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First record of paralytic shellfish toxins in marine pufferfish from the Spanish Mediterranean coast using cell-based assay, automated patch clamp and HPLC-FLD

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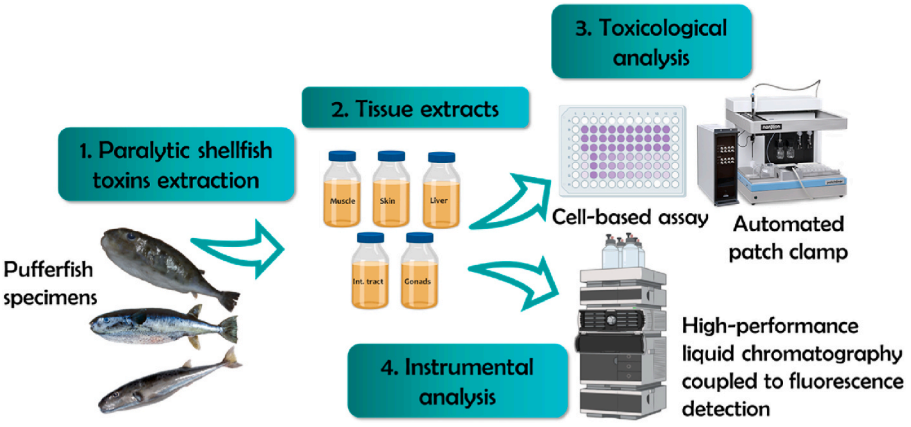
HIGHLIGHTS

- CBA and APC were used to assess the presence of PSTs in different pufferfish species.
- No toxicity was observed in *S. pachygaster*.
- Toxicity was detected in the liver of most *L. lagocephalus*.
- HPLC-FLD analysis confirmed the presence of PSTs in *L. lagocephalus*.
- STX and dcSTX were detected in all positive samples, being dcSTX the major analogue.

GRAPHICAL ABSTRACT



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ABSTRACT

Pufferfish is one of the most poisonous marine organisms, responsible for numerous poisoning incidents and some human fatalities due to its capability to accumulate potent neurotoxins such as tetrodotoxins (TTXs) and paralytic shellfish toxins (PSTs). In this study, tissue extracts (muscle, skin, liver, intestinal tract and gonads) obtained from sixteen pufferfish specimens of the *Lagocephalus lagocephalus* and *Sphoeroides pachygaster* species, collected along the Spanish Mediterranean coast, were analysed for the presence of voltage-gated sodium channel (also known as Na_v channel) blockers using cell-based assay (CBA) and automated patch clamp (APC). No toxicity was observed in any of the *S. pachygaster* specimens, but toxicity was detected in the liver of most *L. lagocephalus* specimens. Instrumental analysis of these specimens, as well as in one *Lagocephalus sceleratus* specimen, by high-performance liquid chromatography coupled to fluorescence detection (HPLC-FLD) was performed, which confirmed the presence of PSTs only in *L. lagocephalus* specimens. This analysis reported the presence of saxitoxin (STX) and decarbamoylsaxitoxin (dcSTX) in all positive samples, being dcSTX the major

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analogue. These results demonstrate the ability of this species to accumulate PSTs, being the first report of the presence of PSTs in Mediterranean *L. lagocephalus* specimens. Furthermore, the presence of high PSTs contents in all five tested tissues of one *L. lagocephalus* specimen pointed the risk that the presence of this toxic fish in the Mediterranean Sea may represent for seafood safety and human health in case of accidental consumption.

1. Introduction

Pufferfish species belong to the order Tetraodontiformes, which mostly reside in the oceans of tropical and subtropical areas, although they can also be found in marine temperate waters, freshwater environments and large river estuaries (Noguchi et al., 2011). The presence of poisonous pufferfish in Asian countries has been documented since historical times (Fuhrman, 1986). The poisonous nature of pufferfish is due to the presence of a highly potent neurotoxin, known as tetrodotoxin (TTX), present in their internal organs. Differences in TTX concentrations have been described according to sex, habitat and season (Bane et al., 2014; Akbora et al., 2020; Kosker et al., 2016, 2019; Gao et al., 2019; Alkassar et al., 2023). Many bacteria, such as *Pseudomonas* sp., *Vibrio* sp. and *Alteromonas* sp., have been observed to produce TTX, but the contribution of microorganisms to TTX bioaccumulation in marine ecosystems is still not fully elucidated (Turner et al., 2018; Magarlamov et al., 2017). In pufferfish, TTX usually co-occurs with its analogues, which have varying toxic potencies depending on their chemical structure (Alkassar et al., 2023). In recent years, the geographical distribution of poisonous pufferfish has become more widespread. In the Mediterranean Sea, six of the thirteen recorded pufferfish species have entered through the Suez Canal (Ulman et al., 2024; Ben Souissi et al., 2014), among them, the invasive *Lagocephalus sceleratus*, which is considered to be the most harmful invasive species in the Mediterranean (Ben Souissi et al., 2014). Most of the pufferfish poisonings and fatalities in the Mediterranean area are mainly attributed to this species (Ulman et al., 2024). The first study of the presence of TTX in *L. sceleratus* tissues from the Mediterranean Sea was carried out by Katikou and co-workers (Katikou et al., 2009). Then, some other researchers (Rambla-Alegre et al., 2017; Saoudi et al., 2011) examined TTX levels in other species, such as *Sphoeroides pachygaster* and *Lagocephalus lagocephalus*. In this study (Rambla-Alegre et al., 2017), the authors demonstrated that *L. sceleratus* is highly toxic, while no TTXs were detected in *L. lagocephalus* and *S. pachygaster* specimens. Saoudi and co-workers (Saoudi et al., 2011) attributed the toxicity of *L. lagocephalus* from the Tunisian coast to TTX. However, instrumental analysis was not performed and therefore, the compound responsible for the toxic effect was not confirmed.

Some pufferfish species, including *L. lagocephalus* (Soliño et al., 2021; Pinto et al., 2019), have been reported to also contain large amounts of saxitoxin (STX) and its analogues, known as paralytic shellfish toxins (PSTs), outside the Mediterranean (Kungsuwan et al., 1997; Zaman et al., 1997; Sato et al., 1997, 2000; Nakashima et al., 2004; Jang and Yotsu-Yamashita, 2007; Landsberg et al., 2006). Nonetheless, one report described possible presence of PSTs in *L. sceleratus* in Greece (Katikou et al., 2009), but these were not confirmed. Up to date, 57 STX analogues have been described (Wiese et al., 2010). PSTs can be classified into subgroups based on the substituent side chains, such as carbamate, sulphate, hydroxyl, hydroxybenzoate or acetate. Each one of these groups contributes to the toxicity of a sample with a different toxicity potency, which can vary by up to two orders of magnitude (Genenah and Shimizu, 1981; Hall et al., 1990). STX and its analogues are responsible for paralytic shellfish poisoning (PSP), attributed to some food intoxications and deaths due to the consumption of contaminated shellfish and pufferfish (Landsberg et al., 2006; Morris et al., 2015; Garcia et al., 2004; Anderson et al., 1996; Rodrigue et al., 1990; Kao and Falconer, 1993). PSTs are produced mainly by several species of the marine dinoflagellates *Alexandrium*, *Gymnodinium* and *Pyrodinium* and some freshwater cyanobacteria (Wiese et al., 2010; Cusick and Sayler, 2013).

STX and TTX have almost equivalent molecular size and share the same mechanism of action. They are known to selectively block the voltage-gated sodium channels (also known as Na_v channels). This blocking results in prevention of sodium ion flow into cells, leading to acute fatalities in animals and humans, and in severe cases death may occur due to respiratory paralysis (Hartshorne and Catterall, 1984; Terlau et al., 1991; Stevens et al., 2011). To date, there is no antidote for intoxications caused by TTX and/or STX, being artificial respiration and fluid therapy the only treatment available (Katikou et al., 2022). The threat of these intoxications is not only a major cause of concern for public health but is also detrimental to the economy. To protect human health, a regulatory limit of 800 μg STX equiv./kg shellfish meat was set in the EU (Regulation (EC)). Currently, no legislation for TTX in bivalves is established. However, the CONTAM panel of the European Food Safety Authority (EFSA) proposed a provisional guideline value of 44 μg TTX equiv./kg shellfish meat, which was considered not to result in adverse effects in humans (EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain) et al., 2017). Regarding fish, the Japanese government has established a regulatory limit of 2 mg TTX equiv./kg of tissue in pufferfish (Noguchi and Ebesu, 2001). There is no limit for STX in fish (Regulation (EC)). Within Europe, the legislation bans the trade of fishes belonging to the Tetraodontidae family or products derived from them (EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain) et al., 2017), although accidental consumption of toxic pufferfish due to fisherman unawareness or from recreational activities cannot be ruled out. Additionally, the EFSA CONTAM panel as well as some other researchers recommend the potential integration of STX, TTX and their analogues within a unified health-based guidance value, taking into account the fact that they exert similar toxic effects via a similar mode of action, as mentioned above, and their toxicities are additive (EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain) et al., 2017; Finch et al., 2018).

The potential spread of TTXs and PSTs, and of organisms potentially accumulating such toxins, through pufferfish living in Mediterranean waters represents an emerging risk for coastal countries. For many years, the official reference method in the EU for detecting PSTs in shellfish was the mouse bioassay (MBA) (Noguchi and Ebesu, 2001). This method provides a composite toxicological assessment and therefore was a useful quantitative monitoring tool. However, the MBA is known to be affected by low sensitivity, poor reproducibility and is subject to matrix interferences and ethical concerns. As an alternative to MBA, cell-based assays (CBAs) using the antagonist effects of the Na_v-inhibitor toxins (STX and TTX) in combination with veratridine (V) and ouabain (O) have been developed (Alkassar et al., 2023). Based on the mode of action of these toxins, these assays are very useful as screening tools, and they also provide a composite toxicological response. The O/V pre-treatment causes an influx of sodium ions into Neuro-2a cells, leading to cellular swelling and subsequent death. Following the addition of STX or TTX, an increase in cell viability in relation to the control without toxin is observed, as these toxins counteract the effect of O and V. However, like the MBA, this assay may also suffer from matrix interferences. Automated patch clamp (APC) electrophysiology has emerged as an effective way to study the direct interaction of these toxins with the Na_v channels. These assays offer both sensitivity and speed (Campàs et al., 2024). Like MBA and CBA, APC provides a useful global toxicological response. However, the APC equipment is expensive. It is necessary to point out that these three toxicological methods are not able to discriminate between TTXs and PSTs, which is a limitation when the purpose is the specific identification of the toxins present in a sample but is an

advantage when toxicity estimations are pursued. Instrumental analysis techniques, such high-performance liquid chromatography coupled to fluorescence detection (HPLC-FLD), have also been developed and validated for the detection of PSTs in shellfish and have also been adopted by the Association of Official Analytical Chemists (AOAC) as the official method for the detection of PSTs in bivalve molluscs, which is regularly used in monitoring programs (UNE-EN 14526, 2017). This method allows identification and quantification of the presence of STX and its analogues with high accuracy and specificity. However, although these techniques provide information about the toxin profile of a sample, they do not estimate its toxicity.

The aim of this work is to evaluate the presence of STX and its analogues in seventeen pufferfish specimens, belonging to the *L. lagocephalus, S. pachygaster* and *L. sceleratus* species, from the Spanish Mediterranean coast. To this purpose, different tissue extracts (muscle, skin, liver, intestinal tract and gonads) were analysed by CBA and APC for toxicity assessment. Then, the toxin profile of these extracts was characterised by pre-column oxidation HPLC-FLD. To the best of our knowledge, this is the first report of the presence of PSTs in *L. lagocephalus* from Spanish Mediterranean waters. By providing new toxicological data on the occurrence of PSTs in pufferfish samples, this study empathises the need for a proper risk assessment in a changing environmental scenario.

2. Materials and methods

2.1. Reagents and materials

Murine neuroblastoma (Neuro-2a) cells were purchased from ATCC LGC standards (Manassas, VA, USA). Foetal bovine serum (FBS), ouabain (O), veratridine (V), phosphate buffered saline (PBS), penicillin–streptomycin, RPMI-1640 medium, sodium pyruvate and thiazolyl blue tetrazolium bromide (MTT) were purchased from Merck KGaA (Gernsheim, Germany).

The APC Patchliner with 8 amplifier channels, 2 HEKA EPC10 Quadro amplifiers, NPC-16 borosilicate recording chips (medium resistance), 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), internal solution (50 mM CsCl, 10 mM NaCl, 60 mM CsF, 20 mM EGTA, 10 mM HEPES/CsOH, pH 7.2) and seal enhancer solution (10 mM HEPES, 130 mM NaCl, 5 mM glucose, 4 mM KCl, 10 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) were obtained from Nanion Technologies GmbH (Munich, Germany).

Glacial acetic acid (HAc), formic acid, ammonium formate, periodic acid, disodium phosphate, sodium hydroxide, ammonium acetate, sodium chloride and hydrogen peroxide 30% (v/v) reagent grade were obtained from Fluka (Zedelgem, Belgium). Acetonitrile and methanol gradient grade for HPLC were purchased from Honeywell (Charlotte, USA). Ultrapure Milli-Q water (18.2 MΩ/cm) was used to prepare the solutions (Millipore Ibérica Ltd., Madrid, Spain). Solid phase extraction (SPE) cartridges: SPE C18 sep-pack (3 mL, 500 mg) and SPE COOH (3 mL, 500 mg) were purchased from JT Baker (VWR International, LLC, Llinars del Vallès, Spain).

Certified PST standards STX (CRM-STX), decarbamoylsaxitoxin (CRM-dcSTX), gonyautoxins 1 & 4 (CRM-GTX1&4), gonyautoxins 2 & 3 (CRM-GTX2&3), decarbamoylgonyautoxins 2 & 3 (CRM-dcGTX2&3), gonyautoxin 5 (CRM-GTX5), *N*-sulfocarbamoylgonyautoxins 2 & 3 (CRM-C1&2), neosaxitoxin (CRM-NEO), decarbamoylneosaxitoxin (CRM-dcNEO), gonyautoxin 6 (CRM-GTX6) and *N*-sulfocarbamoylgonyautoxins 1 & 4 (CRM-C3&4) were purchased from Cifga (Lugo, Spain). Standard mixtures were prepared from the commercial standards: MIX I (STX, dcSTX, GTX2&3, dcGTX2&3, GTX5 and C1&2), MIX II (NEO and GTX1&4), MIX III (dcNEO) and MIX IV (GTX6 and C3&4). For each MIX, six standard calibration levels were prepared by dilution with Milli-Q water in the range LOQ-2000 μg STX equiv./kg. LOQ values were 100 μg STX equiv./kg and 100 μg dcSTX equiv./kg.

2.2. Pufferfish samples and PSTs extraction

Pufferfish extracts were obtained from a previous work (Rambla-Alegre et al., 2017). A total of seventeen pufferfish specimens (eleven *L. lagocephalus*, five *S. pachygaster* and one *L. sceleratus*), all of them female, were caught from 2014 to 2016 along the Spanish Mediterranean coast (Table 1 and Fig. 1). Fish were dissected and separated into muscle, skin, liver, intestinal tract and gonads, and the tissues were stored at −20 °C until required.

The extraction procedure for PSTs from the different pufferfish tissues followed the protocol of Reverté and co-authors (Reverté et al., 2015), originally developed for the extraction of TTX from pufferfish samples. Briefly, 10 g of tissue was mixed with 25 mL of 0.1% HAc and vortexed for 2 min at 1455 *g*. Then, tubes were boiled in a water bath at 100 °C for 10 min with occasional stirring. The samples were cooled down and centrifuged at 1455 *g* for 5 min at 4 °C. The supernatants were collected, and a second extraction was performed with 20 mL of 0.1% HAc. The two supernatants were pooled, and the final volume was set to 50 mL with 0.1% HAc. For the liver samples, an additional liquid-liquid partition with hexane (1:1) was performed to remove fats. For the CBA and APC, the extracts were filtered using 0.45-μm nylon syringe filters. For HPLC-FLD analysis, extracts were filtered through 0.2-μm nylon syringe filters. The final extracts contained 200 mg tissue equiv./mL and were stored at −20 °C until required.

Tissue extracts from a pufferfish specimen negative for PSTs according to HPLC-FLD analysis as well as negative for TTXs according to previous LC-MS/MS analysis (Rambla-Alegre et al., 2017) were used to evaluate possible matrix interferences with CBA and APC. For the evaluation of the recovery with CBA, APC and HPLC-FLD analysis, the muscle tissue was spiked with STX and dcSTX. For CBA and APC spiking experiments were performed at two different levels (200 and 800 μg STX equiv./kg), using the same concentration of STX and dcSTX at each level. In the case of HPLC-FLD, the spiking level was 200 μg STX equiv./kg.

2.3. Cell maintenance and CBA

Neuro-2a cells were maintained in RPMI-1640 medium (supplemented with 10% FBS, 1% penicillin–streptomycin and 1% sodium pyruvate) in an incubator (BINDER Gmbh, Tuttlingen, Germany) at 37 °C in 5% CO_2 humid atmosphere. The CBA was performed as previously described for TTX (Alkassar et al., 2023). Briefly, Neuro-2a cells were trypsinised and suspended in culture medium (containing 5% FBS). Then, Neuro-2a cells were seeded in a 96-well microplate at an approximate density of 35,000 cells/well in 200 μL of culture medium for 24 h at 37 °C in 5% CO_2 humid atmosphere. Prior to exposure to STX

Table 1
General information of pufferfish specimens caught along the Spanish Mediterranean coast.

Sample ID	Species	Collection year	Location
276.14	*L. lagocephalus*	2014	Blanes
286.14	*L. lagocephalus*	2014	L'Hospitalet de l'Infant
288.14	*L. lagocephalus*	2014	L'Ametlla de Mar
289.14	*L. lagocephalus*	2014	L'Ametlla de Mar
290.14	*L. lagocephalus*	2014	Montgat
306.14	*L. lagocephalus*	2014	Vilanova i la Geltrú
307.14	*L. lagocephalus*	2014	Vilanova i la Geltrú
315.14	*L. lagocephalus*	2014	Cambrils
316.14	*L. lagocephalus*	2014	Castelló
382.14	*S. pachygaster*	2014	Alacant
383.14	*L. sceleratus*	2014	Alacant
046.15	*S. pachygaster*	2015	Llançà
430.14	*S. pachygaster*	2015	Blanes
501.15	*L. lagocephalus*	2015	Tarragona
543.15	*S. pachygaster*	2015	Blanes
075.16	*S. pachygaster*	2016	Palamós
346.16	*L. lagocephalus*	2016	Vilanova i la Geltrú

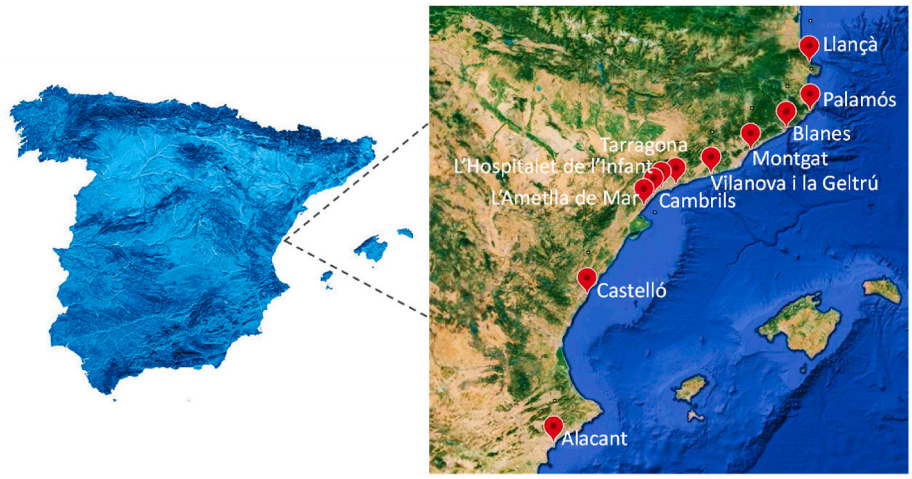

Fig. 1. Sampling points of the pufferfish specimens of this study.

standard solution or pufferfish extracts, some Neuro-2a cells were pre-treated with 20 μL of an O and V mixture in PBS at final concentrations of 0.125 and 0.2 mM, respectively. STX, dcSTX standard solutions or pufferfish extracts were dried under a N_2 stream at 40 °C using a TurboVap evaporator (Zymark corp., Hopkinton, Massachusetts), reconstituted in RPMI medium, serially diluted, and 10 μL was added to the wells with and without O/V pre-treatment. After 24 h, cell viability was measured using the MTT assay. Absorbance at 570 nm was measured with a Synergy LX microplate reader from BioTek (Agilent Technologies,Inc., Santa Clara, CA, USA). Measurements were performed in triplicate.

Calibration curves for STX and dcSTX in the presence of O/V were constructed and fitted to sigmoidal logistic four-parameter equations:

$$y = y_0 + \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

Where a and y_0 are the asymptotic maximum and minimum values respectively, x_0 is the x value at the inflection point and b is the slope at the inflection point.

The toxicity equivalency factor (TEF) of dcSTX was calculated as follows, being the IC_{50} calculated from the previous equation:

$$\text{TEF of dcSTX} = \frac{IC_{50} \text{ of STX standard}}{IC_{50} \text{ of dcSTX standard}}$$

2.4. APC analysis

The electrophysiological analysis was performed as previously described for TTX (Campàs et al., 2024). Briefly, the wells from an NPC-16 borosilicate chip were filled with internal and external solutions followed by the Neuro-2a cell suspension at 100,000 cells/mL. A single cell was immobilised on the hole located at the bottom of the chip at a holding potential of −30 mV. After cell sealing with the enhancer solution, the potential was changed to −100 mV. A stable sealing along time and a resistance between 0.5 and 1 GΩ were used to assume a successful patching. Na_v currents were tested by applying 10 mV increments from −80 mV to 40 mV for 20 ms using two EPC Quatro USB amplifier units (8 probes) controlled and digitalised in real-time with the Patchmaster software (Nanion Technologies GmbH, Munich, Germany). STX, dcSTX standard solutions or pufferfish extracts were injected at different dilutions. In all cases, a volume of 15 μL was injected at a flow rate of 30 μL/s. Measurements were performed at least in triplicate. Calibration curves for STX and dcSTX were constructed and fitted to sigmoidal logistic four-parameter equations and the TEF of dcSTX was calculated as described in the CBA section.

2.5. HPLC-FLD analysis

Analyses were performed using the pre-column oxidation HPLC-FLD Official Method for determination of PSTs in shellfish based on the UNE-EN 14526 (UNE-EN 14526, 2017). Briefly, the method involves an acetic acid extraction followed by a purification step using an SPE C18 cartridge, and the subsequent analysis by HPLC-FLD after oxidation with periodate or peroxide. Most toxins (STX, dcSTX, GTX2&3, dcGTX2&3, GTX5, C1&2) were quantified after SPE C18 clean-up. For the quantitative determination of N-hydroxylated toxins, an additional fractionation step using COOH ion exchange SPE cartridges was performed in order to separate PSTs into three distinct groups (F1, F2 and F3). The C toxins were quantified in F1, the GTX toxins were quantified in F2 and the STX group (STX, dcSTX, NEO and dcNEO) were quantified in F3. Total PSTs toxicity, expressed as μg equiv. STX/kg, was calculated by summing individual toxin concentrations and applying TEFs that are established for each toxin according to the corresponding EFSA Scientific Opinion (European Food Safety Authority (EFSA), 2009). Analyses were carried out using an UPLC Acquity H-Class model and FLR Acquity fluorescence detector (Waters Corporation). A Kinetex C18 4.5 μm, 4.6 × 150 mm column and a C18 guard column 4 × 3 mm from Phenomenex were used. Chromatography conditions used are those described in the rapid method by Hatfield and colleagues (Hatfield et al., 2016).

3. Results

3.1. TEF of dcSTX using Neuro-2a cells

The CBA previously developed for the detection of TTX (Alkassar et al., 2023) was used in this study to detect STX, as both toxins share the same mechanism of action. Fig. 2A shows the viability percentages of Neuro-2a cells pre-treated with O/V and exposed to different concentrations of STX and dcSTX. As expected, these two toxins counteracted the O/V-induced cytotoxicity, resulting in an increase in cell viability in a concentration-dependent manner. However, the sensitivity of the CBA for STX and dcSTX was different. Compared to STX, the dose-response curve of dcSTX shifted to the right, indicating a lower binding affinity of dcSTX for the different Na_v subunits and resulting in an IC_{50} value 5-fold higher than the one obtained for STX (19.63 ng/mL *vs.* 3.73 ng/mL). Therefore, dcSTX was less toxic than STX and its TEF was calculated to be 0.19.

Likewise, the APC method previously developed for the detection of TTX (Campàs et al., 2024) was used to evaluate the blocking activity of STX and dcSTX on Neuro-2a cells. As it can be seen in Fig. 2B, the system was able to detect STX and dcSTX. Again, both toxins effectively block Na_v channels in Neuro-2a cells, although with different binding

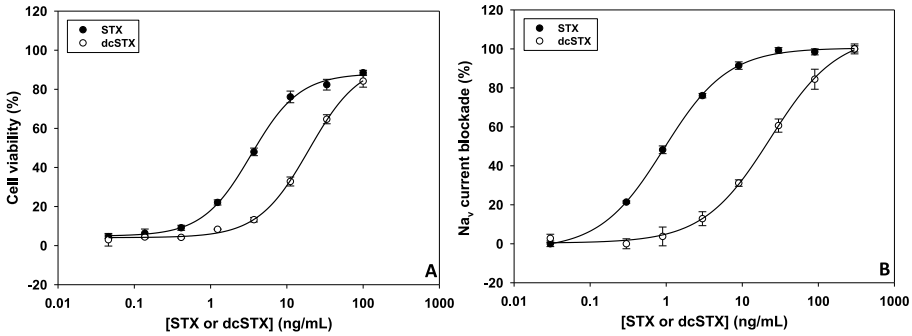

Fig. 2. Dose-response curves for STX and dcSTX on Neuro-2a cells obtained using CBA (**A**) and APC (**B**). Each point shows the average and standard deviation of at least three measurements.

potencies. As expected from the results of the CBA, the binding affinity of dcSTX to the different Na_v subunits was lower than that of STX, being its IC_{50} value 20-fold higher than the one obtained for STX (19.82 ng/mL *vs.* 0.97 ng/mL). Consequently, the TEF of dcSTX was calculated to be 0.05.

3.2. Evaluation of pufferfish matrix effects and recovery

When analysing natural samples, some non-specific cytotoxicity on Neuro-2a cells due to matrix components other than the toxins may be observed. In CBAs for Na_v-activating toxins, such as ciguatoxins (CTXs) or brevetoxins (PbTXs), this effect is usually observed at high matrix concentrations and results in a decrease in cell viability regardless the presence or absence of O/V (Caillaud et al., 2012). Since the effect of the toxin (also resulting in a decrease in cell viability) can only be observed in the wells with O/V pre-treatment, the difference respect to the wells without O/V helps to discriminate the matrix effect from the toxin effect. In the case of Na_v-inhibiting toxins (STX or TTX), previous studies with shellfish (Alkassar et al., 2024; Aballay-Gonzalez et al., 2016) have also shown a decrease in cell viability in the absence of O/V at high matrix concentrations. However, even at safe matrix concentrations (below the concentrations that cause this decrease in cell viability), an additional matrix effect may be observed in the presence of O/V, which is reflected by an increase in cell viability. This matrix effect could lead to lower robustness, lower sensitivity and false positives (since the toxin also cause an increase in cell viability). Thus, prior to the analysis of naturally contaminated samples, the matrix effects caused by each type of tissue on Neuro-2a cells should be evaluated. The tissue extracts (muscle, skin, liver, intestinal tract and gonads) of a *L. lagocephalus* specimen negative for PSTs and TTXs were used to determine the maximum tissue equiv. load capacity (safe tissue concentration where there are no matrix interferences). Fig. 3 shows the viability percentages of Neuro-2a cells with and without O/V pre-treatment and exposed to different tissue extract concentrations.

In the absence of O/V pre-treatment (No O/V), the cell viability started to decrease below 90% at 8.7 mg tissue equiv./mL in the case of skin, intestinal tract and gonads. For the muscle and liver extracts, no cell mortality was observed at any of the tested concentrations. However, in the presence of O/V, a non-specific increase in cell viability (above 10%) was observed at 8.7 mg tissue equiv./mL for skin, liver, intestinal tract and gonads. Nevertheless, with the muscle extract, this matrix interference was not observed at any of the tested concentrations. Therefore, safe tissue concentrations of 2.9 mg tissue equiv./mL for skin, liver, intestinal tract and gonads and 8.7 mg tissue equiv./mL for muscle were chosen.

Then, the recovery from STX/dcSTX-spiked muscle tissue was determined. The STX equiv. contents found in the CBA significantly overestimated the spiked levels with recovery values of 170 ± 10% and 218 ± 11% for 800 and 200 μg STX equiv./kg, respectively. Although

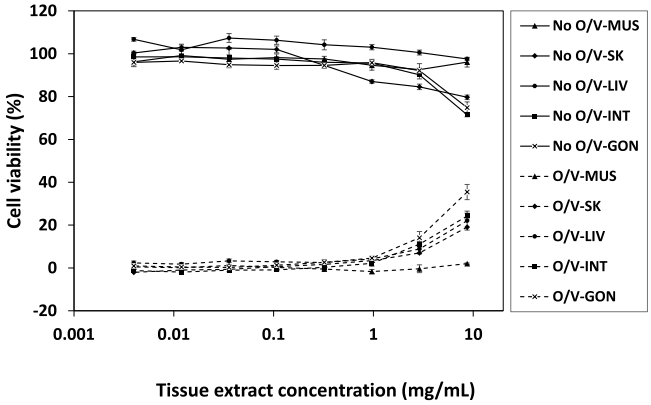

Fig. 3. Cell viability of Neuro-2a exposed to *L. lagocephalus* tissue extracts (STX and TTX-free) in presence and absence of O/V. MUS: muscle, SK: skin, LIV: liver, INT: intestinal tract, and GON: gonads.

the spiked extract was analysed at matrix concentrations that had previously been proved to not interfere with the performance of the assay, these high recovery values seem to indicate that the presence of fish matrix compounds could have an additive effect on the response caused by the toxin. Although this overestimation of the toxin content could result in a false positive, in an official monitoring programme, the CBA would be able to avoid false negative results and identify the positive or suspicious ones, which should be subsequently analysed by HPLC-FLD to be confirmed. Taking into account together the limit of detection (LOD) from the calibration curve (0.50 ng/mL), the tissue concentrations allowed for the different tissues and the recovery values, the effective LODs were calculated to be 26 μg STX equiv./kg for muscle and 79 μg STX equiv./kg for skin, liver, intestinal tract and gonads (effective LOD (μg STX equiv./kg tissue) = LOD in buffer (ng/mL) / safe tissue concentration (mg tissue equiv./mL) × (100 / recovery value) × 1000). These values are well below the value proposed by the European Commission for shellfish (800 μg STX equiv./kg shellfish tissue) and the Japanese regulation for TTX in pufferfish (2000 μg TTX equiv./kg fish tissue).

Regarding APC, the study of pufferfish matrix effects was performed in our previous work (Campàs et al., 2024), which demonstrates that a concentration of muscle, skin, liver and gonads tissue extracts of 10 mg/mL does not interfere with the assay. In this case, the recovery values were determined to be 50 ± 6% and 77 ± 10% for 800 and 200 μg STX equiv./kg, respectively. Taking into account together the LOD from the calibration curve (0.28 ng/mL), the tissue concentration allowed and the recovery value, the effective LOD was calculated to be 36 μg STX equiv./kg.

In the case of HPLC-FLD, recovery values were determined to be 103 ± 6% for 200 μg STX/kg and 102 ± 3% for 200 μg dcSTX/kg. Therefore, the effective LOQs were essentially the same as those stablished in the method, which were 100 μg STX equiv./kg for both STX and dcSTX.

All these toxin recovery values will be used as correction factors to be applied to the quantifications obtained in the analysis of naturally contaminated samples in sections 3.3 and 3.4.

3.3. Analyses of PSTs in pufferfish samples with Neuro-2a cells

The toxicity of the extracts obtained from five tissues (muscle, skin, liver, intestinal tract and gonads) of sixteen out of seventeen pufferfish specimens was assessed by both CBA and APC (the *L. sceleratus* specimen had been analysed in a previous work (Campàs et al., 2024)). Fig. 4A and B show the results obtained in the analysis of a *L. lagocephalus* liver extract (sample ID: 346.16) with CBA and APC, respectively, as an example. The presence of liver extract counteracted the O/V-induced cytotoxicity, resulting in an increase in cell viability in a concentration-dependent manner (Fig. 4A, O/V). No cytotoxicity due to the matrix effect was observed in the absence of O/V (Fig. 4A, no O/V). In Fig. 4B, the Na_v currents in Neuro-2a cells were inhibited when the liver extract was injected at 0.5 mg/mL. The toxin concentrations in μg STX equiv./kg are shown in Table 2. None of the *S. pachygaster* specimens showed toxicity due to PSTs in any of the tissues. Nevertheless, toxicity due to PSTs was detected with CBA in 8 and with APC in 9 out of the 11 *L. lagocephalus* specimens analysed. This toxicity was observed mainly in their livers, with STX equiv. contents ranging from 69 to ~40, 700 μg STX equiv./kg. Only two of the liver extracts exhibited PST contents above the EU regulatory limit of 800 μg STX equiv./kg for shellfish. Nevertheless, one *L. lagocephalus* specimen (sample ID: 346.16) showed STX equiv. contents in all the tissues, which were very high in liver and intestinal tract, and considerable in skin and gonads.

3.4. Toxin profile of pufferfish samples

To confirm the presence of PSTs and to characterise the toxin profile in the different tissue extracts of pufferfish specimens previously analysed by CBA and APC, HPLC-FLD analyses were performed. Fig. 4C shows the results obtained in the analysis of a *L. lagocephalus* liver extract (sample ID: 346.16) with HPLC-FLD, as an example. The STX equiv. contents, calculated by summing individual toxin concentrations and applying the EFSA TEFs and the recovery, are summarised in Table 2. In agreement with CBA and APC, no PSTs were detected in specimens belonging to the *S. pachygaster* species. Regarding *L. lagocephalus*, as also expected from the toxicological analyses, 8 out of the 11 specimens provided positive results for their livers, and in this case 5 of them exceeded the EU regulatory limit of 800 μg STX equiv./kg for shellfish. The total STX equiv. contents in liver ranged from 337 to ~58,100 μg STX equiv./kg. Again, the *L. lagocephalus* specimen 346.16 showed PSTs presence in the five tissues, with very high contents in liver, intestinal tract and gonads. The PST contents detected in liver and intestinal tract were 72- and 27-fold higher than the EU regulation for

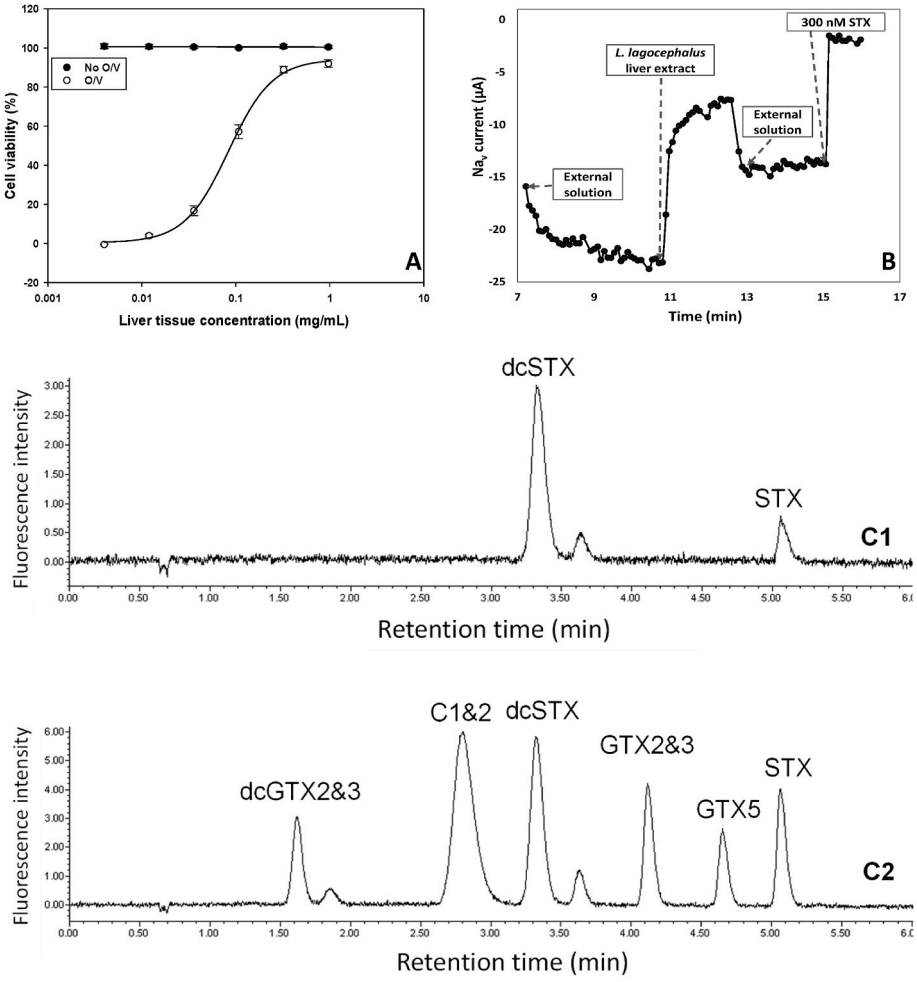

Fig. 4. Results of the analysis of a *L. lagocephalus* liver extract (Sample ID: 346.16) obtained with CBA (**A**), APC (**B**) and HPLC-FLD (**C1**) analyses, and HPLC-FLD chromatogram of certified standards of MIX I (**C2**).

Table 2
STX equiv. contents (μg STX equiv./kg) obtained in different tissues of *S. pachygaster* and *L. lagocephalus* pufferfish specimens using CBA, APC and HPLC-FLD. For HPLC-FLD, STX equiv. contents refer to the sum of STX and analogues after applying the TEF from EFSA for dcSTX.

Sample ID	Species	CBA					APC					HPLC-FLD				
		MUS	SK	LIV	INT	GON	MUS	SK	LIV	INT	GON	MUS	SK	LIV	INT	GON
276.14	*L. lagocephalus*	–	–	184	–	–	–	175	201	–	–	–	–	337	–	–
286.14	*L. lagocephalus*	–	–	160	–	–	–	–	212	–	–	–	–	1353	–	–
288.14	*L. lagocephalus*	–	–	409	981	–	–	–	475	78	–	–	–	1303	–	–
289.14	*L. lagocephalus*	–	–	–	–	–	–	–	69	–	–	–	–	–	–	–
290.14	*L. lagocephalus*	–	–	1061	–	–	–	–	1280	–	–	–	–	1830	–	–
306.14	*L. lagocephalus*	–	–	446	–	–	–	–	464	–	–	–	–	1257	–	–
307.14	*L. lagocephalus*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
315.14	*L. lagocephalus*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
316.14	*L. lagocephalus*	–	–	131	–	–	–	–	165	–	–	–	–	630	–	–
382.14	*S. pachygaster*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
046.15	*S. pachygaster*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
430.14	*S. pachygaster*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
501.15	*L. lagocephalus*	–	–	122	–	–	–	–	205	–	–	–	–	491	–	–
543.15	*S. pachygaster*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
075.16	*S. pachygaster*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
346.16	*L. lagocephalus*	175	605	40,729	10,417	1082	216	1277	15,258	5270	508	283	761	58,115	21,746	1201

shellfish. PSTs were also detected in muscle and skin but at lower concentrations. In all PST-positive extracts, dcSTX was found to be the major toxin and STX the minor toxin (Fig. 4C), with concentrations ranging from 172 to ~41,600 μg dcSTX/kg and from 111 to ~16,500 μg STX/kg (Fig. 5). No other known STX analogues, such as C toxins, gonyautoxins (GTXs) and neoSTX, were detected in any of the samples.

Finally, it is important to mention that the *L. sceleratus* specimen, previously analysed by CBA and APC (Campàs et al., 2024) and by LC-MS/MS for TTXs (Rambla-Alegre et al., 2017), was analysed with HPLC-FLD for PSTs in this work. Although TTX and several of its analogues were found in this sample (Rambla-Alegre et al., 2017), no PSTs were detected in any of the tissues.

4. Discussion

Poisoning due to the consumption of pufferfish is usually caused by the ingestion of TTX (Ulman et al., 2024). However, several cases of poisoning due to consumption of pufferfish containing PSTs have also been reported worldwide (Landsberg et al., 2006; Morris et al., 2015; Garcia et al., 2004; Anderson et al., 1996; Rodrigue et al., 1990; Kao and Falconer, 1993). In this study, tissue extracts (muscle, skin, liver, intestinal tract and gonads) obtained from sixteen pufferfish specimens of the *L. lagocephalus* and *S. pachygaster* species, collected along the Spanish Mediterranean coast, were analysed for the presence of PSTs using CBA and APC, methodologies previously developed by our group to detect TTXs in *L. sceleratus* (Alkassar et al., 2023; Campàs et al., 2024), which can be applied to PSTs. Furthermore, the presence of PSTs in these pufferfishes, as well as in one *L. sceleratus* specimen, was assessed by pre-column oxidation HPLC-FLD.

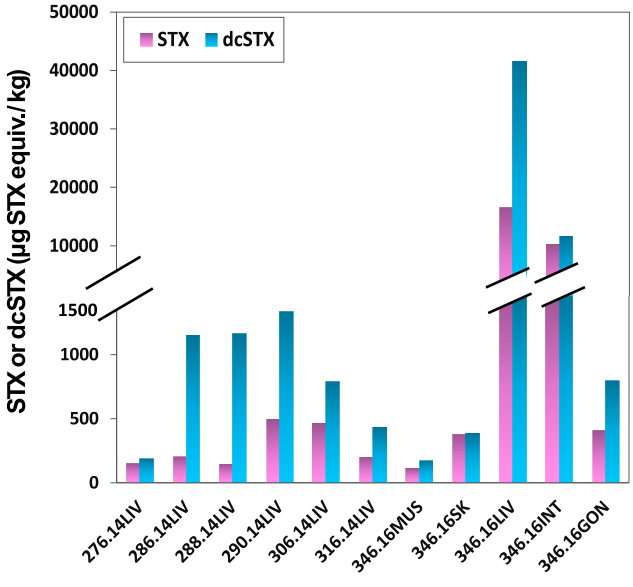

Fig. 5. STX and dcSTX contents in all positive *L. lagocephalus* specimens obtained by pre-column oxidation HPLC-FLD analysis. MUS: muscle, SK: skin, LIV: liver, INT: intestinal tract and GON: gonads.

Both CBA and APC provide an estimation of the overall toxicity of a sample, since they implement living cells. However, the toxicological effect is measured using different experimental variables. In CBA, cell viability is measured using MTT as an indicator of cell viability, while in APC Na_v currents are recorded. Contrary to CBA and APC, the quantification of toxin contents by instrumental analysis techniques is based on the structural recognition of the individual toxin analogues. Therefore, instrumental analysis methods can provide the toxin profile of a sample but do not inform about its toxicity and require the application of TEFs for estimating the total STX equivalents. In this context, TEFs are essential to translate the analytical results obtained from analytical techniques into toxicological values. However, published TEFs may suffer from variability due to the differences between the detection techniques and, even among the same methods, due to the parameters used in the calculation of TEFs and the purity of the standards (Botana et al., 2010). In our study, the TEFs obtained for dcSTX with Neuro-2a cells were different between methods (TEF CBA = 0.19 and TEF APC = 0.05) and lower than the one proposed by EFSA (TEF = 1) (European Food Safety Authority (EFSA), 2009). Therefore, the Neuro-2a cells (CBA and APC) revealed a lower toxicity of dcSTX in relation to STX, which is very different from the method (MBA) used to calculate the TEF proposed by EFSA, which was 1. The discrepancy of TEFs obtained between CBA and APC can be explained by the fact that APC and CBA measure different experimental variables to estimate toxicity, as previously mentioned. Additionally, the toxicological response in APC is derived from single cells, whereas in the CBA it originates from cell monolayers, hence a cell population. Nevertheless, the lower toxic potency of dcSTX compared to STX is consistent with some studies (using Neuro-2a cells or in mice), where varying TEF values of 0.19 (Viallon et al., 2020), 0.27 (Aballay-González et al., 2020), 0.37 (Suzuki and Machii, 2014) and 0.64 (Munday et al., 2013), but always lower than 1, have also been reported. The difference among TEFs can be explained by the varying affinities of STX and its analogues for the different Na_v

subtypes, as previously demonstrated by some researchers (Alonso et al., 2016; Walker et al., 2012). In these studies, the TEF for dcSTX was calculated to be between 4.6 and 0.07, depending on the Na_v subunit. RT-PCR analysis of Na_v subunit expression in Neuro-2a cells showed that Na_v 1.2, 1.3, 1.4 and 1.7 were detected, being the Na_v 1.7 robustly expressed (Lou et al., 2005), which is less affected by PSTs (Alonso et al., 2016) than the other Na_v channel α-subunits. We do not know which subunits are expressed in our Neuro-2a cells; nevertheless, they are sensitive enough for the detection of STX and its analogues. Therefore, the IC_{50} values obtained in this study for STX are similar to those obtained by other authors also using Neuro-2a cells (Viallon et al., 2020; Aballay-González et al., 2020; Melegari et al., 2015; Manger et al., 1993).

Unlike APC, in CBA, as explained before, an additional step, pre-treatment of cells with O/V prior to exposure to extracts, is required. This pre-treatment causes additional stress to the cells, which may make the CBA more susceptible to matrix effects than APC. Consequently, these undesirable interferences may affect the sensitivity of the CBA, which may require the use of lower extract concentrations, leading to a higher LOD in comparison with the APC. Besides, one must not ignore the fact that whereas in APC the sodium influx through the Na_v channels is directly measured, the CBA comprises a series of cellular processes which could be affected by compounds from the matrix other than the toxins themselves. It is not surprising that, since the detection principles are different, the matrix effects may also be different.

Regarding the recovery values, it is important to mention that the extracts were the same for the three techniques and that the recovery values found by pre-column oxidation HPLC-FLD were close to 100%, indicating that no toxin losses occurred during the extraction and clean-up. In the study of the toxin recovery with the CBA, even though the analyses were performed at tissue concentrations where no matrix effect occurred, the results overestimated the spiked STX and dcSTX concentrations. This overestimation has also been observed in previous works that analyse shellfish (Alkassar et al., 2024; Aballay-Gonzalez et al., 2016). Some researchers (Aballay-Gonzalez et al., 2016; Turner et al., 2012) have attributed these interferences to the extraction procedure, since the extracts could contain some neurotoxic compounds and/or free fatty acids and enzymes co-extracted with the toxin. In this case, the discrimination between the presence of toxin or other compounds that could be toxic to the cells is practically impossible. Additionally, many studies (Karunanidhi et al., 2017; Eken et al., 2017; Doğdu et al., 2021) have highlighted the eventually high bioaccumulation of heavy metals (such as Li^{+}, Cd^{+2}, Ca^{+2} and Zn^{+2}) in pufferfish species. At cellular level, these monovalent and divalent cations may act as the Na_v-inhibiting toxins for the binding to Na_v channels, leading to an overestimation of the toxicity (Turner et al., 2012; Doyle et al., 1993; Favre et al., 1995). Therefore, further investigations into the effects of heavy metals or other compounds on the CBA are needed as well as refinements of the extraction methods. In APC, the recovery values results underestimated the spiked STX and dcSTX concentrations. In this case, the matrix compounds other than the toxin may be decreasing the affinity of the toxins for the Na_v channels.

All *S. pachygaster* specimens analysed by CBA, APC and pre-column oxidation HPLC-FLD were found to be negative for PSTs. In fact, in a previous study carried out in our laboratory, the same *S. pachygaster* samples also tested negative for TTX (Rambla-Alegre et al., 2017). The pufferfish *S. pachygaster* in the Mediterranean Sea (Ulman et al., 2022; Malloggi et al., 2023), in the Japanese coast (Masuda et al., 1984; Jeong et al., 1994) and in the East China Sea (Amano et al., 2022) has been classified as non-toxic. Contrary, this species in Pacific waters was reported to be weakly toxic (Noguchi and Arakawa, 2008). Furthermore, in American waters (Florida), several species belonging to the *Sphoeroides* genus have been found to contain high concentrations of TTX, STX and their analogues (Landsberg et al., 2006; Abbott et al., 2009). Additionally, poisoning and death cases associated with specimens belonging to this genus were reported in North America (Landsberg et al., 2006).

In this work, *L. lagocephalus* specimens were also analysed, most of them being toxic. The toxicity of these specimens was mainly observed in their livers, two of them exceeding the level considered to be safe for human consumption according to the European Commission legislation for shellfish (800 μg STX equiv./kg) (European Food Safety Authority (EFSA), 2009). However, PSTs were also detected in all tissues of one *L. lagocephalus* specimen (sample ID: 346.16), which showed high PSTs contents not only in the liver and intestinal tract, but also considerable PSTs contents in skin and gonads. Fortunately, the muscle, which is the tissue most likely to be consumed, contained low PSTs content. It is important to note that some discrepancies have been observed regarding the quantification of PSTs content. For example, a low toxicity was detected in the skin of sample 276.14 and in the liver of the sample 289.14, but only with APC, which is the technique with the lowest LOD. Another example is the intestinal tract of sample 288.14. In this sample, the toxicity detected with the CBA would exceed the regulatory limit of PSTs in shellfish; however, the APC revealed a significantly lower toxicity. It is important to note that HPLC-FLD analysis of this sample did not identify any PST and, besides the fact that HPLC-FLD has the highest LOD, one cannot rule out the possibility that the sample contain an unknown compound with Na_v-inhibiting activity. Regardless the differences in PSTs equiv. found between CBA and APC, these PSTs contents were in line with those found in other marine or freshwater pufferfishes, which ranged from 3.7 to 58,700 μg STX equiv./kg (Soliño et al., 2021; Zhu et al., 2020a). The presence of PSTs in pufferfish was initially attributed to freshwater pufferfish species, such as the genera *Pao* and *Leiodon* (Sato et al., 1997). Later, the co-occurrence of TTX and STX in several marine and freshwater pufferfish species, such as *Sphoeroides*, *Arothron* and *Canthigaster*, was reported, although with variable ratios depending on the species and tissue (Sato et al., 2000; Nakashima et al., 2004; Zhu et al., 2020b). Unlike for TTXs, where the liver is the most toxic tissue almost throughout the year, with the exception of the spawning season when the ovaries become highly toxic, PSTs in some pufferfishes, such as *Fugu pardalis*, *Sphoeroides* genus and *Pao* genus, are consistently much higher in muscle and skin, and in some cases in ovaries (Jang and Yotsu-Yamashita, 2007; Landsberg et al., 2006; Abbott et al., 2009; Zhu et al., 2020a) than in liver. Regarding the Japanese *Takifugu*, PSTs levels were elevated in liver, ovaries and digestive tract (Nakamura et al., 1984). Nakashima and co-workers also found that PSTs were present only in the ovaries of *Arothron firmamentum*, together with TTXs, while the skin contained only TTX (Nakashima et al., 2004). Regarding *L. lagocephalus*, to the best of our knowledge, the presence of PSTs in these species from the Mediterranean Sea had not been previously evaluated. In fact, the presence of PSTs in pufferfish from the Mediterranean Sea, regardless of the species, had never been confirmed. It is important to mention that Katikou and co-workers (Katikou et al., 2009) analysed *L. sceleratus* specimens, which had provided a positive response by MBA, by an enzyme-linked immunosorbent assay (ELISA) assay for PSTs. Although results suggested the presence of PSTs in these samples, it was not confirmed with instrumental analysis methods.

Some works on the analysis of TTXs in *L. lagocephalus* have been performed. Saoudi and colleagues found TTX in the liver, and also muscle and skin but at lower concentrations, of a *L. lagocephalus* specimen caught off the Tunisian coast (Saoudi et al., 2011). However, *L. lagocephalus* specimens caught off the Spanish Mediterranean coast (Rambla-Alegre et al., 2017) which are the same as the ones analysed in this work, were found not to contain TTXs. In other geographical areas, only a couple of studies have evaluated the presence of TTX and PSTs in *L. lagocephalus* specimens, specifically from Madeira (Soliño et al., 2021; Pinto et al., 2019). In these studies, no TTXs were detected in this species, but PSTs were found in liver, intestine and gonads, although not in the muscle. These researchers suggested that the toxicity detected in *L. lagocephalus* was due to the accumulation of PSTs through trophic webs, since this species has an opportunistic trophic behaviour.

As for TTX, the accumulation of PSTs in pufferfish may differ depending on the sex, individual size, geographical area and season (Kosker et al., 2019). The mechanism by which PSTs accumulate in pufferfish species is still unclear. However, it is believed to be exogenous, occurring via the food web and beginning with STX-producing cyanobacteria in freshwater environments and STX-producing dinoflagellates in marine environments (Wiese et al., 2010). Gao and colleagues (Gao et al., 2019) in a recent *in vivo* TTX and STX administration experiment using non-toxic cultured marine and freshwater pufferfish demonstrated the capability of several pufferfish species to selectively accumulate TTX or STX in different tissues. This selectivity in the accumulation of TTX or STX may be related to the pufferfish STX and TTX-binding proteins (PSTBPs) (Yotsu-Yamashita et al., 2001). Yotsu-Yamashita and colleagues (Yotsu-Yamashita et al., 2018) showed that PSTBP is one of the most intriguing proteins related to the toxicity of pufferfish. PSTBP acts as a toxin transporter in the bloodstream and is involved in toxin absorption in the intestine as well as toxin accumulation in the skin, liver and ovaries of pufferfish. Tatsuno and co-workers (Tatsuno et al., 2013) reported the presence of four isoform genes homologous to PSTBP, two of which are expressed in the liver. These genes are found in toxic pufferfish species but not in non-toxic pufferfish species and other fish (Tatsuno et al., 2013). Moreover, Zhu and collaborators (Zhu et al., 2024) suggested that tributyltin-binding protein type 2 (TBT-bp2), which has a similar structure to PSTBP and is presumed to be the evolutionary origin of PSTBP (Hashiguchi et al., 2015), is involved in the diversity of STX selective accumulation in pufferfish. They also found that external factors, including salinity (osmotic pressure) of the environmental water and TTXs/PSTs abundance in the habitat, influence the toxin profile of pufferfish (Zhu et al., 2024).

In the present work, we identified the presence of only two PSTs, STX and dcSTX, the latter being the major toxin found, with abundances ranging from 50 to 89% of the total PSTs, depending on the tissue and the specimen. Conversely, in the studies of the *L. lagocephalus* from Madeira, the authors identified STX as the main analogue, but the presence of dcSTX and neoSTX was also reported. In other pufferfish species, such as the marine *Arothron firmamentum*, Nakashima and colleagues also identified dcSTX as the major component in the ovaries (Nakashima et al., 2004). Sato and co-workers identified STX as the major component, and dcSTX, neoSTX and GTX5 as the minor components in seven marine pufferfish species of the genus *Arothron* collected in Philippines. Furthermore, in freshwater pufferfish (Zaman et al., 1997, 1998), such as *Tetraodon cutcutia* and *Chelonodon patoca*, STX and dcSTX were detected, along with GTX2&3, dcGTX2&3 and three unidentified components (designated as STX-uk, GTX-uk1 and GTX-uk2). The high abundance of dcSTX in the liver of pufferfish has been attributed to a possible enzymatic change, due to the presence of carbomylase and sulfocarbomoylase that would convert STX into dcSTX (Raposo et al., 2020). Some other researchers attributed the high bioaccumulation of dcSTX in pufferfish liver to its slow elimination compared to the other PSTs, which can be rapidly biotransformed or excreted (Costa et al., 2012), since the presence of additional sulphate groups in STX analogues may render them more hydrophilic and therefore more easily eliminated (Hong et al., 2003).

To further understand the results obtained through the toxicological detection techniques and the instrumental analysis technique, the TEFs of dcSTX obtained by CBA (TEF = 0.19) and APC (TEF = 0.05) in our study, as well as the TEF proposed by EFSA (TEF = 1) were applied to the individual contents determined by pre-column oxidation HPLC-FLD. The results are shown in Table 3. When the TEFs of EFSA were applied, the HPLC-FLD values of PSTs where much higher compared to CBA and APC, with 7 out of 12 samples with PSTs above the regulatory limit for shellfish (800 μg STX equiv./kg). The application of the TEFs obtained in our study resulted in a decrease of the toxicity estimation, and only two samples contained PSTs above the regulatory limit for shellfish. It is necessary to mention that, even after application of these TEFs, some discrepancies in particular data points still exist, which can be mainly attributed to the differences in the detection principle between the three techniques. Nevertheless, the better agreement between the PSTs contents obtained from the CBA or APC and those derived from the pre-column oxidation HPLC-FLD analysis after the application of TEFs clearly demonstrates the applicability and the importance of TEFs in obtaining useful toxicological estimations from analytical techniques.

5. Conclusions

In this study, the presence of PSTs in different tissues of pufferfish specimens caught along the Spanish Mediterranean coast was evaluated, using CBA and APC as toxicological methods and pre-column oxidation HPLC-FLD as an instrumental analysis technique. The TEF of dcSTX was determined using the two toxicological approaches, being this analogue less toxic than STX. These two techniques were applied to the analysis of different tissues of pufferfishes of the species *S. pachygaster* and *L. lagocephalus*, demonstrating the non-toxicity of *S. pachygaster* specimens inhabiting the Mediterranean Sea and the presence of STX equiv. contents in the liver of most *L. lagocephalus* specimens. HPLC-FLD analysis, as a confirmatory method to identify the presence of STX and its analogues, was performed. The results demonstrated the presence of STX and dcSTX in all positive samples, mainly in liver, being dcSTX the major analogue. Another remarkable result was the high concentrations of PSTs, which exceeded the current EU regulatory limit for shellfish, found in one *L. lagocephalus* specimen. This work contributes to the better knowledge of the *L. lagocephalus* toxicity. Additionally, the high PSTs contents found in the *L. lagocephalus* specimens evidence the risk that the presence of this toxic fish in the Mediterranean Sea may

Table 3
Contents of STX and dcSTX in *L. lagocephalus* positive samples after application of the EFSA TEFs (obtained from Table 2 and included for comparison purposes) and the ones obtained with Neuro-2a cells (TEF CBA = 0.19 and TEF APC = 0.05) to pre-column oxidation HPLC-FLD individual toxin contents. The contents are expressed as μg STX equiv./kg of pufferfish tissue. *Results of CBA and APC are also obtained from Table 2 and are included for comparison purposes.

Sample ID	CBA*	APC*	HPLC-FLD				
			STX	dcSTX	STX equiv. (TEF EFSA)	STX equiv. (TEF CBA)	STX equiv. (TEF APC)
276.14 LIV	184	201	148	189	337	184	157
286.14 LIV	160	212	205	1148	1353	423	262
288.14 LIV	409	475	140	1162	1302	361	198
290.14 LIV	1061	1280	497	1333	1830	750	564
306.14 LIV	446	464	464	793	1257	615	504
316.14 LIV	131	165	198	432	630	280	220
501.15 LIV	122	205	184	307	491	242	199
346.16 LIV	40,729	15,258	16,489	41,626	58,115	24,398	18,570
346.16 INT	10,417	5270	10,169	11,576	21,745	12,368	10,748
346.16 GON	1128	508	406	795	1201	557	446
346.16 SK	605	1277	377	384	761	450	396
346.16 MUS	175	216	111	172	283	144	120

represent for seafood safety and human health in case of accidental consumption. Further studies are required to accurately predict seasons and tissues with higher probability of PSTs accumulation in this pufferfish species and to identify PST-producing organisms and vectors that are involved in the bioaccumulation process.

CRediT authorship contribution statement

Mounira Alkassar: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Àngels Tudó:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Maria Rambla-Alegre:** Writing – review & editing, Methodology. **Laura Ferreres:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Jorge Diogène:** Writing – review & editing, Supervision, Methodology. **Francesc X. Sureda:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis. **Mònica Campàs:** Writing – review & editing, Writing – original draft, 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.

Data availability

Data will be made available on request.

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