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Detection of tetrodotoxins in juvenile pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from the North Aegean Sea (Greece) by an electrochemical magnetic bead-based immunosensing tool

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Abstract

Two small *Lagocephalus sceleratus* juveniles were captured in picarel targeting catches from North Aegean Sea (Greece) in the autumn of 2017. An electrochemical immunosensing tool using magnetic beads as immobilisation support was developed and applied to the rapid
screening of tetrodotoxins (TTXs), potent neurotoxins that constitute a food safety hazard when present in seafood. This tool revealed the presence of TTXs in both individuals. Results were compared with those provided by mELISA and LC-HRMS, the latter confirming the presence of TTX. Some of the tissues contained TTX contents close to or above 2 mg/kg. L. sceleratus juveniles had been considered as non-toxic and, to our knowledge, this is the first report of high TTX levels in small L. sceleratus individuals. Such specimens can be mistaken with other edible species, posing a threat to consumers. The availability of low-cost and user-friendly tools for TTXs detection will contribute to guarantee seafood safety.

1. INTRODUCTION

The Suez Canal is considered as the major route for migration of indo-pacific marine species from the Red Sea into the Mediterranean, which is also referred to as Lessepsian migration. Following the salinity increase of the Nile estuary (after the construction of the Aswan dam) and the increase of the water temperatures in the last twenty years, the Eastern Mediterranean Sea is gradually becoming a more suitable environment for the establishment, growth and reproduction of alien species from warmer waters, which can compete with the native ones. Today, more than 443 aquatic species from various taxonomic groups have entered the Eastern Mediterranean through the Suez Canal (Galil et al., 2015). Among the most devastating Lessepsian migrant species are the highly toxic silver-cheeked toadfish (Lagocephalus sceleratus (Gmelin, 1789)) (Nader, Indary & Boustany, 2012). Its presence in the Mediterranean was reported for the first time in Gökova Bay, Turkey, in 2003 (Akyol, Ünal, Ceyhan & Bilecenoglu, 2005). Since then, its occurrence in several locations of the Mediterranean has dramatically increased, revealing a rapid spread towards the West of the Mediterranean, reaching Greece in 2005 (Kasapidis, Peristeraki, Tserpes & Magoulas, 2007), Algeria in 2013 (Kara, Ben Lamine & Fancour, 2015), and Spain in 2014 (Katsanevakis et al., 2014). It is one of the fastest expanding
Lessepsian fishes (Peristeraki, Lazarakis, Skarvelis, Georgiadis & Tserpes, 2006). Nowadays, in the SE Mediterranean areas *L. sceleratus* is very abundant and constitutes a true nuisance to the fishermen, damaging the nets and the longlines or even spoiling the catch by attacking the captured fish (Kalogirou, 2013).

However, an even greater concern regarding this species has been raised because of its high toxicity due to tetrodotoxin (TTX) that it contains in its body tissues. Tetrodotoxin is one of the most potent natural neurotoxins, responsible for many human intoxications and fatalities, usually following the consumption of pufferfish (Bane, Lehane, Dikshit, O’Riordan & Furey, 2014). Tetrodotoxin is produced by certain marine endosymbiotic bacteria and enters into other organisms through the food webs (Margarlamov, Melnikova & Chernyshev, 2017). Although, according to the legislation of many Mediterranean countries (e.g. EC, 2004a; 2004b) Tetraodontidae species and their products should not be marketed, cases of TTX poisoning due to the ingestion of *L. sceleratus* have been reported in the Mediterranean (Bentur et al., 2008; Chamandi, Kallab, Mattar & Nader, 2009; Kheifets, Rozhavsky, Solomonovich, Marianna & Soroksky, 2012). The consumption of this fish was probably due to the unawareness of the danger of this species. A second important reason for concern is the possible mixing of the *L. sceleratus* juveniles with other commercial small fish (Kiriake, Ohta, Okayama, Matsuura, Ishizaki & Nagashima, 2016). In the Mediterranean, there are several unofficial reports from North Aegean and Crete (Christidis, Peristeraki, personal observations) of small *L. sceleratus* juveniles intermingled with other small pelagic species (anchovy, sardines, picarel and bogue), primarily in catches coming from beach seines and incidentally in catches from purse seines (Christidis, personal observations). This fact may result in their accidental consumption in case they are not detected by the fishermen and reach the market. Regarding the toxicity of *L. sceleratus*, it generally depends on the maturity stage of the fish (Sabrah, El-Ganainy & Zaky, 2006; Nader et al., 2012), juveniles being considered as non-toxic (Sabrah et al., 2006; Katikou, Georgantelis, Sinouris, Pesti & Fotaras, 2009; Rodríguez, Alfonso, Otero, Katikou, Georgantelis
Since data about the toxicity are scarce, we hypothesise that *L. sceleratus* juveniles may be toxic even at very young stages in certain occasions, a situation that can possibly pose a serious health hazard. Knowledge of the TTX contents in *L. sceleratus* during the early life stages requires careful attention in order to evaluate the overall risk that this species may represent for consumers.

Different methodologies have been developed for the detection of TTXs, being the mouse bioassay (MBA) (Sabrah et al., 2006; Katikou et al., 2009) and liquid chromatography coupled to mass spectrometry analysis (Rambla-Alegre et al., 2017) the most widely used. The MBA provides an overall estimation of the total toxicity of the sample, but it is a non-specific method and cannot clearly discriminate between TTXs and saxitoxins (STXs). Instrumental analysis methods allow the identification and quantification of individual toxin analogues according to their structure and physicochemical properties. Recently, mass spectrometry has been combined with a nanofiber-based solid phase microextraction for *in vivo* sampling and detection of TTX in pufferfish (Tang, Huang, Xu, Ouyang & Liu, 2018).

Immunoassays and immunosensors are attractive candidates for the rapid screening of TTXs due to their high specificity and sensitivity as well as their low cost, ease of use and rapidity. To date, most immunochemical tools for the detection of TTXs are colorimetric immunoassays (Reverté, Soliño, Carnicer, Diogène & Campàs, 2014; Reverté et al., 2015, 2018; Rambla-Alegre et al., 2018), although several optical immunosensors (Leonardo, Reverté, Diogène & Campàs, 2016; Reverté et al., 2017a) and a few electrochemical immunosensors (Kreuzer, Pravda, O’Sullivan & Guilbault, 2002; Neagu, Micheli & Palleschi, 2006; Reverté, Campbell, Rambla-Alegre, Elliott, Diogène & Campàs, 2017b) have also been developed. Biosensors provide more compact and automated tools than conventional immunoassays and, amongst them, electrochemical biosensors stand out because of their inherent high sensitivities, the low cost and possibility for miniaturization of electrodes and potentiostats, as well as their high versatility, reliability and
short analysis times (Leonardo, Toldrà & Campàs, 2017). When developing electrochemical
immunosensors, the immobilisation of the recognition element on the electrode surface plays
an important role, not only in the antibody/antigen interaction but also in the modification of
the sensing surface properties. Coating of the electrode surface with immunoreagents or non-
specific adsorption of other compounds present in the sample may hinder the electron transfer.
These limitations can be overcome by the use of magnetic beads (MBs) as alternative
immobilisation supports, which provide advantages such as a higher surface area available for
biomolecule immobilisation, improved assay kinetics, more efficient washing steps or lower
matrix effects (Pvidori & Alegret, 2010; Pinacho, Sánchez-Baeza, Pvidori & Marco, 2014).
Moreover, by only placing a magnet below the working electrode, the MB-immunocomplex is
immobilised on the electrode surface and the enzyme substrate development takes place close
to the transducer, thus not compromising the sensitivity of the method.
In this work, we report the development of an electrochemical MB-based immunosensing tool
for the detection of TTXs. TTX has been conjugated to maleimide-activated MBs through the
formation of cysteamine self-assembled monolayers (SAMs), thus providing an oriented and
stable TTX immobilisation. After optimisation of the experimental parameters by colorimetry,
TTX-MB immunocomplexes have been combined with electrode arrays as transducer elements
and amperometry as the electrochemical detection method. This rapid and reliable
immunosensing tool has been applied to the analysis of two juvenile pufferfish L. sceleratus
individuals caught in the North Aegean Sea in October 2017. Results have been compared with
those achieved by liquid chromatography coupled to high resolution mass spectrometry (LC-
HRMS) analysis, and the maleimide-based ELISA (mELISA) previously developed at IRTA for the
detection of TTXs in shellfish (Reverté et al., 2018) and in urine samples (Rambla-Alegre et al.,
2018). By combining the immunochemical tools and instrumental methods, the presence of
significant TTX contents in pufferfish at very early stages has been confirmed for the first time.
2. MATERIAL AND METHODS

2.1. Reagents and solutions

TTX standard was purchased from Tocris Bioscience (Bristol, UK) and the standard solution was prepared at 1 mg/mL in 3 mM sodium acetate, pH 4.8. The anti-TTX monoclonal antibody TX-7F (mAb) was produced as described in Kawatsu, Hamano, Yoda, Terano & Shibata (1997). PureCube maleimide-activated MagBeads (MBs) were obtained from Cube Biotech (Monheim, Germany). Pierce maleimide-activated plates were achieved from Thermo Fisher Scientific (Madrid, Spain). Cysteamine hydrochloride, formaldehyde solution, anti-mouse IgG (whole molecule)-horseradish peroxidase antibody produced in rabbit (IgG-HRP), bovine serum albumin (BSA), sodium acetate, potassium phosphate dibasic, potassium phosphate monobasic, ethylenediaminetetraacetic acid (EDTA), Tween-20 and 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate were supplied by Sigma-Aldrich (Tres Cantos, Spain). HPLC-grade acetonitrile (ACN), glacial acetic acid (AA) and methanol (MeOH) were obtained from Chem-la (Zedelgem, Belgium). Ultrapure Milli-Q water (18.2 MΩ/cm) was used for the preparation of solutions (Millipore Iberica Ltd., Madrid, Spain).

2.2. Equipment, electrodes and software

Magnetic separation was performed using a MagneSphere Technology Magnetic Separation Stand (for 12 0.5-mL tubes) and a PolyATract System 1000 Magnetic Separation Stand (for one 15-mL tube) from Promega Corporation (Madison, WI, USA). Colorimetric measurements were performed with a Microplate Reader KC4 from BIO-TEK Instruments, Inc. (Winooski, VT, USA). Gen5 software was used to collect and evaluate data.
Screen-printed carbon electrode arrays (DRP-8x110) and a boxed connector (DRP-CAST8X) were provided by Dropsens S.L. (Oviedo, Spain). Arrays consisted of 8 carbon working electrodes of 2.5 mm in diameter, each with its own carbon counter electrode and silver reference electrode. Amperometric measurements were performed with a PalmSens potentiostat connected to an 8-channel multiplexer (MUX8) (Houte, The Netherlands). Data were collected and evaluated with PalmSens PC software.

LC-HRMS analysis was carried out with an Orbitrap-Exactive HCD and data was processed with Xcalibur 3.1. software (Thermo Fisher Scientific, Bremen, Germany).

2.3. Pufferfish sampling and processing

Two juvenile fish of 53 and 59 mm, morphologically identified as *L. sceleratus*, were captured in Chrousou Bay (Chalkidiki, Greece, North Aegean Sea) in October 2017 (Figure 1). The fish were intermingled in the catch consisting mainly of *Spicara smaris* of a beach seine in depths ranging from 10 to 30 m depth. The specimens were brought to the laboratory and frozen at -20 °C until analysis.

![Figure 1](image_url)  
**Figure 1.** Sampling area of the two juvenile pufferfish *Lagocephalus sceleratus* of this study.
Both pufferfish were dissected into different tissues. For toxin analysis, muscle (M#1) and skin (S#1) from pufferfish 1, and muscle (M#2), skin (S#2) and internal organs (O#2) containing liver and intestinal tract from pufferfish 2 were homogenised using a glass stirring rod. For microbiological analysis, homogenates from muscle (M), skin (S), liver (L) and intestinal tract (IT) tissues from both pufferfish were used. Gonads were not present because of the lack of maturity of both individuals. The remaining skeletal parts were kept at -20 °C for DNA extraction and sequence analysis.

2.4. Pufferfish DNA extraction and sequencing

DNA was extracted from 50 mg of the remaining bone tissue of each L. sceleratus specimen using a DNeasy Blood and Tissue Kit (Qiagen, Barcelona, Spain) following the manufacturer’s protocol. Extracted DNA was analysed by spectrophotometry (GeneQuant, Amersham Biosciences) to measure the concentration and check purity. DNA samples were diluted to 50 ng/µL and one microliter subjected to PCR amplification of the mitochondrial cytochrome oxidase gene using previously described primers (Kochzius et al., 2010). The resulting amplicon was purified (QIAquick PCR Purification Kit, Qiagen, Barcelona, Spain) and sequenced (Sistemas Genómicos, Valencia, Spain). A BLAST analysis was performed to compare similarity of sequences obtained to known sequences from the GenBank database (NCBI).

2.5. Bacterial culture and DNA sequencing

Homogenates of muscle (M), skin (S), liver (L) and intestinal tract (IT) tissues from the two juvenile pufferfish were inoculated on thiosulphate citrate bile sucrose (TCBS) agar (Sharlab, Sentmenat, Spain) for isolation of Vibrio species, and tryptone soy agar (TSA) + 2.5% NaCl plates
(Sharlab, Sentmenat, Spain) for isolation of general heterotroph bacteria. Plates were incubated at 30 °C for 48 h. The dominant colony morphotype from each plate was isolated and purified. Genomic DNA of each purified isolate was extracted using the Wizard Genomic DNA Purification Kit (Promega, Alcobendas, Spain), following the manufacturer’s protocol. The 16S rRNA was PCR amplified using the forward and reverse primers 27F and 1492R (Lane, 1991). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Barcelona, Spain) and sequenced (Sistemas Genómicos, Valencia, Spain). Consensus sequences were compared to those available in GenBank (NCBI) using the BLAST algorithm.

2.6. Tetrodotoxins (TTXs) extraction

A double TTX extraction was performed with 0.1% acetic acid as previously described (Reverté et al., 2015), adjusting the protocol to the small amounts of tissue. Thus, muscle (M#1) and skin (S#1) extracts from pufferfish 1 were obtained at a tissue concentrations of 117 and 86 mg equiv./mL, respectively. Muscle (M#2), skin (S#2) and internal organs (O#2) from pufferfish 2 were obtained at tissue concentrations of 122, 58 and 15 mg equiv./mL. The analysis by the electrochemical MB-based immunosensing tool and mELISA was performed directly with the aqueous extracts. For the LC-HRMS analyses, extracts were evaporated, re-dissolved in MeOH and filtered through 0.2-µm polytetrafluoroethylene (PTFE) filters.

2.7. Immunosensing approach

First, the TTX-MB conjugate was prepared as follows: (1) 6.25 μL of maleimide-activated MBs were transferred to a tube and rinsed with washing buffer (0.1 M PBS, 0.05% Tween®–20, pH 7.2) and vigorous mixing; for the washing steps, the tube was placed on the magnetic separation stand and the washing solution was removed; (2) 500 μL of 1mM cysteamine in binding buffer
(0.1 M PBS, 10 mM EDTA, pH 7.2) was added and incubated for 2 h at room temperature; (3) after three washing steps, 500 μL of TTX solution (25 μg/mL) in binding buffer containing 10% formaldehyde was added and incubated overnight at 4 °C; (4) three washing steps were performed and the TTX-coated MBs were resuspended in 500 μL of binding buffer. When amounts of MB varied, volumes were adjusted proportionally.

Once the TTX-MB conjugate was ready, (5) 50 μL of the conjugate was transferred to a new tube, the supernatant was removed and 25 μL of binding buffer for the optimisation or TTX standard solution for the competition and 25 μL of anti-TTX mAb dilution (from 1/500 to 1/4000 for the optimisation and 1/2000 for the competition) in 1% BSA-binding buffer were added and incubated for 30 min at room temperature; (6) after three washing steps, a blocking step was performed with 100 μL 1% BSA-binding buffer for 30 min; (7) after three washing steps, 50 μL of 1/1000 IgG-HRP dilution in 1% BSA-binding buffer was incubated for 30 min; (8) three washing steps were performed and the immunocomplex was resuspended in 50 μL and 2.5 μL of binding buffer for the colorimetric optimisation and the electrochemical immnosensing tool, respectively.

For the colorimetric optimisation of the protocol: (9) 40 μL of immunocomplex was transferred to a new tube and after supernatant removal, 125 μL of TMB liquid substrate was added and incubated for 10 min; (10) the tube was placed on the magnetic separation stand and 100 μL of TMB liquid substrate was collected for the colorimetric measurement at 620 nm in a microtiter plate. For the electrochemical immnosensing tool: (9) 2.5 μL of immunocomplex was placed on each working electrode of the 8-electrode array with a magnetic support on the back, the magnetic immunocomplex was trapped, and the supernatant was removed; (10) 10 μL of TMB liquid substrate was incubated for 2 min; (11) TMB oxidation was measured by amperometry, applying -0.2 V (vs. Ag) for 10 s, and recording the reduction current.
2.8. LC-HRMS analysis

The quantification of TTXs contents in the juvenile pufferfish extracts was performed following the protocol reported in Rambla-Alegre et al. (2017). Briefly, analytical separation was performed on a HILIC XBridge Amide column; a binary gradient elution was programmed with water (mobile phase A) and acetonitrile/water (mobile phase B), both containing ammonium acetate. ESI parameters and voltages were optimised to: spray voltage of 3.5 kV, capillary temperature of 300 °C, sheath gas flow rate of 40 (arbitrary units) and auxiliary gas flow rate of 10 (arbitrary units), capillary voltage of 30.0 V, tube lens voltage of 130 V and skimmer voltage of 28 V were used. The working mass range was $m/z$ 100-1200 in full scan acquisition mode. The resolution was 50000 ($m/z$ 200, FWHM) at a scan rate of 2 Hz. The automatic gain control (AGC) was set as “balanced (1e6)” with a maximum injection time of 250 ms. Peaks were identified by retention time, exact mass (mass window ± 5 ppm) and isotope pattern ratio.

2.9. mELISA

Pufferfish samples were analysed by mELISA using the protocol previously developed by our group for the determination of TTXs in shellfish (Reverté et al., 2018) and in urine (Rambla-Alegre et al., 2018). Briefly, 100 µL of 1mM cysteamine in binding buffer (0.1 M potassium phosphate, 10 mM EDTA, pH 7.2) was added to maleimide-activated plates and incubated for 3 h, followed by the direct immobilisation of TTX (2 µg/mL) with formaldehyde (3.4%) in the same buffer overnight at 4 °C. A competitive assay was then performed by incubating 50 µL of free TTX/sample dilution and 50 µL of 1/1600 anti-TTX mAb dilution in 1% BSA-binding buffer for 30 min. Afterwards, a blocking step was performed with 200 µL of 1% BSA-binding buffer for 30 min and, finally, 100 µL of IgG-HRP at 1/1000 dilution in 1% BSA-binding buffer was incubated
for 30 min. The colorimetric response was measured at 620 nm after 10 min of TMB liquid substrate incubation.

2.10. Data analysis

Measurements were performed in triplicate for the colorimetric optimisation of the protocol, the electrochemical MB-based immunosensing tool and mELISA, and in duplicate for LC-HRMS analysis. Immunosensing calibration curves were fitted using a sigmoidal logistic four-parameter equation. To evaluate differences between approaches, data were first tested for normality. To compare values from two different groups, the t-test was used for normally distributed data sets. Differences were considered statistically significant at the 0.05 level. SigmaStat 3.1. was used for statistical analysis.

3. RESULTS

3.1. Pufferfish identification

The two juvenile fish captured in the North Aegean Sea in October 2017 were 5-6 cm in length and weighed ~2 g each. Both individuals were dark grey-brownish with black spots of equal size regularly distributed in the dorsal part, a wide silver band present on the lower parts of the flanks, a silver blotch in the front of the eyes, and with the pectoral fin base black and the belly white. Their meristics were in accordance with those provided by Smith and Heemstra (1986) and Golani and Levy (2005) for *L. sceleratus*. DNA extraction and PCR amplification provided PCR products of approximately 800 bp long for both individuals. The BLAST analysis showed that the sequence obtained was 100% similar to *L. sceleratus*, supporting the morphological identification.
3.2. Optimisation of the MB-based immunosensing approach by colorimetry

Maleimide-activated MBs were used for the self-assembling of cysteamine and the subsequent covalent binding of TTX, shifting the competitive immunoassay for TTXs detection from the microtiter plate configuration previously reported by our group (Reverté et al., 2018) to the use of MBs as immobilisation supports. First, TTX concentrations from 0.3 to 10 µg/mL were tested for the toxin conjugation to 2 µL of MBs. Absorbance values increased with TTX concentration and no saturation was observed. Consequently, to achieve a complete TTX coating of the MBs and thus reduce non-specific adsorption and optimise the amount of MBs per assay, higher TTX concentrations (from 12.5 to 50 µg/mL) and lower MB volumes (0.5 µL) were used. Under these conditions, a TTX concentration of 25 µg/mL was observed to be enough to completely coat the MBs, reaching very high absorbance values. These high values are attributed to the high surface area available for TTX immobilisation and the high amount of mAb used (1:500 dilution from stock), which was selected to not limit the detection of the immobilised TTX.

Nevertheless, when performing a competitive assay, lower mAb concentrations may provide higher sensitivities. Thus, different mAb dilutions (from 1:1000 to 1:4000) were tested to optimise the amount of mAb. Free TTX at 10 ng/mL was added to test the sensitivity of the assay using the different mAb concentrations. mAb binding responses of 57, 49 and 33 % were obtained for 1:1000, 1:2000 and 1:4000 mAb dilutions, respectively, demonstrating the clear effect of the mAb concentrations on the competition assay. As a compromise between low antibody concentrations and appropriate absorbance values, 1:2000 mAb dilution was selected for subsequent experiments. The use of MBs as immobilisation supports allows performing all reaction steps in suspension, thus favouring conjugations and immunorecognition events.
3.3. Electrochemical MB-based immunosensing calibration curve

To shift from the colorimetric approach to the electrochemical immunosensing tool, the magnetic immunocomplexes were placed on screen-printed carbon electrode arrays, thus taking benefit from performing the immunorecognition event in suspension but immobilising the immunocomplexes to provide compact and miniaturised devices for the high-throughput detection of TTXs. A schematic representation of the approach is provided in Figure 2. An electrochemical calibration curve was constructed under the conditions previously selected by colorimetry (0.5 µL MB, 25 µg/mL TTX, 1:2000 mAb dilution, 1:1000 IgG-HRP dilution). The curve was background-subtracted (with respect to the controls with no mAb) and fitted to a sigmoidal logistic four-parameter equation (R=0.999) (Figure 3). A limit of detection (LOD), established as the 20% inhibition coefficient (IC$_{20}$), of 1.2 ng/mL and a working range (IC$_{20}$-IC$_{80}$) of 1.2-52.7 ng/mL were obtained. Repeatability (intra-day precision) was appropriate according to Horwitz equation, with relative standard deviation (RSD) values of 15.4 and 6.9% at 25 and 6.3 ng/mL, respectively. Reproducibility (inter-day precision) was also appropriate, with RSD values of 16.0 and 8.2% at the same TTX concentrations. The approach presented high reproducibility, certainly because of the improved assay kinetics and the low non-specific adsorption values provided by the use of MBs. Moreover, the use of MBs as immobilisation supports avoids coating the electrode surface with immunoreagents that could hinder the electron transfer. In comparison with the immunosensor for the detection of TTXs based on dithiols self-assembled directly on gold screen-printed electrodes (Reverté et al., 2017b), the MB-based immunosensor provided a broader working range (1.2-52.7 vs. 2.6-10.2 ng/mL) and a lower LOD (1.2 vs. 2.6 ng/mL). Moreover, the use of cysteamine for TTX coating reduces the cost of the assay compared to the use of carboxylate-dithiols. In terms of the LOD and working range, results were in agreement with the electrochemical immunosensor reported by Neagu and co-workers (Neagu et al., 2006), who immobilised the antibody on the electrode and performed a competition step using TTX-alkaline phosphatase conjugate as a tracer. Merging the advantages of the easy-to-
handle MBs, the high affinity of the antibody and the electrode array configuration, a useful and compact tool for the detection of TTXs has been achieved.

**Figure 2.** Schematic representation of the MB-based immunosensor for the detection of TTXs.

**Figure 3.** TTX calibration curve obtained by the electrochemical MB-based immunosensor. mAb binding is expressed as percentage of the control (no TTX). Error bars show SD values (n=3).
3.4. Detection of TTX contents in pufferfish

First, skin (S) and muscle (M) tissues from both *L. sceleratus* juvenile specimens and internal organs containing liver and intestinal tract (O) of one of the fish were analysed by the electrochemical MB-based immunosensing tool. TTX equiv. contents were detected in all samples (Table 1), and in most cases at levels above the value of 2 mg TTX equiv./kg regarded as a criterion to judge the acceptability of pufferfish as food in Japan (HP of Ministry of Health; Noguchi & Ebesu, 2001). Only the muscle tissue from individual 2 (M#2) showed TTX equiv. contents below this value. These toxin levels are in the range of those found in previous studies of *L. sceleratus* adult specimens: 0.17–239.32 mg/kg, 0.19–87.53 mg/kg, 0.07–10.16 mg/kg and 0.15–6.63 mg/kg in gonads, liver, muscle and skin tissues, respectively (Acar, Ishizaki & Nagashima, 2017; Katikou et al., 2009; Kosker et al., 2016; Rambla-Alegre et al., 2017; Reverté et al., 2015; Rodríguez et al., 2012).

LC-HRMS was then used for confirmatory purposes. The analysis revealed the presence of TTX (4-epiTTX included) in all tissues from both specimens (Figure 4, Table 1), at concentrations that ranged from 478 to 2077 µg/kg. Additionally, some TTX analogues (11-norTTX-6(R/S)-ol, 5-deoxyTTX/11-deoxyTTX, 5,11-dideoxyTTX/6,11-dideoxyTTX and 5,6,11-trideoxyTTX) were identified. Previous works have reported the presence of these and other TTX analogues (e.g. 4,9-anhydroTTX, 11-norTTX-6(R)-ol, 11-norTTX-(S)-ol) in pufferfish (Bane et al., 2014; Rambla-Alegre et al., 2017; Yotsu-Yamashita, Jang, Cho & Konoki, 2011; Yotsu-Yamashita et al., 2013). However, although the LC-HRMS chromatograms showed peaks with the exact mass for these TTX analogues (mass window ≤ ± 1.2 ppm), these identifications were only tentative because their retention times could not be properly assigned (because of the lack of standards available) neither the isotopic profiles fulfilled the established criterion for identification confirmation. Consequently, only TTX/4-epiTTX quantifications are shown in Table 1.
Although it is evident that the electrochemical immunosensing tool provided higher TTX contents than LC-HRMS analysis, when comparing these values it is necessary to take into account the detection principle of the techniques. Whereas LC-HRMS determines individual TTX and TTX analogues contents that are targeted in the analysis, the immunoapproach provides a global response from all TTX and TTX analogues that cross-react with the TTX antibody. This cross-reactivity can differ between the different analogues and it is not necessarily related to their toxicity (Reverté et al., 2015). In the hypothetical case that the TTX analogues tentatively identified were really present in the samples, some of these analogues would be at concentrations even higher than that of TTX. Depending on their concentration and their cross-reactivity with the TTX antibody, they would contribute to a greater or lesser extent to the TTX equivalent contents obtained by the immunosensing tool. LC-HRMS analysis identified only sample M#1 with levels above the value of 2 mg TTX equiv./kg. However, LC-HRMS quantifications could be underestimating the TTXs contents compared to the immunosensing approaches, which identified 4 out of 5 samples with TTX equiv. contents above this level. This different mode of recognition is not detrimental for the immunoapproach, but helps to protect consumer health. In fact, these TTX analogues would also be contributing to the toxicity of the L. sceleratus sample, in a greater or lesser extent depending on their concentration and their toxic potency (Louzao, Abal & Vilariño, 2017). Thus, the electrochemical immunoapproach could be used as a screening tool to prevent false negative results. In case of a positive result, the sample would require complementary analyses for confirmation purposes.
Table 1. TTX contents (µg TTX/kg tissue) in muscle (M), skin (S) and internal organs (O) of the two *L. sceleratus* juveniles by the electrochemical MB-based immunoisensing tool, LC-HRMS and mELISA.

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<th>Electrochemical immunosensing tool</th>
<th>LC-HRMS</th>
<th>mELISA</th>
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<td><strong>M#1</strong></td>
<td>2878</td>
<td>2077</td>
<td>2327</td>
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<tr>
<td><strong>M#2</strong></td>
<td>1395</td>
<td>478</td>
<td>1520</td>
</tr>
<tr>
<td><strong>S#1</strong></td>
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<td>1239</td>
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<tr>
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<td>2780</td>
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</tr>
<tr>
<td><strong>O#2</strong></td>
<td>2882</td>
<td>733</td>
<td>10834</td>
</tr>
</tbody>
</table>

Figure 4. Accurate mass extracted chromatogram of TTX ([TTX+H$^+$]) (peak in grey) in *L. sceleratus* juveniles by LC-HRMS (mass window ± 5ppm).
Pufferfish samples were also analysed by mELISA, which also revealed the presence of TTX equiv. contents in all tissues from both pufferfish individuals (Table 1). Correlations of 81-114% were achieved between the quantifications provided by mELISA and those achieved by the electrochemical MB-based immunosensing tool in the analysis of muscle and skin tissues from both specimens, revealing no significant differences (t=0.078, P=0.940). However, TTX equiv. contents determined by mELISA in the internal organs (O#2) were 3.8-fold higher than those attained by the electrochemical MB-based immunosensor. In a previous work, a disparity in TTX equiv. quantifications between mELISA and LC-HRMS had been observed in the analysis of liver from a *L. sceleratus* individual caught in the Mediterranean Sea (Rambla-Alegre et al., 2017). In the current work, liver was the most abundant tissue in the internal organs sample of the specimens. Rambla-Alegre and co-workers hypothesised that other unknown TTX analogues or liver matrix compounds could be responsible for the disagreement between approaches. Nevertheless, the electrochemical MB-based immunosensing tool showed TTX equiv. contents in better accordance with LC-HRMS analysis for that sample. Both the mELISA and the electrochemical MB-based immunosensing tool being based on the same recognition principle, the TTX overestimation of mELISA seems to be more probably due to undesirable effects of some liver compounds, matrix effects that are certainly reduced by the use of MBs as immobilisation supports.

### 3.5. Bacteria isolation and identification

Several types of bacteria have been demonstrated to be the primary source of TTX, more than 30% belonging to the genera *Vibrio* (Margarlamov et al., 2017). No growth on TCBS agar plates was detected, discarding the presence of *Vibrio* species in the juvenile pufferfish. However, most of the bacteria strains isolated in the TSA agar plates belonged to the genus *Psychrobacter* (Table 1, SM), which belongs to the class *Grammaproteobacteria* of the phylum *Proteobacteria*, like
Vibrio species and other common TTX-producing bacteria such as *Pseudomonas*, *Aeromonas* and *Alteromonas* (Margalamov et al., 2017). Nonetheless, *Psychrobacter* has never been reported as a TTX producer and thus the source of TTXs in these juvenile pufferfish remains unclear. Apart from the possible presence of TTX-producing bacteria, TTX bioaccumulation through the food web could also explain the presence of TTXs in these juvenile *L. sceleratus* specimens from the North Aegean Sea.

3.6. Response to the hypothesis statement

The present study sheds further light on the toxicity status of *L. sceleratus*, detecting for the first time significant TTX contents in very small specimens of the species. These results contradict current knowledge, which considers that *L. sceleratus* juveniles are probably non-toxic (Sabrah et al., 2006; Katikou et al., 2009; Rodríguez et al., 2012). Biological and technical reasons could explain this discrepancy.

Toxicity variability seems to be an inherent trait in pufferfish, this variability depending on the species (Azman, Samsur & Othman, 2014), on the maturity stage and the spawning season (Sabrah et al., 2006), and even on the specific individual (Rodríguez et al., 2012). Geographical location may also be an important factor for the toxicity of several pufferfish species (Azman et al., 2014), *L. sceleratus* included (Rodríguez et al., 2012). Since our specimens were immature, the size, the maturity stage and possibly the seasonality (directly linked to the spawning season) could be excluded from the list of factors that generated this discrepancy. Thus, individuality and locality probably are the main reasons, yet, for the time being, we do not have sufficient evidence regarding the contribution of each factor to this event.

On the other hand, several analytical methods based on different recognition principles have been applied to the analysis of TTX contents in juvenile pufferfish, thus providing different information. As previously mentioned, the MBA provides an estimation of the total toxicity, but
it is not very specific. Immunoapproaches provide more specific and sensitive global responses based on the structural recognition of TTX and its analogues by the antibody, which is not necessarily related to their toxicity. Otherwise, instrumental analysis methods allow identification of individual toxin analogues. However, if some of them are either not known or not targeted in the analysis, or if their toxicity factors have not been previously determined, analytical instrumentation may not properly estimate the potential toxicological risk of a sample. Moreover, the presence of several analogues in multi-toxin profile samples at levels below the limits of quantification of instrumental analysis methods can lead to underestimation or false negative results, in comparison with methods that provide a global response for the presence of TTX. Thus, when comparing TTX contents obtained by the different analytical methods, one should keep in mind the information provided by each one as well as their advantages and limitations. In this work, some TTX analogues others than the parent TTX were tentatively identified but could not be confirmed by LC-HRMS. Consequently, the toxicity of the sample could be underestimated if only TTX contents are taken into account. Otherwise, if the antibody recognises TTX analogues to a different extent than their toxicity, the immunoapproach may not be properly estimating the toxicological risk of the sample.

Another source of controversy can be the definition of toxic. In Europe, all fish of the family Tetraodontidae and products derived from them must not be placed on the markets (EC, 2004a; 2004b). Strong restrictions exist for import of pufferfish in the USA (FDA, 2007). In Japan, a list of edible pufferfish has been published and a value of 2 mg TTX equiv./kg has been established as a criterion to judge the acceptability of pufferfish as food, but *L. sceleratus* is labelled as a non-edible species and is not included in this list (Kawabata, 1978). Thus, while some authors consider juvenile pufferfish as non-toxic when no TTX is detected, which will depend on the limit of detection of the analysis technique, some other works consider *L. sceleratus* as non-toxic when TTX levels are below 2 mg/kg. Reaching TTX contents below or above this value can depend on the recognition principle of the analysis techniques, the consideration of only parent...
TTX or all the different TTX analogues, and the application or not of their toxicity equivalency factors (TEFs).

In any case, the fact that significant TTX contents were detected in such early stages of *L. sceleratus* raises a number of important concerns regarding public health, considering that: a) this species has been well established in the Mediterranean, with progressively increasing abundances all over the basin, and b) *L. sceleratus* juveniles at these stages may intermingle with commercial species such as picarel (*Spicara smaris*) or anchovy (*Engraulis encrasicolus*). Although adult *L. sceleratus* are easy to identify, the situation with small specimens is quite different, as they are not so easily distinguished by non-professionals and non-experienced people. This situation calls for an enhanced vigilance by the fishermen when handling and sorting the catch, so that these fishes will not go unnoticed and reach the market.

Notwithstanding, bearing in mind the small size of these specimens, it is important to contextualise the real hazard that TTX-containing *L. sceleratus* juveniles pose. The minimum lethal dose of TTX for a 50 kg human has been reported to be 2 mg (Noguchi & Ebesu, 2001). Taking into consideration this value and the weight and TTX contents in the *L. sceleratus* juveniles examined in this study, around 500 individuals should be consumed to result in lethal effects in humans. Nevertheless, no clear information exists on the doses that can cause sublethal effects in humans. Currently, a debate in the EU exists regarding acceptable levels for TTXs in the range of 40-200 µg TTX/kg in shellfish (EFSA, 2017; Kasteel & Westerink, 2017). The results of this study bring up the necessity for more extensive research on the toxicity of pufferfish at these early stages and the risk it may pose to consumers. The availability of fast, simple and low-cost analysis tools such as the immunosensing tool presented herein will certainly facilitate this research.
4. CONCLUSIONS

An electrochemical immunosensing tool for the rapid screening of TTX content in juvenile pufferfish has been developed. The use of MBs as TTX immobilisation supports provided remarkable advantages over conventional immunoassays such as improved kinetics, reduced matrix effects, higher reproducibility and versatility in the assay design. The electrochemical approach provides a cost-effective, compact and miniaturised analytical tool that allows the high throughput detection of TTXs. Additionally, in the optimisation of the immunosensing approach, a colorimetric immunoassay has been achieved as an intermediate result, which is a valuable tool for the detection of TTXs by itself.

The applicability of the electrochemical MB-based immunosensing tool to the determination of TTX contents in pufferfish has been demonstrated, highlighting the presence of TTXs in all tissues from the two juvenile L. sceleratus captured in the North Aegean Sea, which confirms our hypothesis. This finding increases the risk that this species may represent for accidental consumers. Results have been compared with those provided by mELISA, showing good correlations, and confirmed by LC-HRMS. LC-HRMS analysis has suggested a multi-TTX profile of the samples and has shown the complementarity of analytical techniques based on different recognition principles. The electrochemical MB-immunosensing tool has been demonstrated to be a reliable screening tool for TTXs. The availability of such user-friendly, rapid and low-cost alternative analytical tools may contribute to protect human health and also to set the basis for further investigation aimed to better understand the factors and the conditions under which small L. sceleratus specimens become toxic.

5. CONFLICTS OF INTEREST

There are no conflicts to declare.
6. ACKNOWLEDGEMENTS

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