1 # Toxicity Equivalency Factors for Tetrodotoxin Analogues
2 # Determined with Automated Patch Clamp on Voltage-Gated
3 # Sodium Channels in Neuro-2a Cells

4 Jaume Reverté[a,b], Maria Rambla-Alegre[a], Andres Sanchez-Henao[a,c], Manolis Mandalakis[d],
5 Panagiota Peristeraki[e], Jordi Molgó[f], Jorge Diogène[a], Francesc X. Sureda[b] and Mònica Campàs[a,*]

6 [a] *IRTA, Ctra. Poble Nou km 5.5, 43540 La Ràpita, Spain.*
7 [b] *Basic Medical Sciences Department, Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili (URV), C/ St. Llorenç 21,*
8 *43201 Reus, Spain.*
9 [c] *University Institute of Animal Health and Food Safety (IUSA), University of Las Palmas de Gran Canaria, 35416 Arucas, Spain.*
10 [d] *Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research (HCMR), 71003 Heraklion, Greece.*
11 [e] *Institute of Marine Biological Resources and Inland Waters, Hellenic Centre for Marine Research (HCMR), 71003 Heraklion, Greece.*
12 [f] *French Alternative Energies and Atomic Energy Commission (CEA), University of Paris-Saclay, INRAE, DMTS, SIMoS, EMR CNRS 9004,*
13 *91191 Gif sur Yvette, France.*
14 **Correspondence: monica.campas@irta.cat*

15 ORCID:

16 Jaume Reverté: https://orcid.org/0009-0002-6914-2851

17 Maria Rambla-Alegre: https://orcid.org/0000-0003-4276-276X

18 Andres Sanchez-Henao: https://orcid.org/0000-0003-3413-4755

19 Manolis Mandalakis: https://orcid.org/0000-0002-7469-4290

20 Panagiota Peristeraki: https://orcid.org/0000-0002-8608-078X

21 Jordi Molgó: https://orcid.org/0000-0002-0693-8994

22 Jorge Diogène: https://orcid.org/0000-0002-6567-6891

23 Francesc X. Sureda: https://orcid.org/0000-0002-7968-3929

24 Mònica Campàs: https://orcid.org/0000-0002-1220-7100

25 **Abstract**

26 Tetrodotoxin (TTX) is a potent marine neurotoxin, responsible for numerous poisoning incidents and some
27 human fatalities. To date, more than thirty TTX analogues have been identified, but their individual
28 toxicities and roles in poisoning remain largely unknown. In this work, the toxicity equivalency factors
29 (TEFs) of five TTX analogues were determined by assessing the blockade of voltage-gated sodium channels
30 (VGSCs) in Neuro-2a cells using automated patch clamp (APC). All TTX analogues were less toxic than TTX.
31 The derived TEFs were applied to the individual TTX analogues concentrations measured in pufferfish
32 samples, using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). A comparison
33 of these results with those obtained from APC analysis demonstrated that TEFs can be effectively used to
34 translate LC-MS/MS analytical data into meaningful toxicological information. This is the first study to
35 utilise APC device for the toxicological assessment of TTX analogues, highlighting its potential as a
36 bioanalytical tool for seafood safety management and human health protection.

37 **Keywords**

38 Tetrodotoxin; tetrodotoxin analogue; toxicology; single-cell analysis; pufferfish; food safety.

39 **1. Introduction**

40 Tetrodotoxins (TTXs) are a family of highly toxic neurotoxins, which received this name because they were
41 first isolated from *Tetraodontidae* fish.[1] In natural samples, TTX coexists with several TTX analogues,
42 sharing a similar overall structure but differing at specific molecular positions. To date, over thirty TTX
43 analogues have been described, yet their individual toxicities and role in poisoning incidents remain
44 largely undefined.[1] The pufferfish *Lagocephalus sceleratus* is one of the main carriers of TTXs, although
45 these toxins are also found in a variety of marine and terrestrial organisms.[2] The presence of TTXs in
46 organisms from different phyla suggests an exogenous origin, which has been linked to various bacteria
47 from the *Proteobacteria* phylum.[3] However, the metabolic pathways for TTXs biosynthesis and/or
48 biotransformation in hosts are still unclear.[4,5] The biological target of TTXs is the voltage-gated sodium
49 channel (VGSC, also known as Na_v), an important membrane protein involved in the generation and
50 propagation of action potentials in neuronal and other excitable tissues.[6] The toxicity mechanism of TTXs
51 relays on the blockade of the VGSCs, inhibiting Na^+ influx into the cell and therefore preventing cell
52 membrane depolarisation.[7] Depending on the dose, TTX can be useful for some medical applications[8-10]
53 or can cause poisoning.[11] In this case, symptoms ranging from mild neurological or gastrointestinal
54 disorders (e.g., oral tingling or vomiting) to severe systemic failures have been described that, in severe
55 cases, can lead to death.[1,12] Currently, there is no specific treatment for TTX poisoning other than palliative
56 care to relieve the symptoms. For this reason, the prevention of TTX poisoning highly depends on the
57 early detection of TTX and TTX analogues in seafood.

58 Tetrodotoxin poisoning events are predominantly reported in Asian countries where pufferfish is eaten
59 as a delicacy.[2] However, poisoning cases have also been reported in non-endemic regions, highlighting a
60 potential new food safety concern if appropriate control measures are not implemented.[13] In Europe,
61 poisonous fish of the *Tetraodontidae, Molidae, Diodontidae and Canthigasteridae* families must not be
62 placed on the market due to the threat they may pose to human health.[14] However, with *L. sceleratus*
63 establishing itself as a new persistent invasive species in the Mediterranean, concerns arise regarding the
64 potential risks associated with the accidental consumption of toxic pufferfish by local populations as a
65 consequence of recreational fishing[15,16] and the possible intermingling of juveniles with other small
66 pelagic species (e.g., anchovy, sardines, picarel and bogue).[17] Moreover, the risk of exposure to TTX

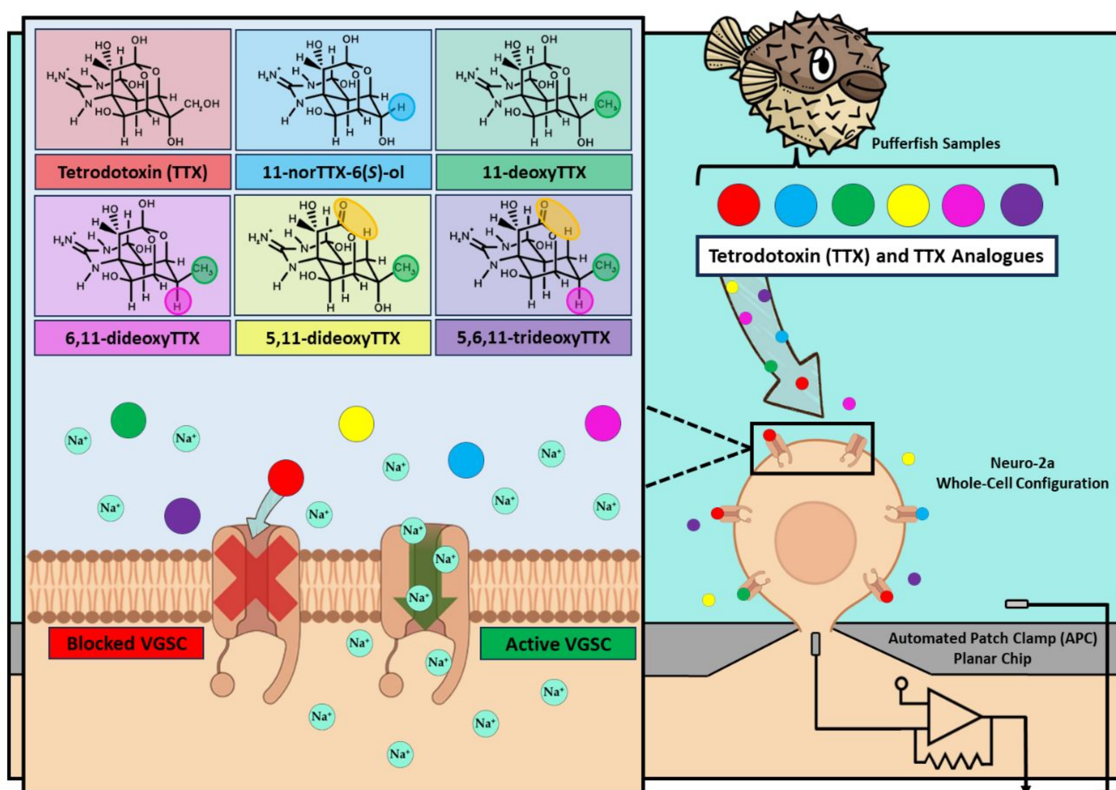
67 through the consumption of common edible seafood species (originally non-toxic) that have been cross-
68 contaminated as a result of accidental pufferfish predation cannot be ignored.[18,19] In addition, over the
69 last few decades, shellfish has been identified as another important TTX carrier in Europe. Although TTX
70 concentrations in shellfish are generally lower than those in pufferfish, they still pose a poisoning risk.
71 Indeed, the first TTX poisoning case originated in Europe was linked to the consumption of contaminated
72 gastropods.[20,21]

73 In general, instrumental analysis techniques, such as liquid chromatography coupled to tandem mass
74 spectrometry (LC-MS/MS), are the gold standard for monitoring regulated toxins in Europe, including
75 lipophilic marine toxins.[22] The main advantage of these techniques is the ability to detect and quantify
76 not only the target toxin but also several of its individual analogues, thus providing a toxin profile for a
77 sample. However, these analyses do not provide direct toxicological insights, requiring the use of toxicity
78 equivalency factors (TEFs) for the different analogues to transform analytical data into practical
79 toxicological information. The scant knowledge about the individual toxicities of emerging marine toxins,
80 including the TTX analogues, emphasises the increasing need for their thorough toxicological evaluation.[23]

81 Historically, animal bioassays, such as the mouse bioassay (MBA), were the primary methods for the
82 toxicological characterisation of marine toxins.[13] However, MBA is now of limited use in many countries
83 due to its low specificity, high variability and the ethical issues concerning the experimentation with live
84 animals.[24] Cell-based assays (CBAs) have been proposed as an alternative approach to assess the toxicity
85 of a sample without involving live animals. The detection of toxins with CBA relies on assessing changes
86 in the morphology or viability of cells after their exposure to them.[25-27] However, cells used for CBA
87 sometimes require pretreatments with auxiliary drugs to ensure optimal assay performance, necessitating
88 cautious interpretation of the toxicological data that they generate. Recently, a new approach that
89 leverages the electrophysiological activity of Neuro-2a cells has emerged as a promising high throughput
90 bioanalytical method for detecting TTXs in pufferfish samples, with potential applications in food safety
91 and broader toxicological studies.[28]

92 In this work, we characterised the toxicity of five TTX analogues using an automated patch clamp (APC)
93 device (Figure 1). By assessing the toxicological response of Neuro-2a cells to 11-norTTX-6(*S*)-ol, 11-
94 deoxyTTX, 6,11-dideoxyTTX, 5,11-dideoxyTTX and 5,6,11-trideoxyTTX, the respective TEF values were

95 determined. Then, we applied these TEFs as correction factors to the concentrations of individual TTX
96 analogues measured in various pufferfish samples from Greece using LC-MS/MS. The overall toxicity levels
97 calculated in TTX equivalents (equiv.) through this indirect approach closely matched those directly
98 measured in the samples using APC, suggesting a consistent agreement between the two techniques. This
99 study represents the first application of APC in evaluating the toxicological potency of TTX analogues.



100 **Figure 1**. The diagram on the right outlines the strategy for assessing the activity of voltage-gated sodium channels
101 (VGSCs) in Neuro-2a cells exposed to TTX or the TTX analogues studied using the APC method. The close-up view on
102 the left illustrates the VGSC blockade by the toxins and the inhibition of Na^+ influx into the cells. Structural differences
103 between TTX and its analogues are highlighted by different colours (upper left).

104 ## 2. Materials and Methods

105 ### *2.1. Reagents*

106 TTX (purity ≥98% by HPLC) was purchased from Tocris Bioscience (Bristol, UK) and a standard solution was
107 prepared at 1 mg/mL in 1% (*v*/*v*) acetic acid. In the APC method, the external solution (140 mM NaCl, 4
108 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-glucose monohydrate, 10 mM HEPES/NaOH, pH 7.4), the
109 internal solution (50 mM CsCl, 10 mM NaCl, 60 mM CsF, 20 mM EGTA, 10 mM HEPES/CsOH, pH 7.2) and

110 the seal enhancer solution (10 mM HEPES, 130 mM NaCl, 5 mM glucose, 4 mM KCl, 10 mM $CaCl_2$, 10 mM
111 $MgCl_2$, pH 7.4, osmolarity: 302 mOsmol) were obtained from Nanion Technologies GmbH (Munich,
112 Germany). Murine neuroblastoma (Neuro-2a) cells were purchased from ATCC LGC Standards (Manassas,
113 VA, USA). Foetal bovine serum (FBS), penicillin/streptomycin solution, Roswell Park Memorial Institute
114 (RPMI-1640) medium, sodium pyruvate and trypsin-EDTA enzyme were purchased from Merk KGaA
115 (Darmstadt, Germany).

116 *2.2. Pufferfish samples and tetrodotoxin extraction*

117 Three pufferfish specimens, two males (PF1, PF2) and one female (PF3), identified as *L. sceleratus* by
118 morphological evaluation, were collected from the Libyan Sea near Crete, Greece in May (PF1, PF2) and
119 March (PF3) of 2019. The specimens were dissected and their tissues, including skeletal muscle, skin, liver,
120 intestinal tract and gonads, were separated. The extraction of TTXs was performed following the protocol
121 described by Reverté and co-workers.[29] Briefly, 10 g of each tissue was homogenised using an Ultraturrax
122 blender at full speed. To each homogenised sample, 25 mL of 0.1% (*v*/*v*) acetic acid was added and the
123 mixture was vortexed for 2 min at 2500 rpm. Subsequently, the tubes were placed in a boiling water bath
124 for 10 min with occasional stirring. The homogenates were then cooled down and centrifuged at 2500
125 rpm for 5 min (4 °C). The supernatants were collected and the pellets were subjected to a second
126 extraction with additional 20 mL of 0.1% (*v*/*v*) acetic acid. The two supernatants were pooled, and their
127 final volume was adjusted to 50 mL with 0.1% (*v*/*v*) acetic acid. For liver samples, a liquid-liquid
128 partitioning of the crude extract with hexane (1:1) was necessary to remove fats. For APC analysis, the
129 extracts were passed through 0.45-µm nylon syringe filters. For LC-MS/MS analysis, filtration was
130 conducted using 3000-Da molecular sieve filters followed by 0.2-µm nylon filters. The final extracts, at
131 200 mg pufferfish tissue per mL, were stored at –20 °C until required.

132 *2.3. Isolation of tetrodotoxin analogues and LC-MS/MS analysis*

133 TTX analogues were sourced from PF2 liver tissue, as detailed in Supporting Information and the work
134 from Alkassar and co-workers.[30] In brief, a 30 g liver tissue extract was reduced to 5 mL using a rotary
135 evaporator system, mixed with 5 mL of acetonitrile and fractionated by hydrophilic interaction liquid
136 chromatography (HILIC) at room temperature utilising a prep-LC column Luna HILIC AXIA (250 mm × 21.2

137 mm, 5 µm particle size; Phenomenex, Torrance, CA, USA). A binary gradient elution at 10 mL/min was
138 applied with Milli-Q water (mobile phase A) and acetonitrile/water (90/10 *v*/*v*, mobile phase B), both
139 containing 30 mM ammonium acetate at pH 5.8. The gradient program started at 100% B, and it was kept
140 isocratic for 5 min; then, phase B was reduced to 95% B at 35 min, further decreased to 82.5% B at 80
141 min, held isocratic for 5 min and returned 100% B at 90 min. A total of ten chromatographic runs were
142 performed on 1 mL aliquots and the TTXs fractions collected (10-mL per run) were pooled together (100
143 mL in total) and stored at –20 °C until required. The fractions were dried under a N_2 stream at room
144 temperature and reconstituted in the external buffer before analysis with APC.

145 The TTXs present in the pooled fractions as well as in the pufferfish samples were analysed by LC-MS/MS,
146 using a triple quadrupole mass spectrometer (Xevo TQ-XS, Waters Corporation, Milford, MA, USA)
147 coupled to a UPLC binary pump system (Acquity UPLC I-plus-Class, Waters Corporation, Milford, MA, USA),
148 as detailed in Supporting Information and the work from Alkassar and co-workers.[30]

149 *2.4. Neuro-2a maintenance and automated patch clamp recording*

150 Neuro-2a cells were cultured and maintained in RPMI-1640 medium supplemented with 10% (*v*/*v*) FBS,
151 1% (*v*/*v*) penicillin-streptomycin and 1% (*v*/*v*) sodium pyruvate at 37 °C in a 5% CO_2 humid atmosphere.
152 All Neuro-2a cells used in this work were between passages 245 and 255.

153 The electrophysiological changes of Neuro-2a cells in response to the TTX analogues were evaluated using
154 a Patchliner (Nanion Technologies GmbH, Munich, Germany), an automated planar patch clamp device.[28]
155 Briefly, eight wells of a medium resistance NPC-16 borosilicate planar chip (Nanion Technologies GmbH,
156 Munich, Germany) were filled up with the internal and external solutions. Then, a suspension of Neuro-
157 2a cells, prepared at 100,000 cells/mL in a 1:1 mixture of RPMI and external solution, was introduced into
158 the chip. A single cell was immobilised on the hole located at the bottom of each well with a holding
159 potential of –30 mV. Then, after adding the enhancer solution, the potential was changed to –100 mV.
160 Successful patching was confirmed by achieving a stable Giga-sealing with resistances (higher than 1 GΩ).

161 Na_v currents were measured by applying the potential in increments of 10 mV from –80 mV to 40 mV
162 using two EPC Quatro USB amplifier units (8 probes) (HEKA Elektronik, Stuttgart, Germany) controlled and
163 digitalised in real time with the Patchmaster software (Nanion Technologies GmbH, Munich, Germany).

164 For preparing the dose-response curves of TTX and TTX analogues, as well as for analysing the pufferfish 165 extracts, 15 µL of sample was injected into each well of the chip at a flow rate of 30 µL/s. Dose-response 166 concentrations ranged as follows: for TTX from 0.07 nM to 300 nM; for 11-norTTX-6(*S*)-ol, 11-deoxyTTX, 167 6,11-dideoxyTTX and 5,11-dideoxyTTX analogues from 0.7 nM to 2500 nM; for 5,6,11-trideoxyTTX from 168 2.44 nM to 10,000 nM. The concentrations of pufferfish tissue equiv. for their analysis ranged from 0.002 169 to 10 mg/mL. All measurements were performed, at least, in quadruplicate.

170 ## 3. Results and Discussion

171 ### *3.1. Toxicological characterisation of tetrodotoxin analogues*

172 The detailed profile of TTXs in the concentrated PF2 liver extract, which served as a source material for 173 the isolation of TTX analogues, was previously characterised by LC-MS/MS[30] and it was shown to contain 174 TTX and thirteen of its analogues. However, after the fractionation process, only five of these analogues 175 (i.e., 11-norTTX-6(*S*)-ol, 11-deoxyTTX, 6,11-dideoxyTTX, 5,11-dideoxyTTX and 5,6,11-trideoxyTTX), which 176 were the most abundant, were isolated in sufficient quantity and abundance (90% to 95%) for the 177 subsequent toxicological characterisation with APC. The TTX analogue abundance in the fractions was 178 calculated as the percentage of the amount of the major TTX analogue with respect to the total amount 179 of all TTX analogues found in those fractions. The TTXs abundance in the fractions used in this work is 180 detailed in Supporting Information (Figure S1).

181 To characterise the toxic potential of the isolated toxins, dose-response curves describing the Na_v current 182 inhibition in Neuro-2a cells exposed to varying concentrations of each TTX analogue were constructed by 183 assessing cells' electrophysiological activity with APC and compared with the dose-response curve of TTX. 184 All data were normalised with respect to the Na_v currents measured for each individual cell after patching 185 stabilisation and before toxin exposure and fitted to a sigmoidal logistic four-parameter equation (Figure 186 2).

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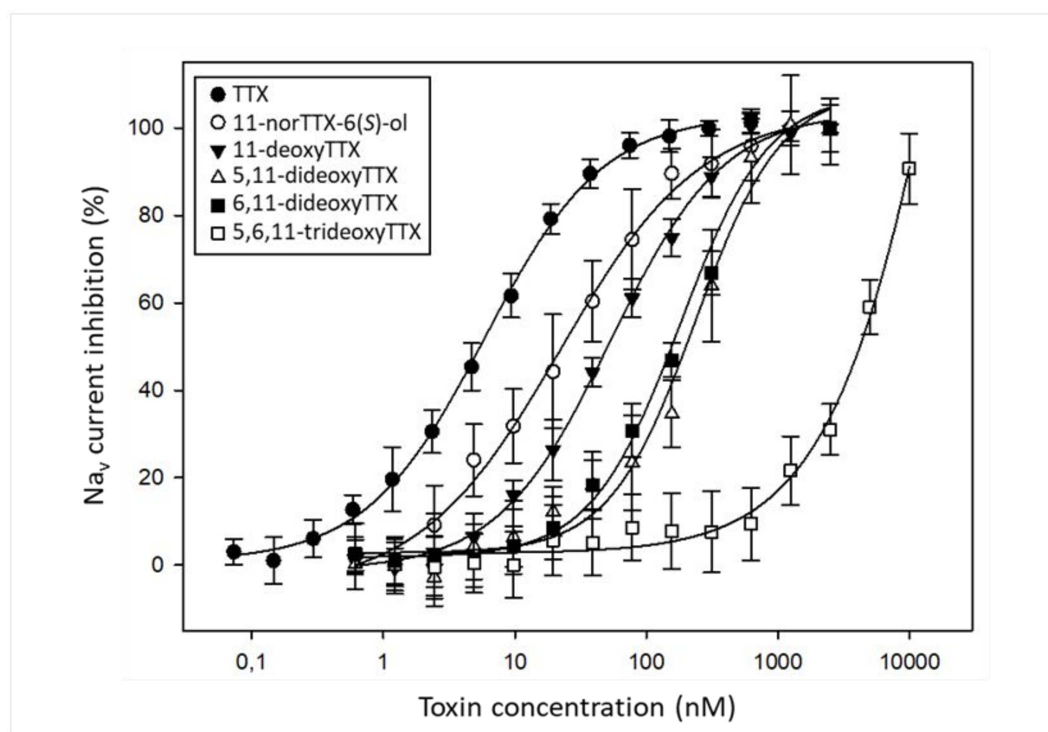

187 **Figure 2.** Dose-response curves describing the sodium current inhibition in Neuro-2a cells exposed to tetrodotoxin
188 (TTX) and TTX analogues through electrophysiological activity assessment with automated patch clamp (APC). Each
189 point represents the average ± standard deviation (at least, n = 4).

190 The half-maximal inhibitory concentration (IC_{50}) of TTX and its analogues were derived from the dose-
191 response curve equations and served as a measure of toxic potency. These values were then used to
192 calculate the toxicity equivalency factors (TEFs) for the different TTX analogues (Table 1), defined as the
193 ratio of the IC_{50} value of TTX to that of each analogue. All TTX analogues presented lower toxicity than TTX
194 (TEF < 1). Among the analogues tested, 11-norTTX-6(*S*)-ol was the most toxic followed by 11-deoxyTTX,
195 6,11-dideoxyTTX, 5,11-dideoxyTTX and 5,6,11-trideoxyTTX. The differences in toxicity can be attributed
196 to the distinct structures among TTX and its analogues, which influence how these molecules interact with
197 the target sites on VGSCs.[31,32] Additionally, changes in the amino acid sequences of the VGSC have been
198 shown to result in different TTX affinities.[33] The five TTX analogues can be categorised into three groups
199 based on the number of structural modifications compared to TTX: 11-norTTX-6(*S*)-ol and 11-deoxyTTX
200 with a single modification, 6,11-dideoxyTTX and 5,11-dideoxyTTX with a double modification, and 5,6,11-
201 trideoxyTTX with a triple modification (Figure 1).

202 **Table 1.** Half-maximal inhibitory concentration (IC_{50}) and toxicity equivalency factors (TEFs) for tetrodotoxin (TTX) and
203 TTX analogues determined by electrophysiological activity assessment of Neuro-2a cells with automated patch clamp
204 (APC).

Toxin	IC_{50} (nM)	TEF
TTX	5.43	1.000
11-norTTX-6(*S*)-ol	22.9	0.238
11-deoxyTTX	50.8	0.107
6,11-dideoxyTTX	157	0.035
5,11-dideoxyTTX	200	0.027
5,6,11-trideoxyTTX	4196	0.001

205 The main difference between the two TTX analogues with a single modification is that the hydroxymethyl
206 group at position C-6 of TTX is fully substituted by a hydrogen in 11-norTTX-6(*S*)-ol, whereas it is replaced
207 by a methyl group in 11-deoxyTTX. Interestingly, the APC analysis revealed that the toxicity of 11-norTTX-
208 6(*S*)-ol and 11-deoxyTTX was 4- and 9-fold lower compared to TTX, respectively. Considering that both
209 analogues were lacking the hydroxyl-group at C-11 position, it is reasonable to suggest that this group is
210 crucial for the efficient interaction of TTXs with VGSCs and its absence leads to significantly lower toxicity.
211 These findings align well with those of previous studies, where it was hypothesised that the specific
212 hydroxyl group was responsible for the formation of a hydrogen bond between TTX and VGSCs.[31,34]
213 Nevertheless, it is important to note that although none of these two TTX analogues have the hydroxyl at
214 C-11 position, 11-norTTX-6(*S*)-ol is twice as toxic as 11-deoxyTTX, suggesting that the exact type of
215 modification at this site has also an important effect on toxicity. It appears that the hydrogen substituent
216 in 11-norTTX-6(*S*)-ol has a less adverse impact on other non-covalent interactions with VGSCs as compared
217 to the bulkier methyl substituent in 11-deoxyTTX, implying that some steric effects also play a role in the
218 toxicity of these analogues.

219 Regarding the two TTX analogues with double modification, both share the same alteration at C-11
220 position as seen in 11-deoxyTTX. Additionally, 6,11-dideoxyTTX has a hydroxyl group at C-6 position
221 replaced by a hydrogen, whereas the ether group at C-5 position of 5,11-dideoxyTTX, which is part of the
222 2,4-dioxaadamantane skeleton, is also replaced by a hydrogen. APC analysis revealed that the toxicity of

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223 6,11-dideoxyTTX and 5,11-dideoxyTTX was 29- and 37-fold lower compared to TTX, respectively. Based
224 on these results, the hydroxyl group at C-6 position seems to have a lower impact on toxicity compared
225 to the hydroxyl group at C-11 position, as evidenced by the greater toxicity reduction between TTX and
226 11-deoxyTTX than between 11-deoxyTTX and 6,11-dideoxyTTX. These results are aligned with the findings
227 of other studies.[35] As for the C-5 modification in the 2,4-dioxaadamantane structure, its impact on toxicity
228 appears to be greater than that of the hydroxyl group modification at C-6 position (i.e., the toxicity of
229 5,11-dideoxyTTX is 22% lower than the toxicity of 6,11-dideoxyTTX). A notable reduction in toxicity in 5-
230 deoxyTTX (that has the same modification at C-5 position than 5,11-dideoxyTTX) was also observed in
231 other works.[36]

232 Finally, 5,6,11-trideoxyTTX combines the three modifications described for 6,11-dideoxyTTX and 5,11-
233 dideoxyTTX. Its toxicity was 773-fold lower than that of TTX, which is aligned with results from previous
234 papers.[37] This huge decrease in toxicity indicates that the combination of the different modifications
235 exerts a synergistic effect. It is clear that C-11, C-6 and C-5 positions are important for the overall toxicity
236 of TTXs to a greater or lesser extent.

237 This is the first time that APC technology has been used to characterise the toxicity of TTX analogues and
238 elucidate their TEFs. Literature on the relative toxicity of TTX analogues is rather limited and fragmented,
239 complicating the comparison of our results with those of previous studies. Nevertheless, all TTX analogues
240 tested by APC exhibited lower toxicity than TTX, aligning with the findings from MBA or CBA methods.[38]
241 The TEFs obtained with APC for 11-norTTX-6(*S*)-ol, 11-deoxyTTX, 6,11-dideoxyTTX and 5,6,11-trideoxyTTX
242 are aligned with some previously obtained with CBA and MBA (Table 2). The only analogue which diverged
243 from the toxicity trend of TTXs was 5,11-dideoxyTTX, with a TEF of 0.027 via APC compared to 0.750 via
244 CBA.[30] The discrepancy in TEFs could stem from differences in the fractions used, since the presence of
245 other compounds may either increase or decrease the toxicological response. However, this does not
246 seem to be the case as the fractions used for the determination of the TEF with CBA[30] and APC (this work)
247 contained similar 5,11-dideoxyTTX abundances (i.e. 98 and 95%, respectively). Since the same cell model
248 has been used for CBA and APC (i.e., Neuro-2a cells), this variability on TEFs is more likely due to the
249 distinct detection principles of the two methods. The detection principle of APC is based on the
250 assessment of the changes in the electrophysiological activity of cells after their exposure to toxins. In

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251 contrast, CBA evaluates the changes in cell viability caused by the toxins in cell cultures pretreated with
252 two auxiliary drugs: ouabain, a Na^+/K^+ ATPase pump blocker, and veratridine, a VGSCs inactivation
253 inhibitor. The combination of these two drugs enhances the accumulation of Na^+ in the cytosol, inducing
254 cell mortality. The blocking of VGSCs by TTX or its analogues counteracts the cytotoxic effect of these two
255 drugs. Therefore, the toxicological response obtained with CBA after exposing the cells to these toxins is
256 a consequence of an induced cellular state that is not physiologically natural and that is influenced by the
257 activity of ouabain and veratridine (any change in ouabain/veratridine concentrations may affect the
258 response of the cells to toxins). As a result, toxicological data obtained with CBA must be interpreted with
259 caution, whereas APC results may more accurately reflect the actual effects encountered in the human
260 body during poisoning events. It is also necessary to mention that we cannot completely rule out the
261 presence of non-targeted co-eluting compounds, other than TTX analogues, in the fractions. Nevertheless,
262 the ouabain/veratridine controls in the analysis of these fractions by CBA from our previous work[30]
263 showed that, if present, these compounds were not interfering with the assay. Therefore, and since APC
264 is less prone to matrix effects than CBA, we assume that the APC system is responding to the toxicological
265 effect of TTX analogues and that the dose-response curves and derived TEFs are appropriate.

266 **Table 2.** Toxicity equivalency factors (TEFs) for the tetrodotoxin (TTX) analogues evaluated with automated patch
267 clamp (APC), cell-based assay (CBA) and mouse bioassay (MBA).

TTX analogue	TEF	Method	Reference
11-norTTX-6(*S*)-ol	0.238	APC	(This work)
	0.404	CBA	30
	0.19	MBA	39
11-deoxyTTX	0.107	APC	(This work)
	0.139	CBA	30
	0.14	MBA	40
6,11-dideoxyTTX	0.035	APC	(This work)
	0.005	CBA	35
	0.02	MBA	41
5,11-dideoxyTTX	0.027	APC	(This work)

	0.750	CBA	30
5,6,11-trideoxyTTX	0.001	APC	(This work)
	0.011	CBA	30
	0.01	MBA	37

268 *3.2. Pufferfish toxicity analysis with automated patch clamp*

269 To understand the impact that multi-TTX mixtures from real biological extracts may have on APC response,
270 fifteen pufferfish samples (five different tissues from three *L. sceleratus* specimens) were analysed with
271 APC and the results were compared with those from CBA and LC-MS/MS analyses performed on the same
272 pufferfish extracts in a previous work[30] (Table 3).

273 **Table 3.** Concentration of tetrodotoxins (TTXs) in five tissues (gonads (G), skeletal muscle (M), liver (L), digestive tract
274 (D) and skin (S)) from three *Lagocephalus sceleratus* pufferfish (PF1, PF2 and PF3) determined by automated patch
275 clamp (APC), cell-based assay (CBA) and liquid chromatography coupled to mass spectrometry (LC-MS/MS). For APC
276 and CBA, toxin concentrations are expressed as µg TTX equiv./kg of pufferfish tissue. For LC-MS/MS, the concentration
277 of only TTX (which also includes 4-*epi*TTX) or all TTXs concentrations are expressed as µg TTX or TTX+TTX analogues/kg
278 of pufferfish tissue.

Sample		APC	CBA*	LC-MS/MS*	
				[TTX]	∑ [TTXs]
PF1	G	**5150** ± 575	**3657** ± 472	**1490**	**2129**
	M	**5738** ± 1160	**5559** ± 1027	**4615**	**7640**
	L	**24,982** ± 1505	**21,454** ± 2598	**20,560**	**38,917**
	D	**25,050** ± 276	**19,584** ± 4105	**19,197**	**34,646**
	S	**6324** ± 312	**8032** ± 454	**5292**	**14,251**
PF2	G	**13,588** ± 1959	**6365** ± 257	**5402**	**10,541**
	M	**22,484** ± 3105	**14,091** ± 1028	**15,066**	**36,486**
	L	**74,215** ± 4259	**51,351** ± 2318	**53,939**	**188,240**
	D	**86,994** ± 8655	**92,425** ± 16,021	**69,950**	**205,770**
	S	**14,147** ± 3096	**16,117** ± 1012	**13,496**	**63,178**

	G	**222,320** ± 48,038	**228,881** ± 12,229	**171,654**	**1,324,439**
	M	**6505** ± 855	**8479** ± 943	**6354**	**11,563**
PF3	**L**	**17,456** ± 2850	**26,760** ± 2443	**16,985**	**57,179**
	D	**87,619** ± 5896	**113,128** ± 9840	**80,447**	**210,873**
	S	**16,031** ± 3239	**14,659** ± 1031	**9179**	**34,746**

279 *Data from Alkassar and co-workers.[30]

280 Pufferfish samples were analysed at a maximum concentration of tissue equiv. of 10 mg/mL. According
281 to our previous works,[28] potential co-extracted compounds, if any, do not interfere with the TTX
282 calibration curve at this matrix concentration and, therefore, there is no need to apply any recovery factor
283 in the determination of TTX concentrations. The same applies to the determination of TTX concentrations
284 by CBA, where the ouabain/veratridine controls indicated the absence of matrix effects at the tested
285 matrix concentrations.[30] All samples analysed with APC showed TTXs concentration exceeding the
286 Japanese official regulatory limit of 2000 µg TTX equiv./kg. Considering the total TTXs concentration, PF3,
287 which was a female, was the most toxic pufferfish followed by PF2 and PF1, which were males. Although
288 limited in number, these results are consistent with those reported in other studies suggesting gender-
289 based toxicity differences, where females typically exhibit higher toxicity levels.[15,42-44] The reason why
290 females are generally more toxic than males is not fully clear. Originally, it was thought that TTXs served
291 exclusively as a defence/protection mechanism against predators.[45] However, several studies suggested
292 that TTXs may also play a role in sexual development and reproduction. For example, females have been
293 proposed to use TTX as a male-attracting pheromone.[46] In this work, the gonads from PF3 (ovaries) was
294 the tissue with the highest concentration of TTXs. The accumulation of TTXs in female gonads has been
295 observed in previous works, and it was hypothesised that the toxin-transferring mechanism during
296 spawning may be useful for conferring protection to eggs.[47,48] Regarding tissue-specific distribution, liver
297 and digestive tract generally contained higher TTXs levels than skeletal muscle and skin, which is
298 consistent with the findings of other studies.[15,44,49-52]

299 Overall, the results from APC, CBA and LC-MS/MS analysis show consistent trends in the distribution of
300 TTXs across the different specimens and tissues. However, the TTXs concentrations estimated as TTX
301 equiv. with APC and CBA are on average 30% and 26% higher, respectively, than those measured for TTX

302 alone by LC-MS/MS. On the contrary, the concentrations derived by LC-MS/MS for the total TTXs
303 concentration (TTX and its analogues) are nearly two times higher than those obtained by APC and CBA.
304 These results may indicate that the toxin profile of a sample is influencing the TTX quantifications due to
305 the non-equally detection of TTX analogues by the three analytical techniques. As mentioned above, APC
306 and CBA are toxicological approaches, and their detection principle is based on the toxicological response
307 of cells to the sample under investigation (composite response) and depends on the toxic potencies of
308 the individual compounds. On the contrary, LC-MS/MS employs a structural detection approach that
309 identifies TTX and its analogues based on their distinct physicochemical properties and provides the molar
310 concentrations of each separate analyte independently of their toxicity. A notable example of this fact is
311 the analysis of the gonads from the PF3 sample. The TTXs concentration determined by APC for this tissue
312 was ≈220,000 µg TTX equiv./kg, whereas by LC-MS/MS the concentration of TTX was ≈160,000 µg TTX/kg
313 and that of TTX analogues was ≈1,160,000 µg TTX analogues/kg. It is evident that, despite the high
314 abundance of 5,6,11-trideoxyTTX (Supporting Information, Figure S2, PF3, G, purple bar), its low toxicity
315 (TEF = 0.001) makes its relative contribution to the toxicological response by APC very low. Despite this
316 fact, the toxic effects from TTX analogues are still considerable and should not be overlooked, as they can
317 play an important role in poisoning especially when pufferfish tissues contain even higher concentrations
318 of these compounds.

319 *3.3. Application of toxicity equivalency factors to LC-MS/MS data*

320 The feasibility of estimating the overall toxicity of a sample by applying the TEF values determined by APC
321 to the individual TTX analogues concentrations measured by LC-MS/MS was evaluated for the fifteen
322 pufferfish samples discussed above. Initially, the APC data were plotted against the LC-MS/MS-measured
323 concentration of TTX (Figure 3A, solid line) or the sum of TTX and its analogues (Figure 3A, dotted line)
324 without applying any TEF value. In the former case, the concentration of TTX was combined with that of
325 4-*epi*TTX, since they are in chemical equilibrium in nature (epimers). Subsequently, TEFs were applied to
326 the LC-MS/MS measurements of the individual TTX analogues, and their sum was again plotted against
327 the APC-derived results (Figure 3B). Given the inability to determine TEFs for all TTX analogues present in
328 the samples, two extreme scenarios were considered: the unknown TTX analogues were assumed to be
329 non-toxic (TEF = 0, Figure 3B solid line) or the unknown TTX analogues were assumed to be as toxic as TTX

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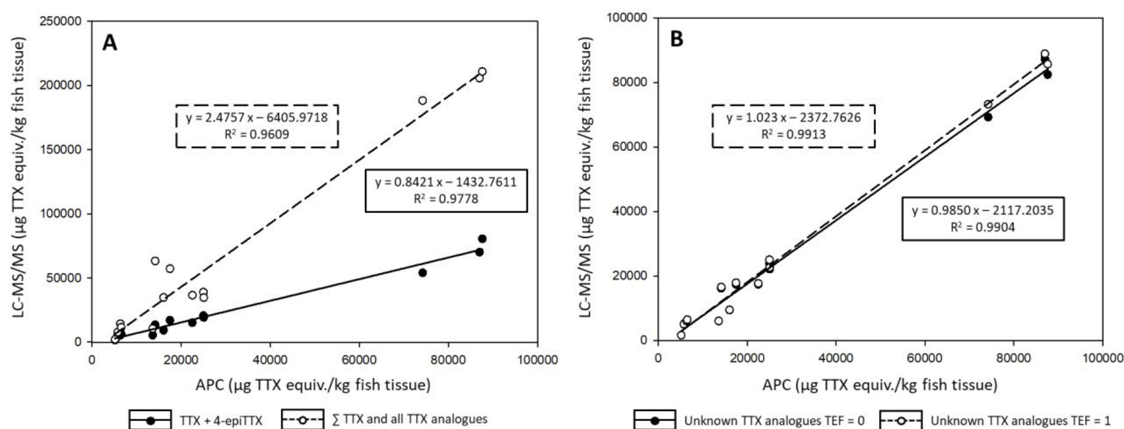
330 (TEF = 1, Figure 3B dotted line). TTXs concentrations after application of TEFs to LC-MS/MS measurements
331 of the individual TTX analogues and corresponding to correlations of Figure 3B are in Supporting
332 Information, Table S1. The gonads from PF3 were excluded from all correlations since the TTXs values
333 determined by both APC and LC-MS/MS were outliers (*z* scores of 3.55 and 3.87, respectively).



334 **Figure 3.** Correlations between the tetrodotoxins (TTXs) concentrations determined with automated patch clamp
335 (APC) and liquid chromatography coupled to mass spectrophotometry (LC-MS/MS) before (**A**) and after (**B**) the toxicity
336 equivalency factors (TEFs) application. All correlations were fitted to a linear regression model and the obtained
337 equations are shown.

338 As illustrated in Figure 3A, LC-MS/MS tends to underestimate the TTXs concentration compared to APC
339 when only the TTX and 4-*epi*TTX are considered (slope of 0.84), suggesting that not all the toxicological
340 response obtained with APC can be explained by the amount of TTX/4-*epi*TTX present in sample. On the
341 contrary, LC-MS/MS leads to an overestimation of TTXs concentration compared to APC when, in addition
342 to TTX/4-*epi*TTX, the other TTX analogues are also taken into account (slope of 2.47). This result confirms
343 that the different TTX analogues do not contribute equally to the toxicological response measured
344 through APC. As shown in Figure 3B, this overestimation is corrected when TEFs are applied. Regardless
345 of whether a TEF of 0 or 1 is applied to the unknown TTX analogues, the slope obtained is close to 1 with
346 a coefficient of determination of 0.99. These findings demonstrate that TEFs derived from APC technique
347 can be effectively applied as correction factors to LC-MS/MS quantifications and enable the translation of
348 those data into practical toxicological information.

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361 **CRediT author statement**

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366 **ASSOCIATED CONTENT**

367 **Supporting Information**

368 [Fractionation method, LC-MS/MS method and data] This material is available free of charge on the ACS
369 Publications website.

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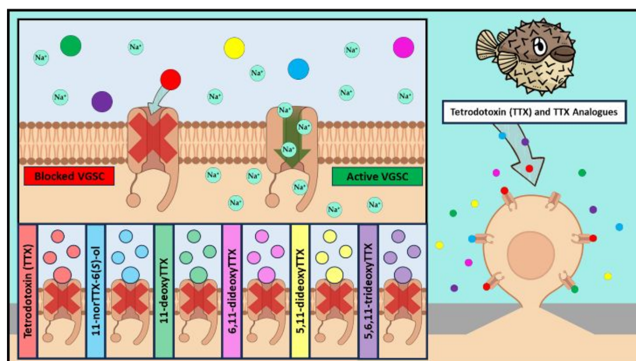
574 **Table of Contents Graphic**



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