Chemico-Biological Interactions 424 (2026) 111871

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Research paper

Mechanistic insights into antibody recognition of tetrodotoxin analogues: Implications for neurotoxicological assessment

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

[a] IRTA, Marine and Continental Waters (AMiC), 43540, La Ràpita, Catalonia, Spain
[b] Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili (URV), 43201, Reus, Spain
[c] University Institute of Animal Health and Food Safety (IUSA), University of Las Palmas de Gran Canaria, 35413, 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

ARTICLE INFO

Keywords:
Tetrodotoxin (TTX)
TTX analogues
Cross-reactivity
Immunoassay
Toxicity neutralisation

ABSTRACT

Tetrodotoxins (TTXs) pose significant food safety risks due to their potent neurotoxicity. Growing concerns about the impact of these toxins on public health have driven the development of new detection methods, with immunoassays showing strong potential. However, limited knowledge of the cross-reactivity of anti-TTX antibodies with analogues may compromise the reliability of these assays in food safety applications. To address this, cross-reactivity factors (CRFs) for five TTX analogues (i.e., 11-norTTX-6(*S*)-ol, 11-deoxyTTX, 6,11-dideoxyTTX, 5,11-dideoxyTTX, and 5,6,11-trideoxyTTX) were assessed using a magnetic bead-based immunoassay. In parallel, the antibody's ability to neutralise the toxicity of TTX analogues was evaluated in Neuro-2a cells using automated patch clamp, a single-cell biosensing platform specifically designed for *in vitro* toxicity assessment and characterisation. Antibody cross-reactivity towards the tested analogues correlated with their relative toxicity, enabling a selective detection of the most hazardous compounds. These findings highlight the dual role of molecular structure in dictating both toxicological potency and immunological recognition, and support the use of immunoassays as effective tools for TTX monitoring in food safety applications.

1. Introduction

Tetrodotoxin (TTX) is one of the most potent neurotoxins found in the marine environment [1]. The presence of this toxin has been reported in a wide variety of organisms across multiple phyla and trophic levels such as fish, gastropods, newts, sea slugs, star fishes, blue-ringed octopuses, and ribbon worms, among others [2], supporting the hypothesis of an exogenous origin, potentially linked to bacterial symbionts [3]. However, the metabolic pathway involved in the biosynthesis of TTX and its biotransformation within hosts remains largely unclear [4,5]. What is certain about TTX is that it plays an important ecological role, offering a defense mechanism against predators and conferring other competitive advantages to TTX-bearing organisms within their ecological niches [6]. However, from a food safety perspective, its presence in seafood poses a significant risk that, if not properly managed, can lead to serious public health concerns [7].

The primary biological target of TTX is the voltage-gated sodium channel (VGSC), a membrane receptor that is highly expressed in excitable cells and plays a crucial role in neuronal information transmission and motor coordination [8]. Due to its high specificity for VGSCs, TTX can effectively block the influx of sodium ions (Na^+) into the cytoplasm in a dose dependent manner [9]. This disruption on the natural function of VGSCs interferes with the generation and propagation of action potentials, impairing neuronal signaling and muscle contraction [10]. At extremely low doses, this effect has been shown to be beneficial in some specialised medical applications and particularly in pain management [11,12]. However, when ingested at high doses, TTX can cause poisoning, with symptoms ranging from mild numbness and motor dysfunction to more severe conditions, such as respiratory failure, cardiac arrest, and, in extreme cases, death [10].

Most TTX poisoning cases have been reported in Asia, particularly in Japan [2]. This is primarily due to the consumption of pufferfish, which

* Corresponding author.
E-mail address: monica.campas@irta.cat (M. Campàs).

https://doi.org/10.1016/j.cbi.2025.111871
Received 3 October 2025; Received in revised form 19 November 2025; Accepted 9 December 2025
Available online 10 December 2025

are known to be one of the main natural carriers of this toxin [10]. Certain toxic pufferfish species, such as *Lagocephalus sceleratus*, endemic to tropical and subtropical areas, have established themselves as persistent invasive species in more temperate regions, including the Mediterranean Sea [13]. Although the trade of these fishes in Europe has been banned due to the risks they pose to food safety and human health [14], their presence in Mediterranean waters remains a concern [15]. In addition to pufferfish, shellfish has emerged as another significant carrier of TTXs in Europe [16], with its consumption linked to the first documented TTX poisoning case in the region [17,18].

In natural samples, more than 30 TTX analogues have been identified, all sharing the same core structure as TTX but differing in the presence or absence of specific functional groups at certain positions [10]. These structural variations can significantly alter the physicochemical properties of the analogues and influence their toxicity by either enhancing or reducing their interaction with VGSCs [9]. Recent studies on the toxicity equivalency factors (TEFs) of TTX analogues, assessed using cell-based assay (CBA) and automated patch clamp (APC), have revealed that while most of these chemical variants are less toxic than TTX, they can still pose a poisoning risk depending on their concentration [9,19]. Therefore, their presence in seafood warrants careful monitoring and should not be overlooked.

Currently, instrumental analysis techniques, such as liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), are the only methods capable of quantifying the individual TTX analogues in a sample providing details on the specific profile as regards TTX analogues [20]. These techniques can achieve high specificity by detecting distinct chemical features that are unique to the molecule of each compound. In contrast, structural methods like immunoassays rely on biorecognition elements that target specific regions of the toxin molecule, which may vary across the different TTX analogues. While the effectiveness of immunoassays for detecting TTX is well established [21], the ability of antibodies to recognise various TTX analogues remains highly unexplored and only a handful of groups have been working on this direction [22–24]. This represents a significant limitation in the applicability of these immunoassays, as the potential failure of the antibody to detect toxic TTX analogues in a sample can lead to false-negative results, ultimately underestimating the risk of poisoning.

To address this issue, the cross-reactivity factors (CRFs) of an anti-TTX antibody towards some TTX analogues purified from the liver of a pufferfish were evaluated using a magnetic bead (MB)-based immunoassay. Furthermore, the capacity of the antibody of neutralising the toxicity of TTX and some analogues on Neuro-2a cells was assessed using an APC system. These approaches will allow us to compare antibody cross-reactivity with the known toxicological properties of different TTX analogues, in order to evaluate whether the immunoassay can provide meaningful insights into the overall toxic potential of TTXs in a sample.

2. Materials and methods

2.1. Reagents

Tetrodotoxin (98 % purity by HLPC) was purchased from Tocris Bioscience (Bristol, UK) and a standard solution was prepared at 1 mg/mL in 1 % (*v/v*) acetic acid. The anti-TTX monoclonal antibody (CABT-L3089) was obtained from Creative Diagnostics (Deltaclon S.L., Madrid, Spain). PureCube maleimide-activated MagBeads were obtained from Cube Biotech (Monheim, Germany). Cysteamine hydrochloride, formaldehyde solution, potassium phosphate dibasic, potassium phosphate monobasic, Tween®−20, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), anti-mouse IgG (whole molecule)-horseradish peroxidase antibody (IgG-HRP) (produced in rabbit) and 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate and Accutase® solution were supplied by Sigma-Aldrich (Tres Cantos, Spain). Murine neuroblastoma (Neuro-2a) cells were purchased from ATCC LGS standards (Manassas, VA, USA). Foetal bovine serum (FBS), penicillin/streptomycin solution, Roswell Park Memorial Institute (RPMI-1640) medium and sodium pyruvate were purchased from Merck KGaA (Darmstadt, Germany). The internal 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 external solution (50 mM CsCl, 10 mM NaCl, 60 mM CsF, 20 mM EGTA, 10 mM HEPES/CsOH, pH 7.2) and the enhancer solution (10 mM HEPES, 130 mM NaCl, 5 mM glucose, 4 mM KCl, 10 mM $MgCl_2$, pH 7.4) were obtained from Nanion Technologies GmbH (Munich, 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 captured in the Libyan Sea (South Crete, Greece) in May (PF1, PF2) and March (PF3) 2019, through scientific experimental fishing conducted by scientists of the Hellenic Centre for Marine Research (HCMR) under the framework of the Greek National Fisheries Data Collection Framework (DCF), which is an officially approved national and EU-supported program. The fish were collected using standard fishing gears, in accordance with relevant national laws and HCMR guidelines.

The fish were dissected and their tissues were separated into skeletal muscle (M), skin (S), liver (L), digestive tract (D) and gonads (G). The TTXs were extracted following the protocol described by Reverté and co-workers [23]. Briefly, 10 g of pufferfish tissue (M, S, L, D or G) was weighed into tubes and homogenised using an Ultraturrax blender at full speed. In each tube, 25 mL of 0.1 % (*v/v*) acetic acid was added and vortexed for 2 min at 2500 rpm. Subsequently, the tubes were placed in a boiling water bath for 10 min with occasional stirring, then cooled for 5 min at room temperature and centrifuged at 2500 rpm for 5 min at 4 °C. The supernatants were collected and a second extraction with additional 20 mL of 0.1 % (*v/v*) acetic acid was performed. The two supernatants were pooled, and the final volume was adjusted to 50 mL with 0.1 % (*v/v*) acetic acid. For liver samples, an additional liquid-liquid partition of the crude extract with hexane (1:1) was required to remove fats. The extracts, with a pufferfish tissue concentration of 200 mg/mL, were filtered through 0.45-μm nylon syringe filters and stored at −20 °C until further use.

2.3. Isolation and purification of tetrodotoxin analogues

The TTX analogues used in this work were obtained from a recent work of our group [19], where 30 g of the PF2 liver tissue was used as a source of TTXs. The liver extract was fractionated using hydrophilic interaction chromatography (HILIC) on a preparative Luna HILIC AXIA column (250 mm × 21.2 mm, 5 μm particle size; Phenomenex, Torrance, CA, USA). The setup included an LC pump system controller (Waters Corp. Milford, MA, USA) coupled to a photodiode array detector (PDA) 996 (Waters Corp.) and a fraction collector FRAC-100 (Pharmacia Biotech, Uppsala, Sweeden). Briefly, the concentrated liver extract was injected in 1 mL portions onto the preparative HILIC column and eluted under a binary gradient of acetonitrile and water containing ammonium acetate. The highly organic starting conditions enabled strong retention of TTX analogues, which were then gradually eluted as the aqueous proportion increased. Fractions of 10 mL were collected every minute throughout each chromatographic run, and equivalent fractions from ten consecutive runs were pooled to increase the overall yield. This procedure allowed the separation of groups of TTX analogues according to their polarity. The composition of all obtained fractions was characterised by LC-MS/MS. The detailed parameters for both fractionation and LC-MS/MS analysis are described in the study by Alkassar and co-workers [19].

2.4. Magnetic bead-based immunoassay

The MB-based immunoassay was performed following the protocol

described by Campàs and co-workers [25]. Initially, the MB-TTX conjugates were prepared: 10 μL of maleimide-activated MBs were transferred to a 1-mL tube and rinsed three times with 1 mL of washing buffer (0.1 M phosphate buffered saline (PBS), 0.05 % (*v/v*) Tween®−20, pH 7.2). For the washing steps, the tube was placed on the magnetic separation stand and the washing solution was removed. Then, 1 mL of 1 mM cysteamine in binding buffer (0.1 M PBS, 10 mM EDTA, pH 7.2) was added and incubated for 2 h at room temperature. After three washing steps, 1 mL of TTX solution (25 μg/mL) in binding buffer containing 10 % (*v/v*) formaldehyde was added and incubated overnight at 4 °C. Finally, three washing steps were performed and the TTX-coated MBs were resuspended in 1 mL of binding buffer. For the immunoassay: 200 μL of the MB-TTX conjugate was transferred to a 0.5-mL tube and placed on the magnetic separation stand to remove the supernatant. Then, 100 μL of TTX standard, TTX analogue or pufferfish extract, along with 100 μL of anti-TTX mAb at 1/2000 dilution in binding buffer containing 1 % (*w/v*) BSA (binding buffer-BSA) were added and incubated for 30 min at room temperature. For the determination of CRFs, a range of TTX and TTX analogue concentrations from 10,000–1 nM was tested. With regard to the analysis of pufferfish samples, six serial dilutions of each extract, from 20 to 0.6 mg tissue equivalents (TE)/mL, were evaluated. After three washing steps, 200 μL of 1/1000 IgG-HRP dilution in binding buffer-BSA was added and incubated for another 30 min at room temperature. Finally, the immunocomplex was washed three times and resuspended in 200 μL of binding buffer. For colorimetric measurements: 50 μL of immunocomplex was transferred to a new 0.5-mL tube and the supernatant was removed. Then, 125 μL of TMB liquid substrate was added and incubated for 10 min. Subsequently, the tube was placed on the magnetic separation stand and 100 μL of the TMB liquid substrate was collected for absorbance measurement at 620 nm using a microplate reader (Agilent® BioTek Synergy LX multi-mode reader). Measurements were performed in triplicate and incubation steps were performed under continuous agitation.

2.5. Automated patch clamp

In the toxicity neutralisation experiments, changes in VGSC currents from Neuro-2a cells upon exposure to TTX, TTX analogues or pufferfish samples pre-incubated with an anti-TTX antibody were evaluated using a Patchliner APC device (Nanion Technologies GmbH, Munich, Germany). The experimental APC analysis details for the detection of TTX were described by Campàs and co-workers [26]. 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. For the preparation of the cell suspension, the cells were detached from the culture flask with Accutase® solution. A single cell was immobilised on the hole located at the bottom of each well with a holding potential of – 30 mV. After adding the enhancer solution, the potential was changed to −100 mV. Successful patching was confirmed by achieving a stable sealing with resistances higher than 1 GΩ. The VGSC currents were measured by applying voltage increments of 10 mV from −80 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). Measurements were performed in quadruplicate at a minimum. 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 the cells used in this work were between passage 245 and 255.

3. Results and discussion

3.1. Cross-reactivity of the antibody with tetrodotoxin analogues

The cross-reactivity of the antibody towards several TTX analogues was investigated using the MB-based immunoassay, where the antibody was incubated with different concentrations of TTX analogues in the presence of TTX-coated MBs. Under this competitive indirect immunoassay format, the higher the affinity of the antibody for a given TTX analogue, the less antibody will bind to the TTX-coated MBs, resulting in low colorimetric signal. The dose-response curves obtained for TTX and five of its analogues (i.e., 11-norTTX-6(*S*)-ol, 11-deoxyTTX, 6,11-dideoxyTTX, 5,11-dideoxyTTX and 5,6,11-trideoxyTTX) are displayed in Fig. 1. At present, commercially available standards for TTX analogues do not exist. Therefore, we isolated these analogues from the liver of a pufferfish using a fractionation process [19]. The purity of the resulting fractions, defined as the percentage of the main TTX analogue relative to the total content of TTX analogues in each fraction, ranged from 90 % to 95 %. This level of purity was considered adequate for evaluating cross-reactivity, as TTX itself was not the predominant contaminant in any of the fractions used (never higher than 1 %). The composition of the fractions used in this work has been detailed in a previous work of our group [9].

The half-maximal inhibitory concentrations (IC_{50}) of TTX and its analogues were derived from the dose-response curve equations and served as a measure of the affinity of the antibody towards the different TTX analogues. These values were then used to calculate the CRFs, defined as the ratio of the IC_{50} value of TTX to that of each analogue (Table 1).

All TTX analogues were successfully detected with the immunoassay. However, the affinity of the antibody towards the different TTX analogues was lower than that of TTX (CRF < 1). Among the TTX analogues investigated, 11-norTTX-6(*S*)-ol exhibited the highest cross-reactivity, followed by 11-deoxyTTX, 6,11-dideoxyTTX, 5,11-dideoxyTTX and 5,6,11-trideoxyTTX (Fig. 2). The absence of the hydroxymethyl group at C-6 position in 11-norTTX-6(*S*)-ol or of the hydroxyl group at C-11 position in 11-deoxyTTX, in comparison to the TTX molecule, resulted in a reduction of antibody cross-reactivity by approximately 90 % and 94 %, respectively. This decrease in cross-reactivity was even more pronounced in analogues with greater structural divergence from TTX (i.e.,

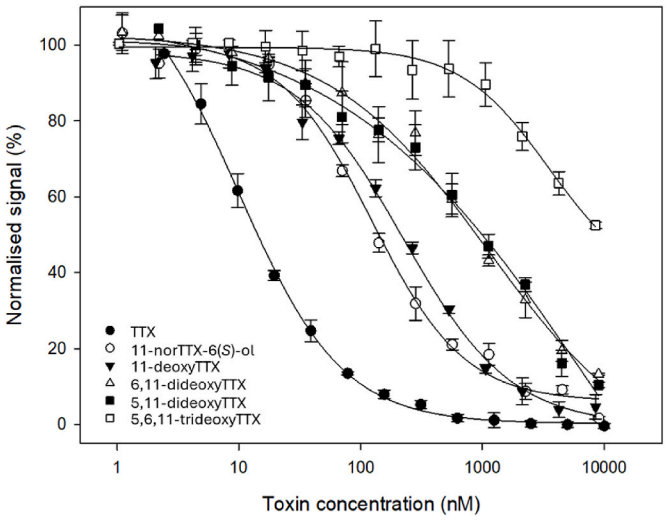

Fig. 1. Dose-response curves obtained from the analysis of TTX and TTX analogues with the MB-based colorimetric immunoassay. All data were background subtracted and normalised with respect to the signal obtained when no toxin is present (maximum signal of the assay). The data set of each individual TTX analogue was fit to a sigmoidal logistic four-parameter equation. Each point represents the average ± standard deviation (n = 3).

Table 1
IC_{50} values and CRFs for TTX and TTX analogues determined with the MB-based colorimetric immunoassay. The TEFs obtained with the same TTX analogues fractions used in this work for the characterisation of the CRFs are shown.

Toxin	IC_{50} (nM)	CRF	TEF[a]
TTX	14.7	1	1
11-norTTX-6(*S*)-ol	142	0.103	0.238
11-deoxyTTX	216	0.068	0.107
6,11-dideoxyTTX	897	0.016	0.035
5,11-dideoxyTTX	993	0.015	0.027
5,6,11-trideoxyTTX	9948	0.001	0.001

[a] Data from Reverté et al., 2024.

6,11-dideoxyTTX and 5,11-dideoxyTTX with two modifications, and 5,6,11-trideoxyTTX with three modifications). These findings suggest that the antibody recognises TTXs based on a tightly confined region of the molecule centred around positions C-11, C-6 and C-5 (Fig. 2). This specific region is also of particular importance for the toxicity of those compounds, as it has been proposed to modulate their interaction with VGSCs [9]. Importantly, the exact same fractions used in this study to characterise CRFs were also employed to assess TEFs in a previous work [9]. Although these fractions are not certified reference standards, their consistent origin ensures the direct comparability of CRF and TEF values. Notably, the CRFs determined in this study follow the same trend as the TEFs reported by Reverté and co-workers [9] (Table 1), and the toxicities observed are consistent with previously published data [19, 27–31]. These findings suggest that the MB-based immunoassay may detect TTX analogues in proportion to their relative toxicity. However, as only five TTX analogues have been evaluated and even though more than thirty have been identified in nature, further research is needed to confirm and extend these observations. For example, some studies have reported that 11-oxoTTX is more toxic than TTX [32], yet it has shown low cross-reactivity with anti-TTX antibodies [24]. Including this analogue in the present study would therefore have been particularly valuable. However, 11-oxoTTX was not detected in any of our pufferfish samples and could not be purified. Therefore, the inclusion of 11-oxoTTX and other analogues in future studies combining TEFs and CRFs would be important to fully assess the potential of the MB-based immunoassay to estimate the toxicity of samples containing TTXs.

Notably, this is the first time that CRFs for 11-deoxyTTX, 6,11-dideoxyTTX, and 5,11-dideoxyTTX have been elucidated. The available literature on CRFs of TTX analogues is somewhat limited and fragmented, which makes it challenging to compare our results with those of previous studies. Nevertheless, our findings are generally well aligned with previously reported data (Table 2). It is important to note that the cross-reactivity of a specific biorecognition element toward its target can be significantly influenced by the configuration of the assay [23]. This is due to the fact that a proper three-dimensional arrangement of immunoassay components is essential for the immunorecognition to take place. Therefore, it is not surprising that the CRFs of the examined analogues show some deviation from literature data, given the differences in immunoassay supports, immobilisation strategies and/or detection principles across studies. For example, the CRF obtained for 11-norTTX-6(*S*)-ol with the MB-based immunoassay was 3.5-fold higher than the value reported by Reverté and co-workers [23], who used maleimide microplates instead of maleimide MBs as the immobilisation support (Table 2). Given that all other parameters of the immunoassay protocol were identical in both works, this difference in cross-reactivity may stem from the vigorous agitation applied during incubation in the MB-based immunoassay. Such agitation likely promotes a more effective three-dimensional arrangement of the reagents, improving the kinetics and sensitivity of the assay [25,33]. A similar increase in cross-reactivity was observed for 5,6,11-trideoxyTTX when using maleimide MBs instead of maleimide plates. The impact that other protocol variations may have on the affinity of the antibody for TTX analogues is difficult to discern. In fact, the CRFs reported so far are certainly influenced by the interplay of several immunoassay parameters. Additionally, as mentioned above, the TTX analogues used in this study were obtained through the fractionation of a fish extract. Therefore, the presence of trace amounts of co-eluting analogues may have had an impact on results. This potential influence could vary between studies, depending on differences in the composition of the original sample used as a source of TTX analogues, the purity of the fractions or how fractionation was conducted. What is undeniable is that the CRFs resulting from a given bioanalytical method are inherently dependent on the assay configuration and should not be considered universal.

3.2. Analysis of tetrodotoxin mixtures with the immunoassay

The impact of the varying cross-reactivities of the antibody towards the TTX analogues on the efficacy of the MB-based immunoassay was further evaluated by analysing laboratory-prepared mixtures of TTX, 11-

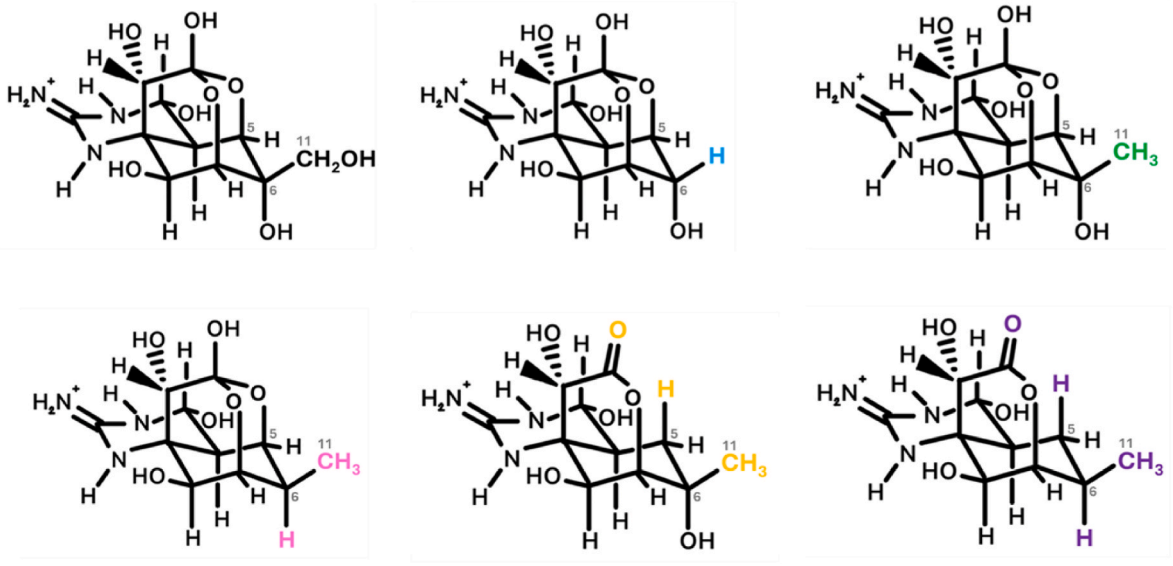

Fig. 2. Structural differences between TTX and its analogues, including: 11-norTTX-6(*S*)-ol (blue), 11-deoxyTTX (green), 6,11-dideoxyTTX (pink), 5,11-dideoxyTTX (yellow) and 5,6,11-trideoxyTTX (purple).

Table 2
Comparison of CRFs of TTX analogues derived from different immunoanalytical approaches based on competitive indirect assay configurations.

TTX Analogue	CRF	Method				Reference
		TTX Immobilisation			Detection	
		Support	Strategy	Disposition		
11-norTTX-6(*S*)-ol	0.103	Maleimide MB	Chemical: Cysteamine, Formaldehyde	Oriented	Colorimetry	This work
	0.179	CM5 Biacore chip	Chemical: EDC/NHS, Ethylenediamine	Oriented	SPR	[23]
	0.029	Maleimide plate	Chemical: Cysteamine, Formaldehyde	Oriented	Colorimetry	[23]
	0.002	Bare plastic plate	Physical: Adsorption (TTX-Ovalbumin)	Random	Colorimetry	[24]
11-deoxyTTX	0.068	Maleimide MB	Chemical: Cysteamine, Formaldehyde	Oriented	Colorimetry	This work
6,11-dideoxyTTX	0.016	Maleimide MB	Chemical: Cysteamine, Formaldehyde	Oriented	Colorimetry	This work
5,11-dideoxyTTX	0.015	Maleimide MB	Chemical: Cysteamine, Formaldehyde	Oriented	Colorimetry	This work
5,6,11-trideoxyTTX	0.001	Maleimide MB	Chemical: Cysteamine, Formaldehyde	Oriented	Colorimetry	This work
	0.022	Bare plastic plate	Physical: Adsorption (TTX-Ovalbumin)	Random	Colorimetry	[24]
	NCR	Maleimide plate	Chemical: Cysteamine, Formaldehyde	Oriented	Colorimetry	[23]
	NCR	CM5 Biacore chip	Chemical: EDC/NHS, Ethylenediamine	Oriented	SPR	[23]
4,9-anhydroTTX	0.083	Bare plastic plate	Physical: Absorption (TTX-Ovalbumin)	Random	Colorimetry	[22]
	NCR	Maleimide plate	Chemical: Cysteamine, Formaldehyde	Oriented	Colorimetry	[23]
	NCR	CM5 Biacore chip	Chemical: EDC/NHS, Ethylenediamine	Oriented	SPR	[23]
11-oxoTTX	0.015	Bare plastic plate	Physical: Adsorption (TTX-Ovalbumin)	Random	Colorimetry	[24]

NCR: No cross-reactivity observed.

norTTX-6(*S*)-ol and 11-deoxyTTX (the two TTX analogues with the highest CRFs). Although 5,6,11-trideoxyTTX is among the most abundant TTX analogues in several of our pufferfish samples, it was not included in these mixture experiments due to its very low cross-reactivity, which would have required high amounts to generate a measurable signal (amounts not achievable with the limited purified material available). The mixtures were designed to simulate hypothetical scenarios where multiple TTXs coexist, with each individual analogue theoretically making an equal contribution to the immunoassay response. To achieve this, the amount of each analogue added in the mixtures was adjusted proportionally to its cross-reactivity, to ensure its contribution matched that of TTX. The relative abundances of TTX and its analogues in the laboratory-prepared mixtures along with their theoretical contributions to immunoassay response based on CRF data are illustrated in Fig. 3A and B, respectively.

As shown in Fig. 3C, when only TTX, 11-norTTX-6(*S*)-ol or 11-deoxyTTX was present, the immunoassay signal aligned with the predicted response based on the actual amount of toxin added in the laboratory-prepared mixtures (40 % mAb binding). However, when two or three of these TTX analogues were combined, a slight discrepancy emerged, with the observed signal (around 60 % mAb binding) being consistently higher than the theoretically expected value. This observation suggests that the efficacy of the immunoassay in detecting TTXs can be somewhat compromised when several TTX analogues are present, likely due to competitive interactions and/or steric hindrance affecting mAb binding. Such a limitation could impact the applicability of the MB-based immunoassay. However, it is important to note that the laboratory-prepared mixtures tested in this study represented intentionally extreme scenarios. These mixtures were prepared so that, after applying the CRFs, some analogues would contribute significantly to the overall immunoassay signal (e.g., up to 33 % or 50 %) (Fig. 3B). In natural biological samples such as the pufferfish samples tested in this work, such situations were not observed (see section 3.3). Even in specimens with complex TTX profiles and relatively high proportions of certain analogues, applying the CRFs substantially reduces their effective contribution to the immunoassay response. In all samples analysed to date, TTX consistently remains the dominant contributor to the signal. Therefore, although this test is interesting from a mechanistic point of view, the impact of the interfering effect observed with these laboratory-prepared mixtures is expected to be minimal under practical, real-world conditions.

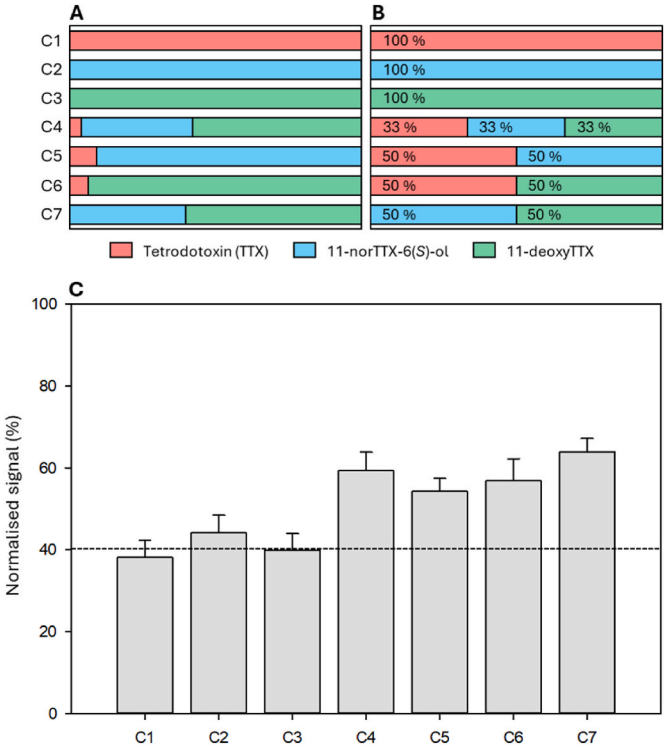

Fig. 3. Composition and analysis of laboratory-prepared mixtures of TTX and TTX analogues. **(A)** Relative abundance of TTX and its analogues in each mixture (C1–C7). **(B)** Adjusted relative abundance of TTX and its analogues in each mixture (C1–C7) after the application of the CRFs (CRF = 1 for TTX, CRF = 0.103 for 11-norTTX-6(*S*)-ol and CRF = 0.068 for 11-deoxyTTX). **(C)** Signal obtained from the analysis of the different laboratory-prepared mixtures (C1–C7) with the MB-based immunoassay. The dotted line represents the expected signal based on the predicted contribution of each individual TTX analogue. All data were background subtracted and normalised with respect to the signal obtained when no toxin is present (maximum signal of the assay). Each bar represents the average ± standard deviation (n = 3).

3.3. Comparative evaluation of MB-based immunoassay and LC-MS/MS for tetrodotoxin analysis in pufferfish

To assess the impact that the varying multi-TTX profiles in naturally contaminated samples may have on the detection efficacy of the immunoassay, 15 pufferfish samples (comprising five different tissues from three *L. sceleratus* specimens) were analysed with the MB-based immunoassay and the results were compared with the analytical data obtained from previous LC-MS/MS analysis of the same extracts [19]. All data from immunoassay and LC-MS/MS analysis are summarised in Table 3. The gonad sample from PF3 were excluded from this comparison, as the values determined by both the immunoassay and LC-MS/MS were identified as outliers (Z-scores of 3.03 and 3.87, respectively).

The pufferfish samples analysed in this study displayed a multi-TTX profile, containing up to 12 distinct TTX analogues [19], with their presence and distribution varying widely across tissues and specimens (Fig. 4A). Nevertheless, TTX was consistently detected in all samples and remained the predominant component in most tissues, except in certain cases where it ranked second after 5,6,11-trideoxyTTX. When comparing the TTXs concentration determined by the immunoassay with the combined levels of TTX and 4-*epi*TTX (epimers in chemical equilibrium) detected by LC-MS/MS (Fig. 4D, solid line), only a slight underestimation of TTX levels by LC-MS/MS relative to the immunoassay was evident (slope = 0.9356). However, when TTX and all its analogues measured by LC-MS/MS are combined (Fig. 4D, dotted line), an almost threefold overestimation of the TTXs levels by LC-MS/MS compared to the immunoassay is observed (slope = 2.7536). This discrepancy can be attributed to the fundamental differences in the detection principles of the two analytical methods [7].

Detection with LC-MS/MS relies on recording the specific mass-to-charge ratios of ions generated by the fragmentation of the TTX analogues in a sample after their separation by chromatographic methods. Reliable detection is ensured as long as these mass-to-charge ratios are well-characterised and integrated into the method. In contrast, the MB-based immunoassay detects TTXs through antibody recognition and binding to specific antigenic determinant on the toxin molecule. The structural variability of these antigenic determinants among TTX analogues influences the affinity of the antibody for its target, making the immunoassay detection response highly dependent on cross-reactivity. Since the cross-reactivity of the antibody towards the different TTX analogues (CRF $\leq$ 0.103) is considerably lower than that of TTX (CRF = 1), discrepancies in the results of the two methods are expected. Though, the key question is whether these discrepancies are systematic and whether the analytical results provided by both methods remain consistent.

To address this point, the CRFs determined in this work were applied to the individual concentrations of TTX analogues measured by LC-MS/MS. Since it was not practically possible to determine CRFs for all TTX analogues present in the pufferfish samples, two extreme scenarios were considered: (i) the antibody was assumed to exhibit no reactivity towards the unknown analogues (CRF = 0; Fig. 4B), or (ii) the antibody was assumed to exhibit the same reactivity towards the unknown TTX analogues as it does with TTX (CRF = 1; Fig. 4C). Subsequently, the summed concentrations of the different TTX analogues, as measured by LC-MS/MS and adjusted using the different CRFs, were compared with the total TTXs concentration determined by the immunoassay (Fig. 4E). The strong correlations obtained (slopes near 1 and coefficient of determination of 0.99) under both scenarios indicate that, despite the initial discrepancies between the two methods, there is a good agreement between them when the CRFs are accounted for (slopes = 1.0111 and 1.0534). This consistency suggests that, even in the presence of multiple TTX analogues in the pufferfish samples, the immunoassay effectively detects TTXs with comparable accuracy to LC-MS/MS. Consequently, any potential interfering effects discussed in the previous section, if present, do not appear to significantly impact the immunoassay results for these pufferfish samples. This highlights the broad applicability of the immunoassay for TTX monitoring, as the TTX profiles observed in this study align well with those reported for pufferfish from the same geographical area and other regions worldwide [34–38].

Table 3
Concentration of TTXs in five tissues (gonads (G), skeletal muscle (M), liver (L), digestive tract (D) and skin (S)) from three *L. sceleratus* pufferfish (PF1, PF2 and PF3) determined with the MB-based colorimetric immunoassay, APC, CBA and LC-MS/MS. The toxin concentrations determined with the immunoassay, APC and CBA analysis are expressed as μg TTX equiv./kg of pufferfish tissue. The toxin concentrations determined with LC-MS/MS analysis are expressed as μg TTX or TTX analogues/kg of pufferfish tissue. No CRF were applied to the LC-MS/MS values from this table. Values express the average ± standard deviation.

Sample		Immunoassay	APC[a]	CBA[b]	LC-MS/MS[b]	
					[TTX + 4-*epi*TTX]	∑ [TTXs]
PF1	G	**1669** ± 370	**5150** ± 575	**3657** ± 472	**1490**	**2129**
	M	**4393** ± 621	**5738** ± 1160	**5559** ± 1027	**4615**	**7640**
	L	**15,379** ± 2183	**24,982** ± 1505	**21,454** ± 2598	**20,560**	**38,917**
	D	**16,941** ± 2141	**25,050** ± 276	**19,584** ± 4105	**19,197**	**34,646**
	S	**4362** ± 639	**6324** ± 312	**8032** ± 454	**5292**	**14,251**
PF2	G	**5061** ± 733	**13,588** ± 1959	**6365** ± 257	**5402**	**10,541**
	M	**11,073** ± 714	**22,484** ± 3105	**14,091** ± 1028	**15,066**	**36,486**
	L	**63,301** ± 5736	**74,215** ± 4259	**51,351** ± 2318	**53,939**	**188,240**
	D	**70,525** ± 8988	**86,994** ± 8655	**92,425** ± 16,021	**69,950**	**205,770**
	S	**12,086** ± 1330	**14,147** ± 3096	**16,117** ± 1012	**13,496**	**63,178**
PF3	G	**179,425** ± 11,740	**222,320** ± 48,038	**228,881** ± 12,229	**171,654**	**1,324,439**
	M	**7921** ± 678	**6505** ± 855	**8479** ± 943	**6354**	**11,563**
	L	**12,427** ± 686	**17,456** ± 2850	**26,760** ± 2443	**16,985**	**57,179**
	D	**81,417** ± 837	**87,619** ± 5896	**113,128** ± 9840	**80,447**	**210,873**
	S	**9389** ± 987	**16,031** ± 3239	**14,659** ± 1031	**9179**	**34,746**

[a] Data from Reverté and co-workers [9].
[b] Data from Alkassar and co-workers [19].

3.4. Evaluation of the toxicity neutralising activity of the antibody

The interplay between structural recognition and toxicity neutralisation is useful for understanding the effectiveness of the immunoassay in detecting TTX and its analogues. The following experiments explore how antibody binding influences TTX toxicity neutralisation, providing insights into the relationship between molecular recognition and functional toxicity across different TTX analogues and pufferfish tissue extracts. Initially, antibody dilutions ranging from 1/50 to 1/12,800 were incubated for 30 min with a 5 nM TTX solution (Fig. 5A). Full neutralisation of TTX toxicity was achieved at the 1/50 dilution, corresponding to an antibody concentration of 30 μg/mL. As antibody concentration decreased, the neutralising effect gradually diminished, becoming nearly undetectable at the 1/12,800 dilution (0.12 μg antibody/mL). A similar experiment was conducted using two of the most toxic TTX analogues based on APC measurements, i.e. 11-norTTX-6(*S*)-ol and 11-deoxyTTX [9]. These analogues were tested at concentrations equivalent to the response generated by 5 nM TTX. However, even at the highest antibody concentration (30 μg/mL), toxicity neutralisation was

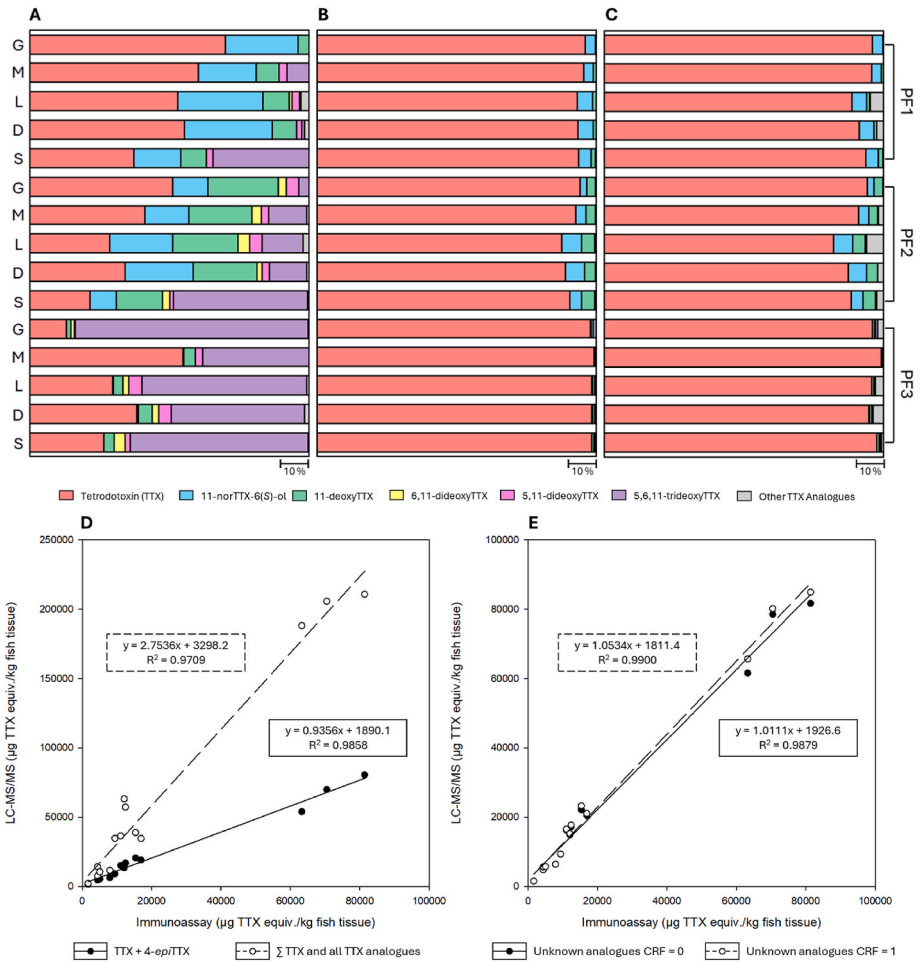

Fig. 4. Application of CRFs to LC-MS/MS pufferfish analysis. **(A)** Relative abundance of TTXs in the pufferfish samples analysed in this work. All those TTX analogues whose CRFs were not assessed were not detailed but expressed as a set (grey bars). **(B)** Adjusted relative abundances of TTXs in the pufferfish samples after the application of the CRFs to the individual TTX analogues contents determined with LC-MS/MS, assuming a CRF of 0 for the TTX analogues with unknown CRF. **(C)** Adjusted relative abundances of TTXs in the pufferfish samples after the application of the CRFs to the individual TTX analogues contents determined with LC-MS/MS, assuming a CRF of 1 for the TTX analogues with unknown CRF. **(D)** Correlation between the TTX contents obtained in the analysis of the samples with the MB-based immunoassay and LC-MS/MS without the application of the CRFs. **(E)** Correlation between the TTX contents obtained in the analysis of the samples with the immunoassay and LC-MS/MS after the application of the CRFs. All correlations fit to a linear regression model and the obtained equations are shown.

only 16 % for 11-norTTX-6(*S*)-ol and 14 % for 11-deoxyTTX. Additionally, the neutralisation experiment was also performed with extracts from different pufferfish tissues. Extract dilutions yielding responses equivalent to 5 nM TTX were incubated with a 1/50 antibody dilution (Fig. 5B). Nearly complete neutralisation (~100 %) was observed in gonads, whereas in skeletal muscle, liver, digestive tract and skin tissues the efficiency did not exceed 80 %. Although the experiment was conducted using tissue extracts at concentrations that theoretically do not cause any matrix effects on APC [26], the possibility that residual matrix components affected the interaction between the antibody and TTXs, either positively or negatively, cannot be ruled out. This could account for the observed variation in neutralising activity across tissues. However, given that the antibody exhibited lower neutralising activity for TTX analogues than for TTX, it is likely that the incomplete neutralisation of toxicity in some pufferfish tissues was partly due to the multi-TTX profiles of those samples. A complete neutralisation would probably be achieved using higher antibody concentrations, but this was not tested due to budget constraints.

These experiments demonstrated that once the antibody binds the TTXs, it prevents them from inducing toxicity in cells. This effect could be attributed to steric hindrance caused by the large size of the antibody-toxin complex. However, since the CRFs closely aligned with the TEFs reported for the same TTX analogues in previous studies [9,19], it is reasonable to hypothesise that the antibody recognises a molecular region of TTXs that coincides with their VGSCs binding site. Immunoassays are structural methods that typically do not provide toxicological or functional insights into a sample [7]. This limitation stems from the fact that antibodies recognise specific molecular regions of a toxin, which may not necessarily correspond to those responsible for its toxic effects. Nevertheless, if the antibody binds to the same region of the toxin that interacts with its biological target, immunoassay data could serve as a valuable indicator of the toxicological potential of the sample. To fully validate this aspect, further comparisons of its analytical performance with other toxicological methods, such as CBA and APC, are necessary.

3.5. Toxicological insights derived from the immunoassay

To evaluate if the immunoassay data accurately reflect the toxicity of a sample, the results obtained from the 15 pufferfish samples with this method were compared with those from previous analysis of the same extracts using CBA [19] and APC [9], two well-established toxicological approaches. A summary of the CBA and APC results is provided in Table 3. The gonads from PF3 sample were again excluded from the comparison, as the concentration of TTXs determined by CBA and APC were identified as outliers (Z-scores of 3.06 and 3.12, respectively).

When TTXs concentrations measured by the immunoassay were

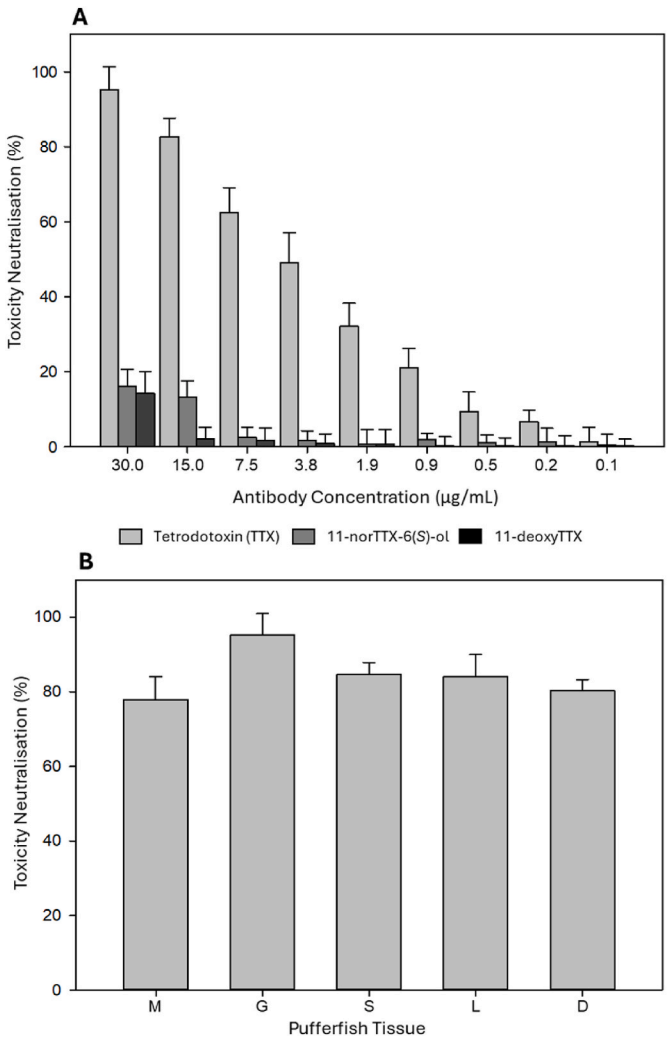

Fig. 5. Neutralisation of the toxicity of TTX and TTX analogues **(A)**, and several tissues of a *L. sceleratus* pufferfish specimen (PF1) **(B)** on Neuro-2a cells with APC. Data were normalised relative to the signal obtained from the analysis of the same samples without preincubation with the antibody. Each bar represents the average ± standard deviation (n = 3).

compared with those obtained using CBA and APC (Fig. 6A and B, respectively), a strong correlation was observed and only a slight overestimation of TTX levels was noticed in CBA and to lesser extent in APC, as indicated by the respective slopes (1.199 for CBA and 1.096 for APC). This difference was not surprising, as the TEFs reported for CBA [19] and APC [9] are not identical (for some analogues, the TEFs derived from CBA are slightly higher than those from APC). Despite these subtle differences, the data obtained from the analysis of naturally contaminated samples using the MB-based immunoassay closely align with the toxicological data inherently provided by CBA and APC methods. Based on the neutralisation experiments and the comparison study presented here, the immunoassay proves to be a valuable tool for assessing the toxic potential of a sample.

4. Conclusions

In this study, the ability of an anti-TTX antibody to detect not only TTX but also several of its analogues was successfully demonstrated using a MB-based immunoassay. When the derived CRFs were applied to individual TTX analogue data from LC-MS/MS analysis, it was proven that the immunoassay is as effective and reliable as high-cost instrumental techniques for assessing total TTXs concentrations in naturally contaminated samples. Additionally, the CRFs of the different TTX analogues examined in this study closely aligned with their reported toxicity. Besides, the antibody exhibited a greater neutralising activity for the more toxic analogues compared to the less toxic ones. Therefore, the response of this method in the analysis of samples was shown to be predominately driven by the most toxic TTX analogues, providing a direct assessment of the actual toxicity of the sample rather than merely quantifying its toxin levels. Although further work with additional TTX analogues is still needed, this study demonstrates the potential of this immunoassay as a promising bioanalytical tool for TTX monitoring programs aimed at food safety assessment and human health protection. Moreover, this work highlights the critical importance of properly validating the cross-reactivity of a biorecognition element towards functionally and/or structurally related targets. Such validation is essential for ensuring the reliability, consistency and broad applicability of new bioanalytical tools targeted toward the analysis of samples with multi-toxin profiles.

CRediT authorship contribution statement

Jaume Reverté: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Mounira Alkassar:** Writing – review & editing, Investigation. **Maria Rambla-Alegre:** Writing – review & editing, Investigation. **Andres Sanchez-Henao:** Writing – review & editing, Investigation. **Manolis Mandalakis:** Writing – review & editing, Resources. **Panagiota Peristeraki:** Writing – review & editing, Resources. **Francesc X.**

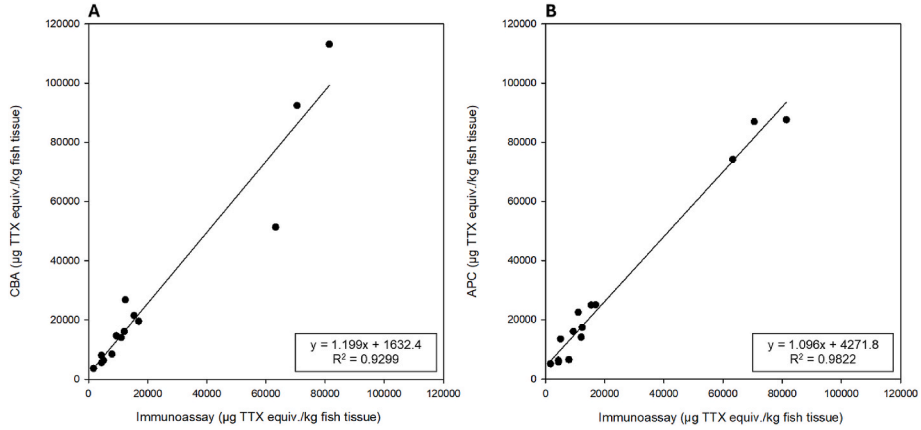

Fig. 6. Correlation between the TTX contents obtained in the analysis of the pufferfish samples with the MB-based immunoassay and CBA **(A)** or APC **(B)**. All correlations fit a linear regression model and the obtained equations are shown.

Sureda: Writing – review & editing, Funding acquisition. **Jorge Diogène:** Writing – review & editing. **Mònica Campàs:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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.

Acknowledgements

This research was funded 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) and the BiOCEANsing (PID2023-149899OB-C21 and PID2023-149899OB-C22) projects. Jaume Reverté acknowledges IRTA for his PhD grant (CPI0422). Mounira Alkassar acknowledges MICIN and AEI for her PhD grant (PRE2019-088181). 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 CERCA Programme/Generalitat de Catalunya.

Data availability

Data will be made available on request.

References

[1] C. Hu, Y. Zhang, Y. Zhou, Y.-J.-Y. Xiang, Z.-F. Liu, Z.-H. Wang, X.-S. Feng, Tetrodotoxin and its analogues in food: recent updates on sample preparation and analytical methods since 2012, J. Agric. Food Chem. 70 (2022) 12249–12269, https://doi.org/10.1021/acs.jafc.2c04106.

[2] V. Bane, M. Lehane, M. Dikshit, A. O'Riordan, A. Furey, Tetrodotoxin: Chemistry, toxicity, source, distribution and detection, Toxins 6 (2014) 693–755, https://doi.org/10.3390/toxins6020693.

[3] T.Y. Magarlamov, D.I. Melnikova, A.V. Chernyshev, Tetrodotoxin-producing bacteria: detection, distribution and migration of the toxin in aquatic systems, Toxins 9 (2017) 166, https://doi.org/10.3390/toxins9050166.

[4] M. Kono, T. Matsui, K. Furukawa, T. Takase, K. Yamamori, H. Kaneda, D. Aoki, J.-H. Jang, M. Yotsu-Yamashita, Examination of transformation among tetrodotoxin and its analogs in the living cultured juvenile puffer fish, kusafugu, *fugu niphobles* by intramuscular administration, Toxicon 52 (2008) 714–720, https://doi.org/10.1016/j.toxicon.2008.08.002.

[5] N. Ueyama, K. Sugimoto, Y. Kudo, K.-I. Onodera, Y. Cho, K. Konoki, T. Nishikawa, M. Yotsu-Yamashita, Spiro bicyclic guanidino compounds from pufferfish: possible biosynthetic intermediates of tetrodotoxin in marine environments, Chem. Eur J. 24 (2018) 7250–7258, https://doi.org/10.1002/chem.201801006.

[6] Y. Zhang, S. Zou, S. Yin, T. Wang, Source, ecological function, toxicity and resistance of tetrodotoxin (TTX) in TTX-bearing organisms: a comprehensive review, Toxin Rev. 42 (2023) 727–740, https://doi.org/10.1080/15569543.2023.2253892.

[7] J. Reverté, M. Alkassar, J. Diogène, M. Campàs, Detection of ciguatoxins and tetrodotoxins in seafood with biosensors and other smart bioanalytical systems, Foods 12 (2023) 2043, https://doi.org/10.3390/foods12102043.

[8] R. Mackieh, R. Abou-Nader, R. Wehbe, C. Mattei, C. Legros, Z. Fajloun, J. M. Sabatier, Voltage-gated sodium channels: a prominent target of marine toxins, Mar. Drugs 19 (2021) 562, https://doi.org/10.3390/md19100562.

[9] J. Reverté, M. Rambla-Alegre, A. Sanchez-Henao, M. Mandalakis, P. Peristeraki, J. Molgó, J. Diogène, F.X. Sureda, M. Campàs, Toxicity equivalency factors for tetrodotoxin analogues determined with automated patch clamp on voltage-gated sodium channels in Neuro-2a cells, J. Agric. Food Chem. 72 (2024) 18192–18200, https://doi.org/10.1021/acs.jafc.4c04321.

[10] P. Katikou, C. Gokbulut, A.R. Kosker, M. Campàs, F. Ozogul, An updated review of tetrodotoxin and its peculiarities, Mar. Drugs 20 (2022) 47, https://doi.org/10.3390/mmd20010047.

[11] G.M. Bucciarelli, M. Lechner, A. Fontes, L.B. Kats, H.L. Eisthen, H.B. Shaffer, From poison to promise: the evolution of tetrodotoxin and its potential as a therapeutic, Toxins 13 (2021) 517, https://doi.org/10.3390/toxins13080517.

[12] S.A. Goldlust, M. Kavoosi, J. Nezzer, M. Kavoosi, W. Korz, K. Deck, Tetrodotoxin for chemotherapy-induced neuropathic pain: a randomized, double-blind, placebo-controlled, parallel-dose finding trial, Toxins 13 (2021) 235, https://doi.org/10.3390/toxins13040235.

[13] O. Akyol, V. Ünal, T. Ceyhan, M. Bilecenoglu, First confirmed record of *Lagocephalus sceleratus* (Gmelin, 1789) in the Mediterranean Sea, J. Fish. Biol. 66 (2005) 1183–1186, https://doi.org/10.1111/j.0022-1112.2005.00667.x.

[14] Regulation (EU), No 2019/627 of the European Union and of the Council of 15 March 2019 laying down uniform practical arrangements for the performance of official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of the European Parliament and of the Council and amending Commission Regulation (EC) no 2074/2005 as regards official controls, Available online, https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32019R0627. June 2025.

[15] R. Bédry, L. de Haro, Y. Bentur, N. Senechal, B.S. Galil, Toxicological risks on the human health of populations living around the Mediterranean Sea linked to the invasion of non-indigenous marine species from the Red Sea: a review, Toxicon 191 (2021) 69–82, https://doi.org/10.1016/j.toxicon.2020.12.012.

[16] P. Antonelli, B. Salerno, P. Bordin, A. Peruzzo, M. Orsini, G. Arcangeli, L. Barco, C. Losasso, Tetrodotoxin in live bivalve mollusks from Europe: is it to be considered an emerging concern for food safety? Compr. Rev. Food Sci. Food Saf. 21 (2022) 719–737, https://doi.org/10.1111/1541-4337.12881.

[17] J.F. Fernández-Ortega, J.M. Morales-de los Santos, M.E. Herrera-Gutiérrez, V. Fernández-Sánchez, P. Rodríguez-Loureo, A. Alfonso-Rancaño, A. Téllez-Andrade, Seafood intoxication by tetrodotoxin: first case in Europe, J. Emerg. Med. 39 (2010) 612–617, https://doi.org/10.1016/j.jemermed.2008.09.024.

[18] P. Rodriguez, A. Alfonso, C. Vale, C. Alfonso, P. Vale, A. Tellez, L.M. Botana, First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell *Charonia lampas lampas* in Europe, Anal. Chem. 80 (2008) 5622–5629, https://doi.org/10.1021/ac800769e.

[19] M. Alkassar, A. Sanchez-Henao, J. Reverté, L. Barreiro, M. Rambla-Alegre, S. Leonardo, M. Mandalakis, P. Peristeraki, J. Diogène, M. Campàs, Evaluation of toxicity equivalency factors of tetrodotoxin analogues with a Neuro-2a cell-based assay and application to pufferfish from Greece, Mar. Drugs 21 (2023) 432, https://doi.org/10.3390/md21080432.

[20] D. Bloch, P. Diel, B. Epe, M. Hellwig, A. Lampen, A. Mally, D. Marko, M.A. Villar-Fernández, S. Guth, A. Roth, et al., Basic concepts of mixture toxicity and relevance for risk evaluation and regulation, Arch. Toxicol. 97 (2023) 3005–3007, https://doi.org/10.1007/s00204-023-03565-6.

[21] L. Reverté, L. Soliño, O. Carnicer, J. Diogène, M. Campàs, Alternative methods for the detection of emerging marine toxins: biosensors, biochemical assays and cell-based assays, Mar. Drugs 12 (2014) 5719–5763, https://doi.org/10.3390/md12125719.

[22] K. Kawatsu, Y. Hamano, T. Yoda, Y. Terano, T. Shibata, Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin, Jpn. J. Med. Sci. Biol. 50 (1997) 133–150, https://doi.org/10.7883/yoken1952.50.133.

[23] L. Reverté, P. de la Iglesia, V. del Río, K. Campbell, C.T. Elliott, K. Kawatsu, P. Katikou, J. Diogène, M. Campàs, Detection of tetrodotoxins in puffer fish by a self-assembled monolayer-based immunoassay and comparison with surface plasmon resonance, LC-MS/MS and mouse bioassay, Anal. Chem. 87 (2015) 10839–10847, https://doi.org/10.1021/acs.analchem.5b02158.

[24] K. Yamaki, K. Sato, Y. Kudo, Y. Cho, K. Konoki, T. Takatani, O. Arakawa, K. Kawatsu, M. Yotsu-Yamashita, The quite low cross-reactivity of Kawatsu's anti-tetrodotoxin monoclonal antibody to 5,6,11-trideoxytetrodotoxin, 11-nortetrodotoxin-6(*S*)-ol, and 11-oxotetrodotoxin, the major tetrodotoxin analogues in pufferfish, Toxicon 226 (2023) 107081, https://doi.org/10.1016/j.toxicon.2023.107081.

[25] M. Campàs, J. Reverté, M. Rambla-Alegre, K. Campbell, A. Gerssen, J. Diogène, A fast magnetic bead-based colorimetric immunoassay for the detection of tetrodotoxins in shellfish, Food Chem. Toxicol. 140 (2020) 111315, https://doi.org/10.1016/j.fct.2020.111315.

[26] M. Campàs, J. Reverté, À. Tudó, M. Alkassar, J. Diogène, F.X. Sureda, Automated patch clamp for the detection of tetrodotoxin in pufferfish samples, Mar. Drugs 22 (2024) 176, https://doi.org/10.3390/md22040176.

[27] J.-H. Jang, M. Yotsu-Yamashita, 6,11-dideoxytetrodotoxin from the puffer fish, *Fugu pardalis*, Toxicon 50 (2007) 947–951, https://doi.org/10.1016/j.toxicon.2007.06.026.

[28] Y. Kudo, J. Finn, K. Fukushima, S. Sakugawa, Y. Cho, K. Konoki, M. Yotsu-Yamashita, Isolation of 6-deoxytetrodotoxin from the pufferfish, *Takifugu pardalis*, and comparison of the effects of the C-6 and C-11 hydroxy groups of tetrodotoxin on its activity, J. Nat. Prod. 77 (2014) 1000–1004, https://doi.org/10.1021/np401097n.

[29] T. Yasumoto, M. Yotsu, M. Murata, H. Naoki, New tetrodotoxin analogs from the newt *Cynops ensicauda*, J. Am. Chem. Soc. 110 (1988) 2344–2345, https://doi.org/10.1021/ja00215a078.

[30] M. Yotsu, Y. Hayashi, S.S. Khora, S. Sato, T. Yasumoto, Isolation and structural assignment of 11-nortetrodotoxin-6(*S*)-ol from the puffer *Arothron nigropunctatus*, Biosci. Biotechnol. Biochem. 56 (1992) 370–371, https://doi.org/10.1271/bbb.56.370.

[31] M. Yotsu-Yamashita, Y. Yamagishi, T. Yasumoto, 5,6,11-trodeoxytetrodotoxin from the puffer fish, *fugu peocilonotus*, Tetrahedron Lett. 36 (1995) 9329–9332, https://doi.org/10.1016/0040-4039(95)02020-P.

[32] S. Saruhasi, K. Konoki, M. Yotsu-Yamashita, The voltage-gated sodium ion channel inhibitory activities of a new tetrodotoxin analogue, 4,4a-anhydrotetrodotoxin, and three other analogues evaluated by colorimetric cell-based assay, Toxicon 119 (2016) 72–76, https://doi.org/10.1016/j.toxicon.2016.05.012.

[33] G. Gaiani, C.K. O'Sullivan, M. Campàs, Magnetic beads in marine toxin detection: a review, Magnetochemistry 5 (2020) 62, https://doi.org/10.3390/magnetochemistry5040062.

[34] T.I. Anastasiou, E.K. Kagiampaki, G. Kondylatos, A. Tselepides, P. Peristeraki, M. Mandalakis, Assessing the toxicity of *Lagocephalus sceleratus* pufferfish from the Southeastern Aegean Sea and the relationship with gonadal hormones, Mar. Drugs 21 (2023) 520, https://doi.org/10.3390/md21100520.

[35] G. Christidis, M. Mandalakis, T.I. Anastasiou, G. Tserpes, P. Peristeraki, S. Somarakis, Keeping *Lagocephalus sceleratus* off the table: sources of variation in the quantity of TTX, TTX analogues, and risk of tetrodotoxication, Toxins 13 (2021) 896, https://doi.org/10.3390/toxins13120896.

[36] M. Ito, R. Furukawa, S. Yasukawa, M. Sato, H. Oyama, T. Okabe, R. Suo, H. Sugita, T. Takatani, O. Arakawa, M. Adachi, T. Nishikawa, S. Itoi, Local differences in the toxin amount and composition of tetrodotoxin and related compounds in pufferfish (*Chelonodon patoca*) and toxic goby (*Yongeichthys cringer*) Juveniles, Toxins 14 (2022) 150, https://doi.org/10.3390/toxins14020150.

[37] A.R. Kosker, M. Karakus, P. Katikou, İ. Dal, M. Durmus, Y. Ucar, D. Ayas, F. Özogul, Monthly variation of tetrodotoxin levels in pufferfish (*Lagocephalus sceleratus*) caught from Antalya Bay, Mediterranean Sea, Mar. Drugs 21 (2023) 527, https://doi.org/10.3390/md21100527.

[38] B.K. Park, H. Lee, I. Kim, K.-S. Choi, W.-O. Lee, N. Kajino, H.-K. Hong, H.-S. Lee, C. Choi, J. Lee, 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 (2024) 105816, https://doi.org/10.1016/j.jfca.2023.105816.