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Circannual Prevalence of tetrodotoxins in trumpet shells: Sea stars as a possible source of contamination and Implications for food safety

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ABSTRACT

Tetrodotoxin (TTX) is a potent neurotoxin, first identified in fish from the Tetraodontidae family but also detected in marine invertebrates. A Human poisoning episode after consumption of trumpet shell *Charonia lampas*, likely caught off the Portuguese mainland southern coast – Algarve, together with the increasing reports of TTX in European waters, led the European Food Safety Authority (EFSA) to recommend maximum safe limits for Human consumption of shellfish meat. However, data on temporal and species incidence of TTX are lacking. In the present study, TTX and its analogues were analysed by liquid chromatography-high resolution mass spectrometry (LC-HRMS) in trumpet shells and in one of their potential prey sources of TTX, the sea star *Astropecten aranciacus*. The estimated toxicity, based on a Toxicity Equivalency Factors approach, of non-edible trumpet shell tissues consistently surpassed EFSA limits (44 μg TTX equivalent (eq.) kg^{-1}) over the studied year. A correlation between TTX concentration and bottom seawater temperature suggests a possible role of this parameter in TTX uptake. TTX levels in edible trumpet shell tissues and all but one sea star individual were below quantification limits. However, several TTX analogues were quantified in the sea stars, resulting in estimated toxicities (monthly averages) ranging from 7 to 64 μg TTX eq kg^{-1} in the digestive glands and from 0.3 to 27 μg TTX eq. kg^{-1} in the stomachs. Therefore, the sea star is a possible TTX source for trumpet shells. Despite the absence of TTX in common edible parts of trumpet shells, whole-shell sales in markets pose a consumer risk, highlighting the need for TTX monitoring and public awareness programs to prevent poisoning.

1. Introduction

Tetrodotoxin (TTX) is a potent neurotoxin recognised for its ability to block voltage-gated sodium channels, which are essential for the excitability of mammalian nerve and muscle tissues (Narahashi & Moore, 1964), leading, in extreme cases, to respiratory and heart failure (How et al., 2003; Knutsen et al., 2017; Noguchi & Ebesu, 2001). TTX is commonly known as the pufferfish toxin, named after its first identification in the Tetraodontidae family of teleost fish (Tahara & Hirata, 1909). It is usually found together with its analogues, which at least 30 have been identified, and show structure-dependent toxicity (Yotsu-Yamashita et al., 1999). TTX is thought to originate from bacteria of the genera *Pseudomonas*, *Pseudoalteromonas* and *Vibrio*, although bacteria from other genera have been suggested as TTX sources (Matsui et al., 1989; Noguchi et al., 1986; Wang & Fan, 2010; Wu et al., 2005; Yang et al., 2010; Yasumoto et al., 1986; Yotsu et al., 1987). TTX-producing bacteria have been isolated from the subcutaneous mucus, ovaries, and the gastrointestinal tract of several marine organisms (Bane et al., 2014; Knutsen et al., 2017; Noguchi et al., 2006a; Wu et al., 2005). The source of TTX in marine animals is still under debate. Two main theories have been proposed: *i*) endogenous production, in which symbiotic bacteria produce TTX in the organism's digestive system, and *ii*) bioaccumulation/exogenous production, in which free-living bacteria produce TTX that is then accumulated in marine animals via a dietary route (Noguchi et al., 2006a, 2006b; Noguchi & Arakawa, 2008).

TTX-related poisoning is well-documented in East Asian countries, especially in Japan, where most cases occur due to the consumption of pufferfish and, to a lesser extent, marine gastropods and crabs (Arakawa et al., 1994; Bane et al., 2014; Knutsen et al., 2017; Kanchanapongkul &

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Krittayapoositpot, 1995; Miyazawa & Noguchi, 2001; Noguchi et al., 2011; Rodriguez et al., 2008; Tsai et al., 2006). This seafood safety issue was initially believed to be restricted to those warm waters (Hort et al., 2020).

However, due to the opening of the Suez Canal, global warming, and the process of tropicalisation, TTX-bearing species have been reported in European waters. The first species reported was puffer fish *Lagocephalus sceleratus* (Gmelin, 1789), detected in the Mediterranean Sea in 2003 (Akyol et al., 2005). The placement of poisonous fish from the families Tetraodontidae, Molidae, Diodontidae, and Canthigasteridae on the European market, as well as fishery products containing biotoxins such as ciguatera or muscle-paralyzing toxins (e.g., TTX), has been prohibited since 1991 (Directive 91/493, 1991). This directive was repealed by Regulation 853/2004, which was subsequently repealed by Regulation 2019/627. This regulation helped to mitigate TTX-related poisonings from the consumption of fishery products in Europe. However, TTX-related poisonings have still been reported since then, which has been associated with fraudulent labelling and accidental or illegal consumption of TTX-bearing fish species (De Haro, 2008; Guardone et al., 2019; Souissi et al., 2014).

In 2007 the consumption of a gastropod trumpet shell (*Charonia lampas*) in Spain led to the first and only known report of TTX-related food poisoning by molluscs in Europe. The poisonous specimen, likely caught off the Algarve coast (south of Portugal), had a TTX concentration of 315 mg TTX eq. kg^{-1} in its digestive gland (Fernández-Ortega et al., 2010; Rodriguez et al., 2008). Other trumpet shell specimens caught at the Algarve coast showed TTX levels in non-edible tissues as high as 42 mg TTX eq. kg^{-1} (Costa et al., 2021; Lage et al., 2022). Edible tissues contained TTX in lower contents (15–73 μg TTX eq. kg^{-1}) (Costa et al., 2021; Lage et al., 2022). However, these studies represent a single point in time of TTX contamination. After this TTX-related poisoning case, several studies across Europe have identified TTX and its analogues in various bivalve molluscs (Bacchiocchi et al., 2021; Blanco et al., 2019; Bordin et al., 2021; Dell'Aversano et al., 2019; Dhanji-Rapkova et al., 2021; Gerssen et al., 2018; Hort et al., 2020; Leão et al., 2018; Réveillon et al., 2021; Turner et al., 2015, 2017 Vlamis et al., 2015) and marine gastropods (Blanco et al., 2019; Costa et al., 2021; Hort et al., 2020; Lage et al., 2022; Nzoughet et al., 2013; Rodriguez et al., 2008). Consequently, the European Food Safety Authority (EFSA) issued a scientific opinion on TTX as an emerging toxin. It was concluded that existing consumption and occurrence data were insufficient to establish a public health risk in Europe but recommended a maximum safe limit of 44 μg TTX equivalent (eq.) kg^{-1} for shellfish meat (Knutsen et al., 2017). However, there is no legal requirement to monitor TTX levels in Europe (Knutsen et al., 2017; Regulation 2019/627). To date, only the Netherlands and France have incorporated the EFSA recommendation into their national plans for monitoring TTX in shellfish (Gerssen et al., 2018; Sinno-Tellier et al., 2023).

In the present study, our primary goal was to assess the temporal variability of TTX and its analogues accumulation in trumpet shells bycatch off the Algarve coast. This is the first study reporting monthly levels of TTX in this marine gastropod over nearly a year. As the origin of TTX contamination of trumpet shells in Europe is still unknown, we explored the presence and potential temporal variations of TTX and its analogues in one of its main preys, the sea star *Astropecten aranciacus* (Morton, 2012). Furthermore, we evaluated how individual specimen features (weight and length), catch depth, and bottom seawater temperature affect TTX concentrations. This study provides preliminary insights into TTX poisoning risks in Europe—although limited by sample size, sampling duration, and regional consumption restrictions— which could help to take decisions regarding the regulation and monitoring of TTX and its analogues.

2. Materials and methods

2.1. Sampling and sample preparation

Trumpet shells, *Charonia lampas* (n = 25) and sea stars, *Astropecten aranciacus* (n = 25) were accidently captured (by-catch) between November 2021 and October 2022 by an artisanal bottom-set net fishery using gillnets (220 mm) and trammel nets (120 mm), operating off the Algarve, the southern coast of Portugal (Fig. 1). Onboard observers recorded the coordinates and depth of capture of each specimen (Supplementary Material Tables S1 and S2, Pais et al., 2025). Bottom seawater temperature (BST) records in the region were provided by Tunipex (2023).

The trumpet shells and sea stars were brought to the laboratory, weighted (nearest g), measured (nearest mm) and dissected on the day of capture (see Supplementary Material, Fig. S1, for photographs of trumpet shell and sea star before and after dissection). The trumpet shells' soft tissue was weighed, and the shell length was measured; the sea stars' whole body was weighed, and arm length (R) and disk radius (r) were measured (Supplementary Material Tables S1 and S2, Pais et al., 2025). The soft tissues of the trumpet shells were subdivided into two fractions: edible (cerebral ganglia, foot muscle, mantle, mouth/-proboscis, and salivary glands) and non-edible (anus/rectum, digestive gland, gill, heart, intestine, kidney, and stomach), while the sea star had their digestive glands, stomachs (both pyloric and cardiac), and stomach content dissected. All tissues were stored at −20 °C.

2.2. TTXs extraction

TTXs extraction was performed according to the Standard Operating Procedure (SOP) of the European Union Reference Laboratory for Marine Biotoxins to determine TTX (EURLMB, 2017). Briefly, 5g of each sample were homogenised in an Ultra-Turrax (T 25 easy clean digital, IKA-Werke GmbH & Co. KG, Germany) with 5 mL of 1 % (*v/v*) acetic acid (LC-MS grade, Fluka Analytical, Steinheim, Germany), vortexed for 3 min, boiled in water for 5 min, cooled to room temperature, vortexed again for 3 min, and centrifuged for 10 min at 4500g and 15 °C (Mega Star 600 R, VWR, Avantor, USA).

A solid-phase extraction (SPE) was performed in the supernatant containing 0.025 % (*v/v*) ammonium hydroxide (LC-MS grade, Fluka Analytical, Steinheim, Germany). The ENVI-Carb cartridge (250 mg/3 mL, Supelclean, Supelco, Sigma-Aldrich, Germany) were previously conditioned with an aqueous solution with 20 % acetonitrile (*v/v*) (LC-MS grade, Merck, Darmstadt, Germany) and 1 % acetic acid (*v/v*), followed by 0.025 % (*v/v*) ammonium hydroxide. After washing with Milli-Q water, TTX and its analogues were eluted using an aqueous solution with 20 % (*v/v*) acetonitrile and 1 % acetic acid (*v/v*). The eluate was diluted three times with acetonitrile before analysis.

2.3. LC-HRMS analysis

LC-HRMS analysis was performed according to Lage et al. (2022). An UltiMate 3000 UHPLC coupled to an Orbitrap Elite mass spectrometer (Thermo Scientific, Germany) equipped with a heated electrospray ionisation source (HESI-II) was used for the chromatographic separation and analysis. TTX and its analogues were separated with an ACQUITY Premier BEH Amide (2.1 × 100 mm, 1.7 mm) column (Waters, USA) at 35 °C. The mobile phase consisted of water (LC-MS grade, J.T. Baker, Center Valley, PA, USA) containing 0.1 % formic acid (*v/v*) (LC-MS grade, Fluka Analytical, Steinheim, Germany) and 10 mM ammonium formate (*w/v*) (A), as well as acetonitrile containing 0.1 % formic acid (*v/v*) and 2 % 10 mM ammonium formate solution (*w/v*) (B). A linear gradient (in *v/v* %) was started with 5 % of B increasing to 95 % in 11 min; held for 1 min before a return to 5 % of B in 1 min; held for 2 min. The flow rate was 0.3 mL/min, and the injection volume was 5 μL. Source conditions were optimised to achieve the highest TTX sensitivity.

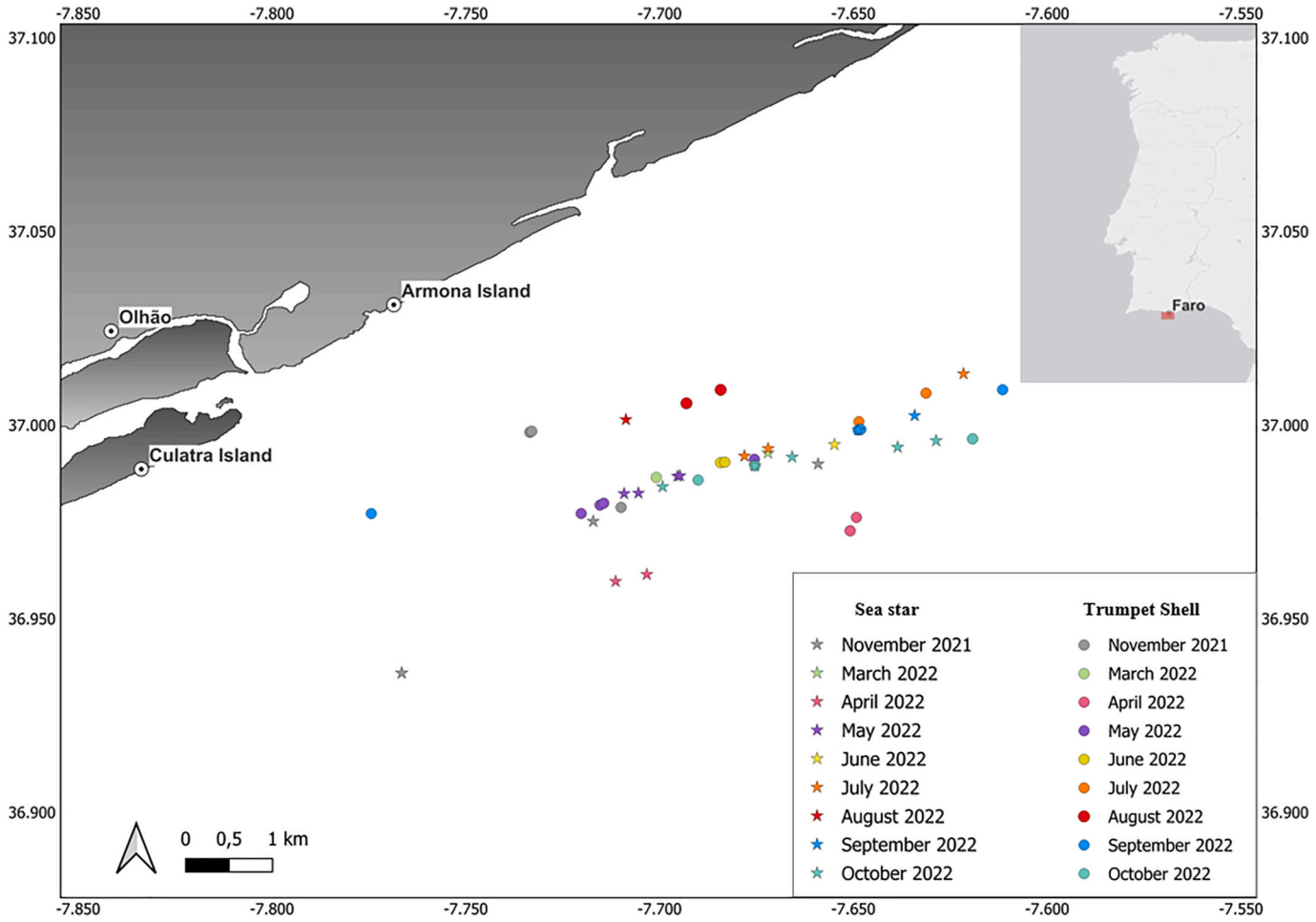

Fig. 1. Location of the sampling points in Algarve offshore coast, southern Portugal. Different colours indicate different months and years. Circles represent trumpet shell samples and stars represent sea star samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Data were acquired under positive polarity using the spray voltage at 3.8 kV, sheath gas at 40 arbitrary units, auxiliary gas at 10 arbitrary units, heater temperature at 300 °C, the capillary temperature at 325 °C, and SLenses RF level at 69.06 %. The LC-HRMS acquisition was performed under full-scan, with positive mode, with a range of *m/z* 100–500. The high-energy collisional dissociation (HCD) spectra of TTX and its analogues (were obtained by running the system under product ion scan by fragmentation of the ion of each analogue over the entire chromatographic separation (LC–HRMS^2) and detection from 50 to 350 *m/z*. The LC–HRMS quantitation was performed by generating accurate mass-extracted ion chromatograms (AM-XIC) obtained from the full-scan positive (ESI+) profiles using the exact mass of each TTX analogue protonated molecular ion $[M+H]^+$ (see Supplementary Material Table S3), and a mass accuracy of ±5 ppm. Signals of TTX and its analogues were assigned based on the exact masses, retention times and fragmentation spectra obtained by HCD (including intensity ratios of MS2 fragments to differentiate between epimers), and comparison of the obtained data with the available in literature (see Supplementary Material, Figs. S2–S7, for details on the collision energy (CE) used for each analogue, along with the corresponding AM-XIC and HCD MS2 spectra of each analogue annotated). The obtained concentrations of TTX analogues are estimates, as the ESI-HRMS response for TTX standard was assumed for all of its analogues. Other general ESI-HRMS parameters were adjusted to ensure an optimum signal for the TTX standard.

A Certified Reference Material (CRM) was used in an aqueous solution of acetic acid (1 mM *v/v,* pH 3.91), containing certified concentrations of TTX, 21.0 ± 1.3 μg g^{-1}; 4,9-anhydroTTX, 5.44 ± 0.40 μg g^{-1}; and 4-*epi*TTX, 1.67 ± 0.15 μg g^{-1}; and trace levels of monoformylTTX, 11-deoxyTTX and 4,4a-anhydroTTX, purchased from CIFGA Laboratorio S.A. (Lugo, Spain). A working solution containing 2 μM of TTX, 0.55 μM of 4,9-anhydroTTX, and 0.16 μM of 4-*epi*TTX was prepared. The quantitation of TTX and of its analogues was obtained from calibration curves spiked with 1, 2, 5, 10, 20, and 40 μL of the working solution per 200 μL of the matrix. LC-HRMS data analysis was performed using Xcalibur 4.1 (Thermo Scientific, Bremen, Germany). The samples toxicity was estimated as the sum of TTX and its analogues concentrations, each multiplied by their respective Toxicity Equivalency Factor (TEF). The TEFs recommended by EFSA were used when available; in cases where they were not, TEFs previously suggested in the literature were applied (Boundy et al., 2020; Knutsen et al., 2017; Kao & Fuhrman, 1963; Kudo et al., 2014, Nakamura & Yasumoto, 1985; Satake et al., 2014; Tsuda et al., 1964; Yotsu-Yamashita et al., 1995) (Supplement Material Table S4, Pais et al., 2025).

The limits of detection (LOD) and quantification (LOQ) were calculated based on the standard deviation (SD) obtained after five injections of each blank matrix spiked with the second-lowest concentration divided by the square root of the number of results (3 × or 10 x SD/$\sqrt{n}$, respectively) (Magnusson and Örnemark, 2014). The matrix effect (ME) was determined after three injections of the standard with the third-lowest concentration of the four matrix-matched calibration curves and calculated using the equation ME (%) = B/A × 100, where A is the average peak area of the standard solution, and B is the average

peak area in the extract spiked with the same concentration.

2.4. Statistical analysis

Data analysis was conducted using R Studio version 4.3.1 (R Project, 2023), with the statistical significance level set at $\alpha = 0.05$. Analysis was conducted solely for the non-edible tissues of the trumpet shell. First, Principal Components Analysis (PCA) was performed to (*i*) investigate the relationships among TTX and its analogues concentrations and (*ii*) their associations with environmental variables (BST and catch depth) and organismal characteristics (soft-tissue weight and shell length). Next, Generalized Linear Models (GLMs) were applied to validate the PCA results regarding the association of TTX concentration with the tested variables. The concentrations were log-transformed, while no transformation was performed for the other variables. The GLMs' performance, homoscedasticity, and normal error distribution were verified. Pearson correlations were carried out between the log-transformed concentrations and environmental variables. Additionally, one-way analysis of variance (ANOVA) was used to test if there were significant monthly concentration changes in TTX.

3. Results

3.1. Limits of detection and quantitation, matrix effects and calibration curves

LOD and LOQ, ME, and calibration curves for TTX were determined via AM-XIC for the four tissue matrices (trumpet shells' edible and non-edible tissues and sea stars' digestive glands and stomachs). The LOD and LOQ were below EFSA's recommended maximum safe limit across all matrices (Table 1). Concerning matrix effects, ion suppression (ME below 100 %) was noted in all matrices. As a result, four calibration curves were prepared, one for each matrix, to account for these effects. The standard curves exhibited a correlation coefficient ≥0.99 (Table 1).

3.2. The gastropod trumpet shell, Charonia lampas

TTX and its analogues were detected in all 25 trumpet shells sampled at the Algarve (Fig. 1) between November 2021 and October 2022, analysed via LC-HRMS. Non-edible tissues exhibited year-round presence of TTX with highly variable concentrations between individuals and sampling months (Fig. 2a). TTX, 4-*epi*TTX, and 4,9-anhydroTTX were identified by comparison with CRMs. Additionally, 12 other analogues were annotated, namely 9-*epi*TTX; 5-deoxyTTX; 6-deoxyTTX; 11-deoxyTTX; 4-*epi*-5,11-dideoxyTTX; 5,11-dideoxyTTX; 6,11-dideoxyTTX; 4-*epi*-5,6,11-trideoxyTTX; 5,6,11-trideoxyTTX; unknown trideoxyTTX; 4,9-anhydrotrideoxyTTX and 4,4a-anhydrotrideoxyTTX (Supplementary Material, Figs. S2–S7). The HCD fragmentation spectra of 9-*epi*TTX are identical to those of 4-*epi*TTX and TTX. However, while TTX and 4-*epi*TTX HCD fragmentation shows an intensity ratio of signals observed at 162.1 and 178.1 of ~4, the same signals observed after fragmentation of 9-*epi*TTX show a ratio ~1, confirming it is a distinct chemical species (Supplementary Material, Fig. S2) (Park et al., 2024; Yaegashi et al., 2022). The three deoxyTTX epimers have identical fragmentation patterns (Supplementary Material, Fig. S3). The annotation of 11-deoxyTTX was based on its trace level observed in TTX CRM and in its retention time compared to the reported in the literature for 5-deoxyTTX, 6-deoxyTTX, and 11-deoxyTTX (Bane et al., 2016; Puilingi et al., 2015). The annotation of 5,11-dideoxyTTX (and its 4-epimer), and 6,11-dideoxyTTX was based on the comparison of their retention times and fragmentation patterns (Supplementary Material, Fig. S5) with those reported by Yotsu-Yamashita et al. (2013). For example, while the intensity of 5,11-dideoxyTTX daughter ion observed at *m/z* 133.1 is higher than those at *m/z* 160.1 and 148.1, in 6,11-dideoxyTTX the signal at *m/z* 133.1 shows intensity lower than the other signals. A smaller chromatographic peak observed before the 6,11-dideoxyTTX, can be assigned to the 4-epimer of this analogue (Yotsu-Yamashita et al., 2013). After accurate mass extraction of trideoxyTTX analogues (*m/z* 272.1241 ± 5 ppm) three peaks with identical fragmentation patterns were detected (Supplementary Material, Fig. S6). Based on the comparison of their retention times to those reported in the literature (Puilingi et al., 2015), the peaks at 6.40 and 6.21 min are assigned to 5,6,11-trideoxyTTX and its 4-epimer, respectively. Although no other trideoxyTTX analogues have been previously annotated in marine organisms, an 8-*epi* form of 5,6,11-trideoxyTTX was identified by Kudo et al. (2014) in the newt *Cynops ensicauda popei*. However, since no fragmentation spectra or chromatograms were provided (Kudo et al., 2014), we were unable to verify if the peak we detected at 7.18 min (*m/z* 272.1241 ± 5 ppm) can be assigned to this analogue (unknown trideoxyTTX). At the *m/z* 254.1135 ± 5 ppm, two peaks (with retention time of 5.55 and 6.15 min) and with fragmentation pattern characteristic of anhydrotrideoxyTTX (Supplementary Material, Fig. S7) were annotated as 4,9-anhydro-5,6,11-trideoxyTTX and 4,4a-anhydro-5,6,11-trideoxyTTX, respectively, based on the retention times and fragmentation spectra reported by Puilingi et al., 2015, Yotsu-Yamashita et al., 2013. Moreover, in a few samples, peaks were observed at *m/z* 290.0983 ± 5 ppm (11-nor tetrodotoxin-6(S)-ol) and *m/z* 336.1038 ± 5 ppm (11-oxo tetrodotoxin). However, due to the weak signal, we were unable to obtain a fragmentation spectra to annotate these compounds. Monthly averages show high concentrations of TTX in the trumpet shells, non-edible tissues, ranging from 536 μg kg^{-1} to 17 mg kg^{-1} (Fig. 2a). TTX concentrations in the non-edible tissues of the trumpet shell individuals vary from non-detected to 45 mg kg^{-1} (Supplementary Material Table S5, Pais et al., 2025). However, TTX was neither the most frequently detected analogue nor the one with the highest concentrations in the trumpet shell non-edible tissues. The most commonly detected analogues and those found in the highest concentrations were 4,9-anhydro-5,6,11-trideoxyTTX; 4,4a-anhydro-5,6,11-trideoxyTTX; 5,6,11-trideoxyTTX; 4-*epi*-5,6,11-trideoxyTTX; and an unknown trideoxyTTX (Fig. 2a). The TTXs profile of the trumpet shell non-edible tissues throughout the year was similar. Exception was the sample of March 2022, where only 6,11-dideoxyTTXand 5,6,11-trideoxyTTX were detected (Fig. 2a). In the PCA biplot (Supplementary Material Fig. S2) showing the TTX analogues concentrations in the non-edible tissues of the trumpet shell, the individuals soft-tissue weight, the shell length, the catch depth and the BST, most of the individual TTX analogue vectors align and overlap, indicating a high autocorrelation. This suggests that their concentrations provide similar information and, thus, the analogues concentrations data could be combined or removed without losing predictive power. Thus, we choose to use only TTX concentration

Table 1
TTX calibration curve parameters (slope, intercept, *r*), limits of detection and quantification (LOD and LOQ, μg.kg^{-1}), matrix effects with correspondent relative standard deviation (ME ± RSD, %) on each trumpet shell and sea star tissue matrix.

Organism	Tissue Type	Slope	Intercept	*r*	LOD	LOQ	ME ± RSD
Trumpet Shell	Non-edible	2.0×10^6	−0.150	0.99	2.17	7.22	31.93 ± 6.13
	Edible	5.0×10^6	−0.009	0.99	4.82	16.10	34.99 ± 5.86
Sea star	Digestive gland	6.0×10^6	−0.019	0.99	1.64	5.47	78.89 ± 26.93
	Stomachs	8.0×10^6	−0.011	0.99	1.84	6.14	88.23 ± 22.39

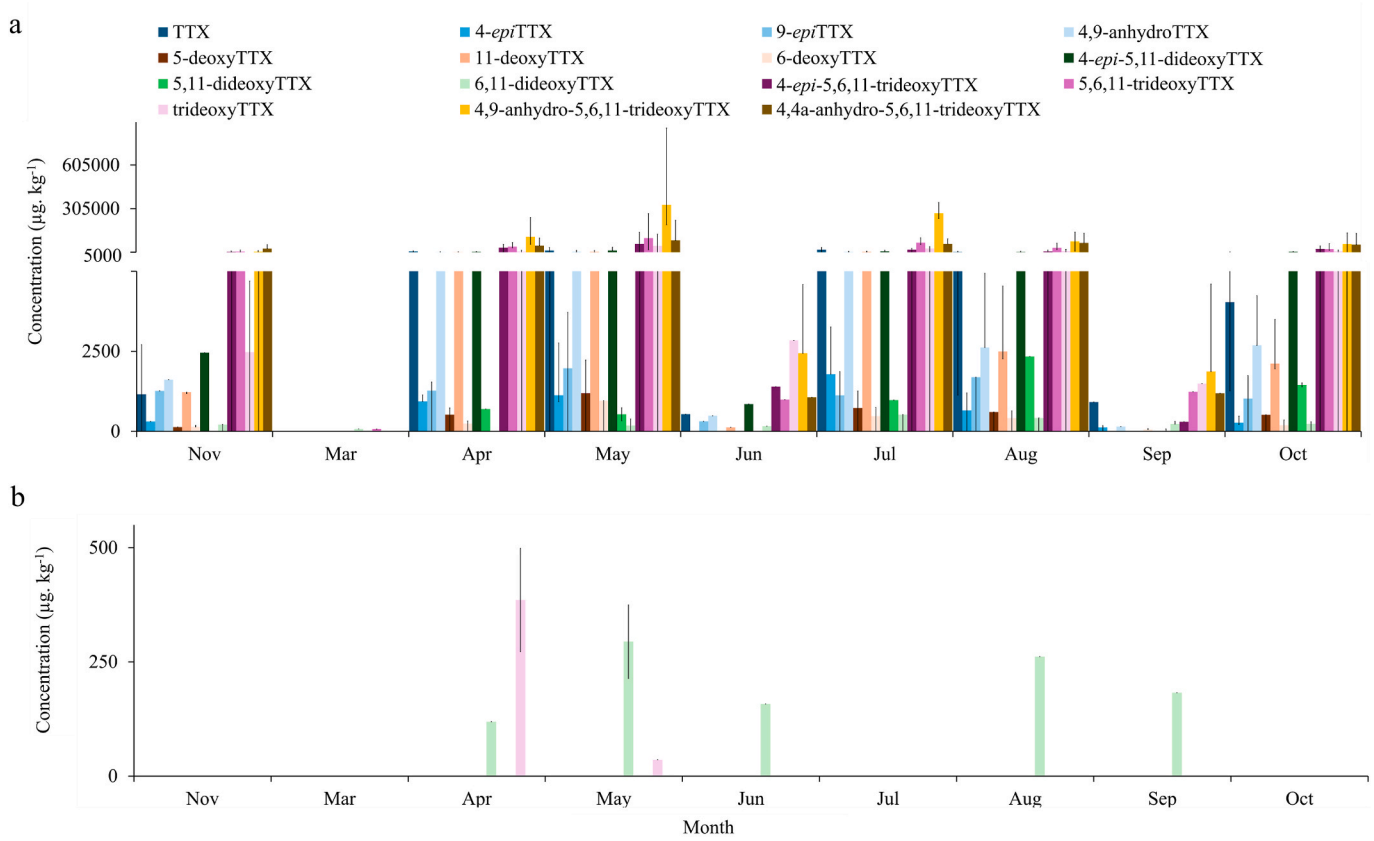

Fig. 2. TTX and its analogues concentrations (mean ± standard deviation) – µg.kg^{-1} in (a) non-edible and (b) edible tissues of trumpet shell harvested off the Algarve coast in November of 2021, n = 3; and March, n = 1; April, n = 3; May, n = 4; June, n = 2; July, n = 2; August, n = 2; September, n = 4; and October, n = 4 of 2022. The lack of a standard deviation bar indicates that TTX or its analogues were only quantifiable in one individual that month. Interruptions in the Y-axis indicate a break in the scale.

(the most toxic analogue) in the following statistical analysis.

There was no statistically significant temporal effect on the concentrations of TTX in the non-edible tissues of trumpet shells. However, a trend was observed: TTX levels were below the LOQ in the non-edible tissues of most trumpet shell specimens caught in March, June, and September (Supplementary Material, Fig. S5). The absence of TTX in these specimens coincided with the periods when the lowest BSTs were recorded in our study. Therefore, there was a significant effect of BST on TTX concentration ($P < 0.01$) and the two variables were positively correlated ($r^2 = 0.29$, n = 25, $P < 0.01$). No relationship was found between the concentrations of TTX with either catch depth, soft tissue weight or the length of the shell of the trumpet shells. Considering the size of our sample set, the accidental nature of the trumpet shells collection (by-catch), and the high variability of TTX concentrations between individuals, caution must be taken when interpreting the statistical results, whether they are significant or not.

The monthly averages of estimated toxicity, based on TEFs, in the non-edible tissues of the trumpet shell exceeded the EFSA-recommended maximum safe limit of 44 µg TTX eq. kg^{-1} throughout the year, except for March (Fig. 3a). However, from March to June, some individuals had estimated toxicities below the EFSA-recommended limit.

TTX was not quantifiable (<LOQ) in any sample of the edible tissues of trumpet shells. However, TTX was detected (>LOD) in one sample collected in April and another collected in May 2022. Two TTX analogues were above the LOQ in the edible tissues, namely 6,11-dideoxyTTX and the unknown trideoxyTTX (Fig. 2b). The 6,11-dideoxyTTXwas detected from March to October 2022 but was only quantifiable in 6 samples from April and June, one in August, and one in September showing concentrations ranging from 119 to 396 µg kg^{-1} (Supplementary Material Table S6, Pais et al., 2025). The unknown trideoxyTTX was detected from November 2021 to July 2022 but was only quantifiable in 2 samples from April, with the respective concentrations of 331 and 106 µg kg^{-1} (Supplementary Material Table S6, Pais et al., 2025). The estimated toxicities of the trumpet shell edible tissues (calculated as described above) were always below the EFSA-recommended maximum safe limit (Fig. 3b).

3.3. *The sea star, Astropecten aranciacus*

TTX was only above the LOQ in the digestive gland of one individual collected in November 2021 out of 25 sea stars by-caught (Fig. 4). The 4,9-anhydro-5,6,11-trideoxyTTX and unknown trideoxyTTX were the TTX analogues most frequently detected and with the highest concentrations in the sea star digestive glands over the year (Fig. 4a). In the stomach of sea stars the 4,9-anhydro-5,6,11-trideoxyTTX was the dominant analogue throughout the year (Fig. 4b). Exception were two samples of July 2022, where only the unknown trideoxyTTX was quantified (Fig. 4b).

In the sea stars, three digestive glands had estimated toxicities above the EFSA recommended maximum safe limit – including the only sample where TTX was quantified (Fig. 5). In the stomach samples, only a September sample had estimated toxicities above this limit.

4. Discussion

4.1. *The gastropod trumpet shell, Charonia lampas is a potential health hazard*

This is the first study evaluating the temporal variability of TTX accumulation in the gastropod trumpet shell, *Charonia lampas*. In this

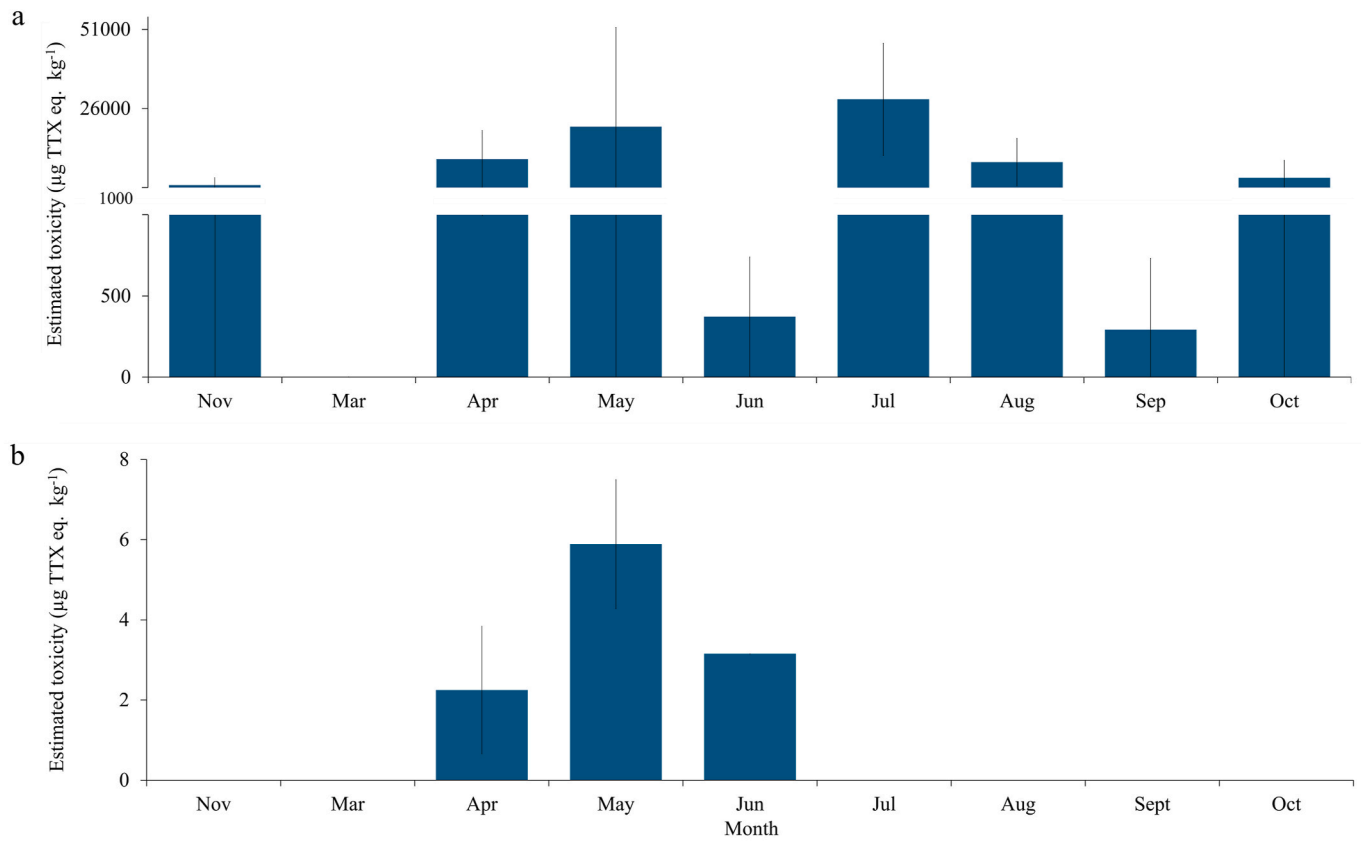

Fig. 3. Estimated toxicity based on Toxicity Equivalency Factors (mean ± standard deviation) – in µg TTX equivalent eq. kg^{-1} in the non-edible tissues of trumpet shell harvest at Algarve Offshore coast in November of 2021, n = 3; and March, n = 1; April, n = 3; May, n = 4; June, n = 2; July, n = 2; August, n = 2; September, n = 4; and October, n = 4 of 2022. The lack of a standard deviation bar indicates that TTX or its analogues were only quantifiable in one individual that month. Interruptions in the Y-axis indicate a break in the scale.

study, non-edible tissue samples of this gastropod consistently exhibited high estimated toxicities (based on TEFs) over nearly one year, reaching values as high as 1693 times above the EFSA recommended maximum safe limit (Knutsen et al., 2017; Rodriguez et al., 2008). In the edible tissues, only TTX analogues with presumed low toxicity were quantified, while TTX itself was either not detected or found to be below LOQ. As a result, the estimated toxicity was consistently below safety limits. TEFs are required to estimate the total toxicity of a sample based on all quantified analogues via LC-HRMS (Knutsen et al., 2017). However, information on TEFs for TTX analogues is limited, and the estimated toxicity of the sample may differ from the actual toxicity. Further toxicity studies are needed to derive TEFs for the various TTX analogues.

Prior studies of specimens collected from the same coast also reported TTX concentrations exceeding the EFSA limit and found that most of the TTX was concentrated in the non-edible tissues (Costa et al., 2021; Lage et al., 2022; Rodriguez et al., 2008). Nevertheless, the lack of consumer awareness of trumpet shell TTX contamination and evisceration techniques that prevent the transfer of TTX from non-edible to edible muscle tissues could pose a significant public health risk. Trumpet shells are sold whole in local markets and are commonly featured as a delicacy in dishes prepared with the whole soft body and served at local restaurants, without awareness of the TTX hazard. Furthermore, processing methods such as cooking or freezing trumpet shells for several hours are unlikely to remove or degrade TTX, as this toxin is heat-stable and water-soluble (Saoudi et al., 2007). TTX is neither regulated nor monitored at the European level (Knutsen et al., 2017; Regulation 2019/627), so these findings highlight a persistent consumer risk. In 76 % of the analysed trumpet shells, TTX concentrations in the non-edible tissues exceeded the EFSA safety threshold, indicating that improper handling could contaminate edible tissues and lead to TTX ingestion.

It is also pertinent to note that due to overfishing and shell collecting, trumpet shells are now considered “endangered or threatened” in the Mediterranean by the European Union (United Nations Environment Programme, 1976)) but continue to be by-caught and marketed in the Atlantic. From a food safety perspective, the accumulation of TTX mainly in the non-edible tissues of the trumpet shells has a parallel in the domoic acid presence in scallops, where most of the toxin is found in the non-edible parts that are removed during shucking (Schrenk et al., 2021). Therefore, a directive akin to the EU Commission Decision which addresses domoic acid levels in scallops, should be applied to TTX accumulation in trumpet shells, as evisceration notably lowers toxin levels, allowing the muscle tissue to be safely marketed (Decision 2002/226/EC, 2002).

4.2. *Higher temperatures might facilitate the incorporation of TTX*

No significant differences in TTX concentrations in the non-edible tissues were observed between months. However, a significant positive correlation was identified between TTX concentration and BST, explaining 29 % of the variance in TTX concentration. Notably, the highest TTX concentrations were recorded in May and July, when BST ranged from 18 to 21 °C. Evidence for a correlation between TTX accumulation and temperature is scant. In the pufferfish *Takifugu rubripes,* TTX *in vitro* uptake rates were higher at 20 °C than at 5 °C (Matsumoto et al., 2007). In tropical regions, where seawater temperatures remain consistently warm throughout the year with minimal seasonal variation, no clear temporal trends in TTX accumulation were observed. In contrast, organisms from temperate regions, which experience seasonal fluctuations in seawater temperature, showed temporal changes in TTX accumulation. TTX concentrations in the gastropods

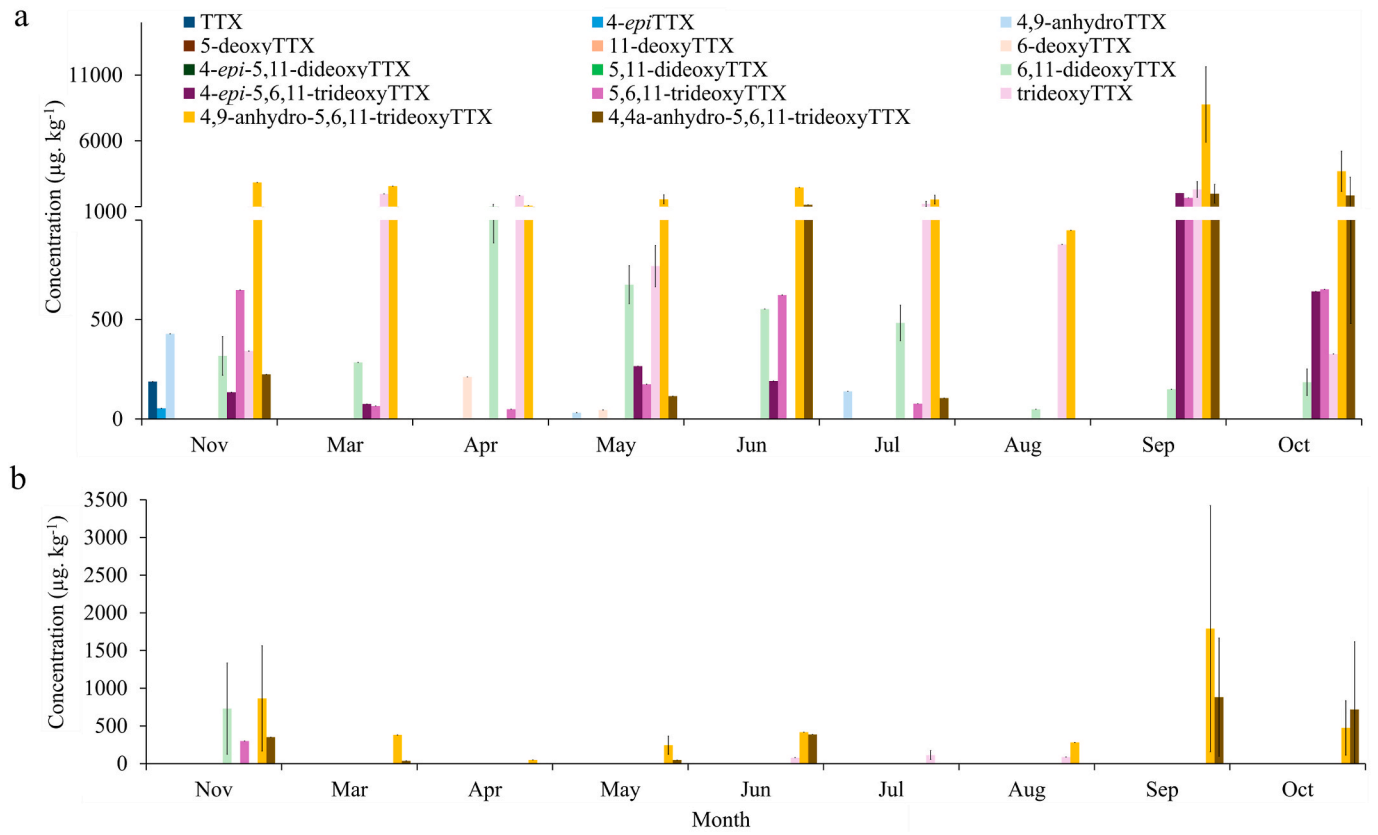

Fig. 4. TTX and its analogues concentrations (mean ± standard deviation) – $\mu g.kg^{-1}$ in (a) digestive glands and (b) stomachs of sea stars harvested off the Algarve coast in November of 2021, n = 3; and March, n = 1; April, n = 3; May, n = 4; June, n = 2; July, n = 2; August, n = 2; September, n = 4; and October, n = 4 of 2022. The lack of a standard deviation bar indicates that TTX or its analogues were only quantifiable in one individual that month. Interruptions in the Y-axis indicate a break in the scale.

Tanea=Natica lineata (Röding, 1798) from Taiwan and *Nassarius glans* from Japan (Linnaeus, 1758) showed no clear temporal trends (Chen & Chou, 1998; Taniyama et al., 2013). In contrast, TTX concentrations in the grey side-gilled sea slug *Pleurobranchea maculata* (nudibranch) from New Zealand declined from a June–July peak until December (Wood et al., 2012). Neither of the gastropod or nudibranch studies evaluated the effect of seawater temperature. In bivalves, from temperate region – England (including mussels – *Mytilus* spp., Pacific oysters – *Crassostrea gigas*, native oysters – *Ostrea edulis*, and hard clams – *Mercenaria mercenaria*), TTX accumulation generally occurred after sea temperatures warmed to approximately 15 °C, with most TTX-positive bivalves found at sea surface temperatures in the 15–20 °C range (Dhanji-Rapkova et al., 2023). The evidence points to a relationship between TTX concentration and temperature, possibly through increased TTX uptake at higher temperatures. However, further studies are needed to determine the role of temperature in TTX accumulation.

4.3. *The sea star Astropecten aranciacus is a possible TTX source for trumpet shells*

Our results indicate high variability in TTX concentrations among the non-edible tissues of trumpet shell specimens, apparently unrelated to size (soft-tissue weight, shell length) or catch depth. These findings are consistent with previous studies that also reported substantial variability in TTX concentrations among trumpet shell specimens, including those collected from the Algarve coast (Costa et al., 2021; Lage et al., 2022; Narita et al., 1981; Rodriguez et al., 2008; Silva et al., 2012, 2019). This variability could result from differences in toxin exposure and retention among specimens, sporadic feeding, their ability to move away from toxin sources, and the slow rate at which gastropods depurate toxins (Shumway, 1995).

Feeding introduces various prey types, which may or may not contain TTX. We found that sea stars *Astropecten aranciacus* contained detectable levels of TTX and its analogues and suggest it could be a possible TTX source for the trumpet shells. This finding also suggests that the origin of TTX in trumpet shells and carnivorous gastropods in general can take place through trophic consumption (Noguchi et al., 2011); *i.e.* feeding of TTX-bearing preys; as (*i*) TTX in trumpet shells was mainly found in the gastrointestinal tissues; and (*ii*) the sea star prey had TTX concentrations lower than those of the predator trumpet shell and were detected less frequently over the year, consistent with possible bioaccumulation. Accordingly, in Japan, fragments of the sea star *A. polyacanthus*, were identified in the stomach content of trumpet shells, and TTX was detected in specimens of this sea star that were collected from the same location as the trumpet shells (Noguchi et al., 1982). Moreover, non-toxic trumpet shells became TTX-positive after being fed toxic sea stars (Narita et al., 1984). Sea stars containing TTX and its analogues have been found in several East Asian *Astropecten* spp. specimens (Lin & Hwang, 2001; Maruyama et al., 1985, 1985, 1985; Noguchi et al., 1982). In European sea stars, TTX and its analogues have been only found in *Ophidiaster ophidianus* caught on São Miguel Island in the Azores archipelago (Silva et al., 2019).

Sea stars probably are not the main source of TTX in trumpet shells in the Algarve, as TTX was only quantifiable in one sea star with a concentration on the digestive gland 88 times lower than the average TTX concentration in the trumpet shell non-edible tissues. Although trumpet shells primarily prey on sea stars, they have also been reported to feed on holothurians and echinoids (Morton, 2012). In the case of sea stars of the genus Astropecten, as they are carnivorous, it has been hypothesized that the TTX may originate from their food sources (Noguchi et al.,

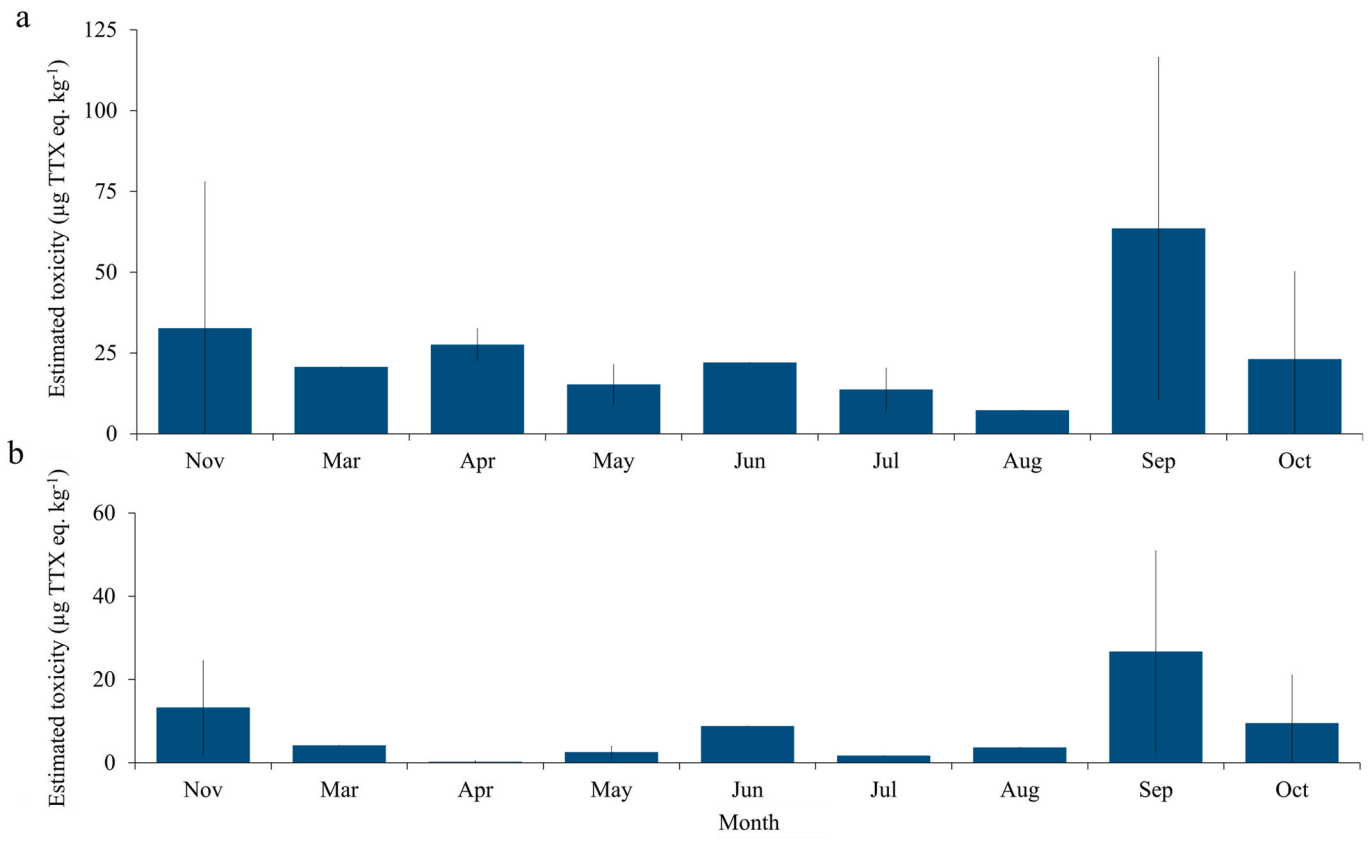

Fig. 5. Estimated toxicity based on Toxicity Equivalency Factors (mean ± standard deviation) – in µg TTX equivalent eq. kg^{-1} in (a) digestive glands and (b) stomachs of sea stars harvested off the Algarve coast in November of 2021, n = 3; and March, n = 1; April, n = 3; May, n = 4; June, n = 2; July, n = 2; August, n = 2; September, n = 4; and October, n = 4 of 2022. The lack of a standard deviation bar indicates that TTX or its analogues were only quantifiable in one individual that month. Interruptions in the Y-axis indicate a break in the scale.

2011), consisting mainly of bivalves, small gastropods, and other small invertebrates (Baeta & Ramón, 2013; Christensen, 1970). Bacteria that produce TTX have also been isolated from sea stars (Lin & Hwang, 2001; Narita et al., 1981, 1987; Noguchi et al., 1982). The origins of TTX in trumpet shells and sea stars must be investigated further. The limited understanding of the biological origin of TTX in Europe is hindering comprehensive assessment of the present and future risks posed by TTX.

4.4. TTX analogues quantified in the absence of TTX

In trumpet shell and sea star samples, 4,9-anhydro-5,6,11-trideoxyTTX; 4,4a-anhydro-5,6,11-trideoxyTTX; 5,6,11-trideoxyTTX; 4-*epi*-5,6,11-trideoxyTTX; and the unknown trideoxyTTX were the TTX analogues most frequently detected and with the highest concentrations, as previously reported for trumpet shells caught off the Algarve coast (Lage et al., 2022; Rodriguez et al., 2008). Thus, unlike previous assumptions (Katikou, 2019), TTX analogues can be detected even without detectable levels of the parent toxin. This was also observed in earlier studies of trumpet shells, marine snails, and pufferfish; where higher content of trideoxyTTX analogues was detected, when TTX was at lower concentrations or undetected (Jang & Yotsu-Yamashita, 2006; 2010; Li et al., 2008; Silva et al., 2012, 2019). The presence of TTX analogues can result from metabolic transformation in trumpet shells and sea stars. Laboratory experiments using pufferfish have demonstrated that TTX is converted into its analogues within a few hours after intramuscular injection (Kono et al., 2008). In the current study, the exact source TTX or TTX-analogue-producing organism was not determined. Even assuming a dietary route for TTX exposure, it is unclear what vectors, possessing distinct capabilities to transform TTX, contribute to the transfer of these compounds over the food web. Further investigation on the conversion of these toxins in trumpet shells, sea stars and other marine organisms is necessary.

5. Conclusion

The gastropod trumpet shell, *Charonia lampas* is a potential health hazard because it can accumulate TTX and its analogues and is simultaneously a gourmet food. Although TTX and its analogues accumulate mainly in non-edible tissues, unsafe levels detected could be accidently transferred to edible parts. This underscores the importance of implementing European regulations to monitor TTX levels and ensure seafood safety and public health protection. Furthermore, consumers should be consistently informed about which parts of the trumpet shells are safe to eat to minimize the risk of contamination.

How trumpet shells acquire TTX is still open to debate. The fact that sea star *Astropecten aranciacus* accumulates TTX and its analogues and is a trumpet shell food item supports the theory of exogenous toxin acquisition via trophic consumption. Furthermore, incorporating TTX appears to be facilitated by higher temperatures, as shown by the positive correlation with bottom seawater temperature. Future studies should address the biological origin and metabolisation of TTX and its analogues.

CRediT authorship contribution statement

Maria F. Pais: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Flávia Carvalho:** Writing – review & editing, Investigation. **Magda Frade:** Writing – review & editing, Investigation. **Pedro Reis Costa:** Writing – review & editing, Conceptualization. **José Paulo da Silva:** Writing – review & editing,

Supervision, Methodology. **Ana Marçalo:** Writing – review & editing, Supervision, Funding acquisition. **Adelino V.M. Canário:** Writing – review & editing, Resources, Funding acquisition, Data curation. **Sandra Lage:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2025.111353.

Data availability

Metadata and data are provided in the supplementary materials file and openly available on Zenodo (https://doi.org/10.5281/zenodo.15013581)

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