Health risk assessment related to pinnatoxins in French shellfish


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Health risk assessment related to pinnatoxins in French shellfish

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ABSTRACT

Pinnatoxins (PnTXs) are a group of emerging marine biotoxins produced by the benthic dinoflagellate *Vulcanodinium rugosum*, currently not regulated in Europe or in any other country in the world. In France, PnTXs were detected for the first time in 2011, in mussels from the Ingril lagoon (South of France, Mediterranean coast). Since then, analyses carried out in mussels from this lagoon have shown high concentrations of PnTXs for several months each year. PnTXs have also been detected, to a lesser extent, in mussels from other Mediterranean lagoons and on the Atlantic and Corsican coasts. In the French data, the main analog is PnTX G (low levels of PnTX A are also present in some samples). No cases of PnTXs poisoning in humans have been reported so far in France or anywhere else in the world. In mice, PnTXs induce acute neurotoxic effects, within a few minutes after oral administration. Clinical signs of toxicity include decreased mobility, paralysis of the hind legs, tremors, jumps and breathing difficulties leading to death by respiratory arrest at high doses. The French agency for food safety (ANSES) recently conducted a review of the state of knowledge related to PnTXs and *V. rugosum*. Based on (i) the clinical signs of toxicity in mice, (ii) the mode of action of PnTXs as nicotinic acetylcholine receptor competitive antagonists and (iii) knowledge on drugs and natural toxins with PnTX-related pharmacology, potential human symptoms have been extrapolated and proposed. In this work, a provisional acute benchmark value for PnTX G of 0.13 µg/kg bw per day has been derived from an oral acute toxicity study in mice. Based on this value and a large shellfish meat portion size of 400g, a concentration lower than 23 µg PnTX G/kg shellfish meat is not expected to result in adverse effects in humans. ANSES recommends taking into account PnTXs in the French official monitoring program for shellfish production and identified data gaps to refine health risk assessment.

KEYWORDS: Pinnatoxins, shellfish, emerging marine biotoxins, risk assessment
1. Introduction

Pinnatoxins (PnTXs) belong to the group of cyclic imines considered as emerging marine biotoxins (Efsa, 2010a), which, to date, includes 40 compounds, without considering acyl esters produced by shellfish metabolism. This group includes different families determined by their structural characteristics: prorocentrolides, spiropriocentrimine, gymnodimines (GYMs), spirolides (SPXs), pinnatoxins (PnTXs), pteriatoxins (PtTXs) and portimine. To date, 8 PnTXs (named A to H) have been identified (reviewed by Molgó et al., 2017). PnTXs are soluble in solvents such as acetone, isopropanol and methanol (Zendong et al. 2014). They are amphoteric compounds, i.e. with both acidic and basic properties, which explains their relative water solubility. Due to their lipophilic properties, they can be detected during the mouse bioassay used to screen for lipophilic marine biotoxins.

Vulcanodinium rugosum is the producer of PnTXs identified by Nézan and Chomerat (2011), based on water samples from a Mediterranean lagoon (Ingril, France). V. rugosum also produce portimine (Selwood et al., 2013; Abadie et al., 2016). This benthic dinoflagellate was a new species belonging to a new genus. No other producer is reported in the literature. The identification of this dinoflagellate in France originates from an atypical situation that occurred in 2006, as part of official monitoring of shellfish production areas. The mouse bioassay used to screen for lipophilic marine biotoxins had revealed unusual neurotoxic effects after the injection of extracts from mussels of the Ingril lagoon. In fact, the symptoms observed for lipophilic toxins are gastrointestinal (diarrhea), and not neurotoxic. Such neurotoxicity could not be related to the presence of regulated marine biotoxins (Amnesic, Paralytic, Diarrhetic toxins) screened for by chemical analysis. In addition, observations of water samples did not conclude to the presence of any microalgae species known to produce neurotoxins.

Vulcanodinium rugosum has also been reported in New Zealand (Rhodes et al., 2010), Australia (Rhodes et al., 2011; Munday et al., 2012), Japan (Smith et al., 2011), China Sea (Zeng et al., 2012), Mexico (Hernandez-Becerril et al., 2013), Arabian Gulf (Al Muftah et al., 2016) and Cuba (Moreira-Gonzalez et al., 2018).

PnTXs in shellfish are mainly analysed by physico-chemical methods using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Biological (mouse bioassay) or biochemical (functional tests) methods can also be used. None of the methods has yet undergone inter-laboratory
validation or standardisation. In addition, there are very few standards (reference substances) available for PnTXs, 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 (Figure 1) has been certified. Since the first identification of *V. rugosum* in France, high concentrations of PnTXs have been measured in mussels from the Ingril lagoon for several months each year. In 2012, the French Research Institute for Exploitation of the Sea (Ifremer) reported that PnTXs 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, 2012). However, *V. rugosum* is rarely detected in water samples collected for the phytoplankton surveillance (in the water column), which can be explained by the benthic nature of this dinoflagellate. The aims of this work were 1) to review the toxicity data of PnTXs to derive an acute oral health-based guidance value, 2) estimate exposure of shellfish consumers to PnTXs, 3) identify if there might be a public health concern regarding the levels of contamination reported in certain French shellfish production areas, and 4) provide recommendations for the monitoring PnTXs in the marine environment.

![Figure 1: Structure of PnTX G (modified from Araoz et al., 2011).](image)

2. *Vulcanodinium rugosum*

As almost all dinoflagellates, *V. rugosum* (Figure 2) is assumed to have two distinct life phases: a vegetative propagation phase (asexual reproduction) and a sexual phase (Zeng et al., 2012). The asexual phase probably corresponds to the pelagic phase, when the cells are present in the water
column. The sexual reproduction corresponds to a benthic phase, which could result 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 phase to the
other are not yet known.

The formation of resistance cysts, which can occur at the end of sexual reproduction, is a crucial step
for survival in adverse environmental conditions due to 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). Therefore,
identification of the resistance forms and knowledge of their distribution area are important for
preventing expansion and contamination. Similarly, determination of the key factors for growth and
toxin production by *V. rugosum* is essential for better understanding the risk associated with this new
species.

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

![Micrograph of a cell of *Vulcanodinium rugosum*](image)

**Figure 2: Micrograph of a cell of *Vulcanodinium rugosum***

3. Hazard characterisation

3.1 Acute *in vivo* toxicity with purified PnTXs
In vivo acute toxicity studies with purified toxins were reviewed using an analysis grid and assessed for quality using ToxRTool (Toxicological data Reliability assessment Tool, https://eur-ecvam.jrc.ec.europa.eu/about-ecvam/archivepublications/toxrtool), a tool that ranks the studies according to the Klimisch rating (Klimisch et al., 1997).

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 by OECD guidelines) and with very few animals tested per dose (1 to 8 mice, when the number is specified). The results are presented in table 1.

Orally, only three studies are available and focused on PnTX E, F, G and H (Munday et al., 2012; Selwood et al., 2014; ANSES-University of Trieste-CNRS report, 2014 also published in Sosa et al., 2020). We did not find any information on the toxicity by the oral route for PtTXs (metabolites of certain PnTXs) and portimine (a toxin also produced by V. rugosum). 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). We identified additional unpublished information regarding PnTX A (personal communication from J. Molgó), but did not find any robust information regarding PtTXs.

Despite the limited number of studies for each PnTX and the low number of mice tested per dose, a consistent set of information enables outlining the main characteristics of the acute toxicity of this toxin group. Firstly, the toxicity of the PnTXs is rapid, with symptoms appearing within minutes of administration (whether oral or IP). This fact was already known, since PnTXs (like other cyclic imines) belong to the fast-acting toxins group (Efsa, 2010a). The second point relies on neurotoxic symptoms, quickly leading to the mouse's death by respiratory arrest. 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; Sosa et al., 2020). 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.

LD50 values vary according to the PnTX analogue and route of administration (Table 1). Orally, LD50 values range from 25 to 2,800 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 route, LD50 range from 13 to 115 \( \mu g/kg \) 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.

LD99 values for some PnTXs and PtTXs have been reported in the literature, but these data were considered not sufficiently reliable due to a lack of information on the protocol used (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 LD50 of 1,570 \( \mu g/kg \) bw. No effect was observed at 500 and 700 \( \mu g/kg \) bw (Selwood et al., 2013). The authors indicated 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 of portimine by oral administration is unknown.

**Table 1: Acute in vivo toxicity in mice of PnTXs and portimine**

1a. Oral administration

<table>
<thead>
<tr>
<th>Toxin (purity)</th>
<th>Route of administration and number of mice</th>
<th>LD50 (( \mu g/kg ) bw)</th>
<th>MTD (( \mu g/kg ) bw)</th>
<th>References</th>
<th>Study quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>PnTX E*</td>
<td>Gavage</td>
<td>2800</td>
<td>600</td>
<td>Munday et al., 2012</td>
<td>ToxRTool: 13 Klimisch: 2</td>
</tr>
<tr>
<td></td>
<td>Number of mice not specified (OECD GL 425)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fed (non-fasted) mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PnTX F*</td>
<td>Gavage</td>
<td>25</td>
<td>9.9</td>
<td>Munday et al., 2012</td>
<td>ToxRTool: 13 Klimisch: 2</td>
</tr>
<tr>
<td></td>
<td>Number of mice not specified (OECD GL 425)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fed (non-fasted) mice</td>
<td>29.9</td>
<td>Not determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16h fasted mice</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PnTX F*</td>
<td>In cream cheese</td>
<td>50</td>
<td>16.0</td>
<td>Munday et al., 2012</td>
<td>ToxRTool: 13 Klimisch: 2</td>
</tr>
<tr>
<td></td>
<td>Number of animals not specified (OECD GL 425)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fed (non-fasted) mice</td>
<td>50</td>
<td>Not determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>In peanut butter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Number of mice not specified (OECD GL 425)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fed (non-fasted) mice</td>
<td>50</td>
<td>Not determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>In dry mouse food</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Number of mice not specified</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toxin (purity)</td>
<td>Route of administration and number of mice</td>
<td>LD50 (µg/kg bw)</td>
<td>MTD (µg/kg bw)</td>
<td>References</td>
<td>Study quality</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------------------------------------------------------------------------------------------------------</td>
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<td>----------------</td>
<td>----------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td></td>
<td>specified (OECD GL 425) 16h fasted mice In cream cheese, 16h fasted mice In peanut butter, 16h fasted mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PnTX G*</td>
<td>Gavage Number of mice not specified (OECD GL 425) Fed (non-fasted) mice</td>
<td>150</td>
<td>75</td>
<td>Munday et al., 2012</td>
<td>ToxRTool: 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CI95: 105-100</td>
<td></td>
<td></td>
<td>Klimisch: 2</td>
</tr>
<tr>
<td>PnTX G (declared purity 100%)</td>
<td>Gavage 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) Mice fasted for 3 hours before administration</td>
<td>208</td>
<td>120</td>
<td>ANSES-University of Trieste-CNRS report, 2014; Sosa et al., 2020</td>
<td>ToxRTool: 17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CI95: 155-281</td>
<td></td>
<td></td>
<td>Klimisch: 2</td>
</tr>
<tr>
<td>PnTX G*</td>
<td>In cream cheese Number of mice not specified (OECD GL 425) Fed (non-fasted) mice</td>
<td>400</td>
<td>153</td>
<td>Munday et al., 2012</td>
<td>ToxRTool: 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CI95: 380-470</td>
<td></td>
<td></td>
<td>Klimisch: 2</td>
</tr>
<tr>
<td>PnTX H*</td>
<td>Gavage Number of animals not specified Fasting not specified</td>
<td>163</td>
<td></td>
<td>Selwood et al., 2014</td>
<td>ToxRTool: 4</td>
</tr>
</tbody>
</table>

* 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

1b. Intraperitoneal (IP) administration

<table>
<thead>
<tr>
<th>Toxin (purity)</th>
<th>Route of administration and number of mice</th>
<th>LD50 (µg/kg bw)</th>
<th>MTD (µg/kg bw)</th>
<th>References</th>
<th>Study quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic PnTX A (purity &gt; 97%)</td>
<td>IP n=18 mice, 9 doses tested (1 to 3 mice/dose)</td>
<td>114.8</td>
<td></td>
<td>J. Molgó (personal communication)</td>
<td>ToxRTool: 16</td>
</tr>
<tr>
<td>Toxin (purity)</td>
<td>Route of administration and number of mice</td>
<td>LD50 (µg/kg bw)</td>
<td>MTD (µg/kg bw)</td>
<td>References</td>
<td>Study quality</td>
</tr>
<tr>
<td>---------------</td>
<td>------------------------------------------</td>
<td>----------------</td>
<td>----------------</td>
<td>------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Fed (non-fasted) mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PnTX E*</td>
<td>IP Number of animals not specified (OECD GL 425)</td>
<td>57 (fed) CI95: 39.7-75.3 48 (16h fasted) CI95: 33.5-63.5</td>
<td>22 Not determined</td>
<td>Munday et al., 2012</td>
<td>ToxRTool: 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45 (fed) CI95: 32-58</td>
<td></td>
<td>Selwood et al., 2010</td>
<td>ToxRTool: 15</td>
</tr>
<tr>
<td>PnTX F*</td>
<td>IP Number of animals not specified (OECD GL 425)</td>
<td>12.7 (fed) CI95: 9.5-14.6 14.9 (16h fasted) CI95: 12.6-15.8</td>
<td>3.2 Not determined</td>
<td>Munday et al., 2012</td>
<td>ToxRTool: 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16 (fed) CI95: 12-23</td>
<td></td>
<td>Selwood et al., 2010</td>
<td>ToxRTool: 15</td>
</tr>
<tr>
<td>PnTX G*</td>
<td>IP Number of animals not specified (OECD GL 425)</td>
<td>48 (fed) CI95: 36.3-68.1 42.7 (16h fasted) CI95: 40-50</td>
<td>18.8 Not determined</td>
<td>Munday et al., 2012</td>
<td>ToxRTool: 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 (fed) CI95: 35-66</td>
<td></td>
<td>Selwood et al., 2010</td>
<td>ToxRTool: 15</td>
</tr>
<tr>
<td>Synthetic PnTX G (purity &gt; 97%)</td>
<td>IP Number of animals not specified</td>
<td>65.8</td>
<td></td>
<td>J. Molgó (personal communication)</td>
<td>ToxRTool: 16</td>
</tr>
<tr>
<td>PnTX H*</td>
<td>IP Number of animals not specified Fasting not specified</td>
<td>67 CI95: 63-79</td>
<td></td>
<td>Selwood et al., 2014</td>
<td>ToxRTool: 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1570 CI95: 1269-3080</td>
<td>No effect at 500 and 700</td>
<td>Selwood et al., 2013</td>
<td>ToxRTool: 4</td>
</tr>
</tbody>
</table>
Toxin (purity) | Route of administration and number of mice | LD50 (µg/kg bw) | MTD (µg/kg bw) | References | Study quality
--- | --- | --- | --- | --- | ---
| | | | | | that a lot of details are missing 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

3.2 Subchronic and chronic toxicity

No repeated dose toxicity studies were identified in the literature.

3.3 Mode of action and extrapolation to humans

We reviewed the data available on the mode of action of PnTXs (Delcourt et al., 2019). PnTXs are high-affinity competitive antagonists of nicotinic acetylcholine receptors (nAChRs) (Araoz et al., 2011; Hellyer et al., 2015). Their lethal effects in mice studies are consistent with the inhibition of muscle nAChRs, inducing respiratory distress and paralysis (Delcourt et al., 2019). PnTX E, F, G and A block nerve stimulation-induced skeletal muscle contraction, but do not alter muscle contraction caused by direct stimulation of isolated adult rodent muscles (Hellyer et al., 2013; Benoit et al., 2019).

The clinical effects observed in humans after exposure to compounds (drugs, natural toxins) whose pharmacology is comparable to that of PnTXs have been described (Delcourt et al., 2019). Possible symptoms in humans due to PnTXs could include muscle weakness (myasthenic-like syndrome), dyspnea, anticholinergic syndrome, dysautonomia, pyramidal syndrome, and seizures.

3.4 Development of a health value

The methodology set in ANSES's guide for the development of toxicological reference values (2017) was followed.

3.4.1 Choice of the key study
Two acute oral toxicity studies in mice with purified PnTX G were identified (Munday et al., 2012; ANSES-University of Trieste-CNRS report, 2014 also published in Sosa et al., 2020). The key study selected was 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; Sosa et al., 2020), which obtained the highest score (17 out of 21 with ToxRTool, corresponding to a Klimisch score of 2 - reliable with restriction).

In this study, groups of three to five 4 weeks-old female SD-1 mice 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. A control group of eight mice was included. Mice were fasted for 3 hours before gavage; food was given back again ad libitum 2 hours after administration during the 24-hour observation period. Lethality, clinical signs of toxicity, histological analysis of several organs and blood biochemistry were recorded.

The results regarding lethality and the observed symptoms are shown in Table 2. 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 between 13 and 18 min). The LD50 for PnTX G was calculated at 208 µg/kg bw (95% confidence interval = 155-281 µg/kg bw).

Before death, the main symptoms were neurotoxic, i.e. 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 (Sosa et al., 2020).

<table>
<thead>
<tr>
<th>Purified PnTX G dose</th>
<th>Lethality</th>
<th>Survival time (h:min)</th>
<th>Clinical signs of toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0/8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8 µg/kg bw</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20 µg/kg bw</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>50 µg/kg bw</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>120 µg/kg bw</td>
<td>0/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>220 µg/kg bw</td>
<td>3/5</td>
<td>00:20; 00:22; 00:22</td>
<td>prostration, tremors, jumps, abdominal</td>
</tr>
</tbody>
</table>
breathing, hypothermia, hind leg paralysis, cyanosis

<table>
<thead>
<tr>
<th>Dose (µg/kg bw)</th>
<th>Mortality</th>
<th>Time of Symptoms</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 µg/kg bw</td>
<td>4/5</td>
<td>00:12; 00:13; 00:17; 00:23</td>
<td>prostration, tremors, jumps, abdominal breathing, hypothermia, hind leg paralysis, cyanosis</td>
</tr>
<tr>
<td>370 µg/kg bw</td>
<td>5/5</td>
<td>00:13; 00:15; 00:16; 00:17; 00:18</td>
<td>prostration, tremors, jumps, abdominal breathing, hypothermia, hind leg paralysis, cyanosis</td>
</tr>
<tr>
<td>450 µg/kg bw</td>
<td>5/5</td>
<td>00:12; 00:12; 00:15; 00:16; 00:29</td>
<td>prostration, tremors, jumps, abdominal breathing, hypothermia, hind leg paralysis, cyanosis</td>
</tr>
</tbody>
</table>

3.4.2 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\(^1\) 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.

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.

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 results obtained in the other acute oral toxicity study of purified PnTX G with non-fasted mice (Munday et al., 2012) were consistent with those of the key study selected conducted by the Department of Life Sciences at the University of Trieste:

i. The LD50 was 150 µg PnTX G/kg bw (95% CI = 105-199 µg/kg bw);

---

\(^1\) 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).
ii. The dose tested without lethality and with no apparent sign of neurotoxicity was 75 µg PnTX G/kg bw.

3.4.3 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:
  - inter-species uncertainty factor: $U_F^A = 10$
  - inter-individual uncertainty factor: $U_F^H = 10$
  - other factors: $U_F^D = 3$ (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 was chosen as the point of departure. The associated overall uncertainty factor was 525:
  - allometric adjustment factor: 7 for the mouse
  - $U_F^A = 2.5$ for the toxicodynamic component
  - $U_F^H = 10$
  - $U_F^D = 3$ (insufficient data).

3.4.4 Proposed health-based guidance value

Due to insufficient data for the hazard characterisation of PnTX G, the guidance value derived is considered as a provisional acute benchmark value.

The provisional acute benchmark value is 0.13 µg PnTX G/kg bw, (Table 3) regardless of the approach used (MTD or BMDL).

Table 3: Table summarising development of the provisional acute benchmark value for PnTX G

<table>
<thead>
<tr>
<th>Critical effect</th>
<th>Critical dose for the mouse</th>
<th>UF</th>
<th>Provisional acute benchmark value for humans</th>
<th>Confidence level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Key study</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

2 MTD approach: \((120 \mu g \text{ PnTX G/kg bw}) / (10 \times 10 \times 3 \times 3) = 120/900 = 0.1333 \mu g \text{ PnTX G/kg bw}\)

BMDL approach: \(69.1 \mu g \text{ PnTX G/kg bw}) / (7 \times 2.5 \times 10 \times 3) = 69.1/525 = 0.1316 \mu g \text{ PnTX G/kg bw}\)
First approach

**Critical effect**: absence of mortality and symptoms in mice 24 hours after a single oral administration by gavage of purified PnTX G

**Key study**: ANSES-University of Trieste-CNRS report, 2014; Sosa et al., 2020

<table>
<thead>
<tr>
<th>Maximum tolerated dose of 120 µg PnTX G/kg bw</th>
<th>Total factor: 900</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( UF_A = 10 )</td>
</tr>
<tr>
<td></td>
<td>( UF_H = 10 )</td>
</tr>
<tr>
<td></td>
<td>( UF_D = 9 ) (3 for insufficient data and 3 for the severity and pattern of the dose-response curve).</td>
</tr>
</tbody>
</table>

0.13 µg PnTX G/kg bw

Moderate

Second approach

**BMDL of 69.1 µg PnTX G/kg bw**

<table>
<thead>
<tr>
<th>Total factor: 525</th>
</tr>
</thead>
<tbody>
<tr>
<td>( UF_A = 2.5 )</td>
</tr>
<tr>
<td>( UF_H = 10 )</td>
</tr>
<tr>
<td>( UF_D = 3 ) (for insufficient data)</td>
</tr>
</tbody>
</table>

0.13 µg PnTX G/kg bw

Moderate

### 3.4.5 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 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**.

### 3.4.6 Confidence level

An 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. In fact, only two acute oral toxicity studies conducted on a single species (mice) and sex (females, which are considered to be more sensitive than males) have been published. The lack of data in another rodent species (rats) and of knowledge on the toxicokinetics and toxicodynamics of PnTXs is underlined.

- 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 (LD50). Signs of neurological toxicity were also observed and are consistent with the known mode of action of PnTXs.

We stress 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.

4. Occurrence of PnTXs in shellfish

4.1 Ingril lagoon

As soon as PnTX G was identified in France (2011), a retrospective analysis of the samples from 2009 to 2012 was conducted by Ifremer, 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 (Table 4). Concentrations in mussels were higher than those observed in clams, when shellfish were sampled simultaneously (Table 5).

To track this phenomenon and maintain monitoring for PnTXs, sampling of Ingril mussels was carried out monthly between 2013 and 2017 by Ifremer. 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).

Table 4: Data on concentrations of PnTX G in mussels from Ingril lagoon from 2010 to 2017 (μg/kg total meat)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>30</td>
<td>32</td>
<td>24</td>
<td>12</td>
<td>27</td>
<td>2</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Min</td>
<td>45.4</td>
<td>36.6</td>
<td>11.0</td>
<td>49.5</td>
<td>22.6</td>
<td>572</td>
<td>44.0</td>
<td>52.9</td>
</tr>
<tr>
<td>Max</td>
<td>1244</td>
<td>568</td>
<td>652</td>
<td>887</td>
<td>918</td>
<td>1143</td>
<td>600</td>
<td>640</td>
</tr>
<tr>
<td>Mean</td>
<td>223</td>
<td>227</td>
<td>209</td>
<td>293</td>
<td>247</td>
<td>858</td>
<td>278</td>
<td>294</td>
</tr>
</tbody>
</table>

Table 5: Data on concentrations of PnTX G in mussels and clams from Ingril lagoon from 2010 to 2012 (μg/kg total meat)

<table>
<thead>
<tr>
<th>Mussels</th>
<th>Clams</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>86</td>
</tr>
<tr>
<td>Min</td>
<td>11.0</td>
</tr>
<tr>
<td>Max</td>
<td>1244</td>
</tr>
<tr>
<td>Mean</td>
<td>217</td>
</tr>
</tbody>
</table>

4.2 Other Mediterranean lagoons

In addition to the monitoring carried out in the Ingril lagoon, screening for PnTX G in wild mussel samples took place the same week in four other Mediterranean lagoons in 2013 (Table 6). Maximum levels of PnTX G increased in the following order: Parc Leucate (11 μg/kg of total meat) > Thau (15 μg/kg) > Le Prevost (54 μg/kg) > Vic (89 μg/kg) > Ingril (887 μg/kg).

Table 6: Data on concentrations of PnTX G in mussels from several Mediterranean lagoons in 2013 (μg/kg total meat)

<table>
<thead>
<tr>
<th>Parc Leucate</th>
<th>Thau</th>
<th>Le Prévost</th>
<th>Vic</th>
<th>Ingril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>12</td>
<td>11</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Min</td>
<td>0.68</td>
<td>1.4</td>
<td>11.8</td>
<td>12.3</td>
</tr>
</tbody>
</table>
4.3 Atlantic and Corsican coasts

In France, 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.

Since 2018, this scheme enables the targeted analysis by chemical methods of PnTXs in mussels and oysters from eleven areas located all along the French metropolitain coasts. During this first year of monitoring, three of these areas (Ingril, Le Scoré in Brittany and the Diana lagoon in Corsica) were affected by the presence of PnTXs, 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.

We believe it is important to point out that the presence of PnTXs in France is not limited to Mediterranean lagoons. Given that global warming is making ecophysiological conditions more favourable to the development of *V. rugosum*, vigilance should be maintained along the Atlantic coast, since the detection of PnTXs in mussels from Brittany points out the presence of the dinoflagellate producer also in these waters.

4.4 Outside France

The literature review concluded that very limited data on PnTX levels in shellfish from Northern and Southern Europe, Canada and New Zealand have been published. Among the eight studies reviewed (MacKenzie et al., 2011; Rundberget, et al., 2011; McCarron et al., 2012; McNabb et al., 2012; Garcia-Altares et al., 2014; Rambla Alegre et al., 2018; Lamas et al., 2019; Otero et al., 2019), 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

<table>
<thead>
<tr>
<th></th>
<th>Max</th>
<th>10.7</th>
<th>14.7</th>
<th>53.9</th>
<th>89.0</th>
<th>887</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>3.4</td>
<td>10.0</td>
<td>26.2</td>
<td>48.4</td>
<td>293</td>
<td></td>
</tr>
</tbody>
</table>
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 a health risk assessment for PnTXs. Some respondents provided contamination data in shellfish from their country. Most of the data were already published in a scientific journal and had been identified in our literature search. Some unpolished data were also provided but without information about the performance of the analytical method used.

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

5 Dietary exposure

5.1. Food consumption data

A consumption survey on seafood products in France (CONSOMER) was conducted in 2016-2017 as part of a research agreement between ANSES and CREDOC (2015-CRD-25). The aim of the survey 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. The database includes answers from 2481 adults. Only data for the Mediterranean population were used in the present work, including 821 adults (Table 7).

Table 7: Consumption data of mussels and clams from CONSOMER survey (Mediterranean population only), daily portion size.

<table>
<thead>
<tr>
<th>Number of consumers</th>
<th>Mean amount in g/day</th>
<th>P95 in g/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mussels</td>
<td>582</td>
<td>91.2</td>
</tr>
<tr>
<td>Clams</td>
<td>140</td>
<td>19.8</td>
</tr>
</tbody>
</table>

5.2 Contamination data

Data on concentrations of PnTX G in mussels and clams from several Mediterranean lagoons from 2010 to 2017 were provided to ANSES by Ifremer (Table 8). Three scenarios were selected, depending on the site where the shellfish were sampled:
- **"All lagoons" scenario**: contamination data of all mussels on the one hand and all clams on the other (from all the lagoons studied);

- **"Ingril" scenario**: contamination data ONLY from the Ingril lagoon, for mussels on the one hand and clams on the other;

- **"Except Ingril" scenario**: contamination data from all lagoons (Vic, le Prévost, Parc Leucate and Thau) except "Ingril", for mussels only (no clams sampled in other lagoons).

### Table 8: Concentrations of PnTX G in mussels and clams from several Mediterranean lagoons from 2010 to 2017

<table>
<thead>
<tr>
<th>Number of samples of mussels</th>
<th>Concentration of PnTX G (µg/kg total meat)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>P95</td>
</tr>
<tr>
<td>All lagoons</td>
<td>196</td>
<td>633</td>
</tr>
<tr>
<td>Ingril</td>
<td>150</td>
<td>712</td>
</tr>
<tr>
<td>Except Ingril*</td>
<td>46</td>
<td>60.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of samples of clams</th>
<th>Concentration of PnTX G (µg/kg total meat)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>P95</td>
</tr>
<tr>
<td>All lagoons</td>
<td>20</td>
<td>57</td>
</tr>
<tr>
<td>Ingril</td>
<td>20</td>
<td>57</td>
</tr>
<tr>
<td>Except Ingril</td>
<td>0</td>
<td>No data</td>
</tr>
</tbody>
</table>

* Number of samples of mussels in other lagoons than Ingril are described in Table 6.

### 5.3. Method to calculate exposure

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) (serving size for acute exposure);
- $L_k$ is the PnTX G contamination of food $k$ ($\mu g/kg$ of fresh weight) ($95^{th}$ percentile for acute exposure);

- $BW_i$ is the body weight of individual $i$ (kg).

### 6 Risk characterisation

#### 6.1 Results

A comparison of acute exposure estimates was made with the provisional acute benchmark value of 0.13 $\mu g$ PnTX G/kg of body weight. A percentage of the population exceeding this value was calculated, firstly in the total population and secondly for consumers of mussels and clams only (Table 9).

#### Table 9: Acute exposure associated with consumption of mussels and clams (CONSOMER, Mediterranean area) for adults (in $\mu g$ PnTX G/kg bw)

<table>
<thead>
<tr>
<th>Total population (Mediterranean area)</th>
<th>Consumers only (Mediterranean area)</th>
<th>Provisional acute benchmark value (PABV) exceeded</th>
</tr>
</thead>
<tbody>
<tr>
<td>n Ave_pop P95_pop</td>
<td>n_cons Ave_cons P95_cons</td>
<td>% &gt; PABV</td>
</tr>
<tr>
<td>All lagoons 821 0.590 1.572</td>
<td>591 0.816 1.733</td>
<td>71</td>
</tr>
<tr>
<td>Ingril 821 0.663 1.767</td>
<td>591 0.917 1.946</td>
<td>71</td>
</tr>
<tr>
<td>Except Ingril (mussels only*) 821 0.056 0.149</td>
<td>582 0.079 0.167</td>
<td>7.3</td>
</tr>
</tbody>
</table>

* 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 mussels and clams from the first column)

Ave_pop: average exposure in the population (whether consumers or not) ($\mu g/kg$ bw)

P95_pop: exposure to the $95^{th}$ percentile calculated in the population ($\mu g/kg$ bw/d)

n_cons: number of adult individuals consuming mussels and clams from the first column (consumers only)

Ave_cons: average exposure in the consumer population only ($\mu g/kg$ bw/day)

P95_cons: exposure to the $95^{th}$ percentile calculated in the consumer population only ($\mu g/kg$ bw/day)

% > PABV: percentage of the general population exceeding the provisional acute benchmark value of 0.13 $\mu g/kg$ bw

% > PABV_cons: percentage of consumer population only exceeding the provisional acute benchmark value of 0.13 $\mu g/kg$ 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 provisional acute benchmark value would be exceeded in 7% of the total Mediterranean population and in 10% of the population of consumers.

6.2 Conclusion in terms of health concern

Considering the percentage of the French population for which the provisional acute benchmark value would be exceeded in the "Except Ingril" scenario, 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 contamination was measured only in the digestive glands, and the distribution of PnTXs between the digestive gland and the total meat is not known.

7. Recommendations for surveillance

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 as the observation of the pelagic phase in the water column is challenging. Official surveillance of shellfish production areas based on the identification and counting of toxic phytoplanktonic species in the water column is therefore ill-suited to this species.

Several monitoring options can be proposed:

1) Regarding dinoflagellate surveillance, monitoring of the benthic population of this organism in risk areas is recommended. It is essential to sample the macroalgae present in the area, in order to collect *V. rugosum* cells. The protocol set up for "Ostreopsis" monitoring and tested in the study by Abadie *et al.* (2018) shows that such methodology is adequate.
2) Alongside the monitoring of *V. rugosum*, more systematic surveillance of the presence of PnTX A and G toxins in molluscs should be considered. The extraction protocol for PnTXs is identical to the one used for official monitoring of lipophilic toxins in the French shellfish production areas (REPHYTOX). Although an additional step for LC-MS/MS analysis is required, the cost remains moderate. A systematic screening for PnTXs in total meat could be added to the REPHYTOX analyses of lipophilic toxins, and not only on samples taken for the EMERGTOX scheme, that focuses only on the digestive gland.

Such proposal will enable estimating and monitoring the presence of PnTXs in bivalve molluscs in national production areas.

### 7.2 Health monitoring, reporting and procedures

To carry on with this issue, ANSES is working on raising awareness among health professionals of the potential neurological symptoms that could be associated with PnTXs poisoning in humans (Delcourt at al., 2019). An epidemiological study is currently underway with the objective to identify potential human cases related to shellfish consumption and PnTX exposure in France.

### 8. Conclusion

This work points out that, in case of high consumption and/or high contamination, the provisional acute benchmark value for PnTX G could be exceeded, suggesting a health concern related to the consumption of shellfish contaminated with PnTXs from Mediterranean lagoons, and particularly from the Ingril area. There is currently no shellfish production intended for sale from Ingril area. Nevertheless, ANSES highlighted the need to avoid all consumption of shellfish from this area. ANSES recommends that PnTXs should be taken into account in the French official monitoring of shellfish production areas.

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, 2019).
9. Data gaps

ANSES recommends undertaking research to estimate more accurately the exposure of shellfish consumers. More data on shellfish contamination by PnTXs are needed, including other species than mussels and clams. More research is also needed to better characterize the oral toxicity of toxins produced by *V. rugosum*. As portimine is produced in large quantities by Mediterranean strains of *Vulcanodinium rugosum*, this toxin should be also considered. This toxin only seems to accumulate at very low levels in shellfish. Additional data are required to confirm its low accumulation in shellfish. Although less toxic than PnTXs in mice by the intraperitoneal route, the toxicological data for portimine are limited and investigation should be carried on. Three of the identified PtTXs (A to C) are structural analogues belonging to the PnTX group but, due to the lack of data available in the literature, they could not be taken into account in this risk assessment. More toxicity and contamination studies are needed.

An extensive acute oral toxicity study on PnTX G in rodents is needed (including description of clinical symptoms, assessment of biological, hematological 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/Sosa et al., 2020), with a 14-day observation period. A 28-day repeated oral administration study (OECD guideline 407) or even a 90-day study (OECD guideline 408) should be conducted. Given the data currently available, the effects on the central and peripheral nervous system as well as on the cardiovascular system should be investigated. Conducting a neurotoxicity study according to OECD guideline 424 is recommended. Preliminary results on *in ovo* exposure of chickens (Couesnon et al., 2014; unpublished results) suggest that PnTXs could affect embryonal growth. Therefore, the effects of PnTXs on offspring should be investigated, according to the OECD guideline 422.

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