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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

49 Lessepsian fishes (Peristeraki, Lazarakis, Skarvelis, Georgiadis & Tserpes, 2006). Nowadays, in
50 the SE Mediterranean areas *L. sceleratus* is very abundant and constitutes a true nuisance to the
51 fishermen, damaging the nets and the longlines or even spoiling the catch by attacking the
52 captured fish (Kalogirou, 2013).

53 However, an even greater concern regarding this species has been raised because of its high
54 toxicity due to tetrodotoxin (TTX) that it contains in its body tissues. Tetrodotoxin is one of the
55 most potent natural neurotoxins, responsible for many human intoxications and fatalities,
56 usually following the consumption of pufferfish (Bane, Lehane, Dikshit, O'Riordan & Furey,
57 2014). Tetrodotoxin is produced by certain marine endosymbiotic bacteria and enters into other
58 organisms through the food webs (Margarlamov, Melnikova & Chernyshev, 2017). Although,
59 according to the legislation of many Mediterranean countries (e.g. EC, 2004a; 2004b)
60 Tetraodontidae species and their products should not be marketed, cases of TTX poisoning due
61 to the ingestion of *L. sceleratus* have been reported in the Mediterranean (Bentur et al., 2008;
62 Chamandi, Kallab, Mattar & Nader, 2009; Kheifets, Rozhavsky, Solomonovich, Marianna &
63 Soroksky, 2012). The consumption of this fish was probably due to the unawareness of the
64 danger of this species. A second important reason for concern is the possible mixing of the
65 *L. sceleratus* juveniles with other commercial small fish (Kiriake, Ohta, Okayama, Matsuura,
66 Ishizaki & Nagashima, 2016). In the Mediterranean, there are several unofficial reports from
67 North Aegean and Crete (Christidis, Peristeraki, personal observations) of small *L. sceleratus*
68 juveniles intermingled with other small pelagic species (anchovy, sardines, picarel and bogue),
69 primarily in catches coming from beach seines and incidentally in catches from purse seines
70 (Christidis, personal observations). This fact may result in their accidental consumption in case
71 they are not detected by the fishermen and reach the market. Regarding the toxicity of
72 *L. sceleratus*, it generally depends on the maturity stage of the fish (Sabrah, El-Ganainy & Zaky,
73 2006; Nader et al., 2012), juveniles being considered as non-toxic (Sabrah et al., 2006; Katikou,
74 Georgantelis, Sinouris, Petsi & Fotaras, 2009; Rodríguez, Alfonso, Otero, Katikou, Georgantelis

75 & Botana, 2012). Since data about the toxicity are scarce, we hypothesise that *L. sceleratus*
76 juveniles may be toxic even at very young stages in certain occasions, a situation that can
77 possibly pose a serious health hazard. Knowledge of the TTX contents in *L. sceleratus* during the
78 early life stages requires careful attention in order to evaluate the overall risk that this species
79 may represent for consumers.

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

89 Immunoassays and immunosensors are attractive candidates for the rapid screening of TTXs due
90 to their high specificity and sensitivity as well as their low cost, ease of use and rapidity. To date,
91 most immunochemical tools for the detection of TTXs are colorimetric immunoassays (Reverté,
92 Soliño, Carnicer, Diogène & Campàs, 2014; Reverté et al., 2015, 2018; Rambla-Alegre et al.,
93 2018), although several optical immunosensors (Leonardo, Reverté, Diogène & Campàs, 2016;
94 Reverté et al., 2017a) and a few electrochemical immunosensors (Kreuzer, Pravda, O'Sullivan &
95 Guilbault, 2002; Neagu, Micheli & Palleschi, 2006; Reverté, Campbell, Rambla-Alegre, Elliott,
96 Diogène & Campàs, 2017b) have also been developed. Biosensors provide more compact and
97 automated tools than conventional immunoassays and, amongst them, electrochemical
98 biosensors stand out because of their inherent high sensitivities, the low cost and possibility for
99 miniaturization of electrodes and potentiostats, as well as their high versatility, reliability and

100 short analysis times (Leonardo, Toldrà & Campàs, 2017). When developing electrochemical
101 immunosensors, the immobilisation of the recognition element on the electrode surface plays
102 an important role, not only in the antibody/antigen interaction but also in the modification of
103 the sensing surface properties. Coating of the electrode surface with immunoreagents or non-
104 specific adsorption of other compounds present in the sample may hinder the electron transfer.
105 These limitations can be overcome by the use of magnetic beads (MBs) as alternative
106 immobilisation supports, which provide advantages such as a higher surface area available for
107 biomolecule immobilisation, improved assay kinetics, more efficient washing steps or lower
108 matrix effects (Pividori & Alegret, 2010; Pinacho, Sánchez-Baeza, Pividori & Marco, 2014).
109 Moreover, by only placing a magnet below the working electrode, the MB-immunocomplex is
110 immobilised on the electrode surface and the enzyme substrate development takes place close
111 to the transducer, thus not compromising the sensitivity of the method.

112 In this work, we report the development of an electrochemical MB-based immunosensing tool
113 for the detection of TTXs. TTX has been conjugated to maleimide-activated MBs through the
114 formation of cysteamine self-assembled monolayers (SAMs), thus providing an oriented and
115 stable TTX immobilisation. After optimisation of the experimental parameters by colorimetry,
116 TTX-MB immunocomplexes have been combined with electrode arrays as transducer elements
117 and amperometry as the electrochemical detection method. This rapid and reliable
118 immunosensing tool has been applied to the analysis of two juvenile pufferfish *L. sceleratus*
119 individuals caught in the North Aegean Sea in October 2017. Results have been compared with
120 those achieved by liquid chromatography coupled to high resolution mass spectrometry (LC-
121 HRMS) analysis, and the maleimide-based ELISA (mELISA) previously developed at IRTA for the
122 detection of TTXs in shellfish (Reverté et al., 2018) and in urine samples (Rambla-Alegre et al.,
123 2018). By combining the immunochemical tools and instrumental methods, the presence of
124 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-lab (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.

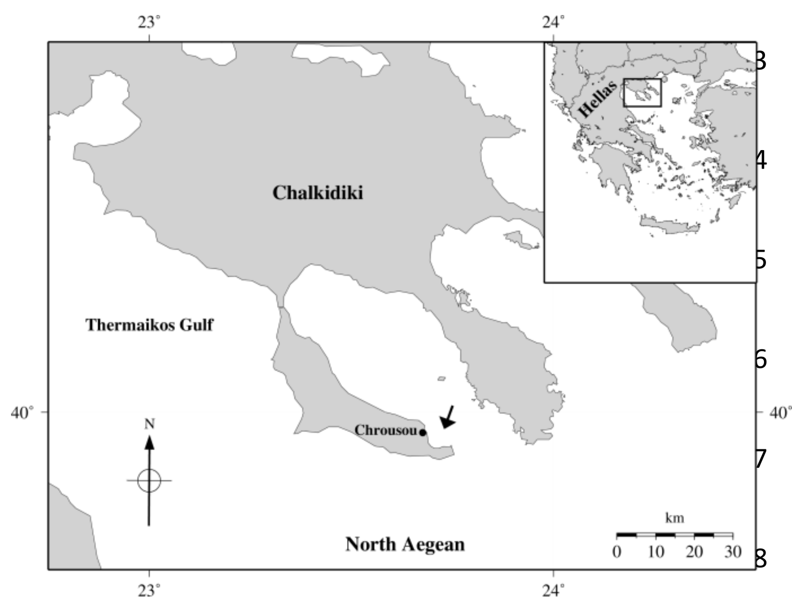


Figure 1. Sampling area of the two juvenile pufferfish *Lagocephalus sceleratus* of this study.

170

171 Both pufferfish were dissected into different tissues. For toxin analysis, muscle (M#1) and skin
172 (S#1) from pufferfish 1, and muscle (M#2), skin (S#2) and internal organs(O#2) containing liver
173 and intestinal tract from pufferfish 2 were homogenised using a glass stirring rod. For
174 microbiological analysis, homogenates from muscle (M), skin (S), liver (L) and intestinal tract (IT)
175 tissues from both pufferfish were used. Gonads were not present because of the lack of maturity
176 of both individuals. The remaining skeletal parts were kept at -20 °C for DNA extraction and
177 sequence analysis.

178

179 **2.4. Pufferfish DNA extraction and sequencing**

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

189

190 **2.5. Bacterial culture and DNA sequencing**

191 Homogenates of muscle (M), skin (S), liver (L) and intestinal tract (IT) tissues from the two
192 juvenile pufferfish were inoculated on thisosulphate citrate bile sucrose (TCBS) agar (Sharlab,
193 Sentmenat, Spain) for isolation of *Vibrio* species, and tryptone soy agar (TSA) + 2.5% NaCl plates

194 (Sharlab, Sentmenat, Spain) for isolation of general heterotroph bacteria. Plates were incubated
195 at 30 °C for 48 h. The dominant colony morphotype from each plate was isolated and purified.
196 Genomic DNA of each purified isolate was extracted using the Wizard Genomic DNA Purification
197 Kit (Promega, Alcobendas, Spain), following the manufacturer´s protocol. The 16S rRNA was PCR
198 amplified using the forward and reverse primers 27F and 1492R (Lane, 1991). PCR products were
199 purified using the QIAquick PCR Purification Kit (Qiagen, Barcelona, Spain) and sequenced
200 (Sistemas Genómicos, Valencia, Spain). Consensus sequences were compared to those available
201 in GenBank (NCBI) using the BLAST algorithm.

202

203 **2.6. Tetrodotoxins (TTXs) extraction**

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

212

213 **2.7. Immunosensing approach**

214 First, the TTX-MB conjugate was prepared as follows: (1) 6.25 µL of maleimide-activated MBs
215 were transferred to a tube and rinsed with washing buffer (0.1 M PBS, 0.05% Tween®−20, pH
216 7.2) and vigorous mixing; for the washing steps, the tube was placed on the magnetic separation
217 stand and the washing solution was removed; (2) 500 µL of 1mM cysteamine in binding buffer

218 (0.1 M PBS, 10 mM EDTA, pH 7.2) was added and incubated for 2 h at room temperature; (3)
219 after three washing steps, 500 µL of TTX solution (25 µg/mL) in binding buffer containing 10%
220 formaldehyde was added and incubated overnight at 4 °C; (4) three washing steps were
221 performed and the TTX-coated MBs were resuspended in 500 µL of binding buffer. When
222 amounts of MB varied, volumes were adjusted proportionally.

223 Once the TTX-MB conjugate was ready, (5) 50 µL of the conjugate was transferred to a new tube,
224 the supernatant was removed and 25 µL of binding buffer for the optimisation or TTX standard
225 solution for the competition and 25 µL of anti-TTX mAb dilution (from 1/500 to 1/4000 for the
226 optimisation and 1/2000 for the competition) in 1% BSA-binding buffer were added and
227 incubated for 30 min at room temperature; (6) after three washing steps, a blocking step was
228 performed with 100 µL 1% BSA-binding buffer for 30 min; (7) after three washing steps, 50 µL of
229 1/1000 IgG-HRP dilution in 1% BSA-binding buffer was incubated for 30 min; (8) three washing
230 steps were performed and the immunocomplex was resuspended in 50 µL and 2.5 µL of binding
231 buffer for the colorimetric optimisation and the electrochemical immunosensing tool,
232 respectively.

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

242

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

267 for 30 min. The colorimetric response was measured at 620 nm after 10 min of TMB liquid
268 substrate incubation.

269

270 **2.10. Data analysis**

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

278

279 **3. RESULTS**

280 **3.1. Pufferfish identification**

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

290

291 **3.2. Optimisation of the MB-based immunosensing approach by colorimetry**

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

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

313

314

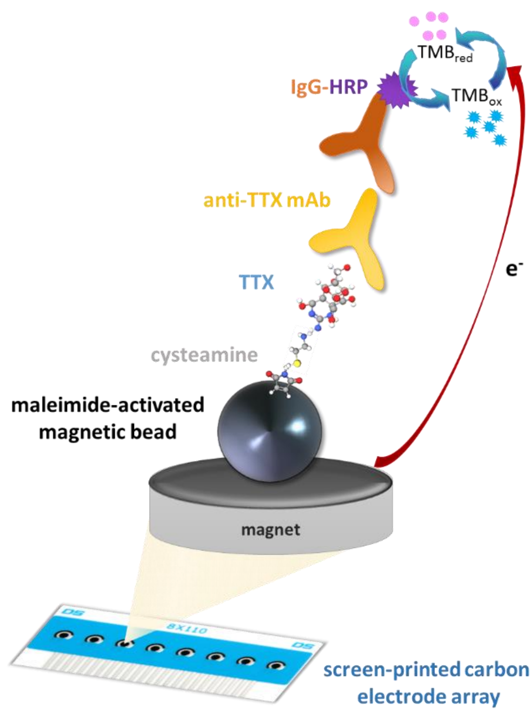
315

316 **3.3. Electrochemical MB-based immunosensing calibration curve**

317 To shift from the colorimetric approach to the electrochemical immunosensing tool, the
318 magnetic immunocomplexes were placed on screen-printed carbon electrode arrays, thus
319 taking benefit from performing the immunorecognition event in suspension but immobilising
320 the immunocomplexes to provide compact and miniaturised devices for the high-throughput
321 detection of TTXs. A schematic representation of the approach is provided in Figure 2. An
322 electrochemical calibration curve was constructed under the conditions previously selected by
323 colorimetry (0.5 µL MB, 25 µg/mL TTX, 1:2000 mAb dilution, 1:1000 IgG-HRP dilution). The curve
324 was background-subtracted (with respect to the controls with no mAb) and fitted to a sigmoidal
325 logistic four-parameter equation (R=0.999) (Figure 3). A limit of detection (LOD), established as
326 the 20% inhibition coefficient (IC_{20}), of 1.2 ng/mL and a working range (IC_{20}-IC_{80}) of 1.2-52.7
327 ng/mL were obtained. Repeatability (intra-day precision) was appropriate according to Horwitz
328 equation, with relative standard deviation (RSD) values of 15.4 and 6.9% at 25 and 6.3 ng/mL,
329 respectively. Reproducibility (inter-day precision) was also appropriate, with RSD values of 16.0
330 and 8.2% at the same TTX concentrations. The approach presented high reproducibility,
331 certainly because of the improved assay kinetics and the low non-specific adsorption values
332 provided by the use of MBs. Moreover, the use of MBs as immobilisation supports avoids coating
333 the electrode surface with immunoreagents that could hinder the electron transfer. In
334 comparison with the immunosensor for the detection of TTXs based on dithiols self-assembled
335 directly on gold screen-printed electrodes (Reverté et al., 2017b), the MB-based immunosensor
336 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
337 ng/mL). Moreover, the use of cysteamine for TTX coating reduces the cost of the assay compared
338 to the use of carboxylate-dithiols. In terms of the LOD and working range, results were in
339 agreement with the electrochemical immunosensor reported by Neagu and co-workers (Neagu
340 et al., 2006), who immobilised the antibody on the electrode and performed a competition step
341 using TTX-alkaline phosphatase conjugate as a tracer. Merging the advantages of the easy-to-

342 handle MBs, the high affinity of the antibody and the electrode array configuration, a useful and

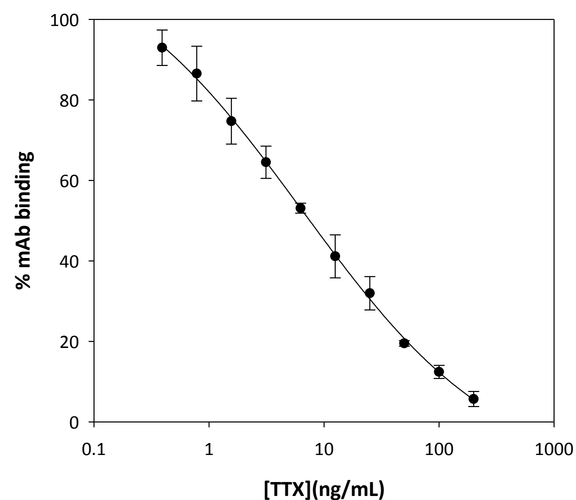
343 compact tool for the detection of TTXs has been achieved.



344

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

346



347

348 **Figure 3.** TTX calibration curve obtained by the electrochemical MB-based immunosensor. mAb

349 binding is expressed as percentage of the control (no TTX). Error bars show SD values (n=3).

350 **3.4. Detection of TTX contents in pufferfish**

351 First, skin (S) and muscle (M) tissues from both *L. sceleratus* juvenile specimens and internal
352 organs containing liver and intestinal tract (O) of one of the fish were analysed by the
353 electrochemical MB-based immunosensing tool. TTX equiv. contents were detected in all
354 samples (Table 1), and in most cases at levels above the value of 2 mg TTX equiv./kg regarded
355 as a criterion to judge the acceptability of pufferfish as food in Japan (HP of Ministry of Health;
356 Noguchi & Ebesu, 2001). Only the muscle tissue from individual 2 (M#2) showed TTX equiv.
357 contents below this value. These toxin levels are in the range of those found in previous studies
358 of *L. sceleratus* adult specimens: 0.17–239.32 mg/kg, 0.19–87.53 mg/kg, 0.07–10.16 mg/kg and
359 0.15–6.63 mg/kg in gonads, liver, muscle and skin tissues, respectively (Acar, Ishizaki &
360 Nagashima, 2017; Katikou et al., 2009; Kosker et al., 2016; Rambla-Alegre et al., 2017; Reverté
361 et al., 2015; Rodríguez et al., 2012).

362 LC-HRMS was then used for confirmatory purposes. The analysis revealed the presence of TTX
363 (4-*epi*TTX included) in all tissues from both specimens (Figure 4, Table 1), at concentrations that
364 ranged from 478 to 2077 µg/kg. Additionally, some TTX analogues (11-norTTX-6(*R*/*S*)-ol, 5-
365 deoxyTTX/11-deoxyTTX, 5,11-dideoxyTTX/6,11-dideoxyTTX and 5,6,11-trideoxyTTX) were
366 identified. Previous works have reported the presence of these and other TTX analogues (e.g.
367 4,9-anhydroTTX, 11-norTTX-6(*R*)-ol, 11-norTTX-(*S*)-ol) in pufferfish (Bane et al., 2014; Rambla-
368 Alegre et al., 2017; Yotsu-Yamashita, Jang, Cho & Konoki, 2011; Yotsu-Yamashita et al., 2013).
369 However, although the LC-HRMS chromatograms showed peaks with the exact mass for these
370 TTX analogues (mass window ≤ ± 1.2 ppm), these identifications were only tentative because
371 their retention times could not be properly assigned (because of the lack of standards available)
372 neither the isotopic profiles fulfilled the established criterion for identification confirmation.
373 Consequently, only TTX/4-*epi*TTX quantifications are shown in Table 1.

374 Although it is evident that the electrochemical immunosensing tool provided higher TTX
375 contents than LC-HRMS analysis, when comparing these values it is necessary to take into
376 account the detection principle of the techniques. Whereas LC-HRMS determines individual TTX
377 and TTX analogues contents that are targeted in the analysis, the immunoapproach provides a
378 global response from all TTX and TTX analogues that cross-react with the TTX antibody. This
379 cross-reactivity can differ between the different analogues and it is not necessarily related to
380 their toxicity (Reverté et al., 2015). In the hypothetical case that the TTX analogues tentatively
381 identified were really present in the samples, some of these analogues would be at
382 concentrations even higher than that of TTX. Depending on their concentration and their cross-
383 reactivity with the TTX antibody, they would contribute to a greater or lesser extent to the TTX
384 equivalent contents obtained by the immunosensing tool. LC-HRMS analysis identified only
385 sample M#1 with levels above the value of 2 mg TTX equiv./kg. However, LC-HRMS
386 quantifications could be underestimating the TTXs contents compared to the immunosensing
387 approaches, which identified 4 out of 5 samples with TTX equiv. contents above this level. This
388 different mode of recognition is not detrimental for the immunoapproach, but helps to protect
389 consumer health. In fact, these TTX analogues would also be contributing to the toxicity of the
390 *L. sceleratus* sample, in a greater or lesser extent depending on their concentration and their
391 toxic potency (Louzao, Abal & Vilariño, 2017). Thus, the electrochemical immunoapproach could
392 be used as a screening tool to prevent false negative results. In case of a positive result, the
393 sample would require complementary analyses for confirmation purposes.

394

395

396

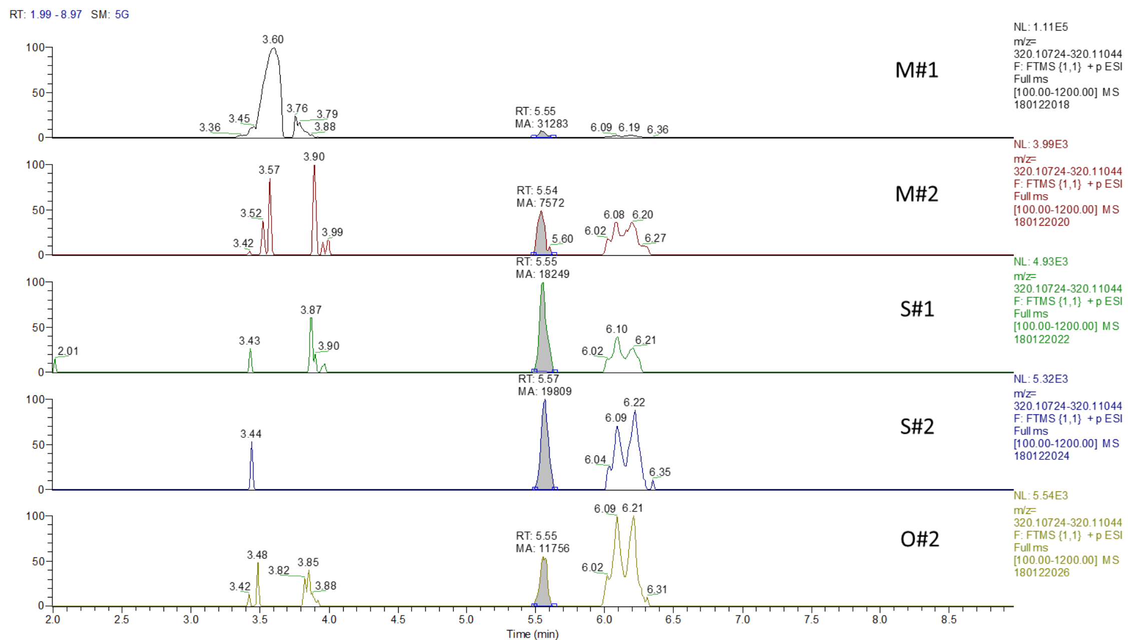
397 **Table 1.** TTX contents (µg TTX/kg tissue) in muscle (M), skin (S) and internal organs (O) of the
398 two *L. sceleratus* juveniles by the electrochemical MB-based immunosensing tool, LC-HRMS and
399 mELISA.

	TTX contents (µg TTX/kg tissue)		
	Electrochemical immunosensing tool	LC-HRMS	mELISA
M#1	2878	2077	2327
M#2	1395	478	1520
S#1	2588	1239	2773
S#2	2780	1188	3175
O#2	2882	733	10834

400

401

402



403 **Figure 4.** Accurate mass extracted chromatogram of TTX ([TTX+H$^+$]) (peak in grey) in *L. sceleratus*
404 juveniles by LC-HRMS (mass window ± 5ppm).

405

406 Pufferfish samples were also analysed by mELISA, which also revealed the presence of TTX equiv.
407 contents in all tissues from both pufferfish individuals (Table 1). Correlations of 81-114% were
408 achieved between the quantifications provided by mELISA and those achieved by the
409 electrochemical MB-based immunosensing tool in the analysis of muscle and skin tissues from
410 both specimens, revealing no significant differences (t=0.078, P=0.940). However, TTX equiv.
411 contents determined by mELISA in the internal organs (O#2) were 3.8-fold higher than those
412 attained by the electrochemical MB-based immunosensor. In a previous work, a disparity in TTX
413 equiv. quantifications between mELISA and LC-HRMS had been observed in the analysis of liver
414 from a *L. sceleratus* individual caught in the Mediterranean Sea (Rambla-Alegre et al., 2017). In
415 the current work, liver was the most abundant tissue in the internal organs sample of the
416 specimens. Rambla-Alegre and co-workers hypothesised that other unknown TTX analogues or
417 liver matrix compounds could be responsible for the disagreement between approaches.
418 Nevertheless, the electrochemical MB-based immunosensing tool showed TTX equiv. contents
419 in better accordance with LC-HRMS analysis for that sample. Both the mELISA and the
420 electrochemical MB-based immunosensing tool being based on the same recognition principle,
421 the TTX overestimation of mELISA seems to be more probably due to undesirable effects of some
422 liver compounds, matrix effects that are certainly reduced by the use of MBs as immobilisation
423 supports.

424

425 **3.5. Bacteria isolation and identification**

426 Several types of bacteria have been demonstrated to be the primary source of TTX, more than
427 30% belonging to the genera *Vibrio* (Margarlamov et al., 2017). No growth on TCBS agar plates
428 was detected, discarding the presence of *Vibrio* species in the juvenile pufferfish. However, most
429 of the bacteria strains isolated in the TSA agar plates belonged to the genus *Pshycrobacter* (Table
430 1, SM), which belongs to the class *Grammaproteobacteria* of the phylum *Proteobacteria*, like

431 *Vibrio* species and other common TTX-producing bacteria such as *Pseudomonas*, *Aeromonas* and
432 *Alteromonas* (Margalamov et al., 2017). Nonetheless, *Psychrobacter* has never been reported as
433 a TTX producer and thus the source of TTXs in these juvenile pufferfish remains unclear. Apart
434 from the possible presence of TTX-producing bacteria, TTX bioaccumulation through the food
435 web could also explain the presence of TTXs in these juvenile *L. sceleratus* specimens from the
436 North Aegean Sea.

437

438 **3.6. Response to the hypothesis statement**

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

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

453 On the other hand, several analytical methods based on different recognition principles have
454 been applied to the analysis of TTX contents in juvenile pufferfish, thus providing different
455 information. As previously mentioned, the MBA provides an estimation of the total toxicity, but

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

472 Another source of controversy can be the definition of toxic. In Europe, all fish of the family
473 Tetraodontidae and products derived from them must not be placed on the markets (EC, 2004a;
474 2004b). Strong restrictions exist for import of pufferfish in the USA (FDA, 2007). In Japan, a list
475 of edible pufferfish has been published and a value of 2 mg TTX equiv./kg has been established
476 as a criterion to judge the acceptability of pufferfish as food, but *L. sceleratus* is labelled as a
477 non-edible species and is not included in this list (Kawabata, 1978). Thus, while some authors
478 consider juvenile pufferfish as non-toxic when no TTX is detected, which will depend on the limit
479 of detection of the analysis technique, some other works consider *L. sceleratus* as non-toxic
480 when TTX levels are below 2 mg/kg. Reaching TTX contents below or above this value can
481 depend on the recognition principle of the analysis techniques, the consideration of only parent

482 TTX or all the different TTX analogues, and the application or not of their toxicity equivalency
483 factors (TEFs).

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

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

505

506

507 **4. CONCLUSIONS**

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

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

528

529 **5. CONFLICTS OF INTEREST**

530 There are no conflicts to declare.

531

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538

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