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3 **Health risk assessment related to pinnatoxins in French shellfish**

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31 **ABSTRACT**

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

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54 **KEYWORDS:** Pinnatoxins, shellfish, emerging marine biotoxins, risk assessment

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61 **1. Introduction**

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

72 *Vulcanodinium rugosum* is the producer of PnTXs identified by Nézan and Chomerat (2011), based on
73 water samples from a Mediterranean lagoon (Ingril, France). *V. rugosum* also produce portimine
74 (Selwood et al., 2013; Abadie et al., 2016). This benthic dinoflagellate was a new species belonging to
75 a new genus. No other producer is reported in the literature. The identification of this dinoflagellate in
76 France originates from an atypical situation that occurred in 2006, as part of official monitoring of
77 shellfish production areas. The mouse bioassay used to screen for lipophilic marine biotoxins had
78 revealed unusual neurotoxic effects after the injection of extracts from mussels of the Ingril lagoon. In
79 fact, the symptoms observed for lipophilic toxins are gastrointestinal (diarrhea), and not neurotoxic.
80 Such neurotoxicity could not be related to the presence of regulated marine biotoxins (Amnesic,
81 Paralytic, Diarrhetic toxins) screened for by chemical analysis. In addition, observations of water
82 samples did not conclude to the presence of any microalgae species known to produce neurotoxins.
83 *Vulcanodinium rugosum* has also been reported in New Zealand (Rhodes et al., 2010), Australia
84 (Rhodes et al., 2011; Munday et al., 2012), Japan (Smith et al., 2011), China Sea (Zeng et al., 2012),
85 Mexico (Hernandez-Becerril et al., 2013), Arabian Gulf (Al Muftah et al., 2016) and Cuba (Moreira-
86 Gonzalez et al., 2018).

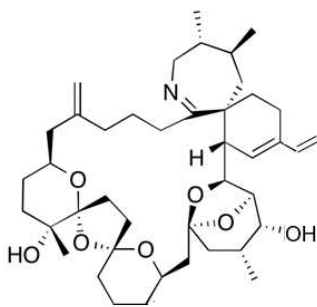
87 PnTXs in shellfish are mainly analysed by physico-chemical methods using liquid chromatography
88 coupled with tandem mass spectrometry (LC-MS/MS). Biological (mouse bioassay) or biochemical
89 (functional tests) methods can also be used. None of the methods has yet undergone inter-laboratory

90 validation or standardisation. In addition, there are very few standards (reference substances)
91 available for PnTXs, which limits and complicates their detection and quantification. Indeed, to date,
92 only PnTX A and G are marketed as calibration solutions and only PnTX G (Figure 1) has been
93 certified.

94 Since the first identification of *V. rugosum* in France, high concentrations of PnTXs have been
95 measured in mussels from the Ingril lagoon for several months each year. In 2012, the French
96 Research Institute for Exploitation of the Sea (Ifremer) reported that PnTXs concentrations varied
97 greatly depending on the years (2010, 2011 and 2012), with a maximum of 1244 µg of PnTX G per kg
98 of shellfish (wet weight) in 2010 (Ifremer, 2012). However, *V. rugosum* is rarely detected in water
99 samples collected for the phytoplankton surveillance (in the water column), which can be explained by
100 the benthic nature of this dinoflagellate.

101 The aims of this work were 1) to review the toxicity data of PnTXs to derive an acute oral health-based
102 guidance value, 2) estimate exposure of shellfish consumers to PnTXs, 3) identify if there might be a
103 public health concern regarding the levels of contamination reported in certain French shellfish
104 production areas, and 4) provide recommendations for the monitoring PnTXs in the marine
105 environment.

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107

108 Figure 1: Structure of PnTX G (modified from Araoz et al., 2011).

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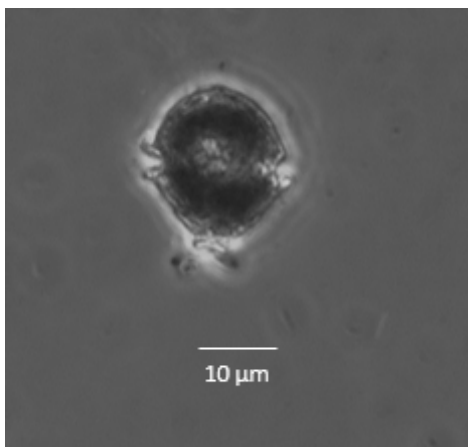
110 **2. *Vulcanodinium rugosum***

111 As almost all dinoflagellates, *V. rugosum* (Figure 2) is assumed to have two distinct life phases: a
112 vegetative propagation phase (asexual reproduction) and a sexual phase (Zeng et al., 2012). The
113 asexual phase probably corresponds to the pelagic phase, when the cells are present in the water

114 column. The sexual reproduction corresponds to a benthic phase, which could result in the formation
115 of a planozygote that produces a resistance cyst. The pelagic and benthic phases are closely related,
116 but the environmental and/or physiological factors controlling the transition from one phase to the
117 other are not yet known.

118 The formation of resistance cysts, which can occur at the end of sexual reproduction, is a crucial step
119 for survival in adverse environmental conditions due to chromosomal mixing, which increases intra-
120 species genetic diversity. Cysts are also one of the ways in which the species spreads, through the
121 transfer of sediments or shellfish from one area to another (cysts are present in the gastrointestinal
122 tract and in intervalvular liquid), as well as via ship ballast water (Garrett et al., 2014). Therefore,
123 identification of the resistance forms and knowledge of their distribution area are important for
124 preventing expansion and contamination. Similarly, determination of the key factors for growth and
125 toxin production by *V. rugosum* is essential for better understanding the risk associated with this new
126 species.

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



131
132 Figure 2: Micrograph of a cell of *Vulcanodinium rugosum*
133

134 3. Hazard characterisation

135 3.1 Acute *in vivo* toxicity with purified PnTXs

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

140 Data on the acute toxicity of purified PnTXs are very limited. Indeed, the available studies were carried
141 out in only one species (mice), one sex (females, which are considered to be more sensitive than
142 males by OECD guidelines) and with very few animals tested per dose (1 to 8 mice, when the number
143 is specified). The results are presented in table 1.

144 Orally, only three studies are available and focused on PnTX E, F, G and H (Munday *et al.*, 2012;
145 Selwood *et al.*, 2014; ANSES-University of Trieste-CNRS report, 2014 also published in Sosa *et al.*,
146 2020). We did not find any information on the toxicity by the oral route for PtTXs (metabolites of certain
147 PnTXs) and portimine (a toxin also produced by *V. rugosum*). Regarding the intraperitoneal (IP) route,
148 four studies have been published on PnTX E, F, G and H and portimine (Munday *et al.*, 2012;
149 Selwood *et al.*, 2010, 2013, 2014). We identified additional unpublished information regarding PnTX A
150 (personal communication from J. Molgó), but did not find any robust information regarding PtTXs.

151 Despite the limited number of studies for each PnTX and the low number of mice tested per dose, a
152 consistent set of information enables outlining the main characteristics of the acute toxicity of this toxin
153 group. Firstly, the toxicity of the PnTXs is rapid, with symptoms appearing within minutes of
154 administration (whether oral or IP). This fact was already known, since PnTXs (like other cyclic imines)
155 belong to the fast-acting toxins group (Efsa, 2010a). The second point relies on neurotoxic symptoms,
156 quickly leading to the mouse's death by respiratory arrest. Clinical signs of toxicity, regardless of the
157 route of administration and the PnTX analogue, include decreased mobility (sometimes preceded by
158 an initial phase of hyperactivity immediately following administration), paralysis of the hind legs and
159 breathing difficulties (Munday *et al.*, 2012), with tremors and jumps also being reported (ANSES-
160 University of Trieste-CNRS Report, 2014; Sosa *et al.*, 2020). In the study by Munday *et al.* (2012), the
161 authors reported that at sublethal doses (without specifying which ones), some mice recovered
162 completely after exhibiting symptoms.

163 LD50 values vary according to the PnTX analogue and route of administration (Table 1). Orally, LD50
164 values range from 25 to 2,800 µg/kg bw (for PnTX F and PnTX E respectively). The analogues tested
165 can be ranked as follows in decreasing order of toxicity: PnTX F > PnTX G ~ PnTX H >> PnTX E. By

166 IP route, LD50 range from 13 to 115 µg/kg bw (for PnTX F and PnTX A respectively) and the
 167 analogues can be ranked as follows: PnTX F > PnTX G > PnTX E > PnTX H > PnTX A.
 168 LD99 values for some PnTXs and PtTXs have been reported in the literature, but these data were
 169 considered not sufficiently reliable due to a lack of information on the protocol used (Uemura *et al.*,
 170 1995; Chou *et al.*, 1996; McCauley *et al.*, 1998; Takada *et al.*, 2001).
 171 Portimine has lower acute IP toxicity than that of PnTXs, with an estimated LD50 of 1,570 µg/kg bw.
 172 No effect was observed at 500 and 700 µg/kg bw (Selwood *et al.*, 2013). The authors indicated that
 173 the signs of toxicity prior to death in mice appeared less rapidly after IP administration compared to
 174 PnTXs (without however mentioning which signs of toxicity were observed). Toxicity of portimine by
 175 oral administration is unknown.

176

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

178 **1a. Oral administration**

Toxin (purity)	Route of administration and number of mice	LD50 (µg/kg bw)	MTD (µg/kg bw)	References	Study quality
PnTX E*	Gavage Number of mice not specified (OECD GL 425) Fed (non-fasted) mice	2800 CI95: 2380-3000	600	Munday <i>et al.</i> , 2012	ToxRTool: 13 Klimisch: 2
PnTX F*	Gavage Number of mice not specified (OECD GL 425) Fed (non-fasted) mice	25 CI95: 19.1-35.1	9.9	Munday <i>et al.</i> , 2012	ToxRTool: 13 Klimisch: 2
PnTX F*	16h fasted mice	29.9 CI95: 25-32	Not determined		
	In cream cheese Number of animals not specified (OECD GL 425) Fed (non-fasted) mice	50 CI95: 39.4-62.8	16.0	Munday <i>et al.</i> , 2012	ToxRTool: 13 Klimisch: 2
	In peanut butter Number of mice not specified (OECD GL 425) Fed (non-fasted) mice	50 CI95: 37.9-71.5	Not determined		
	In dry mouse food Number of mice not	50 CI95: 37.9-71.5	Not determined		

Toxin (purity)	Route of administration and number of mice	LD50 (µg/kg bw)	MTD (µg/kg bw)	References	Study quality
	specified (OECD GL 425) 16h fasted mice In cream cheese, 16h fasted mice In peanut butter, 16h fasted mice	77 CI95: not calculated 50 CI95: 39.4-62.8	Not determined Not determined		
PnTX G*	Gavage Number of mice not specified (OECD GL 425) Fed (non-fasted) mice	150 CI95: 105-100	75	Munday <i>et al.</i> , 2012	ToxRTool: 13 Klimisch: 2
PnTX G (declared purity 100%)	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	208 CI95: 155-281	120	ANSES-University of Trieste-CNRS report, 2014; Sosa <i>et al.</i> , 2020	ToxRTool: 17 Klimisch: 2
PnTX G*	In cream cheese Number of mice not specified (OECD GL 425) Fed (non-fasted) mice	400 CI95: 380-470	153	Munday <i>et al.</i> , 2012	ToxRTool: 13 Klimisch: 2
PnTX H*	Gavage Number of animals not specified Fasting not specified	163 CI95: 139-175		Selwood <i>et al.</i> , 2014	ToxRTool: 4 This low score is due to the fact that a lot of details are missing in the article, but this team has already described the protocol for other PnTXs

179 * Purity verified by NMR according to the authors but percentage not mentioned in the publication

180 CI95: 95% confidence interval

181 MTD (Maximum Tolerated Dose): dose at which no mortality or clinical signs are observed

182

183 1b. Intraperitoneal (IP) administration

Toxin (purity)	Route of administration and number of mice	LD50 (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)	114.8		J. Molgó (personal communication)	ToxRTool: 16

Toxin (purity)	Route of administration and number of mice	LD50 (µg/kg bw)	MTD (µg/kg bw)	References	Study quality
	Fed (non-fasted) mice				
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 <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 2014	ToxRTool: 4 This low score is due to the fact that a lot of details are missing in the article, but this team has already described the protocol for other PnTXs
Portimine*	IP Number of animals not	1570 CI95: 1269-3080	No effect at 500 and 700	Selwood <i>et al.</i> , 2013	ToxRTool: 4 This low score is due to the fact

Toxin (purity)	Route of administration and number of mice	LD50 (µg/kg bw)	MTD (µg/kg bw)	References	Study quality
	specified Fasting not specified				that a lot of details are missing in the article, but this team has already described the protocol for other PnTXs

184 * Purity verified by NMR according to the authors but percentage not mentioned in the publication

185 CI95: 95% confidence interval

186 MTD (Maximum Tolerated Dose): dose at which no mortality or clinical signs are observed

187

188 3.2 Subchronic and chronic toxicity

189 No repeated dose toxicity studies were identified in the literature.

190

191 3.3 Mode of action and extrapolation to humans

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

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

202

203 3.4 Development of a health value

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

206

207 3.4.1 Choice of the key study

208 Two acute oral toxicity studies in mice with purified PnTX G were identified (Munday et al., 2012;
 209 ANSES-University of Trieste-CNRS report, 2014 also published in Sosa *et al.*, 2020). The key study
 210 selected was the one carried out by the Department of Life Sciences at the University of Trieste with
 211 funding from the DGAL and DGS (ANSES-University of Trieste-CNRS report, 2014; Sosa et al., 2020),
 212 which obtained the highest score (17 out of 21 with ToxRTool, corresponding to a Klimisch score of 2 -
 213 reliable with restriction).

214 In this study, groups of three to five 4 weeks-old female SD-1 mice received a single administration of
 215 purified PnTX G by gavage at a dose of 8, 20, 50, 120, 220, 300, 370 or 450 µg/kg bw. A control group
 216 of eight mice was included. Mice were fasted for 3 hours before gavage; food was given back again *ad*
 217 *libitum* 2 hours after administration during the 24-hour observation period. Lethality, clinical signs of
 218 toxicity, histological analysis of several organs and blood biochemistry were recorded.

219 The results regarding lethality and the observed symptoms are shown in Table 2. Administration of
 220 PnTX G resulted in mouse lethality from 220 µg PnTX G/kg bw (3/5 mice, in 22 min). No lethality was
 221 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
 222 (survival time between 13 and 18 min). The LD50 for PnTX G was calculated at 208 µg/kg bw (95%
 223 confidence interval = 155-281 µg/kg bw).

224 Before death, the main symptoms were neurotoxic, i.e. prostration, tremors, jumps, abdominal
 225 breathing, hypothermia, hind leg paralysis and cyanosis. No macroscopic organ changes were
 226 observed during necropsy of the mice treated with PnTX G. Histological analysis of major organs and
 227 tissues revealed only minor changes in the small intestine of mice given PnTX G at doses equal to or
 228 greater than 300 µg/kg bw (moderate mucosal degeneration, villous atrophy). No differences in
 229 biochemical blood parameters were found between treated and control mice (Sosa *et al.*, 2020).

230

231 **Table 2: Lethality and clinical signs of toxicity in mice after single administration by gavage of**
 232 **purified PnTX G (ANSES-University of Trieste-CNRS Report, 2014; Sosa *et al.*, 2020)**

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

233

234 3.4.2 Choice of the critical dose

235 Based on the data from the key study, two approaches were explored:

- 236 - First approach: the **120 µg PnTX G/kg bw** dose was selected as the maximum tolerated dose
237 (MTD), i.e. the dose at which no effect is observed in the studied parameters over a 24-hour
238 period after treatment.
- 239 - Second approach: calculation of a benchmark dose¹ from a response level of 10% mortality
240 (PROAST Web software, RIVM, v. 65.2). The model that best fitted the experimental data
241 according to the Akaike Information Criterion (AIC) was the probit model, for which the BMDL_{95%}
242 was **69.1 µg PnTX G/kg bw** of mouse.

243 It is unusual to use data on mouse mortality from single oral administration to derive a health reference
244 value. This is justified by the fact that the mouse mortality occurs rapidly and there is a very small
245 difference between a dose with no signs of toxicity and a lethal dose.

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

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

- 252 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).

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

255

256 3.4.3 Choice of uncertainty factors

257 Uncertainty factors were identified for both approaches explored when establishing the critical dose:

258 - First approach: the dose of 120 µg PnTX G/kg bw was selected as the maximum tolerated dose
259 (MTD). The associated overall uncertainty factor was 900:

260 ○ inter-species uncertainty factor: $UF_A = 10$

261 ○ inter-individual uncertainty factor: $UF_H = 10$

262 ○ other factors: $UF_D = 3$ (insufficient data) and 3 (to take into account the severity and
263 pattern of the dose-response curve)

264 - Second approach: the BMDL of 69.1 µg PnTX G/kg bw was chosen as the point of departure.

265 The associated overall uncertainty factor was 525:

266 ○ allometric adjustment factor: 7 for the mouse

267 ○ $UF_A = 2.5$ for the toxicodynamic component

268 ○ $UF_H = 10$

269 ○ $UF_D = 3$ (insufficient data).

270

271 3.4.4 Proposed health-based guidance value

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

274 The provisional acute benchmark value is **0.13 µg PnTX G/kg bw**, (Table 3) regardless of the
275 approach used² (MTD or BMDL).

276

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

Critical effect Key study	Critical dose for the mouse	UF	Provisional acute benchmark value for humans	Confidence level
------------------------------	--------------------------------	----	--	---------------------

² MTD approach: $(120 \mu\text{g PnTX G/kg bw}) / (10 \times 10 \times 3 \times 3) = 120/900 = 0.1333 \mu\text{g PnTX G/kg bw}$
BMDL approach: $69.1 \mu\text{g PnTX G/kg bw} / (7 \times 2.5 \times 10 \times 3) = 69.1/525 = 0.1316 \mu\text{g PnTX G/kg bw}$

First approach	<p><i>Critical effect:</i> absence of mortality and symptoms in mice 24 hours after a single oral administration by gavage of purified PnTX G</p> <p><i>Key study:</i> ANSES-University of Trieste-CNRS report, 2014; Sosa et al., 2020</p>	Maximum tolerated dose of 120 µg PnTX G/kg bw	Total factor: 900 $UF_A = 10$ $UF_H = 10$ $UF_D = 9$ (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 Allometric adjustment: 7 for the mouse $UF_A = 2.5$ $UF_H = 10$ $UF_D = 3$ for insufficient data	0.13 µg PnTX G/kg bw	Moderate

278

279 3.4.5 Proposed maximum tolerable concentration of PnTX G in shellfish

280 Based on the provisional acute benchmark value of 0.13 µg PnTX G/kg bw, a default serving size of
281 400 g of shellfish (EFSA 2010b) and a default body weight of 70 kg, the concentration not to be
282 exceeded in shellfish would be **23 µg PnTX G/kg of total meat**.

283

284 3.4.6 Confidence level

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

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

288 The literature review revealed that there were few data on the acute oral toxicity of purified PnTXs and
289 none on repeated administration. In fact, only two acute oral toxicity studies conducted on a single
290 species (mice) and sex (females, which are considered to be more sensitive than males) have been
291 published. The lack of data in another rodent species (rats) and of knowledge on the toxicokinetics
292 and toxicodynamics of PnTXs is underlined.

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

294 The key study is an acute toxicity study with a single oral administration. Its objective was to study
295 mouse mortality and define a median lethal dose (LD50). Signs of neurological toxicity were also
296 observed and are consistent with the known mode of action of PnTXs.

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

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

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

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

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

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

311

312 **4. Occurrence of PnTXs in shellfish**

313 **4.1 Ingril lagoon**

314 As soon as PnTX G was identified in France (2011), a retrospective analysis of the samples from 2009
315 to 2012 was conducted by Ifremer, which revealed the kinetics of shellfish contamination as a function
316 of time. The maximum annual concentrations were 261, 1244, 568 and 652 µg/kg of total mussel meat
317 for 2009, 2010, 2011 and 2012 respectively (Table 4). Concentrations in mussels were higher than
318 those observed in clams, when shellfish were sampled simultaneously (Table 5).

319 To track this phenomenon and maintain monitoring for PnTXs, sampling of Ingril mussels was carried
320 out monthly between 2013 and 2017 by Ifremer. The results show that PnTX G peaks were observed

321 between June and September, but the maximum values varied according to the year (887 in 2013,
 322 918 in 2014, 1143 in 2015, 600 in 2016 and 640 in 2017, expressed in µg/kg of total meat).

323

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

Year	2010	2011	2012	2013	2014	2015	2016	2017
Number of samples	30	32	24	12	27	2	13	10
Min	45.4	36.6	11.0	49.5	22.6	572	44.0	52.9
Max	1244	568	652	887	918	1143	600	640
Mean	223	227	209	293	247	858	278	294

326

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

	Mussels	Clams
Number of samples	86	20
Min	11.0	13.6
Max	1244	95.3
Mean	217	28.1

329

330 **4.2 Other Mediterranean lagoons**

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

335

336 **Table 6: Data on concentrations of PnTX G in mussels from several Mediterranean lagoons in**
 337 **2013 (µg/kg total meat)**

	Parc Leucate	Thau	Le Prévoist	Vic	Ingril
Number of samples	12	11	12	11	12
Min	0.68	1.4	11.8	12.3	49.5

Max	10.7	14.7	53.9	89.0	887
Mean	3.4	10.0	26.2	48.4	293

338

339 **4.3 Atlantic and Corsican coasts**

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

345 Since 2018, this scheme enables the targeted analysis by chemical methods of PnTXs in mussels and
 346 oysters from eleven areas located all along the French metropolitan coasts. During this first year of
 347 monitoring, three of these areas (Ingril, Le Scoré in Brittany and the Diana lagoon in Corsica) were
 348 affected by the presence of PnTXs, mainly PnTX G and to a lesser degree PnTX A (only in Ingril). Of
 349 the three affected areas, the Ingril lagoon remains the most heavily contaminated area (with
 350 concentrations detected every month, varying from 40 to 2614 µg PnTX G/kg of digestive gland). The
 351 levels found at Le Scoré and Diana were low (maximum around 10 µg PnTX G/kg of digestive gland).
 352 The concentrations of PnTX A detected at Ingril ranged from 6 to 32 µg/kg of digestive gland.

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

358

359 **4.4 Outside France**

360 The literature review concluded that very limited data on PnTX levels in shellfish from Northern and
 361 Southern Europe, Canada and New Zealand have been published. Among the eight studies reviewed
 362 (MacKenzie et al., 2011; Rundberget, et al., 2011; McCarron et al., 2012 ; McNabb et al., 2012;
 363 Garcia-Altare et al., 2014; Rambla Alegre et al., 2018; Lamas et al., 2019; Otero et al., 2019), the
 364 highest reported levels of PnTX G in mussels were 115 µg/kg in Norway (Rundberget *et al.*, 2011), 83
 365 µg/kg in Canada (McCarron *et al.*, 2012) and 59 µg/kg in Spain (Garcia-Altare *et al.*, 2014). These
 366 levels are lower than the annual maximum levels reported in France in the Ingril lagoon (between 261

367 and 1244 µg/kg, depending on the year). It should be noted that the study by Rambla-Alegre *et al.*
368 (2018) reported the persistence of PnTX G in samples of canned mussels (up to 12 µg/kg).

369 Lastly, ANSES sent a questionnaire to the European Food Safety Authority (EFSA) network of focal
370 points on 15 February 2018, to which 16 Member States replied. At the time of the request, none of
371 the responding health agencies had conducted a health risk assessment for PnTXs. Some
372 respondents provided contamination data in shellfish from their country. Most of the data were already
373 published in a scientific journal and had been identified in our literature search. Some unpolished data
374 were also provided but without information about the performance of the analytical method used.

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

377

378 **5 Dietary exposure**

379

380 **5.1. Food consumption data**

381 A consumption survey on seafood products in France (CONSOMER) was conducted in 2016-2017 as
382 part of a research agreement between ANSES and CREDOC (2015-CRD-25). The aim of the survey
383 was to assess seafood consumption by an adult population (over 18 years of age) living in coastal
384 areas and with access to local sources of supply. The database includes answers from 2481 adults.
385 Only data for the Mediterranean population were used in the present work, including 821 adults (Table
386 7).

387

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

	Number of consumers	Mean amount in g/day	P95 in g/day
Mussels	582	91.2	200
Clams	140	19.8	40

390

391 **5.2 Contamination data**

392 Data on concentrations of PnTX G in mussels and clams from several Mediterranean lagoons from
393 2010 to 2017 were provided to ANSES by Ifremer (Table 8). Three scenarios were selected,
394 depending on the site where the shellfish were sampled:

- 395 - **"All lagoons" scenario:** contamination data of all mussels on the one hand and all clams on the
 396 other (from all the lagoons studied);
- 397 - **"Ingril" scenario:** contamination data ONLY from the Ingril lagoon, for mussels on the one hand and
 398 clams on the other;
- 399 - **"Except Ingril" scenario:** contamination data from all lagoons (Vic, le Prévost, Parc Leucate and
 400 Thau) except "Ingril", for mussels only (no clams sampled in other lagoons).

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 405

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

	Number of samples of mussels	Concentration of PnTX G (µg/kg total meat)	
		Mean	P95
All lagoons	196	196	633
Ingril	150	249	712
Except Ingril*	46	21.7	60.3
	Number of samples of clams	Concentration of PnTX G (µg/kg total meat)	
		Mean	P95
All lagoons	20	28.1	57
Ingril	20	28.1	57
Except Ingril	0	No data	No data

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

409

410 5.3. Method to calculate exposure

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

$$413 E_i = \sum_{k=1}^n \frac{C_{i,k} \times L_k}{BW_i}$$

414 where:

- 415 - E_i is the total daily exposure of individual i associated with the consumption of food k (µg/kg of body
 416 weight/day);
- 417 - $C_{i,k}$ is the consumption of food k by individual i (kg/d) (serving size for acute exposure);

418 - L_k is the PnTX G contamination of food k ($\mu\text{g}/\text{kg}$ of fresh weight) (95th percentile for acute exposure);
 419 - BW_i is the body weight of individual i (kg).

420

421 **6 Risk characterisation**

422 **6.1 Results**

423 A comparison of acute exposure estimates was made with the provisional acute benchmark value of
 424 0.13 μg PnTX G/kg of body weight. A percentage of the population exceeding this value was
 425 calculated, firstly in the total population and secondly for consumers of mussels and clams only (Table
 426 9).

427

428

429 **Table 9: Acute exposure associated with consumption of mussels and clams (CONSUMER, 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

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

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

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

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

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

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

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

439 % > PABV: percentage of the general population exceeding the provisional acute benchmark value of 0.13 $\mu\text{g}/\text{kg}$
 440 bw

441 % > PABV_cons: percentage of consumer population only exceeding the provisional acute benchmark value of
 442 0.13 $\mu\text{g}/\text{kg}$ bw

443

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

451 **6.2 Conclusion in terms of health concern**

452 Considering the percentage of the French population for which the provisional acute benchmark value
453 would be exceeded in the "Except Ingril" scenario, there may be a health concern related to the
454 consumption of shellfish contaminated with PnTXs from these Mediterranean lagoons. Estimates were
455 not made for the other French sites where PnTXs were detected in mussels (Le Scoré, Diana)
456 because the contamination was measured only in the digestive glands, and the distribution of PnTXs
457 between the digestive gland and the total meat is not known.

458 **7. Recommendations for surveillance**

459 **7.1 Environmental surveillance**

460 Several studies on the ecology of the dinoflagellate *V. rugosum* have been carried out *in vitro* and *in*
461 *situ*. However, its origin and the determinism of its blooms in natural environments remain to be
462 elucidated. While the contamination of molluscs by PnTXs in some areas is undeniable, it remains
463 difficult to establish the relationship with *V. rugosum* blooms as the observation of the pelagic phase in
464 the water column is challenging. Official surveillance of shellfish production areas based on the
465 identification and counting of toxic phytoplanktonic species in the water column is therefore ill-suited to
466 this species.

467 Several monitoring options can be proposed:

- 468 1) Regarding dinoflagellate surveillance, monitoring of the benthic population of this organism in
469 risk areas is recommended. It is essential to sample the macroalgae present in the area, in
470 order to collect *V. rugosum* cells. The protocol set up for "*Ostreopsis*" monitoring and tested in
471 the study by Abadie *et al.* (2018) shows that such methodology is adequate.

472 2) Alongside the monitoring of *V. rugosum*, more systematic surveillance of the presence of
473 PnTX A and G toxins in molluscs should be considered. The extraction protocol for PnTXs is
474 identical to the one used for official monitoring of lipophilic toxins in the French shellfish
475 production areas (REPHYTOX). Although an additional step for LC-MS/MS analysis is
476 required, the cost remains moderate. A systematic screening for PnTXs in total meat could be
477 added to the REPHYTOX analyses of lipophilic toxins, and not only on samples taken for the
478 EMERGTOX scheme, that focuses only on the digestive gland.

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

481

482 **7.2 Health monitoring, reporting and procedures**

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

487

488 **8. Conclusion**

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

496 The risk assessment underlying this conclusion is based on a worst-case scenario for estimating
497 exposure to PnTX G, using the 95th percentile of shellfish contamination. A more realistic risk
498 assessment could be conducted using a probabilistic approach (taking into account the distribution of
499 all the data, on both contamination and consumption), which would require the acquisition of more
500 contamination data in shellfish, particularly oysters (Anses, 2019).

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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 (Coesnon 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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