Journal of
Marine Science and Engineering

MDPI

Article

Evaluation of the Occurrence of Tetrodotoxin in Bivalve Mollusks from the Portuguese Coast

Susana Margarida Rodrigues, Estefanía Pereira Pinto, Patrícia Oliveira, Sónia Pedro and Pedro Reis Costa *

IPMA-Portuguese Institute for the Sea and Atmosphere, Av. Alfredo Magalhães Ramalho 6, 1495-165 Lisbon, Portugal

* Correspondence: prcosta@ipma.pt

Received: 30 June 2019; Accepted: 17 July 2019; Published: 19 July 2019

Abstract: Occurrence of tetrodotoxin (TTX), a potent natural neurotoxin, in marine organisms and seafood from the European Union has been of increasing concern due to its relatively recent detection in bivalve mollusks and gastropods. Following a request of the European Food Safety Authority (EFSA) to EU Member States to obtain more data on TTX occurrence, this study collected 117 samples of bivalve mollusks, including mussels (*Mytilus galloprovincialis*), oysters (*Crassostrea gigas*) and clams (*Ruditapes philippinarum* and *Donax* spp.), from the South and Southwest Portuguese coast between May and October 2018, for TTX determination and microbiological analysis. The analyses carried out by hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-MS/MS) did not detect TTX in any sample, and microbiological analysis did not reveal high concentrations of *Vibrio* spp., which has been linked to the presence of TTX. Although preliminary, results from this study, the first investigating the presence of TTX in bivalve mollusks from the Portuguese coast, suggest that TTX may not represent a risk for human consumption of bivalve mollusks.

Keywords: tetrodotoxin; marine biotoxins; *Vibrio*; shellfish; seafood safety

1. Introduction

Tetrodotoxin (TTX) is a potent neurotoxin that blocks voltage-gated sodium channels in mammalian excitable neuronal cells causing paralytic poisoning and, in the most severe cases, can lead to human fatalities due to respiratory and heart failure [1,2]. TTX and its analogues are natural compounds found in a great variety of organisms, both terrestrial and aquatic, and are particularly well known in pufferfish (Tetraodontidae), as most TTX food poisonings are related to pufferfish/fugu consumption in Southeast Asia.

Particular attention was given to TTX occurrence in European waters after its detection in bivalve mollusks from the UK [3]. A survey study carried out in 2013–2014 and then between 2014 and 2016 revealed TTX concentrations up to 253 μg kg^{-1} in Pacific oysters (*Magallana gigas*, previously known as *Crassostrea gigas*) collected at the south coast of England [3,4]. Following the initial findings of [3], TTX was also detected in mussels and oysters from the Netherlands in 2015 [5], which made the Dutch authorities to issue an advice in 2016 and proposing to apply a zero tolerance for TTX in shellfish, meaning that bivalves would only enter the markets with non-detectable levels of TTX after analysis with analytical methods able to detect a concentration of 20 μg kg^{-1} or lower [6]. In 2017, the European Food Safety Authority (EFSA) stated a scientific opinion, following a request from the European Union, on the risk related to the presence of TTX and TTX analogues in marine bivalves and gastropods [7]. EFSA stated that a concentration lower than 44 μg TTX equiv. kg^{-1} is not expected to result in adverse effects in humans and recommended EU Member States to obtain more data on TTX occurrence.

The biogenic origin of TTX has been under debate, in particular due to the widespread occurrence of TTX in phylogenetically unrelated organisms from terrestrial, freshwater and marine environments. Several studies support that symbiotic bacteria play a role in TTX biosynthesis, and a list of TTX-producing bacterial species isolated from TTX-bearing animals have been updated during the last years [8–10]. The presence of *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and *Pseudomonas* spp. were confirmed in TTX contaminated mussels from the Greek and UK coasts [3,11]. In addition, the hypothesis of phytoplankton species, namely *Prorocentrum minimum*, as a source of TTX to bivalves, has been raised by [12] after correlating TTX in shellfish and algae occurrence in the Greek coast. However, a similar trend between TTX in shellfish and *P. minimum* was not observed in samples from the UK [4]. More recently, TTX-like compounds were identified in cultures of *P. minimum* [11].

In the Portuguese coast, TTX variability in bivalve mollusks has not been studied so far. On the other hand, seasonal dynamics of the microbial community have been described for bivalves. *Vibrio* spp. are particularly abundant during summer, when seawater temperatures are well above 15 °C [13].

The aim of the present study was to investigate the presence and variability of TTX in bivalves from classified production areas (CPA) of the Portuguese coast and to determine the associated bacterial community.

2. Materials and Methods

2.1. Sample Collection

Selected samples of bivalve mollusks were obtained from the national program for Official Control of marine toxins in shellfish: Manila clam (*Ruditapes philippinarum*) from the Sado estuary, mussels (*Mytilus galloprovincialis*) from the Tejo estuary and from the Tavira–Formosa lagoon, oysters (*Crassostrea gigas*) from the Guadiana estuary and donax clams (*Donax* sp) from the sandy beaches of Vila Real de Santo António, designated as an L9 shellfish producing area (Figure 1). Samples were weekly harvested from May to October 2018. A total of 117 samples of bivalves were collected, being 54 mussel samples, 24 oysters, 21 Manila clams and 21 donax clams. Upon collection, shellfish were immediately shipped to IPMA facilities (Lisbon) under refrigerated conditions in a thermally isolated container. For mussels and clams, each sample was composed of at least 20 individuals. In the case of oysters, each sample constituted at least 10 individuals.

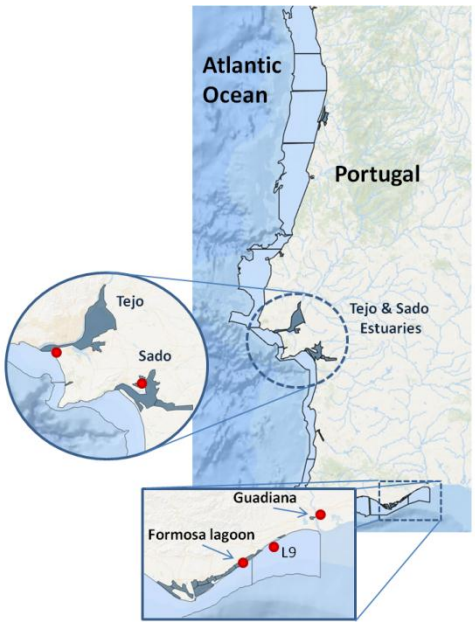

Figure 1. Location of bivalve mollusks sampling sites in the South and Southwest coast of Portugal.

2.2. Toxin Extraction and Liquid Chromatography Analysis

2.2.1. Reagents

All reagents used for toxins extraction and analysis were of analytical grade or higher. Acetic acid glacial (100%, p.a.), methanol (>99.8%, p.a.), LC–MS additive grade ammonium hydroxide solution (NH_4OH, 25% as NH_3), formic acid (98–100%) and acetonitrile (analytical grade) were obtained from Sigma-Aldrich (Sintra, Portugal); ammonium formate (>99% purity) was from Fluka and hydrochloric acid (37%) from Panreac. Water was purified using a Milli-Q 185 Plus system from Millipore. The toxin standard solution for TTX (>99% purity) was sourced from Tocris Bioscience (Bristol, UK).

2.2.2. Toxins Extraction

The bivalve mollusks' soft tissues were removed from the shell, washed with running tap water to remove residues and drained. The samples were then homogenized in a blender and stored at −20 °C until toxin analysis. Toxin extraction was performed according to the Standard Operating Procedure (SOP) for determination of TTX provided by the European Union Reference Laboratory [14]. A portion of 5 g of homogenized shellfish meat was extracted with 5 mL of 1% acetic acid by vortexing for 90 s and heating for 5 min in a boiling water bath. Samples were cooled down until room temperature was achieved and were again vortexed for another 90 s. After that, centrifugation of the samples for 10 min at 3000 *g* was conducted. A clean-up step using Graphitised Carbon SPE was carried out following the method described by [4,14,15]. A total of 5 µL of 25% v/v of NH_3 were added to 1 mL of the supernatant and was centrifuged at 1000 *g* for 1 min before performing the SPE clean-up step. The ENVI-Carb cartridge (Supelclean, Supelco, Sigma-Aldrich, Sintra, Portugal) was first conditioned with 3 mL of 20% MeCN + 1% v/v HOAc and 3 mL of 0.025% v/v NH_3. A 400 µL aliquot of sample extract was loaded onto the cartridge and washed with 700 µL deionized water. Toxins elution and collected were carried out through the addition of 2 mL 20% MeCN + 0.25% acetic acid. The eluted extract was mixed and then diluted by transferring 100 µL to a vial and adding 300 µL of acetonitrile before analysis.

2.2.3. TTX Determination by HILIC-MS/MS

The LC-MS/MS equipment consisted of an Agilent 1290 Infinity coupled to a triple quadrupole mass spectrometer, an Agilent 6470. The chromatographic separation was conducted with a hydrophilic interaction liquid chromatography (HILIC) UHPLC column (1.7 µm, 2.1 mm x 150 mm Waters Acquity Glycan BEH Amide column in conjunction with a Waters VanGuard BEH Amide guard column (Waters, Lisbon, Portugal)). The chromatographic separation was performed using the conditions described in [14]: Elution was achieved using a binary eluent system: Eluent A water/formic acid/NH4OH (500:0.075:0.3 v/v/v) and eluent B acetonitrile/water/formic acid (700:300:0.1 v/v/v). A gradient started at 98% B at 0.4 mL/min for the first 5 min, 98–50% B for the next 2.5 min, and this composition was kept for 1.5 min but the flow rate was linearly increased to 0.5 mL/min until 9.0 min, and then B reverted to 98% by 9.5 min, flow rate ramped to 0.8 mL/min at 10.0 min, held until 10.6 min, and dropped back to 0.4 mL/min until 11.0 min. Two multiple-reaction-monitoring (MRM) transitions from the protonated ions were monitored for TTX and TTX derivatives as described in [14,16,17]: For TTX and 4-*epi*TTX the transitions monitored were the *m/z* 320.1 > 302.1 and *m/z* 320.1 > 162.1, for 11-deoxyTTX and 5-deoxyTTX *m/z* 304.1 > 286.1 and *m/z* 304.1 > 162.1, for 4,9-Anhydro *m/z* TTX 302.1 > 284.1 and *m/z* 302.1 >162.1, for 6,11-dideoxy-TTX *m/z* 288.1 > 272.1 and *m/z* 288.1 > 162.1, finally for the 5,6,11-trideoxy-TTX *m/z* 272.1 > 254.1 and *m/z* 272.1 > 162.1. The optimized source settings were as following: Gas temperature 150 °C, gas flow 13 L min^{-1}, nebulizer 50 psi, sheath gas temperature 400 °C, sheath gas flow 12 L min^{-1} and capillary voltage 2500 V. Linearity of the calibrations curves was validated for TTX standards prepared in different shellfish matrices and the limits of detection (LOD) and quantification (LOQ) were evaluated based on the signal to noise ratios for TTX in fortified shellfish extracts. A five-point calibration curve of TTX with a correlation >0.990 was set up for

quantification, with the quantification being performed using a matrix match calibration and the limit of quantification of 16 µg TTX kg^{-1}.

2.3. *Vibrio* spp. Detection and Enumeration

All samples were analyzed immediately on arrival to the laboratory. Live bivalves were washed and scrubbed under running potable water. Dead and damaged shellfish were discarded. Specimens were aseptically opened, the flesh and intervalvar liquid were extracted into a sterile container using a sterile scalpel and flesh was cut with sterile scissors.

The qualitative *Vibrio* spp. analysis was performed by adding 225 mL alkaline salt peptone water (ASPW; Oxoid Ltd., Basingstoke, Hampshire, UK) to 25 g of sample, homogenized with a Stomacher 400 (Seward Laboratory System, London, UK), and incubated at 37 ± 1 °C for 18 ± 1 h. Following broth incubation, two plates of thiosulphate citrate bile salt sucrose agar (TCBS; Oxoid Ltd., Basingstoke, Hampshire, UK) were streaked from just below the surface of the broth, without mixing, and incubated at 37 ± 1 °C for 21 ± 3 h. Plates were checked for typical colonies and at least five sucrose-negative and positive colonies were selected from each TCBS plate. In order to confirm the isolated strains, biochemical tests were carried out in characteristic colonies. Gram negative and oxidase-positive strains were presumptively identified as *Vibrio* spp. Results were expressed as present or absent in 25 g.

For the quantification of *Vibrio* spp., 10 g of the homogenized sample was added to 90 mL of ASPW (initial decimal dilution) and homogenized with a Stomacher 400 (Seward Laboratory System, London, UK). Serial ten-fold dilutions were also prepared in ASPW. Dilutions were surface plated on TCBS agar and plates incubated at 37 ± 1 °C for 21 ± 3 h. Selection, isolation and testing of characteristics colonies was performed as previously described for *Vibrio* spp. detection. Results were expressed as colony-forming units per gram of sample (CFU/g).

3. Results and Discussion

A total of 117 bivalve mollusk samples, comprising four species (mussels, oysters, and two clam species) collected from shallow water, estuarine environments (Tejo, Sado and Guadiana estuaries, as well as the Formosa Lagoon) and sandy beaches (Vila Real Santo António) from the Southwest and South Portuguese coast were analyzed for TTX by the sensitive HILIC-MSMS method. Although sampling was carried out between May and October, when the highest seawater temperature is reached, which has been pointed out as a relevant factor associated with TTX occurrence [4], TTX or any of its analogues were not detected at quantifiable levels. Among these samples, TTX trace levels were observed in three mussel samples collected in Tavira (Formosa lagoon) in July and August (Figure 2). These results are in agreement with the recent findings in Spain, the nearby country where no TTX have been found in bivalve mollusks in two TTX survey studies [16,18].

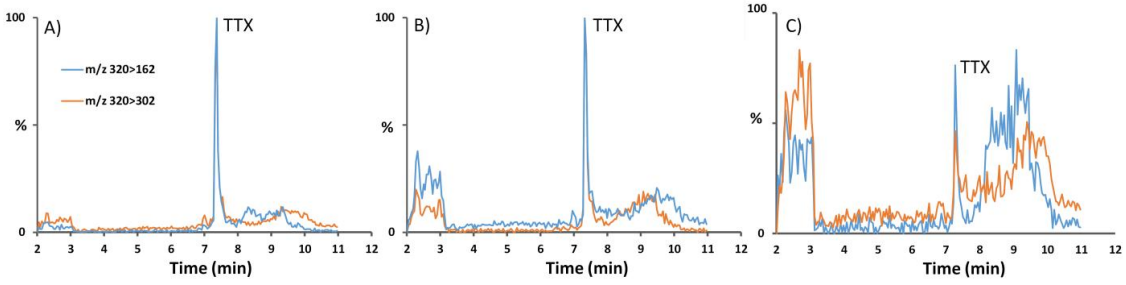

Figure 2. Multiple reaction monitoring (MRM) chromatograms of tetrodotoxin (TTX) obtained by HILIC-MS/MS analysis in (**A**) matrix match TTX standard of 40 µg kg^{-1}, (**B**) TTX spiked mussel extract of 25 µg kg^{-1}, and (**C**) TTX trace levels in mussels from Tavira (South Portuguese coast).

The presence of TTX in marine organisms from the Portuguese coast was previously investigated in gastropods [19]. Even though marine gastropods are well known TTX vectors and have been

involved in several human poisoning cases in different geographic locations from Asia [20], TTX in gastropods from the Portuguese coast was observed at relatively low levels. The highest TTX concentration, 90 μg kg^{-1}, was determined in the sea snail *Monodonta lineata* collected in the Southwest Portuguese coast [19].

The origin of TTX accumulated in bivalve mollusks has been associated with bacterial communities, in particular to species of the genus *Vibrio* [10]. In the present study, *Vibrio* spp. were detected in 96% of samples, with levels ranging from <1.0 × 10 UFC/g to 2.6 × 10^4 UFC/g. The three samples presenting TTX trace levels, collected during the summer months, were positive for *Vibrio* spp., with contamination values between 5.0 × 10^2 UFC/g and 1.9 × 10^3 UFC/g. Samples presenting higher *Vibrio* spp. concentrations did not exhibit TTX, not even at trace levels.

4. Conclusions

This first study on TTX occurrence in bivalve mollusks from the Portuguese coast suggests that TTX does not pose a risk for human consumption of bivalves. However, more comprehensive surveys should be carried out to 1) confirm these initial findings, 2) address the EFSA recommendation regarding obtention of consistent TTX data occurrence, which is essential to perform an appropriated evaluation of the risk, and 3) to better understand the relationship between *Vibrio* spp. and the bacterial community associated with TTX occurrence.

Author Contributions: S.M.R., P.R.C. and S.P. conceived the project; E.P.P., S.M.R. and P.R.C. performed the TTX analysis; P.O. and S.P. performed the microbiological analysis; and all authors were involved in the data analysis, data interpretation, and preparation of the manuscript.

Funding: This research was funded by project SNMB-MONIT and SNMB-INOV: Innovation for a more competitive shellfish sector, co-financed by the Operational Program Mar 2020, Portugal 2020.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Narahashi, T.; Haas, H.G.; Therrien, E.F. Saxitoxin and tetrodotoxin: Comparison of nerve blocking mechanism. *Science* **1967**, *157*, 1438–1440. [CrossRef] [PubMed]
2. Walker, J.R.; Novick, P.A.; Parsons, W.H.; McGregor, M.; Zablocki, J.; Pande, V.S.; Du Bois, J. Marked difference in saxitoxin and tetrodotoxin affinity for the human nociceptive voltage-gated sodium channel (Nav1.7). *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 18102–18107. [CrossRef] [PubMed]
3. Turner, A.D.; Powell, A.; Schofield, A.; Lees, D.N.; Baker-Austin, C. Detection of the pufferfish toxin Tetrodotoxin in European bivalves, England, 2013 to 2014. *Eurosurveillance* **2015**, *20*, 21009. [CrossRef] [PubMed]
4. Turner, A.D.; Dhanji-Rapkova, M.; Coates, L.; Bickerstaff, L.; Milligan, S.; O'Neill, A.; Faulkner, D.; McEneny, H.; Baker-Austin, C.; Lees, D.N.; et al. Detection of tetrodotoxin shellfish poisoning (TSP) toxins and causative factors in bivalve molluscs from the UK. *Mar. Drugs* **2017**, *15*, 277. [CrossRef] [PubMed]
5. Gerssen, A.; Bovee, T.H.F.; Klijnstra, M.D.; Poelman, M.; Portier, L.; Hoogenboom, R.L.A.P. First report on the occurrence of tetrodotoxins in bivalve molluscs in the Netherlands. *Toxins* **2018**, *10*, 450. [CrossRef] [PubMed]
6. Front Office and Public Safety 2015. Risk Assessment of Tetrodotoxins in Shellfish. Available online: https://www.nvwa.nl/documenten/consument/eten-drinken-roken/schelpdieren/risicobeoordeling/beoordeling-tetrodotoxines-in-schelpdieren (accessed on 26 June 2019). (In Dutch).
7. EFSA. Scientific opinion on the risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. *EFSA J.* **2017**, *15*, e04752.
8. Chau, R.; Kalaitzis, J.A.; Neilan, B.A. On the origin and biosynthesis of Tetrodotoxin. *Aquat. Toxicol.* **2011**, *104*, 61–72. [CrossRef] [PubMed]
9. Pratheepa, V.; Vasconcelos, V. Microbial diversity associated with tetrodotoxin production in marine organisms. *Environ. Toxicol. Pharmacol.* **2013**, *36*, 1046–1054. [CrossRef] [PubMed]
10. Magarlamov, T.Y.; Melnikova, D.I.; Chernyshev, A.V. Tetrodotoxin-producing bacteria: Detection, distribution and migration of the toxin in aquatic systems. *Toxins* **2017**, *9*, 166. [CrossRef] [PubMed]

11. Rodriguez, P.; Alfonso, A.; Alonso, E.; Rubiolo, J.A.; Roel, M.; Vlamis, A.; Katikou, P.; Jackson, S.A.; Menon, M.L.; Dobson, A.; et al. The association of bacterial C9-based TTX-like compounds with *Prorocentrum minimum* opens new uncertainties about shellfish seafood safety. *Sci. Rep.* **2017**, *7*, 40880. [CrossRef] [PubMed]
12. Vlamis, A.; Katikou, P.; Rodriguez, I.; Rey, V.; Alfonso, A.; Papazachariou, A.; Zacharaki, T.; Botana, A.M.; Botana, L.M. First Detection of Tetrodotoxin in Greek Shellfish by UPLC-MS/MS Potentially Linked to the Presence of the Dinoflagellate *Prorocentrum minimum*. *Toxins* **2015**, *7*, 1779–1807. [CrossRef] [PubMed]
13. Anacleto, P.; Pedro, S.; Nunes, M.L.; Rosa, R.; Marques, A. Microbiological composition of native and exotic clams from Tagus estuary: Effect of season and environmental parameters. *Mar. Pollut. Bull.* **2013**, *74*, 116–124. [CrossRef] [PubMed]
14. EULRMB. Determination of Tetrodontoxin by HILIC-MS/MS, European Reference Laboratory for Marine Biotoxins, Ed 01062107. 2017. Available online: http://www.aecosan.msssi.gob.es/en/CRLMB/docs/docs/metodos_analiticos_de_desarrollo/HILIC-LCMSMS_SOP_for_TTX_in_mussels.pdf (accessed on 26 June 2019).
15. Boundy, M.J.; Selwood, A.I.; Harwood, D.T.; McNabb, P.S.; Turner, A.D. Development of a sensitive and selective liquid chromatography-mass spectrometry method for high throughput analysis of paralytic shellfish toxins using graphitised carbon solid phase extraction. *J. Chromatogr. A* **2015**, *1387*, 1–12. [CrossRef] [PubMed]
16. Leao, J.M.; Lozano-Leon, A.; Giráldez, J.; Vilariño, O.; Gago-Martínez, A. Preliminary results on the evaluation of the occurrence of tetrodotoxin associated to marine *Vibrio* spp in bivalves from the Galician Rias (Northwest Spain). *Mar. Drugs* **2018**, *16*, 81. [CrossRef] [PubMed]
17. Yotsu-Yamashita, M.; Abe, Y.; Kudo, Y.; Ritson-Williams, R.; Paul, V.J.; Konoki, K.; Cho, Y.; Adachi, M.; Imazu, T.; Nishikawa, T.; et al. First identification of 5, 11-dideoxytetrodotoxin in marine animals, and characterization of major fragment ions of tetrodotoxin and its analogs by high resolution ESI-MS/MS. *Mar. Drugs* **2013**, *11*, 2799–2813. [CrossRef]
18. Blanco, L.; Lago, J.; González, V.; Paz, B.; Rambla-Alegre, M.; Cabado, A.G. Occurrence of tetrodotoxin in bivalves and gastropods from harvesting areas and other natural spaces in Spain. *Toxins* **2019**, *11*, 331. [CrossRef] [PubMed]
19. Silva, M.; Azevedo, J.; Rodriguez, P.; Alfonso, A.; Botana, L.M.; Vasconcelos, V. New Gastropod Vectors and Tetrodotoxin Potential Expansion in Temperate Waters of the Atlantic Ocean. *Mar. Drugs* **2012**, *10*, 712–726. [CrossRef] [PubMed]
20. Noguchi, T.; Onuki, K.; Arakawa, O. Tetrodotoxin poisoning due to pufferfish and gastropods and their intoxication mechanism. *ISRN Toxicol.* **2011**, *2011*, 276939. [CrossRef] [PubMed]