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Research article

Analytical and functional profiles of paralytic shellfish toxins extracted from *Semele proficua* and *Senilia senilis* from Angola

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ABSTRACT

Paralytic shellfish poisoning is a foodborne illness that typically derive from the consumption of shellfish contaminated with saxitoxin-group of toxins produced by dinoflagellates of the genus Gymnodinium, Alexandrium and Pyrodinium. N-sulfocarbamoyl, carbamate and dicarbamoyl are the most abundant. In 2007 and 2008 some episodes of PSP occurred in Angola where there is not monitoring program for shellfish contamination with marine biotoxins. Therefore, ten samples extracted from Semele proficua from Luanda Bay and Senilia senilis from Mussulo Bay, were analyzed by HPLC finding saxitoxin, decarbamoylsaxitoxin and other three compounds that have an unusual profile different to the known hydrophilic PSP toxins were found in different amounts and combinations. These new compounds were not autofluorescent, and they presented much stronger response after peroxide oxidation than after periodate oxidation. The compounds appear as peaks eluted at 2.5 and 5.6 min after periodate oxidation and 8.2 min after peroxide oxidation. Electrophysiological studies revealed that none of the three unknown compounds had effect at cellular level by decreasing the maximum peak inward sodium currents by blocking voltage-gated sodium channels. Thus, not contributing to PSP intoxication. The presence in all samples of saxitoxin-group compounds poses a risk to human health and remarks the need to further explore the presence of new compounds that contaminate seafood, investigating their activity and developing monitoring programs.

1. Introduction

Paralytic shellfish poisoning (PSP) is one of the most important food poisonings developed after the consumption of shellfish contaminated with the marine toxins saxitoxins (STX) and its analogues.

These toxins responsible of PSP are metabolites produced by dinoflagellates from the genus *Pyrodinium bahamense, Gymnodinium* and *Alexandrium*, specifically, *Alexandrium tamarense, Alexandrium fundyense* and *Alexandrium catenella* [1]. The genus *Gymnodinium* appears frequently in temperate and tropical waters and the genus *Pyrodinium* in tropical waters [2]. STX is the most important toxin

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Abbreviations

CGCs	Cerebellar granule cells
dcSTX	Decarbamoylsaxitoxin
dcNeoSTX	Decarbamoylneosaxitoxin
GTX	Gonyautoxins
NeoSTX	Neosaxitoxin
PSP	Paralytic shellfish poisoning
STX	Saxitoxin
VGSCs	Voltage-gated sodium channels

produced by these organisms and was the first PSP toxin recognized from the dinoflagellate *Alexandrium catenella* [3]. Besides STX, these dinoflagellates produce a series of STX analogues which are mainly hydrophilic molecules, although in a recent study three analogues with an hydrophobic component have been isolated from Australian strains of *Gymnodinium catenatum* known as GC derivates [4]. The group of toxins involved in PSP is represented in Fig. 1 and include STX and its related compounds; neosaxitoxin (NeoSTX), decarbamoylsaxitoxin (dcSTX), decarbamoylneosaxitoxin (dcNeoSTX), gonyautoxins 1 to 6 (GTX) and their dicarbamoyl analogues (dcGTX) and four C-toxins (C 1–4) [5]. Recently, several analogues of STX possessing a hydroxybenzoate moiety have also been identified [4,6]. Due to their mechanism of action blocking the voltage-gated sodium channels (VGSCs) in excitable cells [7,8], the main symptoms caused by PSP toxins are neurological, manifesting as tingling sensation of the lips, numbness of extremities, gastrointestinal problems and difficulty in breathing [9].

In 2006, the presence of *Gymnodinium catenatum* and *Pyrodinium bahamense* has been reported in the Angola coast [10]. Currently there is not monitoring program for bivalve mollusk contaminated with marine biotoxins in Angola, which is very important taking into account that this area has a high consumption of marine products [11]. Samples of the clams *Semele proficua* from Luanda Bay and samples of *Senilia senilis* from Mussulo Bay collected between June 2007 and February 2008 [12] were used to develop a preliminary assessment of PSP toxins content in bivalves in this area. The study allowed to conclude that these samples exhibited an unusual profile that did not match any of the 10 oxidation products expected from the known hydrophilic PSP toxins usually reported in marine dinoflagellates. Instead, three major compounds which were not autofluorescent and whose retention times did not match the retention times of standards after separation in C18 columns were found [12].

With the aim to further explore the contribution of these compounds to the PSP intoxications, the ten samples from the previously described clams were received and analyzed in 2009 by HPLC to establish the amount and the type of toxins contained in each sample and once known the toxic content, the same samples were studied by electrophysiology to determine their contribution to the PSP intoxication based on their activity blocking the VGSC on primary cultured neurons.

2. Materials and methods

2.1. Chemicals, solutions and toxin standards

Plastic tissue-culture dishes were purchased from Falcon (Madrid, Spain). Foetal bovine serum was obtained from Gibco (Glasgow, UK) and Dulbecco's Modified Eagle's medium (DMEM) was from Biochrom (Berlin, Germany). All other chemicals were reagent grade and purchased from Sigma-Aldrich (Madrid, Spain). Certified toxin standards were obtained from the Institute for Marine Bioscience, National Research Council of Canada (NRCC, Halifax, Nova Scotia, Canada): STX, NeoSTX, GTX1,4, GTX2,3, C1,2, GTX5, dcSTX, dcNeo, and dcGTX2,3. Reagents were of analytical grade and solvents were HPLC grade. Primary toxin standards were prepared to have concentrated stock standard solutions and stored following NRCC recommendations. To perform oxidation of the extracts and quantitation, toxin standard mixes (I-IV) were prepared as recommended [20] and are illustrated in Table 1.

	R1	R2	R3	R4
STX	H	H	H	$OCONH_2$
NeoSTX	OH	H	H	$OCONH_2$
dcSTX	H	H	H	OH
dcNeoSTX	OH	H	H	OH
GTX1	OH	OSO_3	H	$OCONH_2$
GTX2	H	OSO_3	H	$OCONH_2$
GTX3	H	H	OSO_3	$OCONH_2$
GTX4	OH	H	OSO_3	$OCONH_2$
GTX5	H	H	H	$OCONHSO_3$
GTX6	OH	H	H	$OCONHSO_3$

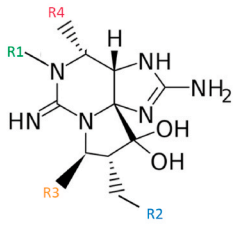

Fig. 1. Chemical structure of the toxins involved in PSP.
Abbreviations: STX: Saxitoxin, NeoSTX: neosaxitoxin, dcSTX: decarbamoyl saxitoxin, dcNeoSTX: decarbamoyl neosaxitoxin, GTX1 to 6: gonyautoxins 1 to 6, R1 to 4: radical 1 to 4.

2.2. HPLC toxins identification

A LC-18 Supelco reversed-phase column was used (15 cm x 4,6 mm and 5 μm particle size) with a TR-C-160-1 Teknokroma guard column. HPLC equipment was a Shimadzu fluorescence detector (model RF-10AXL), with excitation and emission wavelengths of 340 nm and 395 nm respectively. Column was thermostated at 35 °C in a Shimadzu oven (model CTO-20AC) and Shimadzu autosampler (model SIL-20AC) was thermostated at 6 °C. Mobile phase (A): ammonium phormiate 0.1 M adjusted at pH = 6 ± 0,1 with acetic acid 0,1 M; (B): ammonium phormiate 0,1 M dissolved in 5 % acetonitrile and also adjusted at pH = 6 ± 0.1 with acetic acid 0.1 M. Mobile phase was pumped through two Shimadzu pumps (model LC-10AD) at a flowrate of 2 mL/min. Gradients were: 0–5 % of B for 5 min, 5–70 % of B for the next 4 min and 0 % of B the following 6 min to equilibrate the column.

Individual toxins and mixtures were identified with pre-column oxidation HPLC Lawrence method [13]. Toxins and mixtures were oxidized using either peroxide or periodate, injecting 25 and 100 μL volumes respectively. Chromatographic data were revised to verify retention times and relative peak area responses for toxins under both oxidation conditions. The toxicity equivalence factors (TEFs) described by Oshima [5] were used. Individual toxin concentrations were reported as μg STX dihydrochloride eq/kg, and the total PSP toxicity was calculated by summing the individual contribution of toxins and given as μg STX dihydrochloride eq/kg.

2.2.1. Extraction, cleanup and oxidation of samples

The extraction and extract oxidation procedures of Lawrence [14] and AOAC Method 2005.06 were followed as closely as possible. Samples were extracted with acetic acid followed by SPE-C18 clean-up and oxidation with periodate and peroxide before HPLC analysis. For toxins with more than one oxidation product (GTX1,4, GTX2,3, dcSTX, dcGTX2,3, and NEO), to consider a sample positive it was also required the presence of at least the most significant secondary peak.

If sample contains C1,2, dcSTX, GTX2,3, GTX5 and STX, they were directly quantified after SPE-C18 cleaning; if sample contains GTX1,4, C3,4 (not available standard), NEO and GTX6 (not available standard) must be purified through SPE-COOH ionic exchange cartridges, to obtain three separated fractions (F1–F3) (Table 2) with toxins to be oxidized and analyzed by HPLC-FLD. Fraction F1 contains the N-sulfocarbamoyl C-toxins (C1,2), F2 contains the gonyautoxins (GTX) group of toxins (GTX1-5, and dcGTX2,3) and the carbamates (STX, dcSTX, and NEO) elute in F3 [15].

Matrix modifier was prepared from oyster extract free of PSP toxins, using the same extraction procedure and leaving it to precipitate in the refrigerator for 2–3 days. Oyster extract can be stored at −20 °C for two months, but the modifier must be prepared fresh every two weeks. The absence of PSP toxins was previously certified by oxidation with peroxide and periodate.

2.3. Cell cultures

Primary cultures of cerebellar granule cells (CGCs)were obtained from cerebella of 7-day-old mice (from the animal care facilities of the University of Santiago de Compostela) as previously described [16,17]. In brief, cells were dissociated by mild trypsinization at 37 °C, followed by trituration in a DNAse solution (0.004 % w/v) containing a soybean trypsin inhibitor (0.05 % w/v). Cells were suspended in DMEM containing 25 mM KCl, 31 mM glucose, and 0.2 mM glutamine supplemented with p-amino benzoate, insulin, penicillin and 10 % foetal calf serum. The cell suspension was seeded in 18 mm glass coverslips precoated with poly-D-lysine and incubated in 12 multiwell plates for 6–11 days *in vitro* (div) in a humidified 5 % CO_2/95 % air atmosphere at 37 °C. Cytosine arabinoside, 20 μM, was added before 48 h in culture to prevent glial proliferation.

2.4. Sample preparation

Samples of the clams *Semele proficua* and *Senilia senilis* [12] were dissolved in 50 μl 0.03 M acetic acid and after dilution 1/100 with extracellular medium used in electrophysiological experiments.

Table 1
Toxin standard mixes used in this study.

Toxin mixture	Toxins
I	dcGTX2,3, dcSTX, STX (for peroxide oxidation)
II	C1,2, GTX2,3, GTX5 (for peroxide oxidation)
III	NeoSTX, GTX1,4 (for periodate oxidation)
IV	dcNeoSTX (for periodate oxidation)

Abbreviations.
STX: Saxitoxin.
C 1,2: C-toxins 1 and 2.
NeoSTX: neosaxitoxin.
dcSTX: decarbamoyl saxitoxin.
dcGTX: decarbamoyl gonyautoxins.
dcNeoSTX: decarbamoyl neosaxitoxin.
GTX: gonyautoxins.

Table 2
PSP toxins classified into fractions.

	F1	F2	F3
Peroxide oxidation	C1,2	GTX2,3 GTX5 dcGTX2,3	STX dcSTX
Periodate oxidation	C3,4	GTX2,3 GTX1,4 dcGTX2,3 GTX6	STX dcSTX NeoSTX

Abbreviations.
STX: Saxitoxin.
C 1,2: C-toxins 1 and 2.
C 3,4: C-toxins 3 and 4.
NeoSTX: neosaxitoxin.
dcSTX: decarbamoyl saxitoxin.
dcGTX: decarbamoyl gonyautoxins.
GTX: gonyautoxins.

2.5. Electrophysiology

Voltage gated sodium currents from single cells were studied at room temperature (22–25 °C) by electrophysiological recordings in whole cell mode using a computer-controlled current and voltage clamp amplifier (Multiclamp 700 B, Molecular Devices). Signals were recorded and analyzed using a Pentium computer equipped with Digidata 1440 data acquisition system and pClamp10 software (Molecular Devices, Sunnyvale, CA). pClamp10 was used to generate current and voltage-clamp commands and to record the resulting

Table 3
Different amount of toxins present in each sample analyzed.

SAMPLE	μg/kg	μg STX Eq/kg
Sample 12	STX: 8.21 Other: 2.5 min: 7.87	16.08
Sample 15	STX: 9.78 Other: 5.6 min: 7.8 8.2 min: 4.9	22.48
Sample 16	STX: 7.83 Other: 2.5 min: 5.3 5.6 min: 6.3 8.2 min: 5.2	24.63
Sample 17	STX: 7.53 Other: 5.6 min: 6.04	13.57
Sample 18	STX: 22.0 dcSTX: 4.86 Other: 5.6 min: 9.61	36.47
Sample 19	STX: 20.37 dcSTX: 5.03 Other: 5.6 min: 8.1 8.2 min: 5.1	38.6
Sample 20	STX: 60.22 dcSTX: 11.57 Other: 5.6 min:17.3 8.2 min: 5.0	94.10
Sample 23	STX: 6.99	6.99
Sample 24	STX: 11.32 dcSTX: 5.14 Other: 2.5 min: 7.4 5.6 min: 7.7	31.56
Sample 25	STX: 7.31 Other: 2.5 min: 4.7 5.6 min: 6.4	18.41

Abbreviations.
STX: Saxitoxin.
dcSTX: decarbamoyl saxitoxin.
GTX: gonyautoxins.

data. Signals were prefiltered at 10 kHz and digitized at 20 ms intervals. Recording electrodes were fabricated from borosilicate glass microcapillaries (outer diameter 1.5 mm), and the tip resistance was 5–10 mV. The internal pipette solution contained (in mM): 108 Cs gluconate, 1.7 NaCl, 0.9 EGTA, 9 HEPES, 1.8 $MgCl_2$, 4 Na_2ATP and 0.3 NaGTP, pH 7.2 [18]. The extracellular medium contained (in mM): 154 NaCl, 5.6 KCl, 3.6 $NaHCO_3$, 1.3 $CaCl_2$, 1 $MgCl_2$, 5 glucose and 10 HEPES (pH 7.4). Moreover, 20 mM TEA and 1 mM 4-AP were added in the extracellular recording solution to block voltage-dependent potassium currents. Voltage dependent sodium currents were elicited in CGCs by applying a series of 25 ms depolarizing pulses in 5 mV increments, from a holding potential of −100 mV [19]. Current-voltage (I–V) relationships for voltage-gated sodium currents were obtained by measuring the peak amplitude of the current for each given membrane potential during the voltage steps and dose response curves for the inhibition of voltage-gated sodium currents obtained by plotting the inhibition of peak sodium current amplitude.

2.6. Statistical analysis

Data analysis was performed using GraphPad Prism 8. All data are expressed as mean ± SEM of n determinations. Statistical comparison was by ANOVA followed by post hoc Dunnett's tests. The p values ≤ 0.05 were considered statistically significant. IC_{50} values were determined by fitting the data with a log (inhibitor) vs response model.

3. Results

Ten samples from the clams *Semele proficua* from Luanda Bay and *Senilia senilis* from Mussulo Bay were used to determine their content of PSP toxins and the same sample with a known toxic content, with a previously identified unknown compounds [12] were analyzed by electrophysiology to clarify their contribution to PSP intoxication based on their effects on VGSC.

3.1. HPLC quantification

Table 3 shows the toxic profile present in each sample obtained by HPLC analysis. All samples contained STX in different amounts that appeared in the chromatograms as a peak eluted at 9.4 min after peroxide oxidation. Four out of ten samples analyzed also contained the dcSTX analogue, represented in chromatograms as a characteristic peak eluted at 4.3 min after peroxide oxidation. The rest of the samples contained different combinations of these two characteristic toxins with three unknown compounds in different amounts and combinations. Only sample 23 showed to contain STX as unique toxic measurable compound.

3.2. Effects of the samples in voltage-gated sodium channels of cerebellar granule cells

Once the content of STX, dcSTX and other compounds were quantified in each sample, with the aim to elucidate the contribution and the toxicity of the unknown PSP-like compounds on PSP intoxication, the *in vitro* effects of each sample in VGSC were evaluated in cerebellar granule cells. First, the direct effect of STX pure standard was evaluated after bath application of 0.0001, 0.01, 0.1, 1, 2.5, 5, 10, 25, 100 nM STX. Fig. 2A represents the dose-response curve for the effect of the commercial standard of STX in the peak amplitude (I_{Na}) of voltage-dependent sodium currents. In this neuronal cell model, STX caused a dose-dependent inhibition of the maximum peak inward sodium currents obtaining an IC_{50} of 2.6 x 10^{-9} M (95 % CI: 1.2 x 10^{-9} to 5.4 x 10^{-9} M). By addition of 100 nM STX sodium currents were almost completely blocked. In the same way, the effects of the dcSTX pure standard in sodium currents were studied obtaining an IC_{50} of 5.1 x 10^{-8} M (95 % CI: 1.9 x 10^{-8} to 1.4 x 10^{-7} M) as represented in Fig. 2B, showing as expected a lower effect inhibiting sodium currents than STX which is known to be the most potent analogue of the PSP group.

Since the toxic profile of sample 23 revealed, as represented in Fig. 3A, only the presence of saxitoxin (6.99 μg/kg STX), it was possible to assess the effect in VGSC of the sample and compare it with the effect elicited by the standard STX. The activity of the

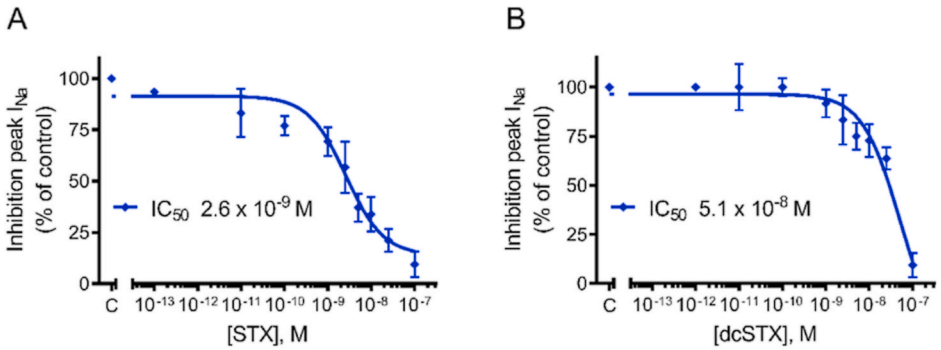

Fig. 2. A. Concentration-response graph for the peak inhibition of sodium currents by addition of different concentrations of STX pure standard to the recording chamber. B. Concentration-response graph for the peak inhibition of sodium currents after bath application of different concentrations of dcSTX. Abbreviations; STX, Saxitoxin; dcSTX, dicarbamoyl saxitoxin.

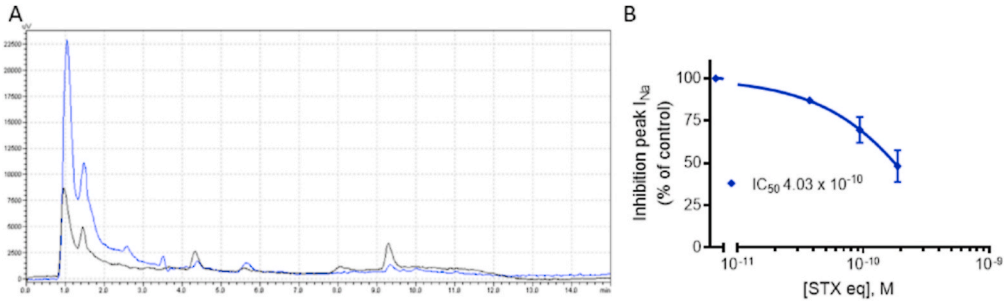

Fig. 3. A. HPLC-MS chromatogram obtained after peroxide oxidation (black trace) and periodate oxidation (blue trace) of sample 23 containing STX shown as a peak eluted at 9.4 min in peroxide oxidation. B. Concentration-response graph indicating the effect of sample 23 in sodium current amplitude. Abbreviations; STX, Saxitoxin.

sample in VGSC was analyzed by the addition of 1, 2.5 and 5 μl of the *Semele proficua* clam extract to the extracellular medium. As shown in Fig. 3B, non-lineal fit of the toxin-sample concentration against inhibition of peak sodium current amplitude yielded an estimated IC_{50} of 4.03 x 10^{-10} M (95 % CI from 8.5 x 10^{-9} to 1.9 x 10^{-11}).

The effects in VGSC of the rest of the samples containing STX, dcSTX and the three unknown compounds were also evaluated in cerebellar granule cells with the aim to identify their contribution to PSP intoxication. Sample 12, 15, 16, 17 and 25 only contained STX in different amounts together with different combinations of the three unknown compounds (Table 3).

As shown in Fig. 4, HPLC analysis of sample 12 showed a toxin profile containing 8.21 μg/kg of STX after peroxide oxidation and one additional unknown component eluting at 2.5 min after periodate oxidation equivalent to 7.87 μg/kg of STX (Fig. 4A, blue trace). The toxin content of sample 12 elicited a dose-dependent inhibition of the maximum peak inward sodium currents as represented in Fig. 4B after addition of 1, 2.5, 5, 10 and 15 μl of the extract obtaining an IC_{50} of 1.58 x 10^{-9} M (95 % CI from 1.95 x 10^{-10} to 1.2 x 10^{-8}). However, considering that sample 12 containing STX and one PSP-like compound had the same activity as STX standard we can conclude that the effect of the sample on sodium current inhibition strictly agrees with its STX content indicating no activity for the PSP-like compound eluted at 2.5 min after periodate oxidation.

HPLC analysis of sample 15, represented in Fig. 5A, showed to contain 22.6 μg STX Eq/kg of total toxic content which corresponds to 9.78 μg/kg STX after peroxide oxidation and two unknown peaks eluted at 5.6 min after periodate oxidation and 8.2 min after peroxide oxidation, equivalent to 12.8 μg/kg STX. Fig. 5B shows that the concentration–response curve of the inhibition of the maximum peak inward sodium currents yielded an estimated IC_{50} of 8.9 x 10^{-11} M (95 % CI from 4.1 x 10^{-9} to 1.9 x 10^{-12} M). The higher activity by inhibiting sodium currents of this sample was not statistically significant in contrast to control sodium currents.

Toxin content of Sample 16 is represented in Fig. 6A, with 24.63 μg STX Eq/kg of total toxic content, 7.83 μg/kg STX total content as represented in the peroxide oxidation trace of the chromatogram and three unknown peaks corresponding to the three different unknown compounds present in the sample eluted at 2.5 and 5.6 min after periodate oxidation (5.3 and 6.3 μg STX Eq/kg respectively) and a third peak eluted at 8.2 min in peroxide oxidation (5.2 μg STX Eq/kg) leading to a total toxic content of these unknown compounds of 16.86 μg STX Eq/kg. The toxin content of the sample resulted in the concentration–response curve shown in Fig. 6B yielded an estimated IC_{50} for the decrease of the maximum peak inward sodium currents of 5.3 x 10^{-10} M (95 % CI from 2.6 x 10^{-9} to 1.05 x 10^{-10} M).

Sample 17 chromatographic profile is represented in Fig. 7A and contained 13.57 μg STX Eq/kg of total toxic content. From this toxin content, 7.53 μg/kg were identified as STX after peroxide oxidation while an unknown peak eluted at 5.6 min after periodate oxidation with a toxin amount of 6.04 μg STX Eq/kg was identified. Fig. 7B shows the concentration–response curve for the effect of

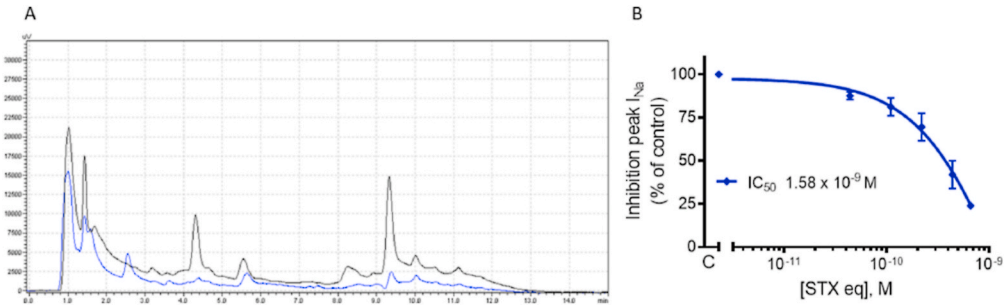

Fig. 4. A. High-Performance Liquid Chromatography with Mass Spectrometry (HPLC-MS) chromatogram obtained after peroxide (black trace) and periodate oxidation (blue trace) of sample 12 containing 8.21 μg/kg STX and one peak at 2.5 min in periodate oxidation equivalent to 7.87 μg/kg STX, corresponding to one of the three unknown compounds detected in the sample. B. Concentration-response graph indicating the effect of the toxic profile of sample 12 in voltage gated sodium channels. Abbreviations: STX, Saxitoxin; HPLC-MS.

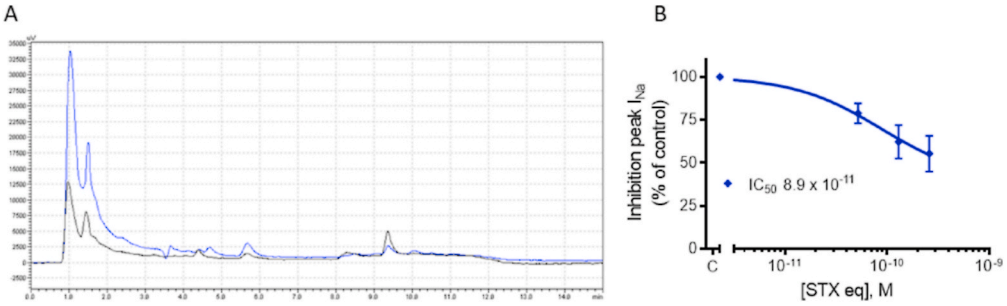

Fig. 5. A. High-Performance Liquid Chromatography with Mass Spectrometry (HPLC-MS) chromatogram obtained after peroxide oxidation (black trace) and periodate oxidation (blue trace) of sample 15 that present STX and two unknown compounds that appeared as peaks at 5.6 min in periodate oxidation and 8.2 min in peroxide oxidation B. Concentration-response graph indicating the effect of sample 15 whose toxic profile contains 9.78 μg/kg of STX and two new compounds equivalent to 12.8 μg/kg STX. Abbreviations; STX, Saxitoxin.

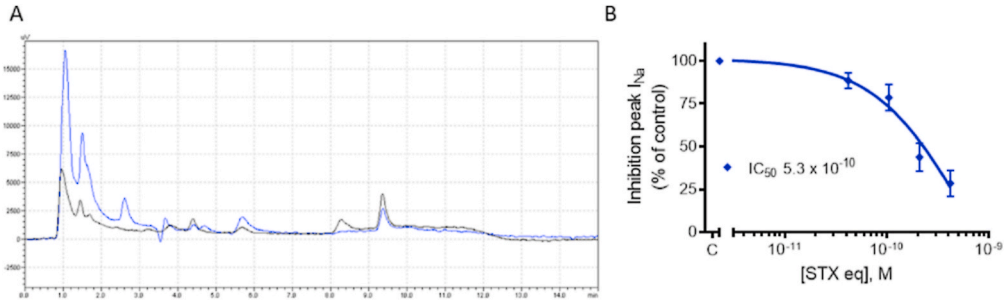

Fig. 6. A. High-Performance Liquid Chromatography with Mass Spectrometry (HPLC-MS) chromatograms obtained after peroxide (black trace) and periodate oxidation (blue trace) of Sample 16, the presence of STX and other three compounds that appeared as peaks at 2.5 and 5.6 min in periodate oxidation and 8.2 min in peroxide oxidation. B. Concentration-response graph showing the effect of Sample 16 on the decrease of the maximum peak inward sodium current. Abbreviations; STX, Saxitoxin.

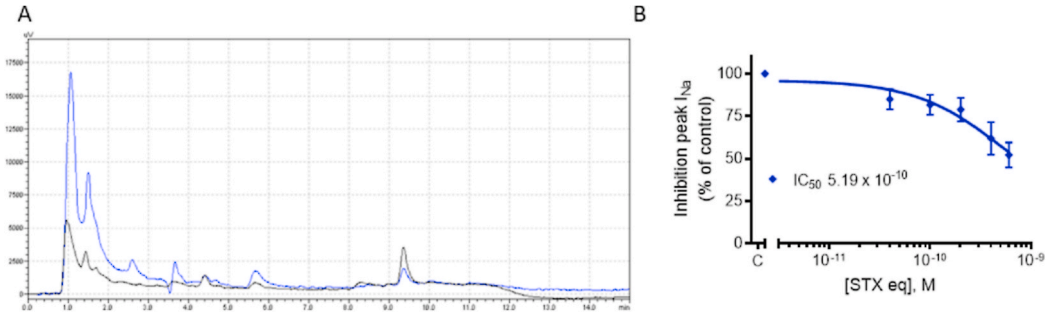

Fig. 7. A. High-Performance Liquid Chromatography with Mass Spectrometry (HPLC-MS) chromatogram obtained after peroxide oxidation (black trace) and periodate oxidation (blue trace) of sample 17, the presence of STX and other compound that appeared as a peak at 5.6 min in periodate oxidation. B. Concentration-response graph indicating the effect of STX and the unknown compound eluted at 5.6 min in periodate oxidation on VGSC. Abbreviations; STX, Saxitoxin; HPLC-MS.

this sample in sodium channels which allowed to calculate an estimated IC_{50} of 5.19 x 10^{-10} M (95 % CI from 4.9 x 10^{-11} to 5.4 x 10^{-9} M).

Sample 25, as represented in Fig. 8A, also contains two out of the three compounds detected, with a total toxin content of 18.4 μg/STX Eq/kg. After peroxide oxidation 7.31 μg/kg were identified as STX while 4.7 μg/STX Eq/kg correspond to the compound eluting at 2.5 min and 6.4 μg/STX Eq/kg corresponds to the compound eluting at 5.6 min after periodate oxidation. In this case, the estimated IC_{50} for inhibition of voltage-gated sodium currents amplitude is represented in Fig. 8B and was 3.5 x 10^{-10} M (95 % CI from 1.1 x 10^{-10} to 1.1 x 10^{-9} M).

Samples 18, 19, 20 and 24 contained both STX and dcSTX in combination with the unknown compounds, identified as peaks at 2.5 and 5.6 min in periodate oxidation and 8.2 min in peroxide oxidation.

Chromatograms showing the toxic profile of each sample were illustrated in Fig. 9A, C, 9E and 9G, as well as their concentration-

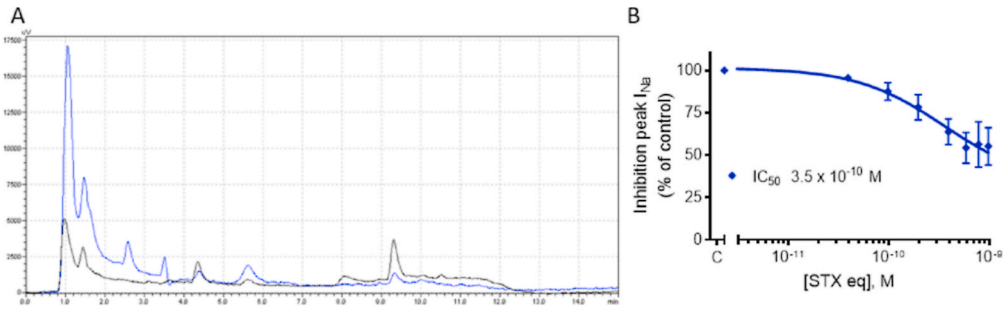

Fig. 8. A. High-Performance Liquid Chromatography with Mass Spectrometry (HPLC-MS) chromatogram obtained after peroxide (black trace) and periodate oxidation (blue trace) of sample 25, the presence of STX and two compounds that appeared at 2.5 and 5.6 min in periodate oxidation. B. Concentration-response graph indicating the effect decreasing the maximum peak inward sodium currents. Abbreviations; STX, Saxitoxin.

response graph for their effect inhibiting the maximum peak inward sodium currents were represented in Fig. 9B, D, 9F and 9H.

In sample 18 only the compound eluted at 5.6 min after periodate oxidation appeared (Table 3), obtaining an IC_{50} for the inhibition of the peak inward sodium current of 1.89 x 10^{-10} M (95 % CI from 2.21 x 10^{-11} to 1.6 x 10^{-9} M). Both, sample 19 and 20 contained the two unknown compounds eluted at 5.6 and 8.2 min with higher total toxin content for sample 20 (94.10 μg STX Eq/kg) as previously summarized in Table 3. Thus, the IC_{50} for the inhibition of the peak inward sodium currents were 1.98 x 10^{-10} M (95 % CI from 8.8 x 10^{-11} to 4.4 x 10^{-10} M) for sample 19 and 7.2 x 10^{-10} M (95 % CI from 2.4 x 10^{-10} to 2.1 x 10^{-9} M) for sample 20. The similar IC_{50} value obtained for samples 18 and 19 may be due to their similar STX and dcSTX content (Table 3).

Finally, sample 24 contained 5.14 μg/kg of dcSTX, 11.32 μg/kg of STX and two out of the three unknown compounds (Table 1). The effect elicited in the inhibition of the peak inward sodium currents yielded an IC_{50} of 1.02 x 10^{-10} M (95 % CI from 1.35 x 10^{-11} to 7.68 x 10^{-10} M).

All these *in vitro* results led to conclude that these PSP-like compounds found in the analyzed samples did not show any significant effect blocking the VGSC, since the inhibitory activity in sodium channels correlated with that elicited by the presence in samples of STX, dcSTX or both in combination.

4. Discussion

Saxitoxin (STX)-group of toxins have been detected in filter-feeding bivalve mollusks such as oysters, mussels, scallops, and clams from different parts of the world [21–23]. They are mainly produced by dinoflagellates belonging to the genus *Alexandrium, Gymnodinium catenatum* and *Pyrodinium bahamense* [24]. STX-group toxins cause PSP in humans, characterized by symptoms varying from a slight tingling sensation or numbness around the lips to fatal respiratory paralysis [25,26]. In lethal cases respiratory arrest occurs 2–12 h after consumption of shellfish contaminated with these toxins [26]. More than 30 different STX analogues have been identified and demonstrated to play a critical role in PSP intoxication. STX, NeoSTX, GTX and dc-STX [5] seem to be the most toxic ones. Toxicity data for PSP toxins is limited and comprises mostly studies on their acute toxicity following intraperitoneal administration. For monitoring purposes using HPLC techniques, toxicity equivalency factors have been applied to express the detected analogues as STX equivalents [27,28]. The mechanism of action of these toxins is the blockade of VGSC, therefore mainly producing neurological symptomatology. The most potent toxin of this group is STX which has numerous analogues and a lethal oral dose in humans of 1–4 mg (5.000–20,000 mouse units) while in mice its intraperitoneal LD_{50} varies from 3 to 10 μg/kg body weight (bw) and orally is 263 μg/kg body weight [29]. PSPs cause important economic losses worldwide and lead to a huge negative impact on the commerce of fishery products in the market [9]. Advanced methods are needed to detect the presence of PSP toxins, since the mechanisms of production of the toxic tides are unpredictable and the toxin must be detected in food. PSP toxin levels are currently regulated in EU legislation Regulation EC 854/2004 [30].

For decades, the official reference method in the European Union for STX detection was the mouse bioassay (MBA) [31]. However, this method involves ethical and animal welfare concerns and lacks specificity. Consequently, MBA was replaced by the Association of Official Analytical Chemists (AOAC) official method 2005.06, known as Lawrence method [13]. Lawrence method was validated and accepted as an alternative method of analysis for official control testing, being included in the EU legislation (Regulation EC 2074/2005) and became from January 1, 2019 the AOAC reference official method for PSP toxins detection [32] followed in this study for the detection of the compounds.

The mechanism of action of PSP toxins blocking the sodium channels make them dangerous neurotoxins with highly specific effects in the nervous system of humans, by interfering with nerve impulse transmission. STX was identified in different amounts in all the samples analyzed while dcSTX was identified in four out of the ten samples. Currently, more than eighteen GC analogues have been identified [33,34]. In this study, three of these compounds were identified by HPLC in peroxide and periodate oxidation, showing different retention times from the standards, 2.5, 5.6 and 8.2 min. With the aim to know whether these compounds contributed to PSP intoxication, the effect of each sample in VGSC was evaluated by electrophysiological recordings.

The evaluation of the effect caused by pure STX and dcSTX standards allowed to compare the relative potency of the compounds in their main cellular effect, the voltage-gated sodium channel blockade. STX showed a higher effect blocking sodium currents with an

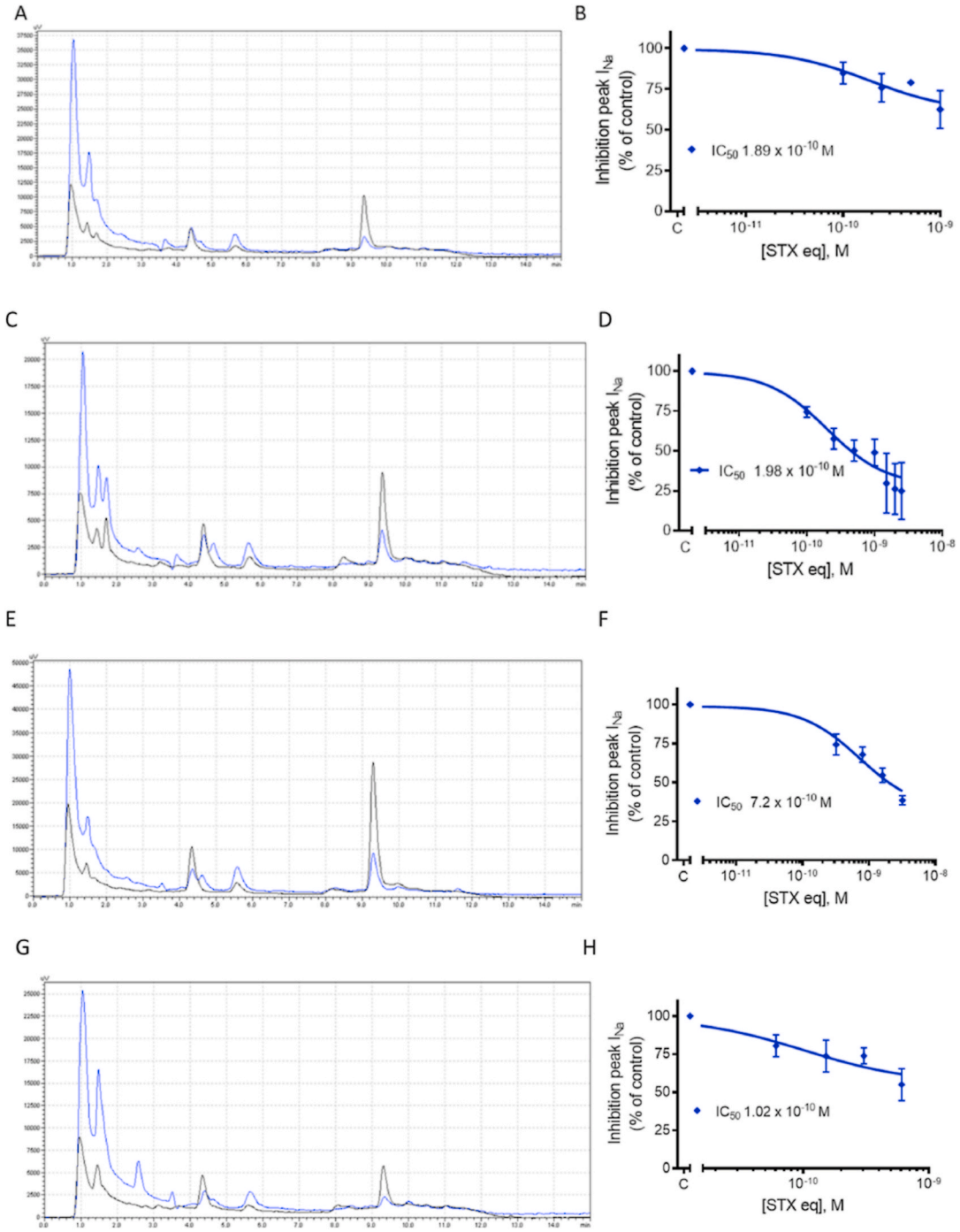

Fig. 9. Left panel: High-Performance Liquid Chromatography with Mass Spectrometry (HPLC-MS obtained after peroxide oxidation (black traces) and periodate oxidation (blue traces) of samples 18 (A), 19 (C), 20 (E) and 24 (G). Right panel: Concentration-response graph indicating the effect decreasing the maximum peak inward sodium currents of samples 18 (B), 19 (D), 20 (F) and 24 (H).

IC_{50} of 2.6 x 10^{-9} M compared to that of dcSTX whose IC_{50} for the blocking of sodium currents was 5.1 x 10^{-8} M. These results were in accordance with the previously reported results, where STX was demonstrated to be the most potent analogue blocking sodium currents of the group of PSP toxins, and also had been demonstrated to have higher affinity than dcSTX for sodium channels [35,36].

Sample 20 was the sample with the highest toxin content with 89.26 μg/STX Eq/kg distributed in STX, dcSTX and two out of the three unknown compounds eluted at 5.6 and 8.2 min. However, its potency blocking sodium currents was not higher than the other samples that contained both main toxins.

Among the 10 samples analyzed, sample 16 presented the most varied toxic profile since it contained a combination of STX and the three unknown compounds. The effect of this sample in sodium currents demonstrates that the unknown compounds did not affect the functionality of the channels since the IC_{50} for the inhibition of the maximum peak inward sodium currents was 5.3 x 10^{-10} M for sample 16 containing 7.83 μg STX/kg. This IC_{50} was very similar to that obtained for sample 17 (5.19 x 10^{-10} M) that contained 7.53 μg STX/kg and only one of the unknown compounds and the IC_{50} of sample 25 (3.5 x 10^{-10} M) which also had 7.3 μg/kg STX and two unknown compounds. All these data remark that the effects in sodium channels were due to STX and none of the three unknown compounds had remarkable activity in sodium currents. However, the results obtained showed slight differences in their activity blocking sodium currents among the samples, but the decrease of the maximum peak inward sodium current amplitude was higher in samples with higher STX and dcSTX contents corroborating the hypothesis of the non-activity of these compounds on sodium currents.

In view of all these results, the PSP-like compounds that were present in the analyzed samples did not show any activity blocking VGSCs in cerebellar granule cells, consequently not contributing to the PSP intoxication. Nevertheless, from this study is very important to highlight that, to a greater or lesser extent, all the samples analyzed contained PSP toxins in different amounts and combinations revealing a potential risk to human health and emphasizing the need for a monitoring program. Future sampling and monitoring of toxin content of seafood samples, is essential to anticipate possible poisoning episodes, thus guaranteeing the health of consumers, since it can be harmless compounds as in the case of these three compounds, or compounds that can become toxic after different chemical reactions in fish.

5. Conclusions

No effects on sodium channels or toxicity of the unknown compounds were found in the samples of the specimens *Senilia senilis* and *Semele proficua*. However, it is essential to establish a monitoring program for specimens likely to contain PSP toxins to ensure consumer safety given the high prevalence of SXT in all the analyzed samples.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Sandra Raposo-García: Writing – review & editing. **Ana M. Botana:** Resources, Formal analysis, Data curation. **Verónica Rey:** Formal analysis, Data curation. **Celia Costas:** Resources. **Luis Rodríguez-Santos:** Resources. **M. Carmen Louzao:** Resources. **Carmen Vale:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Luis M. Botana:** Writing – review & editing, Supervision, Resources, 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.

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