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

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

[a] *IRTA, Ctra. Poble Nou km 5.5, 43540 La Ràpita, Spain.*

[b] *Basic Medical Sciences Department, Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili (URV), C/ St. Llorenç 21, 43201 Reus, Spain.*

[c] *University Institute of Animal Health and Food Safety (IUSA), University of Las Palmas de Gran Canaria, 35416 Arucas, Spain.*

[d] *Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research (HCMR), 71003 Heraklion, Greece.*

[e] *Institute of Marine Biological Resources and Inland Waters, Hellenic Centre for Marine Research (HCMR), 71003 Heraklion, Greece.*

[f] *French Alternative Energies and Atomic Energy Commission (CEA), University of Paris-Saclay, INRAE, DMTS, SIMoS, EMR CNRS 9004, 91191 Gif sur Yvette, France.*

**Correspondence: monica.campas@irta.cat*

Abstract

Tetrodotoxin (TTX) is a potent marine neurotoxin, responsible for numerous poisoning incidents and some human fatalities. The pufferfish *Lagocephalus sceleratus* is one of the main carriers of this toxin in nature, which coexists with a variety of TTX analogues. To date, more than thirty TTX analogues have been identified, but their individual toxicities and roles in poisoning remain largely unknown. In this work, the toxicity equivalency factors (TEFs) of five TTX analogues were determined by assessing the blockade of voltage-gated sodium channels (VGSCs) in Neuro-2a cells using automated patch clamp (APC). Among the TTX analogues tested, 11-norTTX-6(*S*)-ol exhibited the highest toxicity, followed by 11-deoxyTTX, 6,11-dideoxyTTX, 5,11-dideoxyTTX and 5,6,11-trideoxyTTX. All analogues were less toxic than TTX. The derived TEFs were applied to the individual TTX analogues concentrations measured in fifteen pufferfish samples from Greece, using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). A comparison of these results with those obtained from APC analysis demonstrated that TEFs can be effectively used to translate LC-MS/MS analytical data into meaningful toxicological information. This is the first study to utilise APC device for the toxicological assessment of TTX analogues, highlighting its potential as a bioanalytical tool for seafood safety management and human health protection.

Keywords

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

1. Introduction

Tetrodotoxins (TTXs) are a family of highly toxic neurotoxins, which received this name because they were first isolated from *Tetraodontidae* fish (Katikou et al., 2022). In natural samples, TTX coexists with several TTX analogues, sharing a similar overall structure but differing at specific molecular positions. To date, over thirty TTX analogues have been described, yet their individual toxicities and role in poisoning incidents remain largely undefined (Katikou et al., 2022). The pufferfish *Lagocephalus sceleratus* is one of the main carriers of TTXs, although these toxins are also found in a variety of marine and terrestrial organisms (Bane et al., 2014). The presence of TTXs in organisms from different phyla suggests an exogenous origin, which has been linked to various bacteria from the *Proteobacteria* phylum (Magarlamov et al., 2017). However, the metabolic pathways for TTXs biosynthesis and/or biotransformation in hosts are still unclear (Kono et al., 2008; Ueyama et al., 2018). The biological target of TTXs is the voltage-gated sodium channel (VGSC, also known as Na_v), an important membrane protein involved in the generation and propagation of action potentials in neuronal and other excitable tissues (Catterall, 2012). The toxicity mechanism of TTXs relays on the blockade of the VGSCs, inhibiting Na^+ influx into the cell and therefore preventing cell membrane depolarisation (Tukker et al., 2023). Depending on the dose, TTX can be useful for some medical applications (Nieto et al., 2012; Bucciarelli et al., 2021; González-Cano et al., 2021) or can cause poisoning (Guardone et al., 2019). In this case, symptoms ranging from mild neurological or gastrointestinal disorders (e.g., oral tingling or vomiting) to severe systemic failures have been described that, in severe cases, can lead to death (Bentur et al., 2008; Katikou et al., 2022). Currently, there is no specific treatment for TTX poisoning other than palliative care to relieve the symptoms. For this reason, the prevention of TTX poisoning highly depends on the early detection of TTX and TTX analogues in seafood.

Tetrodotoxin poisoning events are predominantly reported in Asian countries where pufferfish is eaten as a delicacy (Bane et al., 2014). However, poisoning cases have also been reported in non-endemic regions, highlighting a potential new food safety concern if appropriate control measures are not implemented (Reverté et al., 2023). In Europe, poisonous fish of the *Tetraodontidae, Molidae, Diodontidae and Canthigasteridae* families must not be placed on the market due to the threat they may pose to human health (Regulation (EC) No 854/2004). However, with *L. sceleratus* establishing itself as a new persistent invasive species in the Mediterranean (Akyol et al., 2005; Katikou et al., 2009; Rambla-Alegre et al., 2017; Coro et al., 2018; Guardone et al., 2018; Akbora et al., 2020; Christidis et al., 2021; Anastasiou et al., 2023; Kosker et al., 2023), concerns arise regarding the potential risks associated with the accidental consumption of toxic pufferfish by local populations as a consequence of recreational fishing (Anastasiou et al., 2023; Ulman et al., 2024) and the possible intermingling of juveniles with other small pelagic species (e.g., anchovy, sardines, picarel and bogue) (Leonardo et al., 2019). Moreover, the risk of exposure to TTX through the consumption of common edible seafood species (originally non-toxic) that have been cross-contaminated as a result of accidental pufferfish predation (Malloggi et al., 2023; Tinacci et al., 2023) cannot be ignored. In addition, over the last few decades, shellfish has been identified

as another important TTX carrier in Europe (Rodriguez et al., 2008; Silva et al., 2012; Turner et al., 2015; Vlamis et al., 2015; Gerssen et al. 2018; Leão et al., 2018; Reverté et al., 2018; Blanco et al., 2019; Dell'Aversano et al., 2019; Campàs et al., 2020; Hort et al., 2020; Bacchiocchi et al., 2021; Bordin et al., 2021; Réveillon et al., 2021; Alkassar et al., 2024). Although TTX concentrations in shellfish are generally lower than those in pufferfish, they still pose a poisoning risk. Indeed, the first TTX poisoning case originated in Europe was linked to the consumption of contaminated gastropods (Rodriguez et al., 2008; Fernández-Ortega et al., 2010).

In general, instrumental analysis techniques, such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), are the gold standard for monitoring regulated toxins in Europe, including lipophilic marine toxins (Regulation (EC) No 15/2011). The main advantage of these techniques is the ability to detect and quantify not only the target toxin but also several of its individual analogues, thus providing a toxin profile for a sample (Jang et al., 2010; Rambla-Alegre et al., 2017; Rambla-Alegre et al., 2018; Alkassar et al., 2023; Hong et al., 2023; Park et al., 2024). However, these analyses do not provide direct toxicological insights (Reverté et al., 2023), requiring the use of toxicity equivalency factors (TEFs) for the different analogues to transform analytical data into practical toxicological information. The scant knowledge about the individual toxicities of emerging marine toxins, including the TTX analogues, emphasises the increasing need for their thorough toxicological evaluation (Botana et al., 2017).

Historically, animal bioassays, such as the mouse bioassay (MBA), were the primary methods for the toxicological characterisation of marine toxins (Reverté et al., 2023). However, MBA is now of limited use in many countries due to its low specificity, high variability and the ethical issues concerning the experimentation with live animals (Campàs et al., 2021). Cell-based assays (CBAs) have been proposed as an alternative approach to assess the toxicity of a sample without involving live animals. The detection of toxins with CBA relies on assessing changes in the morphology or viability of cells after their exposure to them (Kogure et al., 1988; Manger et al., 1993; Manger et al., 1995). However, cells used for CBA sometimes require pretreatments with auxiliary drugs to ensure optimal assay performance, necessitating cautious interpretation of the toxicological data that they generate. Recently, a new approach that leverages the electrophysiological activity of Neuro-2a cells has emerged as a promising high throughput bioanalytical method for detecting TTXs in pufferfish samples, with potential applications in food safety and broader toxicological studies (Campàs et al., 2024).

In this work, we characterised the toxicity of five TTX analogues using an automated patch clamp (APC) device (Figure 1). By assessing the toxicological response of Neuro-2a cells to 11-norTTX-6(*S*)-ol, 11-deoxyTTX, 6,11-dideoxyTTX, 5,11-dideoxyTTX and 5,6,11-trideoxyTTX, the respective TEF values were determined. Then, we applied these TEFs as correction factors to the concentrations of individual TTX analogues measured in various pufferfish samples from Greece using LC-MS/MS. The overall toxicity levels calculated in TTX equivalents (equiv.) through this indirect approach closely matched those directly measured in the samples using APC, suggesting a consistent agreement between the two techniques. This study represents the first application of APC in evaluating the toxicological potency of TTX analogues.

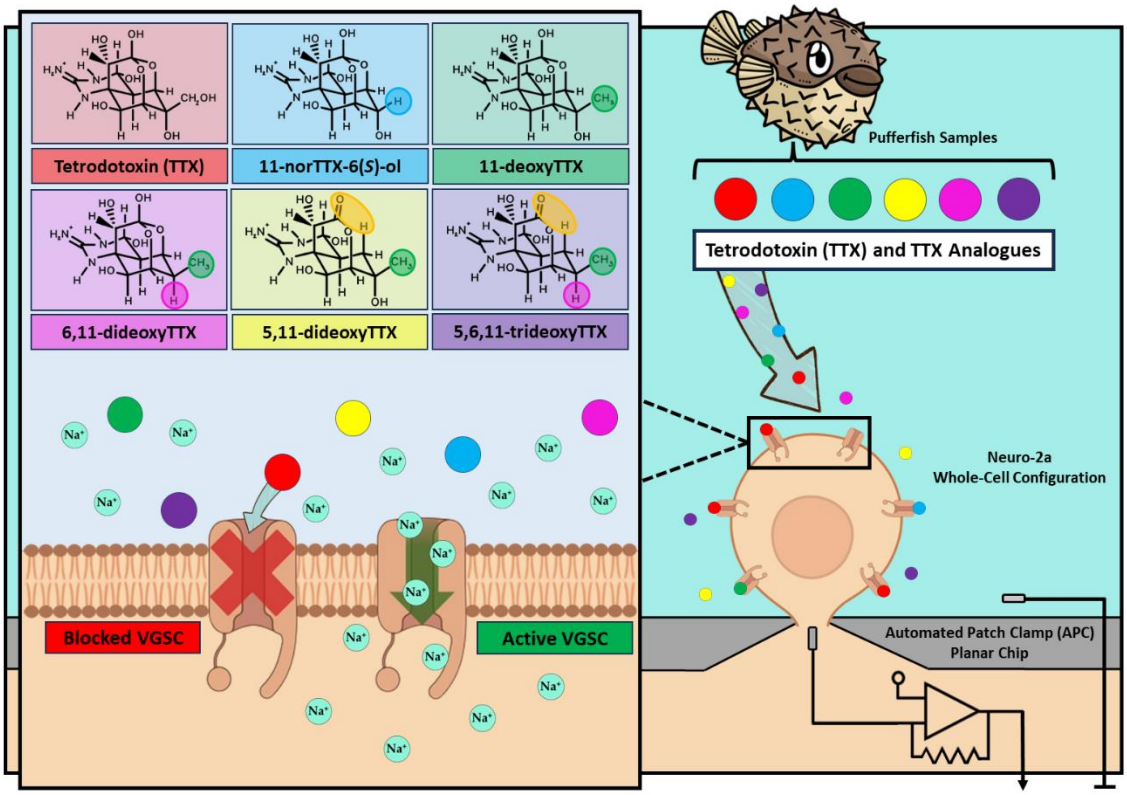


Figure 1. The diagram on the right outlines the strategy for assessing the activity of voltage-gated sodium channels (VGSCs) in Neuro-2a cells exposed to TTX or the TTX analogues studied using the APC method. The close-up view on the left illustrates the VGSC blockade by the toxins and the inhibition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

[Colour should be used for Figure 1 in print]

2. Materials and Methods

2.1. Reagents

TTX (purity ≥98% by HPLC) was purchased from Tocris Bioscience (Bristol, UK) and a standard solution was prepared at 1 mg/mL in 1% (*v*/*v*) acetic acid. In the APC method, the external solution (140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-glucose monohydrate, 10 mM HEPES/NaOH, pH 7.4), the internal solution (50 mM CsCl, 10 mM NaCl, 60 mM CsF, 20 mM EGTA, 10 mM HEPES/CsOH, pH 7.2) and the seal enhancer solution (10 mM HEPES, 130 mM NaCl, 5 mM glucose, 4 mM KCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, pH 7.4, osmolarity: 302 mOsmol) were obtained from Nanion Technologies GmbH (Munich, Germany). Murine neuroblastoma (Neuro-2a) cells were purchased from ATCC LGC Standards (Manassas, VA, USA). Foetal bovine serum (FBS), penicillin/streptomycin solution, Roswell Park Memorial Institute (RPMI-1640) medium, sodium pyruvate and trypsin-EDTA enzyme were purchased from Merk KGaA (Darmstadt, Germany).

2.2. Pufferfish samples and tetrodotoxin extraction

Three pufferfish specimens, two males (PF1, PF2) and one female (PF3), identified as *L. sceleratus* by morphological evaluation, were collected from the Libyan Sea near Crete, Greece in May (PF1, PF2) and March (PF3) of 2019. The specimens were dissected and their tissues, including skeletal muscle, skin, liver, intestinal tract and gonads, were separated. The extraction of TTXs was performed following the protocol described by Reverté and co-workers (Reverté et al., 2015). Briefly, 10 g of each tissue was homogenised using an Ultraturrax blender at full speed. To each homogenised sample, 25 mL of 0.1% (*v*/*v*) acetic acid was added and the mixture was vortexed for 2 min at 2500 rpm. Subsequently, the tubes were placed in a boiling water bath for 10 min with occasional stirring. The homogenates were then cooled down and centrifuged at 2500 rpm for 5 min (4 °C). The supernatants were collected and the pellets were subjected to a second extraction with additional 20 mL of 0.1% (*v*/*v*) acetic acid. The two supernatants were pooled, and their final volume was adjusted to 50 mL with 0.1% (*v*/*v*) acetic acid. For liver samples, a liquid-liquid partitioning of the crude extract with hexane (1:1) was necessary to remove fats. For APC analysis, the extracts were passed through 0.45-µm nylon syringe filters. For LC-MS/MS analysis, filtration was conducted using 3000-Da molecular sieve filters followed by 0.2-µm nylon filters. The final extracts, at 200 mg pufferfish tissue per mL, were stored at –20 °C until analysed.

2.3. Isolation and purification of tetrodotoxin analogues

TTX analogues were sourced from PF2 liver tissue, as detailed in Alkassar et al., 2023. In brief, a 30 g liver tissue extract was reduced to 5 mL using a rotary evaporator system, mixed with 5 mL of acetonitrile and fractionated by hydrophilic interaction liquid chromatography (HILIC) at room temperature utilising a prep-LC column Luna HILIC AXIA (250 mm × 21.2 mm, 5 µm particle size; Phenomenex, Torrance, CA, USA). A binary gradient elution at 10 mL/min was applied with Milli-Q water (mobile phase A) and acetonitrile/water (90/10 *v*/*v*, mobile phase B), both containing 30 mM ammonium acetate at pH 5.8. The gradient program started at 100% B, and it was kept isocratic for 5 min; then, phase B was reduced to 95% B at 35 min, further decreased to 82.5% B at 80 min, held isocratic for 5 min and returned 100% B at 90 min. A total of ten chromatographic runs were performed on 1 mL aliquots and the TTXs fractions collected (10-mL per run) were pooled together (100 mL in total) and stored at -20 °C until used. The TTXs present in the pooled fractions were analysed by LC-MS/MS, using a triple quadrupole mass spectrometer (Xevo TQ-XS, Waters Corporation, Milford, MA, USA) coupled to a UPLC binary pump system (Acquity UPLC I-plus-Class, Waters Corporation, Milford, MA, USA). Further experimental details are provided in a previous work (Alkassar et al., 2023). The fractions were dried under a N_2 stream at room temperature and reconstituted in the external buffer before being analysed with APC.

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

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

The electrophysiological changes of Neuro-2a cells in response to the TTX analogues were evaluated using a Patchliner (Nanion Technologies GmbH, Munich, Germany), an automated planar patch clamp device (Campàs et al., 2024). Briefly, eight wells of a medium resistance NPC-16 borosilicate planar chip (Nanion Technologies GmbH, Munich, Germany) were filled up with the internal and external solutions. Then, a suspension of Neuro-2a cells, prepared at 100,000 cells/mL in a 1:1 mixture of RPMI and external solution, was introduced into the chip. A single cell was immobilised on the hole located at the bottom of each well with a holding potential of –30 mV. Then, after adding the enhancer solution, the potential was changed to –100 mV. Successful patching was confirmed by achieving a stable Giga-sealing with resistances (higher than 1 GΩ).

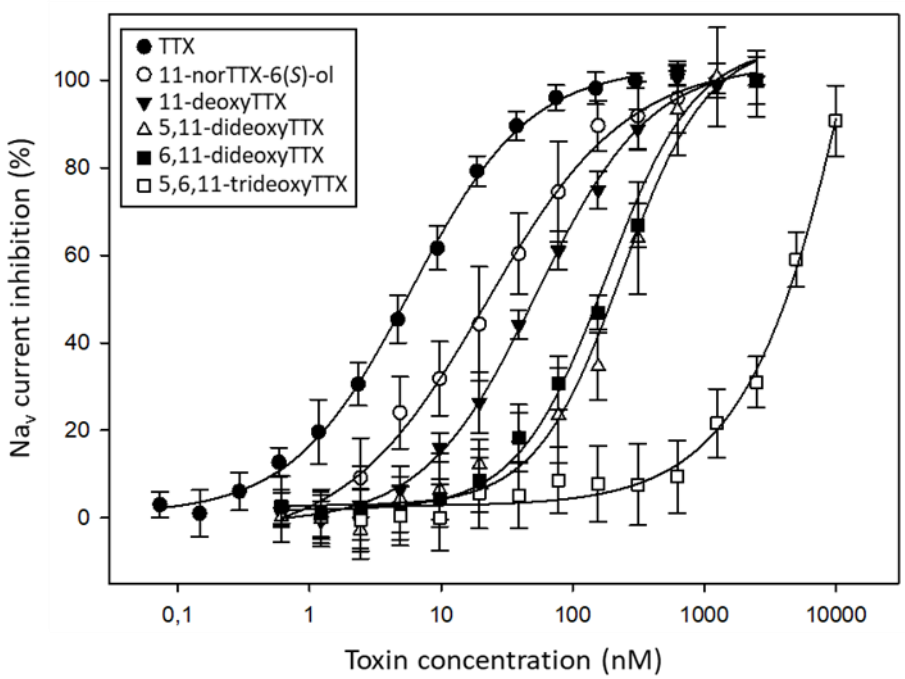
The Na_v currents were measured by applying the potential in increments of 10 mV from –80 mV to 40 mV using two EPC Quatro USB amplifier units (8 probes) (HEKA Elektronik, Stuttgart, Germany) controlled and digitalised in real time with the Patchmaster software (Nanion Technologies GmbH, Munich, Germany). For preparing the dose-response curves of TTX and TTX analogues, as well as for analysing the pufferfish extracts, 15 µL of sample was injected into each well of the chip at a flow rate of 30 µL/s. Dose-response concentrations ranged as follows: for TTX from 0.07 nM to 300 nM; for 11-norTTX-6(*S*)-ol, 11-deoxyTTX, 6,11-dideoxyTTX and 5,11-dideoxyTTX analogues from 0.7 nM to 2500 nM; for 5,6,11-trideoxyTTX from 2.44 nM to 10,000 nM. The concentrations of pufferfish tissue equivalents for their analysis ranged from 0.002 to 10 mg/mL. All measurements were performed, at least, in quadruplicate.

3. Results and Discussion

3.1. Toxicological characterisation of tetrodotoxin analogues

The detailed profile of TTXs in the concentrated PF2 liver extract, which served as a source material for the isolation of TTX analogues, was previously characterised by LC-MS/MS (Alkassar et al., 2023) and it was shown to contain TTX and thirteen of its analogues. However, after the fractionation process, only five of these analogues (i.e., 11-norTTX-6(*S*)-ol, 11-deoxyTTX, 6,11-dideoxyTTX, 5,11-dideoxyTTX and 5,6,11-trideoxyTTX), which were the most abundant, were isolated in sufficient quantity and grade of purity (90% to 95%) for the subsequent toxicological characterisation with APC. The grade of purity of the fractions corresponding to these TTX analogues was calculated as the percentage of the major TTX analogue with respect to the total amount of all TTX analogues found in those fractions. The composition of the fractions used in this work is detailed in the “Supplementary material” section (Figure S1).

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



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

199 [Colour should not be used for Figure 2 in print]

200 The half-maximal inhibitory concentration (IC_{50}) of TTX and its analogues were derived from the dose-
201 response curve equations and served as a measure of toxin potency. These values were then used to
202 calculate the toxicity equivalency factors (TEFs) for the different TTX analogues (Table 1), defined as the
203 ratio of the IC_{50} value of TTX to that of each analogue. All TTX analogues presented lower toxicity than TTX
204 (TEF < 1). Among the analogues tested, 11-norTTX-6(*S*)-ol was the most toxic followed by 11-deoxyTTX,
205 6,11-dideoxyTTX, 5,11-dideoxyTTX and 5,6,11-trideoxyTTX. The differences in toxicity can be attributed to
206 the distinct structures among TTX and its analogues, which influence how these molecules interact with
207 the target sites on VGSCs (Yotsu-Yamashita et al., 1999; Bane et al., 2016). Additionally, changes in the
208 amino acid sequences of the VGSC have been shown to result in different TTX affinities (Tsukamoto et al.,
209 2017). The five TTX analogues can be categorised into three groups based on the number of structural
210 modifications compared to TTX: 11-norTTX-6(*S*)-ol and 11-deoxyTTX with a single modification, 6,11-
211 dideoxyTTX and 5,11-dideoxyTTX with a double modification, and 5,6,11-trideoxyTTX with a triple
212 modification (Figure 1).

213

214

215

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

The main difference between the two TTX analogues with a single modification is that the hydroxymethyl group at position C-6 of TTX is fully substituted by a hydrogen in 11-norTTX-6(*S*)-ol, whereas it is replaced by a methyl group in 11-deoxyTTX. Interestingly, the APC analysis revealed that the toxicity of 11-norTTX-6(*S*)-ol and 11-deoxyTTX was 4- and 9-fold lower compared to TTX, respectively. Considering that both analogues were lacking the hydroxyl-group at C-11 position, it is reasonable to suggest that this group is crucial for the efficient interaction of TTXs with VGSCs and its absence leads to significantly lower toxicity. These findings align well with those of previous studies, where it was hypothesised that the specific hydroxyl group was responsible for the formation of a hydrogen bond between TTX and VGSCs (Yotsu-Yamashita et al., 1999; Choudhary et al., 2003). Nevertheless, it is important to note that although none of these two TTX analogues have the hydroxyl at C-11 position, 11-norTTX-6(*S*)-ol is twice as toxic as 11-deoxyTTX, suggesting that the exact type of modification at this site has also an important effect on toxicity. It appears that the hydrogen substituent in 11-norTTX-6(*S*)-ol has a less adverse impact on other non-covalent interactions with VGSCs as compared to the bulkier methyl substituent in 11-deoxyTTX, implying that some steric effects also play a role in the toxicity of these analogues.

Regarding the two TTX analogues with double modification, both share the same alteration at C-11 position as seen in 11-deoxyTTX. Additionally, 6,11-dideoxyTTX has a hydroxyl group at C-6 position replaced by a hydrogen, whereas the ether group at C-5 position of 5,11-dideoxyTTX, which is part of the 2,4-dioxaadamantane skeleton, is also replaced by a hydrogen. APC analysis revealed that the toxicity of 6,11-dideoxyTTX and 5,11-dideoxyTTX was 29- and 37-fold lower compared to TTX, respectively. Based on these results, the hydroxyl group at C-6 position seems to have a lower impact on toxicity compared to the hydroxyl group at C-11 position, as evidenced by the greater toxicity reduction between TTX and 11-deoxyTTX than between 11-deoxyTTX and 6,11-dideoxyTTX. These results are aligned with the findings of other studies (Kudo et al., 2014). As for the C-5 modification in the 2,4-dioxaadamantane structure, its impact on toxicity appears to be greater than that of the hydroxyl group modification at C-6 position (i.e., the toxicity of 5,11-dideoxyTTX is 22% lower than the toxicity of 6,11-dideoxyTTX). A notable reduction in

toxicity in 5-deoxyTTX (that has the same modification at C-5 position than 5,11-dideoxyTTX) was also observed in other works (Satake et al., 2014).

Finally, 5,6,11-trideoxyTTX combines the three modifications described for 6,11-dideoxyTTX and 5,11-dideoxyTTX. Its toxicity was 773-fold lower than that of TTX, which is aligned with results from previous papers (Yotsu-Yamashita et al., 1995). This huge decrease in toxicity indicates that the combination of the different modifications exerts a synergistic effect. It is clear that C-11, C-6 and C-5 positions are important for the overall toxicity of TTXs to a greater or lesser extent.

This is the first time that APC technology has been used to characterise the toxicity of TTX analogues and elucidate their TEFs. Literature on the relative toxicity of TTX analogues is rather limited and fragmented, complicating the comparison of our results with those of previous studies. Nevertheless, all TTX analogues tested by APC exhibited lower toxicity than TTX, aligning with the findings from MBA or CBA methods (Knutsen et al., 2017). The TEFs obtained with APC for 11-norTTX-6(*S*)-ol, 11-deoxyTTX, 6,11-dideoxyTTX and 5,6,11-trideoxyTTX are aligned with some previously obtained with CBA and MBA (Table 2). The only analogue which diverged from the toxicity trend of TTXs was 5,11-dideoxyTTX, with a TEF of 0.027 via APC compared to 0.750 via CBA (Alkassar et al., 2023). The discrepancy in TEFs could stem from differences in the grade of purity of the TTX analogues used, since the presence of other compounds may either increase or decrease the toxicological response. However, this does not seem to be the case as the purities of the 5,11-dideoxyTTX fractions used for the determination of the TEF with CBA (Alkassar et al., 2023) and APC (this work) were similarly high (i.e. 98 and 95%, respectively). Since the same cell model has been used for CBA and APC (i.e., Neuro-2a cells), this variability on TEFs is more likely due to the distinct detection principles of the two methods. The detection principle of APC is based on the assessment of the changes in the electrophysiological activity of cells after their exposure to toxins. In contrast, CBA evaluates the changes in cell viability caused by the toxins in cell cultures pretreated with two auxiliary drugs: ouabain, a Na^+/K^+ ATPase pump blocker, and veratridine, a VGSCs inactivation inhibitor. The combination of these two drugs enhances the accumulation of Na^+ in the cytosol, inducing cell mortality. The blocking of VGSCs by TTX or its analogues counteracts the cytotoxic effect of these two drugs. Therefore, the toxicological response obtained with CBA after exposing the cells to these toxins is a consequence of an induced cellular state that is not physiologically natural and that is influenced by the activity of ouabain and veratridine (any change in ouabain/veratridine concentrations may affect the response of the cells to toxins). As a result, toxicological data obtained with CBA must be interpreted with caution, whereas APC results may more accurately reflect the actual effects encountered in the human body during poisoning events.

Table 2. Toxicity equivalency factors (TEFs) for the tetrodotoxin (TTX) analogues evaluated with automated patch 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	(Alkassar et al., 2023)
	0.19	MBA	(Yotsu et al., 1992)
11-deoxyTTX	0.107	APC	(This work)
	0.139	CBA	(Alkassar et al., 2023)
	0.14	MBA	(Yasumoto et al., 1988)
6,11-dideoxyTTX	0.035	APC	(This work)
	0.005	CBA	(Kudo et al., 2014)
	0.02	MBA	(Jang et al., 2007)
5,11-dideoxyTTX	0.027	APC	(This work)
	0.750	CBA	(Alkassar et al., 2023)
5,6,11-trideoxyTTX	0.001	APC	(This work)
	0.011	CBA	(Alkassar et al., 2023)
	0.01	MBA	(Yotsu-Yamashita et al., 1995)

3.2. Pufferfish toxicity analysis with automated patch clamp

To understand the impact that multi-TTX mixtures from real biological extracts may have on APC response, fifteen pufferfish samples (five different tissues from three *L. sceleratus* specimens) were analysed with APC and the results were compared with those from CBA and LC-MS/MS analyses performed on the same pufferfish extracts in a previous work (Alkassar et al., 2023) (Table 3).

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

Sample		APC	CBA*	LC-MS/MS*	
				[TTX]	∑ [TTXs]
PF1	G	**5150** ± 575	**3657** ± 472	**1490** ± 101	**2129**
	M	**5738** ± 1160	**5559** ± 1027	**4488** ± 1161	**7640**
	L	**24,982** ± 1505	**21,454** ± 2598	**18,616** ± 668	**38,917**
	D	**25,050** ± 276	**19,584** ± 4105	**17,907** ± 2299	**34,646**
	S	**6324** ± 312	**8032** ± 454	**5036** ± 356	**14,251**
PF2	G	**13,588** ± 1959	**6365** ± 257	**5193** ± 119	**10,541**
	M	**22,484** ± 3105	**14,091** ± 1028	**14,313** ± 2572	**36,486**
	L	**74,215** ± 4259	**51,351** ± 2318	**48,745** ± 7611	**188,240**
	D	**86,994** ± 8655	**92,425** ± 16,021	**66,731** ± 10,052	**205,770**
	S	**14,147** ± 3096	**16,117** ± 1012	**12,550** ± 27	**63,178**
PF3	G	**222,320** ± 48,038	**228,881** ± 12,229	**161,798** ± 3420	**1,324,439**
	M	**6505** ± 855	**8479** ± 943	**5902** ± 260	**11,563**
	L	**17,456** ± 2850	**26,760** ± 2443	**15,763** ± 624	**57,179**
	D	**87,619** ± 5896	**113,128** ± 9840	**71,809** ± 4773	**210,873**
	S	**16,031** ± 3239	**14,659** ± 1031	**8334** ± 836	**34,746**

*Data from Alkassar and co-workers (Alkassar et al., 2023).

All samples analysed with APC showed TTXs concentration exceeding the Japanese official regulatory limit of 2000 µg TTX equiv./kg. Considering the total TTXs concentration, PF3, which was a female, was the most toxic pufferfish followed by PF2 and PF1, which were males. Although limited in number, these results are consistent with those reported in other studies suggesting gender-based toxicity differences, where females typically exhibit higher toxicity levels (Ikeda et al., 2010; Itoi et al., 2012; Christidis et al., 2021; Anastasiou et al., 2023). The reason why females are generally more toxic than males is not fully clear. Originally, it was thought that TTXs served exclusively as a defence/protection mechanism against predators (Williams et al., 2010). However, several studies suggested that TTXs may also play a role in sexual development and reproduction. For example, females have been proposed to use TTX as a male-attracting pheromone (Okita et al., 2013). In this work, the gonads from PF3 (ovaries) was the tissue with the highest concentration of TTXs. The accumulation of TTXs in female gonads has been observed in previous works, and it was hypothesised that the toxin-transferring mechanism during spawning may be useful for conferring protection to eggs (Wang et al., 2011; Wood et al., 2012). Regarding tissue-specific distribution, liver and digestive tract generally contained higher TTXs levels than skeletal muscle and skin,

which is consistent with the findings of other studies (Rodriguez et al., 2012; Kosker et al., 2016; Reverté et al., 2017; Akbora et al., 2020; Christidis et al., 2021; Anastasiou et al., 2023).

Overall, the results from APC, CBA and LC-MS/MS analysis show consistent trends in the distribution of TTXs across the different specimens and tissues. However, the TTXs concentrations estimated as TTX equiv. with APC and CBA are on average 30% and 26% higher, respectively, than those measured for TTX alone by LC-MS/MS. On the contrary, the concentrations derived by LC-MS/MS for the total TTXs concentration (TTX and its analogues) are nearly two times higher than those obtained by APC and CBA. These results may indicate that the toxin profile of a sample is influencing the TTX quantifications due to the non-equally detection of TTX analogues by the three analytical techniques. As mentioned above, APC and CBA are toxicological approaches, and their detection principle is based on the toxicological response of cells to the sample under investigation (composite response) and depends on the toxic potencies of the individual compounds. On the contrary, LC-MS/MS employs a structural detection approach that identifies TTX and its analogues based on their distinct physicochemical properties and provides the molar concentrations of each separate analyte independently of their toxicity. A notable example of this fact is the analysis of the gonads from the PF3 sample. The TTXs concentration determined by APC for this tissue was ≈220,000 μg TTX equiv./kg, whereas by LC-MS/MS the concentration of TTX was ≈160,000 μg TTX/kg and that of TTX analogues was ≈1,160,000 μg TTX analogues/kg. It is evident that, despite the high abundance of 5,6,11-trideoxyTTX (Supplementary material, Figure S2, PF3, G, purple bar), its low toxicity (TEF = 0.001) makes its relative contribution to the toxicological response by APC very low. Despite this fact, the toxic effects from TTX analogues are still considerable and should not be overlooked, as they can play an important role in poisoning especially when pufferfish tissues contain even higher concentrations of these compounds.

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

The feasibility of estimating the overall toxicity of a sample by applying the TEF values determined by APC to the individual TTX analogues concentrations measured by LC-MS/MS was evaluated for the fifteen pufferfish samples discussed above. Initially, the APC data were plotted against the LC-MS/MS-measured concentration of TTX (Figure 3A, solid line) or the sum of TTX and its analogues (Figure 3A, dotted line) without applying any TEF value. In the former case, the concentration of TTX was combined with that of 4-*epi*TTX, since they are in chemical equilibrium in nature (epimers). Subsequently, TEFs were applied to the LC-MS/MS measurements of the individual TTX analogues, and their sum was again plotted against the APC-derived results (Figure 3B). Given the inability to determine TEFs for all TTX analogues present in the samples, two extreme scenarios were considered: the unknown TTX analogues were assumed to be non-toxic (TEF = 0, Figure 3B solid line) or the unknown TTX analogues were assumed to be as toxic as TTX (TEF = 1, Figure 3B dotted line). TTXs concentrations after application of TEFs to LC-MS/MS measurements of the individual TTX analogues and corresponding to correlations of Figure 3B are in Supplementary material, Table S1. The gonads from PF3 were excluded from all correlations since the TTXs values determined by both APC and LC-MS/MS were outliers (z-scores of 3.55 and 3.87, respectively).

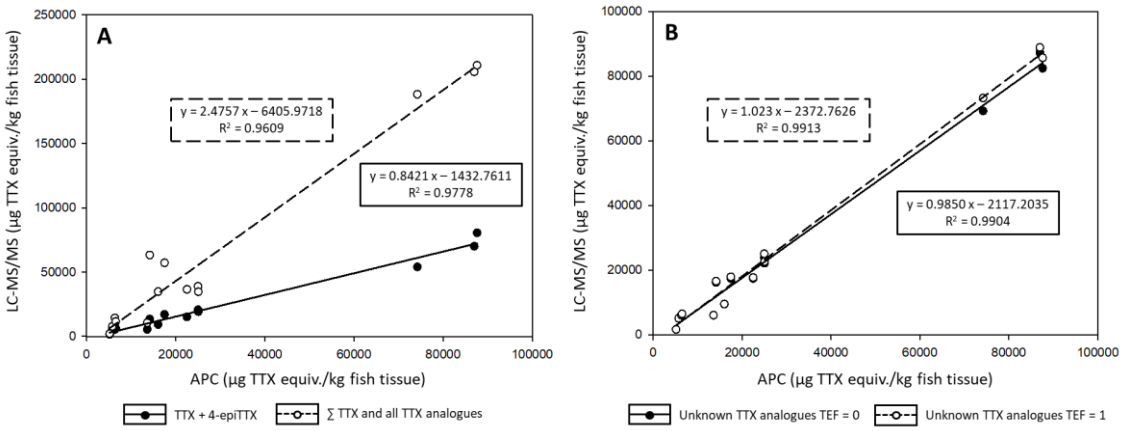


Figure 3. Correlations between the tetrodotoxins (TTXs) contents determined with automated patch clamp (APC) and liquid chromatography coupled to mass spectrophotometry (LC-MS/MS) before (**A**) and after (**B**) the toxicity equivalency factors (TEFs) application. All correlations were fitted to a linear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

[Colour should not be used for Figure 3 in print]

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

4. Conclusions

In this study, the toxicity of five TTX analogues was characterised through the assessment of the changes in the electrophysiological activity of Neuro-2a cells using APC, following their exposure to various doses of these VGSC blockers. All tested TTX analogues exhibited lower toxicity compared to TTX, with TEFs ranging from 0.238 to 0.001. The most toxic analogue was 11-norTTX-6(*S*)-ol, followed by 11-deoxyTTX, 6,11-dideoxyTTX, 5,11-dideoxyTTX and 5,6,11-trideoxyTTX. Naturally contaminated pufferfish samples displaying diverse multi-TTX profiles were analysed using APC and the results were compared with the TTXs concentrations measured by LC-MS/MS. The differences in the TTXs concentrations determined by the two analytical techniques were greatly influenced by the presence of TTX analogues, which were not equally detected by the different techniques. These discrepancies were corrected after applying TEFs to the individual TTX analogues concentrations determined by LC-MS/MS, indicating that the TEFs derived

in this study can effectively serve as correction factors to convert instrumental analysis data into meaningful toxicological information. The robust performance of the APC device across the different specimens analysed underscores its potential as a valuable, new bioanalytical tool for assessing the toxic potency of marine toxins and enhancing consumers protection against seafood-borne TTX poisonings.

Acknowledgements

This work would not have been possible without the intervention of Marisa Vinyals from Almirall, S.A. The authors want to thank Almirall, S.A. for the kind transfer of use of the automated patch clamp equipment. The authors would also like to express their gratitude to Alison Obergrussberger and her team from Nanion Technologies GmbH for their technical assistance.

Funding sources

This research was supported by the Ministerio de Ciencia e Innovación (MICIN) and the Agencia Estatal de Investigación (AEI) (Spain) through the CELLECTRA (PID2020-112976RB-C21 and PID2020-112976RB-C22) project. J.R. acknowledges IRTA for his PhD grant (CPI0422). Andres Sanchez-Henao acknowledges the Canary Islands Research Agency (ACIISI) for his research staff training aid through the Catalina Ruiz Program (APCR2022010011). The authors also acknowledge the support from the CERCA Program/Generalitat de Catalunya.

CRediT author statement

Conceptualisation, M.C.; methodology, J.R., M.R.-A., F.X.S. and M.C.; investigation, J.R., A.S.-H., M.M. and P.P.; writing—original draft preparation, J.R. and M.C.; writing—review and editing, all authors; supervision, M.C.; project administration, M.C.; funding acquisition, F.X.S and M.C. All authors have read and agreed to the published version of the manuscript.

References

Akbora, H.D., Kunter, I., Erçetin, T., Elagöz, A.M., Çiçek, B.A., 2020. Determination of tetrodotoxin (TTX) levels in various tissues of the silver cheeked puffer fish (*Lagocephalus sceleratus* (Gmelin, 1789)) in Northern Cyprus Sea (Eastern Mediterranean). Toxicon 175, 1–6.

Akyol, O., Ceyhan, V.Ü.T., Bilecenoglu, M., 2005. First confirmed record of *Lagocephalus sceleratus* (Gmelin, 1789) in the Mediterranean Sea. J. Fish Biol. 66, 1183-1186.

Alkassar, M., Sanchez-Henao, A., Reverté, J., Lourdes, B., Rambla-Alegre, M., Leonardo, S., Mandalakis, M., Peristeraki, P., Diogène, J., Campàs, C., 2023. Evaluation of toxicity equivalency factors of tetrodotoxin analogues with a Neuro-2a cell-based assay and application to puffer fish from Greece. Mar. Drugs 21, 432.

Alkassar, M., Reverté, J., Fragoso, A., Torréns, M., Klijnstra, M., Gerssen, A., Diogène, J., Campàs, M., 2024. β-cyclodextrin polymer as tetrodotoxins scavenger in oyster extracts. Microchem. J. X, 110585.

Anastasiou, T.I., Kagiampaki, E., Kondylatos, G., Tselepides, A., Peristeraki, P., Mandalakis, M., 2023. Assessing the toxicity of *Lagocephalus sceleratus* pufferfish from the Southeastern Aegean Sea and the relationship of tetrodotoxin with gonadal hormones. Mar. Drugs 21, 520.

Bacchiocchi, S., Campacci, D., Siracusa, M., Dubbini, A., Leoni, F., Tavoloni, T., Accoroni, S., Gorbi, S., Giuliani, M.E., Stramenga, A., Piersanti, A., 2021. Tetrodotoxins (TTXs) and *vibrio alginolyticus* in mussels from central Adriatic Sea (Italy): Are they closely related? Mar. Drugs 19, 304.

Bane, V., Lehane, M., Dikshit, M., O'Riordan, A., Furey, A., 2014. Tetrodotoxin: Chemistry, toxicity, source, distribution and detection. Toxins 6, 693-755.

Bane, V., Brosnan, B., Barnes, P., Lehane, M., Furey, A., 2016. High-resolution mass spectrometry analysis of tetrodotoxin (TTX) and its analogues in puffer fish and shellfish. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 33, 1468-1489.

Bentur, Y., Ashkar, J., Lurie, Y., Levy, Y., Azzam, Z.S., Litmanovich, M., Golik, M., Gurevych, B., Golani, D., Aisenman, A., 2008. Lessepsian migration and tetrodotoxin poisoning due to *Lagocephalus sceleratus* in the eastern Mediterranean. Toxicon 52, 964-968.

Blanco, L., Lago, J., González, V., Paz, B., Rambla-Alegre, M., Cabado, A.G., 2019. Occurrence of tetrodotoxin in bivalves and gastropods from harvesting areas and other natural spaces in Spain. Toxins 11, 331.

Bordin, P., Dall'Ara, S., Tartaglione, L., Antonelli, P., Calfapietra, A., Varriale, F., Guiatti, D., Milandri, A., Dell'Aversano, C., Arcangeli, G., Barco, L., 2021. First occurrence of tetrodotoxins in bivalve mollusks from Northern Adriatic Sea (Italy). Food Control 120, 107510.

Botana, L.M., Hess, P., Munday, R., Nathalie, A., DeGrasse, L.S., Feeley, M., Suzuki, T., van den Berg, M., Fattori, V., Garrido Gamarro, E., Tritscher, A., Nakagawa, R., Karunasagar, I., 2017. Derivation of toxicity equivalency factors for marine biotoxins associated with bivalve molluscs. Trends Food Sci. Technol. 59, 15-24.

Bucciarelli, G.M., Lechner, M., Fontes, A., Kats, L.B., Eisthen, H.L., Shaffer, H.B., 2021. From poison to promise: The evolution of tetrodotoxin and its potential as a therapeutic. Toxins 13, 517.

Campàs, M., Reverté, J., Rambla-Alegre, M., Campbell, K., Gerssen, A., Diogène, J., 2020. A fast magnetic bead-based colorimetric immunoassay for the detection of tetrodotoxins in shellfish. Food Chem. Toxicol. 140, 111315.

Campàs, M., Alkassar, M., Gaiani, G., Leonardo, S., Rambla-Alegre, M., Diogène, J., 2021. The wide spectrum of methods available to study marine neurotoxins. In: Advances in Neurotoxicology. Academic Press, Cambridge, MA, USA, pp. 275–315.

Campàs, M., Reverté, J., Tudó, À., Alkassar, M., Diogène, J., Sureda, F.X., 2024. Automated patch clamp for the detection of tetrodotoxin in pufferfish samples. Mar. Drugs 22, 176.

Catterall, W.A., 2012. Voltage-gated sodium channels at 60: Structure, function and pathophysiology. J. Physiol. 590, 2577-2589.

Choudhary, G., Yotsu-Yamashita, M., Shang, L., Yasumoto, T., & Dudley, S.C.Jr., 2003. Interactions of the C-11 hydroxyl of tetrodotoxin with the sodium channel outer vestibule. Biophys. J. 84, 287–294.

Coro, G., Gonzalez Vilas, L., Magliozzi, C., Ellenbroek, A., Scarponi, P., Pagano, P., 2018. Forecasting the ongoing invasion of Lagocephalus sceleratus in the Mediterranean Sea. Ecol. Model. 371, 37-49.

Christidis, G., Mandalakis, M., Anastasiou, T.I., Tserpes, G., Peristeraki, P., Somarakis, S., 2021. Keeping *Lagocephalus sceleratus* off the table: Sources of variation in the quantity of TTX, TTX analogues, and risk of tetrodotoxication. Toxins 13, 896.

Dell'Aversano, C., Tartaglione, L., Polito, G., Dean, K., Giacobbe, M., Casabianca, S., Capellacci, S., Penna, A., Turner, A.D., 2019. First detection of tetrodotoxin and high levels of paralytic shellfish poisoning toxins in shellfish from Sicily (Italy) by three different analytical methods. Chemosphere 215, 881–892.

Fernández-Ortega, J.F., Moreales-de los Santos, J.M., Herrera-Gutiérrez, M.E., Fernández-Sánchez, V., Rodrígez Loureo, P., Alfonso Rancaño, A.A., Téllez-Andrade, A., 2010. Seafood intoxication by tetrodotoxin: first case in Europe. J. Emerg. Med. 2010, 39, 612-617.

459 Gerssen, A., Bovee, T.H.F., Klijnstra, M.D., Poelman, M., Portier, L., Hoogenboom, R.L.A.P., 2018. First
460 report on the occurrence of tetrodotoxins in bivalve mollusks in The Netherlands. Toxins, 10, 450.

461 González-Cano, R., Ruiz-Cantero, M.C., Santos-Caballero, M., Gómez-Navas, C., Tejada, M.Á., Nieto, F.R.,
462 2021. Tetrodotoxin, a Potential Drug for Neuropathic and Cancer Pain Relief? Toxins, 13, 483.

463 Guardone, L., Gasperetti, L., Maneschi, A., Ricci, E., Susini, F., Guidi, A., Armani, A., 2018. Toxic invasive
464 pufferfish (Tetraodontidae family) along Italian coasts: Assessment of an emerging public health risk. Food
465 Control 91, 330-338.

466 Guardone, L., Maneschi, A., Meucci, V., Gasperatti, L., Nucera, D., Armani, A., 2019. A global retrospective
467 study on human cases of tetrodotoxin (TTX) poisoning after seafood consumption. Food Rev. Int. 36, 645-
468 667.

469 Hong, H-.K., Kajino, N., Park, B.K., Shin, J-.S., Lee, J., Choi, K-.S., 2023. Detection of tetrodotoxin (TTX) and
470 its analogues in mud snails *Nassarius livescens* occurring on a sandy beach in Jeju Island, Korea, using
471 liquid chromatography-tandem mass spectrometry (LC-MS/MS). Fish Sci. 89, 863-873.

472 Hort, V., Arnich, N., Guérin, T., Lavison-Bompard, G., Nicolas, M., 2020. First detection of tetrodotoxin in
473 bivalves and gastropods from the French Mainland coasts. Toxins 12, 599.

474 Ikeda, K., Emoto, Y., Tatsuno, R., Wang, J.J, Ngy, L., Taniyama, S., Takatani, T., Arakawa, O., 2010.
475 Maturation-associated changes in toxicity of the pufferfish *Takifugu poecilonotus*. Toxicon 55, 289-297.

476 Itoi, S., Yoshikawa, S., Tatsuno, R., Suzuki, M., Asahina, K., Yamamoto, S., Takanashi, S., Takatani, T.,
477 Arakawa, O., Sakakura, Y., Sugita, H., 2012. Difference in the localization of tetrodotoxin between the
478 female and male pufferfish *Takifugu niphobles*, during spawning. Toxicon 60, 1000-1004.

479 Jang, J.-H., Yotsu-Yamashita, M., 2007. 6,11-dideoxytetrodotoxin from the puffer fish, *Fugu pardalis*.
480 Toxicon 50, 947–951.

481 Jang, J.-H., Lee, J.-S., Yotsu-Yamashita, M., 2010. LC/MS analysis of tetrodotoxin and its deoxy analogs in
482 the marine puffer fish *Fugu niphobles* from the Southern Coast of Korea, and in the brackishwater puffer
483 fishes *Tetraodon nigroviridis* and *Tetraodon biocellatus* from Southeast Asia. Mar. Drugs 8, 1049-1058.

484 Katikou, P., Georgantelis, D., Sinouris, N., Petsi, A., Fotaras, T., 2009. First report on toxicity assessment of
485 the Lessepsian migrant pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters (Aegean
486 Sea, Greece). Toxicon 54, 50–55.

487 Katikou, P., Gokbulut, C., Kosker, A.R., Campàs, M., Ozogul, F., 2022. An updated review of tetrodotoxin
488 and its peculiarities. Mar. Drugs 20, 47.

489 Kogure, K., Tamplin, M.L., Simidu, U., Colwell, R.R., 1988. A tissue culture assay for tetrodotoxin, saxitoxin
490 and related toxins. Toxicon 26, 191–197.

491 Kono, M., Matsui, T., Furukawa, K., Takase, T., Yamamori, K., Keneda, H., Aoki, D., Jang, J.-H., Yotsu-
492 Yamashita, M., 2008. Examination of transformation among tetrodotoxin and its analogs in the living
493 cultured juvenile puffer fish, kusafugu, *Fugu niphobles* by intramuscular administration. Toxicon 52, 714-
494 720.

495 Kosker, A.R., Özogul, F., Ayas, D., Durmus, M., Ucar, Y., Regenstein, J.M., Özogul, Y., 2016. Tetrodotoxin
496 levels in pufferfish (*Lagocephalus sceleratus*) caught in the Northeastern Mediterranean Sea. Food Chem.
497 210, 332-337.

498 Kosker, A.R., Karakus, M., Katikou, P., Dal, I., Durmus, M., Ucar, Y., Ayas, D., Özogul, F., 2023. Monthly
499 variation of tetrodotoxin levels in pufferfish (*Lagocephalus sceleratus*) caught from Antalya Bay,
500 Mediterranean Sea. Mar. Drugs 21, 527.

501 Knutsen, H.K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Caccatelli, S., Cottrill, B., Dinovi,
502 M., Elder, L., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L.R., Knutsen, K.H, Nebbia, C.S., Oswald, I.P.,
503 Petersen, A., Rogiers, V.M., Rose, M., Roudot, A.C., Schwerdtle, T., Vleminckx, C., Vollmer, G., Wallace, H.,

2017. Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. EFSA J. 15, 4752.

Kudo, Y., Finn, J., Fukushima, K., Sakugawa, S., Cho, Y., Konoki, K., Yotsu-Yamashita, M., 2014. Isolation of 6-deoxytetrodotoxin from the pufferfish, *Takifugu pardalis*, and a comparison of the effects of the C-6 and C-11 hydroxy groups of tetrodotoxin on its activity. J. Nat. Prod. 77, 1000–1004.

Leão, J.M., Lozano-Leon, A., Giráldez, J., Vilariño, Ó., Gago-Martínez, A., 2018. Preliminary results on the evaluation of the occurrence of tetrodotoxin associated to marine Vibrio spp. in bivalves from the Galician Rias (Northwest of Spain). Mar. Drugs, 16, 81.

Leonardo, S., Kiparissis, S., Rambla-Alegre, M., Almarza, S., Roque, A., Andree, K.B., Christidis, A., Flores, C., Caixach, J., Campbell, K., Elliott, C.T., Aligizaki, K., Diogène, J., Campàs, M., 2019. Detection of tetrodotoxins in juvenile pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from the North Aegean Sea (Greece) by an electrochemical magnetic bead-based immunosensing tool. Food Chem. 290, 255-262.

Magarlamov, T.Y., Melnikova, D.I., Chernyshev, A.V., 2017. Tetrodotoxin-producing bacteria: Detection, distribution and migration of the toxin in aquatic systems. Toxins 9, 166.

Malloggi, C., Rizzo, B., Giusti, A., Guardone, L., Gasperetti, L., Dall' Ara, S., Armani, A., 2023. Accidental discovery of a Tetraodontidae (*Sphoeroides marmoratus*) within a cuttlefish (*Sepia officinalis*) bought in a fish shop in Italy: risk assessment associated with the presence of tetrodotoxin. Ital. J. Food Saf. 12, 11117.

Manger, R.L., Leja, L.S., Lee, S.Y., Hungerforg, J.M., Wekell, M.M., 1993. Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Anal. Biochem. 214, 190–194.

Manger, R.L., Leja, L.S., Lee, S.Y., Hungerford, J.M., Hokama, Y., Dickey, R.W., Granade, H.R., Lewis, R., Yasumoto, T., Wekell, M.M., 1995. Detection of sodium channel toxins: Directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts. J. AOAC Int. 78, 521–527.

Nieto, F.R., Cobos, E.J., Tejada, M.Á., Sánchez-Fernández, C., González-Cano, R., Cendán, C.M., 2012. Tetrodotoxin (TTX) as a therapeutic agent for pain. Mar. Drugs, 10, 281-305.

Okita, K., Yamazaki, H., Sakiyama, K., Yamane, H., Niina, S., Takatani, T., Arakawa, O., Sakakura, Y., 2013. Puffer smells tetrodotoxin. Ichthyol. Res. 60, 386-389.

Park, B.K., Lee, H., Kim, I., Choi, K-.S., Lee, W-.O., Kajino, N., Hong, H-.K., Lee, H-.S., Choi, C., Lee, J., 2024. Development and validation of an LC-MS/MS method for determination of tetrodotoxin and its analogues in various pufferfish tissues and its application in Korean pufferfish. J. Food Compos. Anal. 125, 105816.

Rambla-Alegre, M., Reverté, L., del Río, V., de la Iglesia, P., Palacios, O., Flores, C., Caixach, J., Campbell, K., Elliott, C.T., Izquierdo-Muñoz, A., Campàs, M., Diogène, J., 2017. Evaluation of tetrodotoxins in puffer fish caught along the Mediterranean coast of Spain. Toxin profile of *Lagocephalus sceleratus*. Environ. Res. 158, 1–6.

Rambla-Alegre, M., Leonardo, S., Barguil, Y., Flores, C., Caixach, J., Campbell, K., Elliott, C., Maillaud, C., Boundy, M.J., Harwood, D.T., Campàs, M., Diogène, J., 2018. Rapid screening and multi-toxin profile confirmation of tetrodotoxins and analogues in human body fluids derived from a pufferfish poisoning incident in New Caledonia. Food Chem. Toxicol. 112, 188-193.

Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organization of official controls on products of animal origin intended for human consumption. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32004R0854 (accessed on 27 March 2024).

Regulation (EC) No 15/2011 of the Commission Regulation of 10 January 2011 as regards recognized testing methods for detecting marine biotoxins in live bivalve molluscs. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32011R0015 (accessed on 27 March 2024).

549 Réveillon, D., Savar, V., Schaefer, E., Chevé, J., Halm-Lemeille, M.-P., Hervio-Heath, D., Travers, M.-A.,
550 Abadie, E., Rolland, J.-L., Hess, P., 2021. Tetrodotoxins in French bivalve mollusks-analytical methodology,
551 environmental dynamics and screening of bacterial strain collections. Toxins 13, 740.

552 Reverté, L., de la Iglesia, P., del Río, V., Campbell, K., Elliott, C.T, Kawatsu, K., Katikou, P., Diogène, J.,
553 Campàs, M., 2015. Detection of tetrodotoxins in puffer fish by a self-assembled monolayer-based
554 immunoassay and comparison with surface plasmon resonance, LC-MS/MS and mouse bioassay. Anal.
555 Chem. 87, 10839-10847.

556 Reverté, L., Campbell, K., Rambla-Alegre, M., Elliott, C.T., Diogène, J., Campàs, M., 2017. Immunosensor
557 array platforms based on self-assembled dithiols for the electrochemical detection of tetrodotoxins in
558 puffer fish. Anal. Chim. Acta 989, 95-103.

559 Reverté, L., Rambla-Alegre, M., Leonardo, S., Bellés, C., Campbell, K., Elliott, C.T., Gerssen, A., Klijnstra,
560 M.D., Diogène, J., Campàs, M., 2018. Development and validation of a maleimide-based enzyme-linked
561 immunosorbent assay for the detection of tetrodotoxin in oysters and mussels. Talanta 176, 659-666.

562 Reverté, J., Alkassar, M., Diogène, J., Campàs, M., 2023. Detection of ciguatoxins and tetrodotoxins with
563 biosensors and other smart bioanalytical systems. Foods 12, 2043.

564 Rodriguez, P., Alfonso, A., Vale, C., Alfonso, C., Vale, P., Tellez, A., Botana, L.M., 2008. First toxicity report
565 of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell *Charonia lampas lampas* in Europe. Anal.
566 Chem. 80, 5622–5629.

567 Rodriguez, P., Alfonso, A., Otero, P., Katikou, P., Georgantelis, D., Botana, L.M., 2012. Liquid
568 chromatography-mass spectrometry method to detect tetrodotoxin and its analogues in the puffer fish
569 *Lagocephalus sceleratus* (Gmelin, 1789) from European Waters. Food Chem. 132, 1103-111.

570 Satake, Y., Adachi, M., Tokoro, S., Yotsu-Yamashita, M., Isobe, M., Nishikawa, T., 2014. Synthesis of 5- and
571 8-deoxytetrodotoxin. Chem. Asian J. 9, 1922-1932.

572 Silva, M., Azevedo, J., Rodriguez, P., Alfonso, A., Botana, L.M., Vasconcelos, V., 2012. New Gastropod
573 Vectors and Tetrodotoxin Potential Expansion in Temperate Waters of the Atlantic Ocean. Mar. Drugs 10,
574 712–726.

575 Tinacci, L., Malloggi, C., Giusti, A., Galli, F., Dall'Ara, S., Armani, A., 2023. Seafood cross-contamination by
576 tetrodotoxin (TTX): management of an unusual route of exposure. J. Consum. Prot. Food Saf. 19, 119-122.

577 Tsukamoto, T., Chiba, Y., Wakamori, M., Yamada, T., Tsunogae, S., Cho, Y., Sakakibara, R., Imazu, T.,
578 Tokoro, S., Satake, Y., Adachi, M., Nishikawa, T., Yotsu-Yamashita, M., Konoki K, 2017. Differential binding
579 of tetrodotoxin and its derivatives to voltage-sensitive sodium channel subtypes (Na_v 1.1 to Na_v 1.7). Br.
580 J. Pharmacol. 174, 3881-3892.

581 Turner, A.D., Powell, A., Schofield, A., Lees, D.N., Baker-Austin, C., 2015. Detection of the pufferfish toxin
582 tetrodotoxin in European bivalves, England, 2013 to 2014. Eurosurveillance 20, 21009.

583 Tukker, A.M., Vrolijk, M.F., van Kleef, R., Sihm, D., Westerink, R.H.S., 2023. Mixture effects of tetrodotoxin
584 (TTX) and drugs targeting voltage-gated sodium channels on spontaneous neuronal activity *in vitro*.
585 Toxicol. Lett. 373, 53-61.

586 Ueyama, N., Sugimoto, K., Kudo, Y., Onodera, K-I., Yuku, C., Konoki, K., Nishikawa, T., Yotsu-Yamashita,
587 M., 2018. Spiro bicyclic guanidino compounds from pufferfish: Possible biosynthetic intermediates of
588 tetrodotoxin in marine environments. Chem. Eur. J. 24, 7250-7258.

589 Ulman, A., Abd Rabou, A.F.N., Al Mabruk, S., Bariche, M., Bilecenoğlu, M., Demirel, N., Galil, B.S.,
590 Hüseyinoğlu, M.F., Jimenez, C., Hadjioannou, L., Kosker, A.R., Peristeraki, P., Saad, A., Samaha, Z.,
591 Stoumboudi, M.T., Temraz, T.A., Karachle, P.K., 2024. Assessment of human health impacts from invasive
592 pufferfish (attacks, poisonings and fatalities) across the Eastern Mediterranean. Biology, 13, 208.

593 Vlamis, A., Katikou, P., Rodriguez, I., Rey, V., Alfonso, A., Papazachariou, A., Zacharaki, T., Botana, A.M.,
594 Botana, L.M., 2015. First detection of tetrodotoxin in Greek shellfish by UPLC-MS/MS potentially linked to
595 the presence of the dinoflagellate *Prorocentrum minimum*. Toxins 7, 1779–1807.

596 Wang, J., Araki, T., Tatsuno, R., Nina, S., Ikeda, K., Hamasaki, M., Sakakura, Y., Takatani, T., Arakawa, O.,
597 2011. Transfer profile of intramuscularly administered tetrodotoxin to artificial hybrid specimens of
598 pufferfish, *Takifugu rubripes* and *Takifugu niphobles*. Toxicon 58, 565-569.

599 Williams, B.L., 2010. Behavioral and chemical ecology of marine organisms with respect to tetrodotoxin.
600 Mar. Drugs 8, 381-398.

601 Wood, S.A., Casas, M., Taylor, D.I., McNabb, P. Salvitti, L., Ogilvie, S., Cary, S.C., 2012. Depuration of
602 tetrodotoxin and changes in bacterial communities in Pleurobranchea maculata adults egg masses
603 maintained in captivity. J. Chem. Ecol. 38, 1342-1350.

604 Yasumoto, T., Yotsu, M., Murata, M., 1988 New tetrodotoxin analogues from the newt *Cynops ensicauda*.
605 J. Am. Chem. Soc. 110, 2344–2345.

606 Yotsu, M., Hayashi, Y., Khora, S.S., Sato, S., Yasumoto, T., 1992. Isolation and structural assignment of 11-
607 nortetrodotoxin-6(*S*)-ol from the puffer *Arothron-nigropunctatus*. Biosci. Biotechnol. Biochem. 56, 370–
608 371.

609 Yotsu-Yamashita, M., Yamagishi, Y., Yasumoto, T., 1995. 5,6,11-Trideoxytetrodotoxin from the puffer fish,
610 *Fugu poecilonotus*. Tetrahedron Lett. 36, 9329–9332.

611 Yotsu-Yamashita, M., Sugimoto, A., Takai, A., Yasumoto, T., 1999. Effects of specific modifications of
612 several hydroxyls of tetrodotoxin on its affinity to rat brain membrane. J. Pharmacol. Exp. Ther. 289, 1688-
613 1696.

Supplementary material

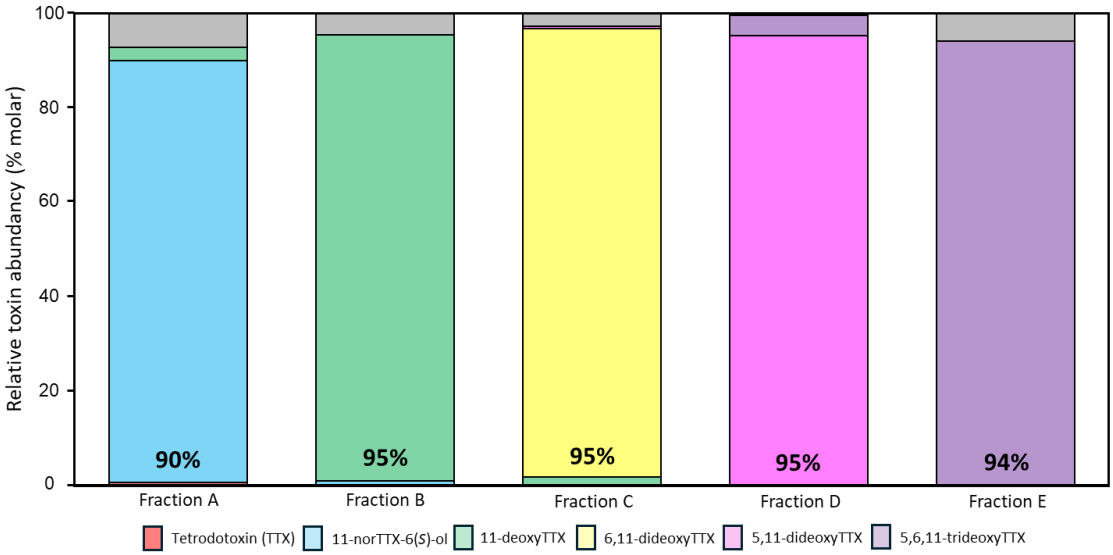


Figure S1. Relative abundance of TTX and TTX analogues in the different fractions used in this work (A-E), which have been obtained from the fractionation of a liver of a pufferfish (PF2). The TTX analogues other than TTX, 11-norTTX-6(*S*)-ol, 11-deoxyTTX, 6,11-dideoxyTTX, 5,11-dideoxyTTX or 5,6,11-trideoxyTTX have not been individually detailed but they have been expressed as a set in the toxin profile of the fractions (grey bars).

[Colour should be used for Figure S1 in print]

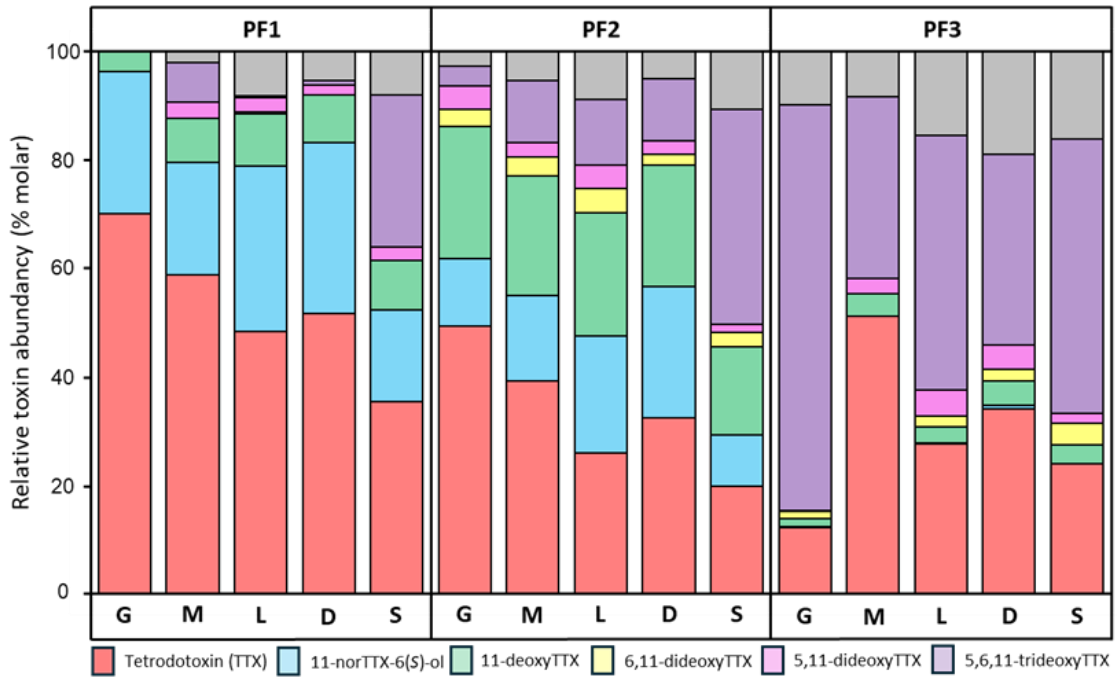


Figure S2. Toxin profile of the gonads (G), skeletal muscle (M), liver (L), digestive tract (D) and skin (S) of three pufferfishes from Greece (PF1, PF2 and PF3). The analogues with unknown TEFs have not been individually detailed but they have been expressed as a set in the toxin profile (grey bars).

[Colour should be used for Figure S2 in print]

Table S1. Concentration of tetrodotoxins (TTXs) in five tissues (gonads (G), skeletal muscle (M), liver (L), digestive tract (D) and skin (S)) from three *Lagocephalus sceleratus* pufferfish (PF1, PF2 and PF3) after application of TEFs to LC-MS/MS measurements of the individual TTX analogues. The concentrations are expressed as µg TTX equiv./kg of pufferfish tissue.

Sample		LC-MS/MS	
		∑ [TTXs] $TEF_{unknown} = 0$	∑ [TTXs] $TEF_{unknown} = 1$
PF1	G	1631	1631
	M	5063	5063
	L	23,893	25,018
	D	22,127	22,622
	S	6018	6021
PF2	G	6022	6025
	M	17,386	17,682
	L	69,254	73,297
	D	87,300	88,921
	S	16,179	16,529
PF3	G	176,353	180,008
	M	6422	6422
	L	17,380	17,884
	D	82,469	85,700
	S	9408	9485

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.