg. | 50 (fed) CI95: 35-66 | | Selwood *et al.*, 2010 | ToxRTool: 15 |
| Synthetic PnTX G (purity > 97%) | IP Number of animals not specified | 65.8 | | J. Molgó (personal communication) | ToxRTool: 16 |
| PnTX H* | IP Number of animals not specified Fasting not specified | 67 CI95: 63-79 | | Selwood *et al.,* 2014 | ToxRTool: 4 because there were few details in the article, but this team has already described the protocol for other PnTXs |
| Portimine* | IP Number of animals not specified Fasting not specified | 1570 CI95: 1269-3080 | No effect at 500 and 700 | Selwood *et al.,* 2013 | ToxRTool: 4 because there were few details in the article, but this team has already described the protocol for other PnTXs |

* Purity verified by NMR according to the authors but percentage not mentioned in the publication
CI95: 95% confidence interval
MTD (Maximum Tolerated Dose): dose at which no mortality or clinical signs are observed

As previously mentioned, PnTXs have a rapid mode of action *in vivo*, with death in mice occurring within minutes (less than 30 minutes) of administration, whether IP or oral. The symptoms observed are neurotoxic (paralysis, respiratory distress).

The WG noted that PnTX F appears to be the analogue with the highest toxicity.

### 3.3.2. Acute *ex vivo* and *in vitro* toxicity

*Ex vivo* (mouse hemidiaphragm) and *in vitro* toxicity studies were also reviewed by the "Pinnatoxins" WG and are described in the expert report.

### 3.3.3. Subchronic and chronic toxicity

No repeated dose toxicity studies were identified in the literature.

### 3.3.4. Mode of action and cellular target

- Acetylcholine and cholinergic networks

Acetylcholine (ACh) is the main neurotransmitter in the peripheral nervous system of vertebrates. This molecule is also found in the central nervous system.

In the peripheral nervous system, cholinergic networks mainly concern:
  i.   the vegetative (or autonomic) nervous system, i.e. ganglionic transmission in the sympathetic and parasympathetic pathways;
  ii.  the muscular system (skeletal muscles);
  iii. the medulla-adrenal gland, whose cholinergic synapses control catecholamine secretion.

In the central nervous system, ACh acts as an excitatory neurotransmitter via the nicotinic receptors (nAChRs) and as an inhibitor via the muscarinic cholinergic receptors (mAChRs). The central cholinergic networks are numerous and diffuse, projecting into different regions of the brain such as the hippocampus, cerebral cortex and amygdala. They mainly concern cognitive functions (memory and learning), the limbic system (emotions, mood, behaviour) and motor control.

- Physiological and molecular targets of PnTXs

The data in the literature suggest that PnTXs share a common mode of action and it seems unlikely that some of them could exert effects on a molecular or physiological target that differ from those of the others (Araoz *et al.,* 2011). Characterising the mode of action of PnTXs has unambiguously revealed that they target skeletal muscle nAChRs by causing a concentration-dependent blockage of nerve stimulation-induced muscle contraction. PnTX E, F and G block nerve stimulation-induced muscle contraction, but do not alter muscle contraction caused by direct stimulation of isolated adult rodent muscles (Hellyer *et al.,* 2013). This muscle paralysis is due to the antagonistic effect of PnTXs on nAChRs.

### 3.3.5. Development of the health value

The WG followed the methodology presented in ANSES's TRV Development Guide (2017a).

- Choice of the key study

The WG identified two acute oral toxicity studies in mice with purified PnTX G (Munday *et al.,* 2012; ANSES-University of Trieste-CNRS report, 2014).

The WG selected as the key study the one carried out by the Department of Life Sciences at the University of Trieste with funding from the DGAL and DGS (ANSES-University of Trieste-CNRS report, 2014), which obtained a score of 17 out of 21 with ToxRTool, corresponding to a Klimisch score of 2 (reliable with restriction).

In this study, groups of three to five female SD-1 mice aged 4 weeks received a single administration of purified PnTX G by gavage at a dose of 8, 20, 50, 120, 220, 300, 370 or

450 µg/kg bw. There was a control group of eight mice. Mice were fasted for 3 hours before gavage; food was given again 2 hours after administration and was *ad libitum* during the 24-hour observation period. The parameters studied included lethality, clinical signs of toxicity, histological analysis of certain organs and biochemical blood tests.

The results regarding lethality and the observed symptoms are shown in Table 3.

Administration of PnTX G resulted in mouse lethality from 220 µg PnTX G/kg bw (3/5 mice, in 22 min). No lethality was observed at the doses of 8, 20, 50 and 120 µg/kg bw. All mice (5/5) died at the dose of 370 µg/kg bw (survival time was less than or equal to 18 min). The $LD_{50}$ for PnTX G was calculated at 208 µg/kg bw (95% confidence interval = 155-281 µg/kg bw).

Before death, the main signs of toxicity were prostration, tremors, jumps, abdominal breathing, hypothermia, hind leg paralysis and cyanosis.

No macroscopic organ changes were observed during necropsy of the mice treated with PnTX G.

Histological analysis of major organs and tissues revealed only minor changes in the small intestine of mice given PnTX G at doses equal to or greater than 300 µg/kg bw (moderate mucosal degeneration, villous atrophy).

No differences in biochemical blood parameters were found between treated and control mice.

**Table 3: Lethality and clinical signs of toxicity in mice after single administration by gavage of purified PnTX G (ANSES-University of Trieste-CNRS Report, 2014)**

| Purified PnTX G dose | Lethality | Survival time (h:min) | Clinical signs of toxicity |
|---|---|---|---|
| Control | 0/8 | - | - |
| 8 µg/kg bw | 0/3 | - | - |
| 20 µg/kg bw | 0/3 | - | - |
| 50 µg/kg bw | 0/3 | - | - |
| 120 µg/kg bw | 0/3 | - | - |
| 220 µg/kg bw | 3/5 | 00:20; 00:22; 00:22 | prostration, tremors, jumps, abdominal breathing, hypothermia, hind leg paralysis, cyanosis |
| 300 µg/kg bw | 4/5 | 00:12; 00:13; 00:17; 00:23 | prostration, tremors, jumps, abdominal breathing, hypothermia, hind leg paralysis, cyanosis |
| 370 µg/kg bw | 5/5 | 00:13; 00:15; 00:16; 00:17; 00:18 | prostration, tremors, jumps, abdominal breathing, hypothermia, hind leg paralysis, cyanosis |
| 450 µg/kg bw | 5/5 | 00:12; 00:12; 00:15; 00:16; 00:29 | prostration, tremors, jumps, abdominal breathing, hypothermia, hind leg paralysis, cyanosis |

- Choice of the critical dose

Based on the data from the key study, two approaches were explored:
- First approach: the **120 µg PnTX G/kg bw** dose was selected as the maximum tolerated dose (MTD), i.e. the dose at which no effect is observed in the studied parameters over a 24-hour period after treatment.
- Second approach: calculation of a benchmark dose[5] from a response level of 10% mortality (PROAST Web software, RIVM, v. 65.2). The model that best fitted the experimental data according to the Akaike Information Criterion (AIC) was the probit model, for which the $BMDL_{95\%}$ was **69.1 µg PnTX G/kg bw** of mouse.

*The CES ERCA notes that it is unusual to use data on mouse mortality from single oral administration to derive a health reference value. This is justified by the fact that the mouse mortality occurs rapidly and there is a very small difference between a dose with no signs of toxicity and a lethal dose.*

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[5] The benchmark dose is a dose producing a measurable effect corresponding to a given response level compared to a control group. The lower limit of its 95% or 90% confidence interval ($BMDL_{95\%}$ or $BMDL_{90\%}$) is most often used. This approach is based on modelling of the experimental data taking into account the entire dose-response curve (ANSES 2017a).

*A response level of 10% (for lethality) is considered high but was exceptionally selected in this expert appraisal because the results obtained with a response level of 1% were regarded as too uncertain due to the small number of mice tested in the study.*

The WG noted and took into consideration the results obtained in the other acute oral toxicity study in non-fasted mice with purified PnTX G (Munday *et al.*, 2012) as they were consistent with those of the study conducted by the Department of Life Sciences at the University of Trieste:

i.  The $LD_{50}$ was 150 µg PnTX G/kg bw (95% CI = 105-199 µg/kg bw);

ii. The dose tested without lethality and with no apparent sign of neurotoxicity was 75 µg PnTX G/kg bw.

- Choice of uncertainty factors

Uncertainty factors were identified for both approaches explored when establishing the critical dose:
- First approach: the dose of 120 µg PnTX G/kg bw was selected as the maximum tolerated dose (MTD). The associated overall uncertainty factor was 900:
  o inter-species uncertainty factor: $UF_A$ = 10
  o inter-individual uncertainty factor: $UF_H$ = 10
  o other factors: $UF_D$ = 3 (insufficient data) and 3 (to take into account the severity and pattern of the dose-response curve)
- Second approach: the BMDL of 69.1 µg PnTX G/kg bw of mouse was chosen as the point of departure. The associated overall uncertainty factor was 525:
  o allometric adjustment factor: 7 for the mouse
  o $UF_A$ = 2.5 for the toxicodynamic component
  o $UF_H$ = 10
  o $UF_D$ = 3 (insufficient data).

- Proposed provisional acute benchmark value

The usual terminology "acute reference dose (ARfD)" was not used in this expert appraisal to avoid any confusion with how this term is used in a regulatory context, particularly for plant protection or biocidal substances.

Due to insufficient data for the hazard characterisation of PnTX G, the value developed to characterise the risk was referred to as a **provisional acute benchmark value**.

The provisional acute benchmark value is **0.13 µg PnTX G/kg bw**, regardless of the approach used[6] (MTD or BMDL).

- Proposed maximum tolerable concentration of PnTX G in shellfish

Based on the provisional acute benchmark value of 0.13 µg PnTX G/kg bw, a default serving size of 400 g of shellfish (EFSA 2010) and a default body weight of 70 kg, the concentration not to be exceeded in shellfish would be **23 µg PnTX G/kg of total meat**.

- Confidence level

The overall confidence level **moderate** was assigned to the provisional acute benchmark value based on the following criteria:

- Level of confidence in the type and quality of the body of data: **Low**

The literature review revealed that there were few data on the acute oral toxicity of purified PnTXs and none on repeated administration. The WG identified only two acute oral toxicity studies in a single species (mice) and sex (females, which are considered to be more sensitive than males) for

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[6] MTD approach: (120 µg PnTX G/kg bw) / (10 x 10 x 3 x 3) = 120/900 = 0.1333 µg PnTX G/kg bw
BMDL approach: 69.1 µg PnTX G/kg bw) / (7 x 2.5 x 10 x 3) = 69.1/525 = 0.1316 µg PnTX G/kg bw

the hazard characterisation. The CES ERCA underlined the lack of data in another rodent species (rats), as well as the lack of knowledge on the toxicokinetics and toxicodynamics of PnTXs.

- Level of confidence in the choice of the critical effect and the mode of action: **Moderate**

The key study is an acute toxicity study with a single oral administration. Its objective was to study mouse mortality and define a median lethal dose ($LD_{50}$). Signs of neurological toxicity were also observed and are consistent with the known mode of action of PnTXs.

*The CES ERCA stresses that it is unusual to use data on mouse mortality from single oral administration to derive a health reference value. This is justified by the fact that the mouse mortality occurs rapidly and there is a very small difference between a dose with no signs of toxicity and a lethal dose.*

- Level of confidence in the choice of the key study: **Moderate/High**

The key study was analysed using ToxRTool and obtained a total score of 17 (out of 21), corresponding to a score of 2 using the Klimisch method (reliable study with restriction).

- Level of confidence in the choice of the critical dose: **High**

Two approaches were explored for selecting the critical dose from the key study data. The first approach retained a maximum tolerated dose (MTD), at which there is no mortality or signs of toxicity in mice during the 24-hour observation period (which is considered sufficient because PnTXs are fast-acting toxins, with neurotoxic signs in mice occurring within 30 minutes of administration). The second approach modelled the dose-response relationship (lethality) to calculate a BMDL.

This overall confidence level may be reassessed when new acute oral toxicity data become available for PnTX G or other toxins produced by *V. rugosum*.

- In the absence of available data on repeated oral administration in laboratory animals with purified PnTX, the WG was unable to propose a chronic toxicity reference value.

**Table 4: Table summarising development of the provisional acute benchmark value for PnTX G**

| | Critical effect<br>Key study | Critical dose for the mouse | UF | Provisional acute benchmark value for humans | Confidence level |
|---|---|---|---|---|---|
| First approach | *Critical effect*: absence of mortality and symptoms in mice 24 hours after oral administration by gavage of purified PnTX G<br><br>*Key study*: ANSES-University of Trieste-CNRS report, 2014 | Maximum tolerated dose of 120 µg PnTX G/kg bw | Total factor: 900<br>$UF_A = 10$<br>$UF_H = 10$<br>$UF_D = 9$ (3 for insufficient data and 3 for the severity and pattern of the dose-response curve). | 0.13 µg PnTX G/kg bw | Moderate |
| Second approach | | BMDL of 69.1 µg PnTX G/kg bw | Total factor: 525<br>Allometric adjustment: 7 for the mouse<br>$UF_A = 2.5$<br>$UF_H = 10$<br>$UF_D = 3$ for insufficient data | 0.13 µg PnTX G/kg bw | Moderate |

### 3.3.6. Transposition to humans

To date, there have been no reported cases of human poisoning with PnTXs. This part therefore aims to describe the clinical effects observed in humans after exposure to compounds (drugs, natural toxins) whose pharmacology is comparable to that of pinnatoxins. Similarly, a specific description of certain disorders (myasthenia, channelopathies, etc.) and their symptoms, which could be observed after exposure to PnTXs, was produced on the basis of molecular pharmacology and *in vivo* toxicology studies.

- Clinical signs of toxicity in mice and possible correspondence in humans

The clinical signs of toxicity reported in animals come from publications (Munday, 2008; Selwood *et al.,* 2010; Munday *et al.,* 2012; ANSES-University of Trieste-CNRS, 2014). These signs are similar according to the studies but are insufficiently described. In addition, most of the studies did not include a control group.

Table 5 suggests a possible correspondence in humans for the signs of toxicity reported during exposure in mice.

**Table 5: Clinical signs of toxicity in mice and possible correspondence in humans**

| Clinical signs of toxicity in mice | Anatomical or physiological support | Possible correspondence in humans |
|---|---|---|
| Hyperactivity Agitation | Start of respiratory impairment and hypercapnic agitation? Start of an epileptic seizure? | Motor agitation in frontal-lobe epilepsy seizure? Central confusion and agitation as in cytisine poisoning? |
| Loss of motor activity | Damage to the neuromuscular junction | Myasthenic syndrome analogous with the disease *myasthenia gravis* Flaccid paralysis caused by curare |
| Respiratory depression | Damage to the diaphragm neuromuscular junction | Respiratory impairment in myasthenia |
| Respiratory arrest | Complete block on diaphragm neuromuscular junction | Respiratory arrest corresponding to a myasthenic crisis |
| Epilepsy | Central damage via nicotinic receptors | Epilepsy by GABA release or by hereditary mutation |
| Leg extension | Spinal interneuron damage Central damage | Pyramidal syndrome Babinski sign |
| Reversibility if no death occurs or in the case of prostigmine injection | Removal of the post-synaptic neuromuscular block | Fluctuation in the degree of myasthenic syndrome Or temporary removal of the block with prostigmine Removal of the action of curare |
| Exophthalmos | Increased intraocular pressure | Action of curare-like suxamethonium Action of lupin |

- Known effects of medicinal and/or toxic nicotinic antagonists (curare-like and ganglioplegic compounds)

Muscle-type nAChR antagonists are curariform agents that can be used in therapy to relax skeletal muscles during surgery, for tracheal intubation, and in resuscitation to facilitate artificial ventilation in some difficult cases. These compounds do not cross the blood-brain barrier.

Ganglioplegic (ganglionic-blocking) compounds inhibit the influence of the vegetative nervous system (orthosympathetic and parasympathetic) on the organs it innervates.

These compounds have mainly been used in therapy as anti-hypertensive agents. Alkaloids of the quinolizidine family, such as sparteine or piperidine, are natural compounds with ganglioplegic effects. These compounds are found in plants of the Fabaceae family such as laburnum (broom), and in lupin seeds.

As with PnTXs, curare-like compounds bind to the alpha-7 subunit of the acetylcholine receptor. Their effects are modified by physiological and pathological factors, and drug interactions. They produce flaccid muscle paralysis leading to respiratory arrest. The ganglioplegic properties of curare show signs of allergic anaphylactic shock from cutaneous or systemic histaminoid reactions, ganglioplegic impairment, bradycardia, tachycardia, heart rhythm disorders, hypertension, pulmonary oedema and bronchospasm.

Exposure to drugs and natural alkaloids with ganglioplegic effects leads to dysautonomia, including changes in blood pressure and heart rate. Exposure to high doses may result in the patient's death by cardiorespiratory arrest. Chronic toxicity effects have also been reported and may lead to neuromuscular pathologies. Lastly, some alkaloids are believed to pass into breast milk in animals and cause developmental disorders in exposed offspring.

- Known effects of autoimmune nicotinic receptor antagonists: anti-acetylcholine receptor antibodies and myasthenia gravis

Myasthenia gravis is the consequence of a post-synaptic neuromuscular block. It is an autoimmune disease – for which an experimental model has been obtained using the AChR as an antigen – caused by anti-AChR antibodies. These are antibodies that bind to the AChR, mostly outside the ACh binding site. These antibodies have a complex mode of action.

The symptoms of myasthenia gravis are purely motor. They fluctuate, are aggravated by exertion, and improved by rest. Symptoms are triggered by the loss of the "safety margin" at the junction between the nerve and muscle: the endplate potential falls below the threshold necessary to trigger the action potential. In almost half of cases, the first manifestations are purely ocular with ptosis and diplopia, but after a year of progression in 80 to 90% of patients, other areas are affected: pharyngeal-laryngeal muscles and/or limb muscles and/or respiratory muscles. Myasthenia is then generalised. The fluctuation of symptoms and the possible normality of the clinical examination lead to signs being trivialised and significant diagnostic delay, especially since fatigue is a common reason for seeking a consultation in general practice.

Myasthenia, the neuromuscular junction disease, is the most likely clinical model that could correspond to PnTX poisoning. The clinical signs of myasthenia gravis are those of a fluctuating motor deficit aggravated by exertion. A delayed diagnosis risks respiratory distress, especially if the patient is at risk. Diagnosis is difficult and requires hospital technical facilities.

- Known central effects in humans of nicotinic receptor mutations

Epilepsy is caused by a channelopathy related to a mutation of the nicotinic alpha-7 receptor. By analogy, the blocking by PnTXs of the alpha-7 receptor could lead to a seizure in humans, as observed in toxicity studies in mice.

- Known central and peripheral effects of activation on the spinal cord

Blocking nAChR in the intramedullary canal could lead to leg extension (equivalent to a pyramidal sign or Babinski sign) accompanied by blood pressure deregulation in the animal. In the PnTX G toxicity studies in mice, leg extension was observed.

### 3.4. Contamination of the environment by *V. rugosum* and PnTXs

#### 3.4.1. Geographical distribution of *V. rugosum* and PnTXs

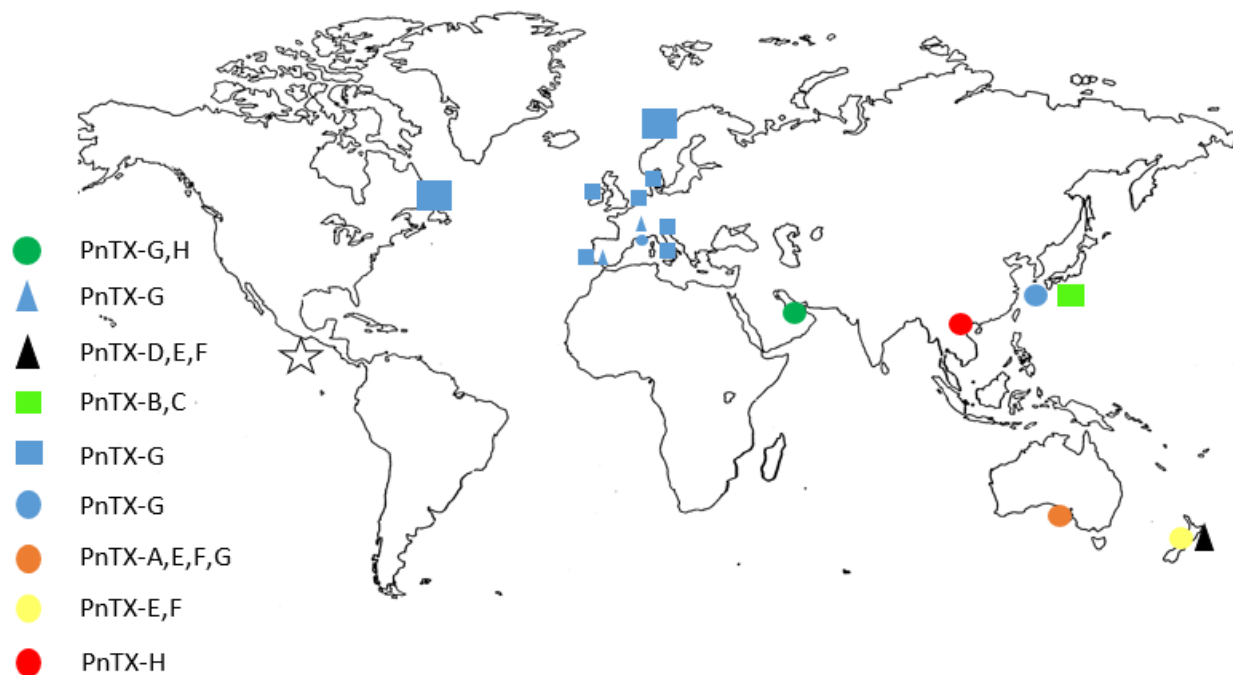The global distribution of *V. rugosum* and PnTXs reported to date is shown in Figure 1.

**Figure 1: Reported presence of PnTXs and/or *Vulcanodinium rugosum* in the world (modified from E. Abadie, Ifremer)**

The colours of the symbols on the map indicate which PnTX (PnTX A to H) was detected in the cells of *V. rugosum* in the geographical area studied (circles), only in shellfish (squares), or only in passive samplers (triangles). A star marks the presence of cells of *V. rugosum* but no PnTX analysis was performed.

### 3.4.2. Ingril lagoon

In June 2006, as part of the official surveillance of shellfish production areas (REPHY), mouse bioassays were carried out on mussel samples from this lagoon during an alert related to the presence of *Dinophysis acuminata*, a lipophilic toxin-producing species (DSP).

Mice had a survival time of 27 to 32 minutes with neurological symptoms (different from those observed during PSP paralytic toxin episodes). This phenomenon was observed for four weeks. No microalgae species whose toxins might explain the symptoms in mice (neurological type) were identified in water samples.

Due to this neurotoxicity, it was decided at the beginning of June 2007 to carry out mouse bioassays to monitor a possible contamination episode, even in the absence of potentially toxic phytoplankton species (deviation from the REPHY protocol). The neurotoxicity phenomenon in mice was detected until 10 September 2007. No toxic phytoplankton species that might explain this phenomenon were identified.

The following years (2008 and 2009) only confirmed the phenomenon and showed its temporal expansion (extension towards the autumn and early winter period). In 2008, this toxicity was detected from 19 May to 4 November, and for 2009 from 15 June to 17 November (weekly analyses throughout the period when the mouse bioassay caused mice deaths). The onset of the phenomenon seemed to be limited by water temperatures below 20°C.

The mouse bioassay as part of official surveillance was stopped on 1 January 2010, but was maintained until 31 December 2017 as part of the monitoring of the emergence of marine biotoxins.

As soon as PnTX G was identified in 2011, a retrospective analysis of the samples from 2009 to 2012 was conducted, which revealed the kinetics of shellfish contamination as a function of time. The maximum annual concentrations were 261, 1244, 568 and 652 µg/kg of total mussel meat for 2009, 2010, 2011 and 2012 respectively. Concentrations in mussels were higher than those observed in clams, when shellfish were sampled simultaneously.

To track this phenomenon and maintain monitoring for PnTXs, sampling of Ingril mussels was carried out monthly between 2013 and 2017. The results show that PnTX G peaks were observed between June and September, but the maximum values varied according to the year (887 in 2013, 918 in 2014, 1143 in 2015, 600 in 2016 and 640 in 2017, expressed in µg/kg of total meat).

### 3.4.3. Other Mediterranean lagoons

In addition to the monitoring carried out at the Ingril lagoon, screening for PnTX G in wild mussel samples took place at four other Mediterranean lagoons in 2013.
A statistically significant difference in PnTX G concentration was observed between lagoons, with maximum values increasing in the direction Parc Leucate (11 µg/kg of total meat) → Thau (15 µg/kg) → Prevost (54 µg/kg) →Vic (89 µg/kg)→ Ingril (887 µg/kg).

### 3.4.4. Atlantic and Corsican coasts

The EMERGTOX scheme (for monitoring the emergence of marine biotoxins in shellfish) was set up by the DGAL to complement national surveillance schemes for regulated toxins (REPHYTOX, the DGAL's surveillance plan). Its purpose is to bring to light any possible hazard associated with the presence in shellfish of known regulated and unregulated lipophilic toxins, either identified in France or that could be introduced into France via ballast water or commercial trade between countries.

Of the eleven areas monitored, three (Ingril, Le Scoré in Brittany and the Diana lagoon in Corsica) were affected by the presence of PnTXs in 2018, mainly PnTX G and to a lesser degree PnTX A (only in Ingril). Of the three affected areas, the Ingril lagoon remains the most heavily contaminated area (with concentrations detected every month, varying from 40 to 2614 µg PnTX G/kg of digestive gland). The levels found at Le Scoré and Diana were low (maximum around 10 µg PnTX G/kg of digestive gland). The concentrations of PnTX A detected at Ingril ranged from 6 to 32 µg/kg of digestive gland.

The WG believes it is important to point out that the presence of PnTXs in France is not limited to Mediterranean lagoons. And given that global warming is making ecophysiological conditions more favourable to the development of *V. rugosum,* vigilance should be maintained with regard to the Atlantic coast, since the detection of PnTXs in mussels indicates the presence of this dinoflagellate in these waters.

### 3.4.5. Outside France

The literature review conducted by the WG identified very limited published data on PnTX levels in shellfish from Northern and Southern Europe, Canada and New Zealand.

Among the six studies reviewed, the highest reported levels of PnTX G in mussels were 115 µg/kg in Norway (Rundberget *et al.*, 2011), 83 µg/kg in Canada (McCarron *et al.*, 2012) and 59 µg/kg in Spain (Garcia-Altares *et al.*, 2014). These levels are lower than the annual maximum levels reported in France in the Ingril lagoon (between 261 and 1244 µg/kg, depending on the year).

It should be noted that the study by Rambla-Alegre *et al.* (2018) reported the persistence of PnTX G in samples of canned mussels (up to 12 µg/kg).

Lastly, ANSES sent a questionnaire to the European Food Safety Authority (EFSA) network of focal points on 15 February 2018, to which 16 Member States replied. At the time of the request, none of the responding health agencies had conducted health risk assessments for PnTXs. In addition to the publications already identified by the WG, unpublished data on shellfish contamination were mentioned, although it was not possible for the WG to assess the reliability of the analytical methods used.

In conclusion, the concentrations of PnTX G measured in mussels from Ingril are the highest reported in the world to date.

### 3.5. Dietary exposure

#### 3.5.1.  Food consumption data

Three food consumption studies were used to calculate dietary exposure.

i.  General population: INCA3. The Third Individual and National Study on Food Consumption (INCA3) is a cross-sectional survey designed to estimate the food consumption and eating habits of individuals living in France. It was conducted with 3157 adults and 2698 children between the ages of 0 and 17 from February 2014 to September 2015.

ii.  High consumers of seafood products: CALIPSO. The CALIPSO study (2006) was conducted with 1011 individuals over 18 years of age who are heavy consumers of seafood (at least twice a week) and reside in one of the four coastal sites or its surroundings (within a radius of 20-25 km) selected for the survey: Le Havre, Toulon-Hyères, La Rochelle and Lorient.

iii.  Coastal consumers of seafood products: CONSOMER. A consumption study on seafood products (CONSOMER) was conducted in 2016-2017 as part of a research agreement between ANSES and CREDOC (2015-CRD-25). The aim of the study was to assess seafood consumption by an adult population (over 18 years of age) living in coastal areas and with access to local sources of supply.

#### 3.5.2.  Contamination data

Data on PnTX concentrations in mussels and clams from several Mediterranean lagoons from 2010 to 2017 were provided to ANSES by Ifremer.

Two contamination values were calculated, one for mussels and another for clams: the average contamination and the 95th percentile (P95) of the distribution (the value at which 95% of the measured contamination is lower and 5% is higher).

Three scenarios were selected, depending on the site where the measurements were taken:

- **"All lagoons" scenario**: average/P95 contamination of all mussels on the one hand and all clams on the other (from all the lagoons studied);

- **"Ingril" scenario**: average/P95 of the contamination measured in the Ingril lagoon at the points "Ingril - Port des Pauvres" and "Ingril Sud", for mussels on the one hand and clams on the other;

- **"Except Ingril" scenario**: average/P95 of the contamination measured in all lagoons EXCEPT "Ingril - Port des Pauvres" and "Ingril Sud", for mussels only (no contamination of clams measured, except Ingril).

### 3.6. Risk characterisation

#### 3.6.1.  Calculating exposure

- Method

From the individual consumption data and the contamination data, exposure was calculated according to the following equation:

$$E_i = \sum_{k=1}^{n} \frac{C_{i,k} \times L_k}{BW_i}$$

where:
- $E_i$ is the total daily exposure of individual i associated with the consumption of food k (µg/kg of body weight/day);
- $C_{i,k}$ is the consumption of food k by individual i (kg/d) (either the serving size or the daily consumption);
- $L_k$ is the PnTX G contamination of food k (µg/kg of fresh weight) (either the average contamination, or the P95 depending on the calculated exposure);
- $BW_i$ is the body weight of individual i (kg).

- Scenarios tested

For acute exposure, a comparison was made with the provisional acute benchmark value of 0.13 µg PnTX G/kg of body weight. Depending on the contamination scenario considered, a percentage of the population exceeding this value was calculated, firstly in the total population of each study and secondly among consumers alone. It should be noted that with regard to the "total population", this is actually the <u>Mediterranean</u> population (except in the case of INCA3). The different scenarios studied according to the consumption studies are summarised in Table 6.

**Table 6: Summary of populations and scenarios studied based on the consumption studies**

|  | **Population** | | **Site** | **Consumption** | |
| --- | --- | --- | --- | --- | --- |
|  | Adults | Children | Mediterranean | Mussels | Mussels + Clams |
| INCA3 | x | x |  | x |  |
| CONSOMER | x |  | x | x | x |
| CALIPSO | x |  | x | x | x |

### 3.6.2. Results

All the results are presented in detail in the WG report.
Table 7 shows the results of the acute exposure scenario associated with adult consumption of mussels and clams, based on CONSOMER data (Mediterranean area), which are considered to be the most representative data for this risk assessment.

**Table 7: Acute exposure associated with consumption of mussels and clams (CONSOMER, Mediterranean area) for adults (in µg PnTX G/kg bw)**

|  | **Total population** (Mediterranean area) | | | **Consumers only** (Mediterranean area) | | | **Provisional acute benchmark value (PABV) exceeded** | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | n | Ave_pop | P95_pop | n_cons | Ave_cons | P95_cons | % > PABV | % > PABV_cons |
| All lagoons | 821 | 0.590 | 1.572 | 591 | 0.816 | 1.733 | 71 | 100 |
| Ingril | 821 | 0.663 | 1.767 | 591 | 0.917 | 1.946 | 71 | 100 |
| Except Ingril (mussels only*) | 821 | 0.056 | 0.149 | 582 | 0.079 | 0.167 | 7.3 | 10.23 |

\* analyses of clams were only carried out for the Ingril lagoon

n: number of adult individuals in the population monitored during the survey (this population includes consumers and non-consumers of products from the first column)

Ave_pop: average exposure in the population (whether consumers or not) (µg/kg bw)

P95_pop: exposure to the 95th percentile calculated in the population (µg/kg bw/d)

n_cons: number of adult individuals consuming products from the first column (consumers only)

Ave_cons: average exposure in the consumer population only (µg/kg bw/day)

P95_cons: exposure to the 95th percentile calculated in the consumer population only (µg/kg bw/day)

% > PABV: percentage of the general population exceeding the provisional acute benchmark value of 0.13 µg/kg bw

% > PABV_cons: percentage of consumer population only exceeding the provisional acute benchmark value of 0.13 µg/kg bw

These results show that, in the tested scenarios including the contamination data from the Ingril lagoon, the provisional acute benchmark value would be exceeded in 71% of adults according to the consumption data from the CONSOMER study for the Mediterranean area (in the scenario including non-consumers of mussels and clams). If only individuals consuming mussels and clams are considered, this proportion increases to 100%.
In the tested scenario excluding the contamination data from the Ingril lagoon, the PABV would be exceeded in 7% of the total Mediterranean population and in 10% of the population of consumers.

### 3.6.3. Conclusion in terms of health concern

Given the cases in which the provisional acute benchmark value was exceeded in the "Except Ingril" scenario, the WG considers that there may be a health concern related to the consumption of shellfish contaminated with PnTXs from these Mediterranean lagoons.

Estimates were not made for the other French sites where PnTXs were detected in mussels (Le Scoré, Diana) because the analyses focused on the digestive glands, and the distribution of PnTXs between the digestive gland and total meat is not known.

## 3.7. Recommendations for surveillance

### 3.7.1. Environmental surveillance

Several studies on the ecology of the dinoflagellate *V. rugosum* have been carried out *in vitro* and *in situ*. However, its origin and the determinism of its blooms in natural environments remain to be elucidated.

While the contamination of molluscs by PnTXs in some areas is undeniable, it remains difficult to establish the relationship with *V. rugosum* blooms. This is because it is difficult to observe the pelagic phase of this species in the water column. Official surveillance of shellfish production areas based on the identification and counting of toxic phytoplankton species in the water column is therefore ill-suited to this species.

Several surveillance options can be proposed:

1) Regarding dinoflagellate surveillance, it is important to establish monitoring of the benthic population of this organism in risk areas. It is essential to sample the macroalgae present in the area, in order to collect the cells of this dinoflagellate. The "*Ostreopsis*" protocol tested in the study by Abadie *et al.* (2018) shows that such surveillance is possible.

2) Alongside this monitoring of *V. rugosum*, more systematic surveillance of the presence of PnTX A and G toxins in molluscs should be considered. The PnTX extraction protocol is identical to that for the lipophilic toxins currently screened for in REPHYTOX. Only one additional step of determination by LC-MS/MS needs to be considered, whose analytical cost remains moderate. Systematic screening for PnTXs (in total meat) could be carried out during REPHYTOX lipophilic toxin analyses (and not only on samples taken as part of the EMERGTOX scheme, whose analyses focus on the digestive gland).

   This would make it possible to estimate and monitor the presence of PnTXs in bivalve molluscs in national production areas.

### 3.7.2. Health monitoring, reporting and procedures

- What should be reported?

The combination of ganglioplegia/fluctuating myasthenia-type neuromuscular signs in humans should alert the clinician, especially if they are associated with central signs such as pyramidal syndrome and/or epileptic seizures.

Definition of a suspected human case: any person who has consumed cooked or raw shellfish (oysters, mussels, clams, etc.) in the previous 24 hours, or who has been in direct contact with lagoon areas (swimming, diving) or in their immediate vicinity (angling, boating, kayaking, diving, windsurfing, kitesurfing), and who, up to 24 hours after this contact (sea water or spray), has presented with at least one of the following symptoms:

- muscle weakness (myasthenia gravis)
- respiratory disorders (difficulty breathing)
- sleepiness
- anticholinergic syndrome (dry mouth, constipation, elimination of intestinal noise, mydriasis, accommodation disorders, increased intraocular pressure, decreased tear secretion, sinus tachycardia, urinary retention)

- low/high blood pressure
- brady/tachycardia
- pyramidal syndrome
- epilepsy

These symptoms, subsequent to ingestion of shellfish contaminated with PnTXs, correspond to poisoning by nicotinic cholinergic receptor antagonists (curariform effects with or without ganglioplegic effects).

To date, in the Mediterranean, no food poisoning by PnTXs has been detected. If such cases of serious poisoning (hospitalisation) occur, they should be reported immediately to a Poison Control and Monitoring Centre (CAPTV) and the Regional Health Agency (ARS).

- Who should do the reporting?

Users concerned directly, as well as private medical practitioners, emergency hospital workers and seaside pharmacies, who are likely to receive suspected patients (respiratory disorders, muscle weakness), should report them to a CAPTV.

- Who should cases be reported to?

Every case should be reported to a Poison Control and Monitoring Centre (CAPTV).

For the *départements* of Hérault and Aude, in which the especially-affected Mediterranean lagoons are located, this is the Toulouse CAPTV at: +33 (0)5 61 77 74 47, and on the reporting portal at the following address: http://www.signalement-sante.gouv.fr

The Toulouse CAPTV is responsible for verifying and confirming reports of suspected clustered cases. It then forwards this information (number of cases and geographical location) to the ARS Occitanie.


### 3.8. Conclusions and recommendations of the Working Group and the CES ERCA


#### 3.8.1.  Are there any toxicological data for PnTXs and is there a recognised LD$_{50}$ for acute effects in particular? Are there any data for the chronic risk in the form of a toxicity reference value (TRV)?

Pinnatoxins are a group of eight toxins (PnTX A to H) among which PnTX G is taken as a reference, being the one almost exclusively found in shellfish in France (with the exception of PnTX A, also detected at Ingril in low concentrations). It should be noted that differences in acute toxicity have been observed between the analogues, and that PnTX G is not regarded as the most toxic. According to current knowledge, PnTX F appears to be the most toxic.

Following the literature review, acute toxicological data show the neurotoxicity of PnTXs, which therefore constitute a potential hazard to humans. Two experimental studies determined an LD$_{50}$ in mice by the oral route.

The data from these studies were used to propose a provisional acute benchmark value of 0.13 µg PnTX G/kg of body weight. Based on this value, a default serving size of 400 g of shellfish (EFSA, 2010b) and a default body weight of 70 kg, the concentration not to be exceeded in shellfish would be 23 µg PnTX G/kg of total meat.

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

Portimine, produced in large quantities by Mediterranean strains of *Vulcanodinium rugosum*, is related to the problem under consideration. This toxin only seems to accumulate at very low levels in shellfish. The toxicological data for portimine are more limited and it appears to be less toxic than PnTXs in mice by the intraperitoneal route.

Three of the identified PtTXs (A to C) are structural analogues belonging to the PnTX group, but the lack of data available in the literature means that they could not be taken into account in this risk assessment.

### 3.8.2. Given these toxicological data and in view of the context, is there a public health concern regarding the levels of contamination identified by Ifremer in certain French shellfish production areas?

Since the presence of PnTX G in the Ingril lagoon was reported by Ifremer in 2012, this toxin has been observed every year, at the time of the *Vulcanodinium rugosum* blooms, in samples of mussels (up to 1244 µg/kg of total meat) and clams (up to 95 µg/kg of total meat). In France, these two species of molluscs are the only ones to have been studied and there are no data available for the other species consumed.

Studies conducted on an ad hoc basis in 2012-2014 also showed the presence of PnTX G in other Mediterranean lagoons.

Lastly, the scheme for monitoring the emergence of marine biotoxins in shellfish (EMERGTOX) has shown the presence of PnTX G on the Atlantic and Corsican coasts.

The mode of action of PnTXs established *in vivo* and *in vitro* (clinical signs of toxicity and lethality, mechanistic) has enabled the experts to propose hypotheses for transposition to humans.

Using the available consumption data (INCA3, CONSOMER and CALIPSO), dietary exposure was estimated under various scenarios. The results obtained show that in some cases (high consumption and/or high contamination), the provisional acute benchmark value could be exceeded.

The WG therefore believes that there may be a health concern related to the consumption of shellfish contaminated with PnTXs from Mediterranean lagoons. This concern is particularly strong for the Ingril area, although as far as the WG knows, there is no shellfish production intended for sale. Nevertheless, the WG recommends avoiding all consumption of shellfish from this area.

### 3.8.3. What methods could be recommended for monitoring PnTXs in the marine environment, with a view to including these toxins in the vigilance scheme for lipophilic marine biotoxins in shellfish, led by the DGAL?

The WG recommends including screening for PnTXs in the analysis of lipophilic toxins in shellfish conducted as part of the official surveillance of production areas (REPHYTOX), and setting up monitoring of the benthic phase of the dinoflagellate *Vulcanodinium rugosum* within the health component of REPHY.

### 3.8.4. Research prospects

#### 3.8.4.1. Improve knowledge of the ecology of *Vulcanodinium rugosum* and the accumulation of its toxins in the food chain

Further studies should be conducted on:
- The tissue distribution of PnTXs in molluscs: preliminary unpublished results from Ifremer show that unlike other toxins such as okadaic acid, PnTXs do not seem to concentrate mainly in the digestive gland.
- Contamination of other marine organisms: the literature review (MacKenzie *et al.,* 2011) led to the finding that gastropods have very high concentrations of PnTX E and F (>1000 µg/kg of total meat). Additional studies could be carried out in risk areas (the Mediterranean lagoons), bearing in mind that some of these gastropods are consumed.
- The accumulation of the different toxins produced by *V. rugosum* along the marine food chain.
- The kinetics of accumulation and detoxification in molluscs. It should also be investigated whether a depuration phase is possible.
- The metabolites of PnTXs produced by molluscs and their potential toxicity.
- PnTX contamination of shellfish sampled from the French coast, in order to refine the dietary exposure estimates made as part of this expert appraisal, which were based on data acquired between 2010 and 2014 (2017 for Ingril).
- The thermal stability of PnTXs (effect of cooking).

### 3.8.4.2. Toxicology

- Characterise the fate of PnTXs after ingestion (ADME[7])

Unpublished results (personal communications from V. Fessard and J. Molgó) indicate that PnTXs are able to cross most biological barriers (intestinal, blood-brain, placental). However, the ADME parameters could depend on the analogue in question. These preliminary results should be supplemented by *in vivo* kinetic studies taking the various analogues into account. Data on the metabolism and distribution of these toxins are needed.
The WG recommends focusing firstly on PnTX G and then on the other analogues and portimine.

- Better characterise the oral toxicity of toxins produced by *V. rugosum*

The WG recommends conducting an extensive acute oral toxicity study (including description of clinical symptoms, assessment of biological, haematological and anatomopathological parameters as in the repeated dose studies, for a range of doses close to the maximum tolerated dose in the 2014 ANSES-University of Trieste-CNRS report study) on PnTX G in rodents, with a 14-day observation period.
The WG recommends conducting a 28-day repeated oral administration study (OECD guideline 407) or even a 90-day study (OECD guideline 408). Given the data currently available, the WG recommends supplementing these studies by investigating central and peripheral neurotoxic effects, as well as effects on the cardiovascular system. Conducting a neurotoxicity study according to OECD guideline 424 is recommended.
This recommendation applies first to PnTX G and then to the other PnTXs and associated toxins identified in raw or cooked shellfish (according to the results of the thermostability study).

Preliminary results (Couesnon *et al.,* 2014) as well as personal communications on *in ovo* exposure of chickens suggest that PnTXs could affect embryo growth. The WG recommends that potential effects on offspring be investigated, according to OECD guideline 422.

- Human epidemiology

Depending on the results of the toxicokinetic studies in rodents, it may be worthwhile screening for PnTXs in the blood, urine and faeces of populations living near the Mediterranean lagoons and consuming mussels and clams.
The WG also recommends conducting a study of poisoning cases in the area concerned, involving hospitals near the Mediterranean lagoons, and general practitioners' surgeries in the area. This study, coordinated by the toxicovigilance scheme, could be based on the reporting form proposed by the WG and could involve Ifremer for the analysis of toxins in shellfish for the period concerned.

## 4. AGENCY CONCLUSIONS AND RECOMMENDATIONS

The French Agency for Food, Environmental and Occupational Health & Safety (ANSES) endorses the conclusions of the Working Group on "Pinnatoxins" (PnTX) 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 the emerging hazard posed by PnTXs. The analysis of the data available in the literature enabled a provisional acute benchmark value to be proposed for PnTX G, identified as the pinnatoxin most commonly detected in French shellfish. This value was used to characterise the risk to consumers for different scenarios: exposure associated with shellfish consumption was estimated on the basis of PnTX G contamination data in mussels and clams from Mediterranean lagoons and consumption data available at ANSES and representative of the adult population in this geographical area. The expert appraisal showed that depending on the scenarios tested, there may be a health concern in the event of high consumption and/or high contamination. This concern is particularly strong for shellfish coming from the Ingril

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[7] ADME: absorption, distribution, metabolism, excretion

area. As far as ANSES knows, there is no shellfish production intended for sale from this area. Nevertheless, ANSES recommends that the public authorities ensure that all consumption of shellfish from this area is avoided.

The risk assessment underlying this conclusion is based on a worst-case scenario for estimating exposure to PnTX G, using the 95th percentile of shellfish contamination. A more realistic risk assessment could be conducted using a probabilistic approach (taking into account the distribution of all the data, on both contamination and consumption), which would require the acquisition of more contamination data in shellfish, particularly oysters.

ANSES emphasises:

1/ the unknowns with regard to the spatial distribution of *Vulcanodinium rugosum* and its presence in shellfish production areas in metropolitan France on the one hand, and its proven ubiquitous nature in Europe, from the south Atlantic coast of Spain to the Norwegian coasts, on the other;

2/ the unknowns regarding the toxigenicity of *V. rugosum* in most shellfish production areas in metropolitan France;

3/ the confirmed observation of the toxin production capability of some toxigenic *V. rugosum*, at levels of health concern revealed by the risk assessment, in the "Ingril lagoon" area, a stretch of water contiguous with the major shellfish production basin of the Thau Lagoon (Hérault).

In this context, ANSES recommends:

1/ at Ingril and Thau, establishing regular surveillance of dinoflagellates and analytical monitoring of PnTX concentrations in shellfish whose production is authorised, according to procedures to be established by risk managers, in particular with regard to setting an acceptable contamination guideline value. Based on the provisional acute benchmark value and a default serving size of 400 g of shellfish, ANSES identified that the concentration not to be exceeded in shellfish would be 23 µg PnTX G/kg of total meat.

2/ For the other production areas (or for recreational fishing), in particular in the framework of the REPHYTOX and EMERGTOX scheme steering committees, considering whether to draw up maps showing where *V. rugosum* is established in the benthic zones of all the shellfish growing areas along the coast of metropolitan France, with a view to conducting regular surveillance. Given the proven potential for the long-term establishment of this dinoflagellate in the environment and its toxigenicity in Mediterranean lagoon-type environments, particular attention should be paid to surveillance in these areas.

Lastly, ANSES underlines the importance of undertaking research aimed at supplementing knowledge on PnTXs and other toxins produced by *V. rugosum*, in particular studies on their toxicity and their accumulation in shellfish species consumed by humans.

Dr Roger Genet

**KEYWORDS**

Pinnatoxins, Pteriatoxins, Portimine, *Vulcanodinium rugosum*, Cyclic imines, Emerging toxins, Shellfish