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Evaluation of tetrodotoxins in puffer fish caught along the Mediterranean coast of Spain. Toxin profile of *Lagocephalus sceleratus*

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Abstract

Although consumption of Tetraodontidae species is prohibited in the EU, intoxications are still reported. The evaluation of tetrodotoxins (TTXs) by mass spectrometry (LC-MS/MS) and a screening immunoassay (mELISA) in tetraodontid fishes caught along the Western Mediterranean Sea revealed high concentrations of TTXs in *Lagocephalus sceleratus* while no TTXs were identified in *L. lagocephalus* and *Sphoeroides pachygaster* individuals. The high TTXs content found in the *L. sceleratus* analysed herein demonstrate the occurrence of highly toxic puffer fish in the Western Mediterranean Sea. Being *L. sceleratus* a recent invasive species in the Mediterranean, surveillance, risk assessment and risk management measures are necessary. The strategy used within this research work could be a valuable tool for future food safety monitoring.

**Keywords:** tetrodotoxin (TTX); *Lagocephalus sceleratus*; puffer fish; mELISA; mass spectrometry; LC-MS/MS; LC-HRMS.
1. Introduction

Tetraodontidae is a family of marine and estuarine fish that includes 130 species grouped in 19 genera that are potential carriers of tetrodotoxins (TTXs) (Smith, 1986). Most puffer fish are from tropical waters but some have been found in temperate waters. *Sphoeroides pachygaster*, is an expanding species that most probably reached the Mediterranean from the Atlantic Ocean and *Lagocephalus lagocephalus* is a circumglobal species, considered as native in the Mediterranean (Froese et al., 2016). However, *L. sceleratus* only recently entered the Mediterranean Sea through the Suez Chanel (Lessepsian migration) and is considered an invasive species. *L. sceleratus* was first reported in the Mediterranean Sea in February 2003 at Gökova Bay (southern Aegean Sea, Turkey) (Akyol et al., 2005), in November 2004 at Jaffa along the Israeli coast (Golani and Levy, 2005), and in July 2005 in the Cretan Sea, Greece (Kasapidis et al., 2007). Since then, *L. sceleratus* has been recorded with increasing frequency in Greece, Cyprus, Malta, Algeria, and Turkey and is considered as one of the fastest expanding Lessepsian invaders (Acar et al., 2017; Deidun et al., 2015; Kara et al., 2015; Katikou et al., 2009; Kosker et al., 2016; Rodriguez et al., 2012; Rousou et al., 2014). In Spain, one individual of *L. sceleratus* was captured in July 2014 in Denia (Alicante, Western Mediterranean) (Katsanevakis et al., 2014) and is the object of the present publication.

Tetrodotoxin is a neurotoxin responsible for human intoxications and fatalities, commonly following the consumption of puffer fish (Bane et al., 2014). Structurally, TTX consists of a guanidinium moiety connected to a highly oxygenated carbon skeleton that possesses a 2,4-dioxadamantane portion containing five hydroxyl groups (Isbister and Kiernan, 2005). In Japanese waters, the presence of pufferfish belonging to the Tetraodontidae family is very common. In fact, Japanese people are well-known consumers of *fugu*, which is considered a gastronomic delicacy. In humans, according to
case studies, between 0.18 and 0.2 mg of TTX have been reported to cause severe symptoms, and a fatality was reported after an ingestion of around 2 mg of TTX (Noguchi and Ebesu, 2001).

Additionally, the Ministry of Health, Labour and Welfare published a guide with the edible parts and species of puffer fish that are allowed for consumption (HP of Ministry of Health). However, TTX poisoning cases still occur in this and other Asian countries (Noguchi and Arakawa, 2008; Yotsu-Yamashita et al., 2011). In Europe, the current legislative requirements (European Commission, 2004a, European Commission, 2004b) establish that poisonous fish of the family Tetraodontidae and products derived from them must not be placed on the European markets. Despite this fact, the possibility for accidental consumption of these species is possible. Since 2007, when the first toxic European episode was reported in Málaga (Spain) by the consumption of trumpet shells of the species Charonia lampas lampas containing TTXs (Rodriguez et al., 2008), several episodes along the Mediterranean coastal countries have been reported due to the consumption of puffer fish (Bentur et al., 2008; Kheifets et al., 2012). Very recently the presence of TTXs has been reported in gastropods from Portugal (Silva et al., 2012) as well as in bivalve mollusk shellfish grown at the south coast of England (Turner et al., 2015), along the Greek coast (Vlamis et al., 2015) and in the Netherlands (RASFF, 2016). Following these events, the European Food Safety Authority (EFSA) has recently published an opinion on the risks to public health related to the presence of TTX and TTX analogues in marine bivalves and gastropods (EFSA, 2017).

The aim of this work was to characterise the toxin profile of different Tetraodontidae species including the L. sceleratus caught in Denia (Alicante, Western Mediterranean). To this purpose, different puffer fish tissues were analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), liquid chromatography coupled to
high resolution mass spectrometry (LC-HRMS), and a self-assembled monolayer-based enzyme-linked immunosorbent assay (mELISA).

2. Materials and Methods

2.1 Standards and reagents

TTX standard was from Tocris Bioscience (Bristol, UK). The TTX standard solution was prepared at 1 mg/mL in 10 mM acetic acid. For LC-MS/MS analyses, hypergrade acetonitrile (ACN) was used for separation and gradient. HPLC methanol (MeOH) was used for washing the injection needle. Both ACN and MeOH were purchased from Fisher Scientific (Loughborough, UK). Ultrapure water (resistivity >18 MΩ·cm) was obtained from a Milli-Q water purification system (Millipore Iberica Ltd., Madrid, Spain). Ammonium acetate, acetic acid and formic acid were purchased from Sigma-Aldrich (Tres Cantos, Spain). For mELISA experiments: the anti-TTX monoclonal antibody was produced as previously described (Kawatsu et al., 1997) and prepared as in the literature (Campbell et al., 2013); Pierce maleimide-activated plates were obtained from Thermo Fisher Scientific (Madrid, Spain); dithiolalkanearomaticPEG6-COOH (dithiol-carboxylate) was purchased from Sensopath Technologies (Bozeman, USA), and anti-mouse IgG (whole molecule)-horseradish peroxidase antibody (IgG-HRP), bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), and 3,3',5,5'-tetramethylbenzidine (TMB) were all supplied by Sigma-Aldrich (Tres Cantos, Spain).

2.2 Sample preparation

Fourteen oceanic puffer fish (L. lagocephalus, Linnaeus, 1758), one silver-cheeked toadfish (L. sceleratus, Gmelin, 1789) and five blunthead puffer (S. pachygaster, Müller
and Troschel, 1848) were caught from 2014 to 2016 over the Spanish coast along the Mediterranean Sea (Figure 1). All puffer fish were dissected and gonads (only in females), liver, skin and muscle were retrieved. Extraction of TTXs was performed as previously described (Reverté et al., 2015). Briefly, a double extraction of each organ with 0.1% acetic acid was performed. In the case of liver, an additional liquid-liquid partition with hexane was required. The analysis by the immunoassay was performed with the aqueous extracts, but for the LC-MS/MS and LC-HRMS analyses, extracts were evaporated, re-dissolved in methanol and filtered through 0.2-μm nylon filters.

![Figure 1](image)

**Figure 1.** Map of Spain including locations where puffer fish have been caught and received at IRTA from 2014 to 2016.● *L. lagocephalus*, ▲ *S. pachygaster* ★ *L. sceleratus*.


2.3 Sample analysis

Fourteen *L. lagocephalus*, one *L. sceleratus* and five *S. pachygaster* were first analysed for the presence of TTXs by LC-MS/MS. LC-HRMS was subsequently used for confirmatory purposes on samples containing TTXs. Additionally the mELISA was applied to the analysis of *L. sceleratus* since this technique provides complementary
information. TTX standard was used for LC-MS/MS, LC-HRMS and mELISA analysis. For the other analogues, for which no reference standards are available, quantification was carried out through external calibration using TTX standard as a reference.

LC-MS/MS analysis was performed by a TSQ Quantum system (Thermo Fisher Scientific, Bremen, Germany) as previously described (Reverté et al., 2015). 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; extracts were analysed with the mass spectrometer operating in positive mode, [M+H]^+; two multiple reaction monitoring (MRM) transitions were monitored; identification was supported by toxin retention time and MRM ion ratios. 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. Data was processed with Xcalibur 2.0.7 SP1 software (ThermoFisher Scientific, Bremen, Germany). Six level calibration curves between 1-250 ng/mL (0.1-25 mg/kg) showed good intra-batch performance and linear adjustment (r^2) ≥ 0.9992.

LC-HRMS analysis was carried out with an Orbitrap-Exactive HCD (Thermo Fisher Scientific, Bremen, Germany). The chromatography and ESI source parameters used were the same as LC-MS/MS methodology. The working mass range was m/z 100-1,200 in full scan acquisition mode. The resolution was 50,000 (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. Data was processed with Xcalibur 3.1 software (ThermoFisher Scientific, Bremen, Germany).
The recently developed mELISA, based on the immobilization of TTX through dithiol-carboxylate self-assembled monolayers (Reverté et al., 2015) was used to determine the TTX equivalent contents in the *L. sceleratus* tissues. Briefly, carboxylate–dithiol was self-assembled on maleimide-activated plates; carboxylic groups of dithiols were activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS) for subsequent reaction with ethylenediamine; remaining carboxylic groups were deactivated with ethanolamine; TTX was immobilized through formaldehyde cross-linking (amino-amino reaction); competition between free (standard or extract) and immobilised TTX for the anti-TTX monoclonal antibody took place; a blocking step with BSA was performed; immunoglobulin G-horseradish peroxidase (IgG-HRP) was added; TMB liquid substrate was incubated; and absorbance was read at 620 nm.

3. Results and Discussion

The twenty puffer fish were first analysed by LC-MS/MS. Whereas TTX presence was not detected in any of the *L. lagocephalus* and *S. pachygaster* tissues (limit of detection (LOD) = 0.05 mg/kg), TTXs were found in all of the *L. sceleratus* tissues (results are summarized in Table 1). Figure 2 shows the toxin profile of the *L. sceleratus* gonads obtained by LC-MS/MS, as an example. TTX was co-existing with various analogues such as 4-epiTTX and 4,9-anhydroTTX, which are the chemical equilibrium TTX analogues, in all four tissues (gonads, liver, skin and muscle). In addition, four deoxy analogues (5-deoxyTTX, 11-deoxyTTX, 5,11-dideoxy/6,11-dideoxy and 5,6,11-trideoxyTTX) and two 11-nor analogues (11-norTTX-6(R)-ol and 11-norTTX-(S)-ol) were also identified (Bane et al., 2014; Yotsu-Yamashita et al., 2011; Yotsu-Yamashita et al., 2013).
Table 1. TTX and analogues contents (mg TTX or analogue/kg tissue) in *L. sceleratus* by LC-MS/MS and LC-HRMS. Ion composition, theoretical accurate and measured m/z and mass accuracy of main signals obtained from ESI-Orbitrap fragmentation spectra.

<table>
<thead>
<tr>
<th></th>
<th>TTX</th>
<th>4-ep/TTX</th>
<th>11-norTTX-6(R)-ol</th>
<th>11-norTTX-6(S)-ol</th>
<th>4,9-anhydroTTX</th>
<th>5-deoxyTTX</th>
<th>11-deoxyTTX</th>
<th>5,11-dideoxyTTX/6,11-dideoxyTTX</th>
<th>5,6,11-trideoxyTTX</th>
<th>4,9-anhydro-5,6,11-trideoxyTTX</th>
<th>4,4a-anhydro-5,6,11-trideoxyTTX</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LC-MS/MS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRM1</td>
<td>320.1&gt;302.1</td>
<td>320.1&gt;302.1</td>
<td>290.1&gt;272.1</td>
<td>290.1&gt;272.1</td>
<td>302.1&gt;256.1</td>
<td>304.1&gt;286.1</td>
<td>304.1&gt;286.1</td>
<td>288.1&gt;270.1</td>
<td>272.1&gt;254.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MRM2</td>
<td>320.1&gt;162.2</td>
<td>320.1&gt;162.2</td>
<td>290.1&gt;162.2</td>
<td>290.1&gt;162.2</td>
<td>302.1&gt;162.2</td>
<td>304.1&gt;162.2</td>
<td>304.1&gt;162.2</td>
<td>288.1&gt;224.0</td>
<td>272.1&gt;162.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Gonads</strong></td>
<td>21.8</td>
<td>4.3</td>
<td>1.1</td>
<td>16.3</td>
<td>0.5</td>
<td>0.9</td>
<td>1.1</td>
<td>0.4</td>
<td>94.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>2.3</td>
<td>0.7</td>
<td>0.3</td>
<td>1.3</td>
<td>0.2</td>
<td>ND</td>
<td>0.2</td>
<td>ND</td>
<td>12.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Skin</strong></td>
<td>1.2</td>
<td>0.3</td>
<td>0.1</td>
<td>1.1</td>
<td>ND</td>
<td>ND</td>
<td>0.1</td>
<td>ND</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Muscle</strong></td>
<td>0.7</td>
<td>0.3</td>
<td>0.2</td>
<td>0.6</td>
<td>0.1</td>
<td>ND</td>
<td>0.1</td>
<td>ND</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

|                  |         |          |                    |                    |                |             |             |                                 |                   |                                |                                |
| **LC-HRMS**      |         |          |                    |                    |                |             |             |                                 |                   |                                |                                |
| **Ion Composition [M+H]^+** |         |          |                    |                    |                |             |             |                                 |                   |                                |                                |
| Theoretical accurate m/z | 320.1088 | 320.1088 | 290.0983           | 290.0983           | 302.0983      | 304.1139    | 304.1139    | 288.1190                           | 272.1241           | 254.1135                       | 254.1135                       |
| Measured m/z     | 320.1086 | 320.1088 | 290.0982           | 290.0981           | 302.0980      | 304.1137    | 304.1138    | 288.1191                           | 272.1240           | 254.1134                       | 254.1134                       |
| Mass Accuracy (ppm) | -0.63    | -0.25    | -0.33              | -0.57              | -0.75         | -0.61       | -0.51       | 0.24                             | -0.17              | -0.28                          | -0.32                          |
| **Gonads**       | 20.0    | 3.5      | 0.7                | 13.1               | 4.7           | 0.1         | 4.2         | 2.9                             | 167.2              | 427.7                          | 94.0                           |
| **Liver**        | 4.6     | 0.5      | ND                 | 1.4                | 1.0           | 0.1         | 0.4         | 0.3                             | 43.0               | 78.2                          | 35.3                           |
| **Skin**         | 1.8     | 0.2      | ND                 | 0.5                | 0.4           | ND          | 0.1         | ND                             | 3.0                | 15.4                          | 1.3                            |
| **Muscle**       | 0.9     | 0.1      | ND                 | 0.3                | 0.2           | ND          | 0.1         | ND                             | 1.4                | 7.4                           | 0.9                            |

ND: not detected; -: not searched. The LOD of LC-MS/MS and LC-HRMS was 0.05 mg/kg using 20 µL and 10 µL injection volume, respectively.
Figure 2. Multiple reaction monitoring chromatogram of transition monitored obtained following the analysis of TTX and its analogues in the *L. sceleratus* gonads by LC-MS/MS.

A multi-toxin LC-HRMS analysis method was developed to confirm and quantify the TTX and analogues described in the *L. sceleratus* by LC-MS/MS, as well as to identify other possible analogues described in the literature (Bane et al., 2014). Figure 3 shows the toxin profile of the *L. sceleratus* gonads by LC-HRMS, as an example. In general, quantifications by LC-HRMS (LOD = 0.05 mg/kg) agreed with those previously obtained by LC-MS/MS (Table 1). It is important to mention that LC-HRMS analysis allowed the identification and quantification of some analogues (4,9-anhydro-5,6,11-trideoxyTTX
and 4,4a-anhydro-5,6,11-trideoxyTTX) not included in the LC-MS/MS analysis method. These two compounds have been already identified in puffer fish and newt from Japan and Solomon Islands (Kudo et al., 2012; Puilingi et al., 2015).

Figure 3. Accurate mass extracted chromatogram of TTX ([TTX+H]+) and its analogues in the *L. sceleratus* gonads by LC-HRMS (mass window: ± 5 ppm).

*Lagocephalus sceleratus* was also analysed by mELISA (LOD = 0.23 mg/kg). As in the LC-MS/MS and LC-HRMS analysis, TTX was detected in all tissues. Whereas LC-MS/MS and LC-HRMS analyses determine individual TTX and TTX analogues contents, mELISA provides a global response from all TTX and TTX analogues that cross-react with the TTX antibody. Table 2 shows the TTX equivalent contents provided by mELISA and the sums of the LC-MS/MS and LC-HRMS quantifications, values resulting after application of the known cross-reactivity factors (CRFs) of the different TTX analogues.
previously determined (Reverté et al., 2015) to the individual LC-MS/MS and LC-HRMS quantifications. Cross-reactivity factors (in percentage) are 29.9 for 5-deoxyTTX and 11-deoxyTTX, 2.9 for 11-norTTX-6(R)-ol and 11-norTTX-6(S)-ol, $<10^{-2}$ for 4,9-anhydroTTX, and $<10^{-4}$ for 5,6,11-trideoxyTTX; CRF for 4-epiTTX was assumed to be 1; CRFs for 5,11-dideoxyTTX/6,11-dideoxy and the two new 5,6,11-trideoxyTTX analogues were not available and thus these analogues were not included in the sum of TTXs. Tetrodotoxin equivalent contents determined by mELISA in the gonads, skin and muscle were in the range of the quantifications obtained by LC-MS/MS and LC-HRMS, but a considerable disparity was observed in the liver analyses between the quantifications by the mELISA method in relation to those provided by the chromatographic methods. The presence of four analogues with no available CRF may justify this difference between the mELISA and the instrumental analysis methods. However, 5,11-dideoxyTTX/6,11-dideoxy was found in small amounts, and the two 5,6,11-trideoxyTTX analogues can be supposed to have a very low CRF in relation to TTX because of their similar structure to the 5,6,11-trideoxyTTX. For that reason, it may be hypothesised that their presence may not significantly change the TTX quantification, but it cannot be discarded that there is a possible antigen-antibody affinity interaction. Other unknown TTX analogues or liver matrix compounds could also be responsible for the disagreement between the mELISA technique and the LC-MS/MS and LC-HRMS approaches.

**Table 2.** TTX equivalent contents (mg TTX equiv./kg tissue) in *L. sceleratus* by LC-MS/MS, LC-HRMS and mELISA.

<table>
<thead>
<tr>
<th></th>
<th>Σ LC-MS/MS</th>
<th>Σ LC-HRMS</th>
<th>mELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonads</td>
<td>25.95</td>
<td>25.22</td>
<td>33.55</td>
</tr>
<tr>
<td>Liver</td>
<td>3.08</td>
<td>5.36</td>
<td>28.30</td>
</tr>
<tr>
<td>Skin</td>
<td>1.65</td>
<td>2.08</td>
<td>3.50</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.01</td>
<td>0.98</td>
<td>2.53</td>
</tr>
</tbody>
</table>
When comparing TTX content in the different tissues of *L. sceleratus*, all techniques showed the same trend from higher to lower TTX content: gonads > liver > skin > muscle. The higher TTXs content in gonads and liver compared to skin and muscle had also been observed in silver-cheeked toadfishes from Greece (Acar et al., 2017; Katikou et al., 2009; Kosker et al., 2016; Reverté et al., 2015; Rodriguez et al., 2012). Tetrodotoxin level in gonads was above 10-fold the Japanese acceptability criterion value of 2 mg TTX equiv./kg fish tissue for human consumption (HP of Ministry of Health; Mahmud et al., 1999; Noguchi and Ebesu, 2001). In fact, TTX levels were in the range of those found in previous studies: 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 et al., 2017; Katikou et al., 2009; Kosker et al., 2016; Reverté et al., 2015; Rodriguez et al., 2012). This *L. sceleratus* was caught in November. These high TTX values may be in agreement with the results described by Kosker and co-workers (Kosker et al., 2016), who reported that TTX levels in *L. sceleratus* species caught from the Mediterranean Sea were higher in autumn and winter seasons.

In accordance with Rodriguez and collaborators (Rodriguez et al., 2012), 5,6,11-trideoxyTTX was the major analogue detected by LC-MS/MS. In our case, this analogue was followed by TTX and the isomer 11-norTTX-6(S)-ol in all four tissues. Regarding LC-HRMS results, 4,9-anhydro-5,6,11-trideoxyTTX was the major analogue followed by 5,6,11-trideoxyTTX and 4,4a-anhydro-5,6,11-trideoxyTTX, which were found in higher amounts than TTX. Although the 5,6,11-trideoxyTTX analogues were not included in the LC-MS/MS method, they were detected by LC-HRMS and in quite high amounts. These analogues should be included in future analysis of *L. sceleratus* in the LC-MS/MS method, regardless that they may be considered of low toxicity and may not significantly
change the TTX equivalent content. A consensus on the LC-MS/MS method between the number of analogues included in the analysis and sensitivity of the method is necessary. This work constitutes the first report that describes the toxin profile of *L. sceleratus* reaching Spanish waters and complements previous information on TTX present in puffer fish. The LC-MS/MS approach is a suitable technique to identify and quantify TTXs in puffer fish. The LC-HRMS method, which provides a high mass accuracy, has allowed the identification of new TTX analogues. Up to 11 TTX analogues were identified in *L. sceleratus*. Additionally, the mELISA has demonstrated to be a useful method to quantify the total TTX equivalent content in puffer fish. This method does not require expensive instrumentation, provides results in short time and can be used as a TTX screening tool.

Present regulation in the EU is scarce for TTXs, and no maximum permitted levels are established by the legislation. In the recently published EFSA opinion, a concentration below 44 µg TTX equivalents/kg shellfish meat, based on large portion size of 400 g, was considered not to result in adverse effects in human; however, more data is needed to provide a more reliable exposure assessment (EFSA, 2017). The LOD for TTX of the LC-MS/MS and LC-HRMS methods on this work (0.05 mg/kg) is in the same order of magnitude than other LODs previously reported 0.08 mg/kg (Rodriguez et al., 2012) and 0.01 mg/kg (Kosker et al., 2016) and slightly higher than 0.007 mg/kg described by Acar et al, 2017. Nevertheless, this LOD (and also the LOD for mELISA, 0.23 mg/kg) covers the Japanese acceptance criterion to consider puffer fish safe for consumption (2 mg TTX equiv/kg). The protocols for the mass spectrometry analysis and mELISA described in this work can be adjusted to fit for more restrictive criteria.

This work provides additional data of TTX in *L. sceleratus* that may contribute to better assess the risk for TTXs in puffer fish. Fish belonging to this group, although banned for
consumption, may not be well-known by fishermen and the risk of using these fish in soups or other formats may exist.

4. Conclusions

Herein we provide a new evidence for the presence of puffer fish containing high amounts of TTXs in the Western Mediterranean Sea, thereby demonstrating the potential hazard for TTX in this area. Whereas *L. lagocephalus* and *S. pachygaster* individuals were determined as non-containing TTX, *L. sceleratus* caught in Denia was determined to be highly toxic and a detailed toxin profile is provided. Tetrodotoxin levels in gonads tissues were up to 10-fold above the Japanese criterion to judge the acceptability of puffer fish for human consumption. The use of an immunoassay as a screening tool for TTX (mELISA) and a confirmatory analysis by liquid chromatography coupled to mass spectrometry is a useful strategy to quantify TTXs in puffer fish and assess the risk they may represent for consumers.

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