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Seasonal distribution of Tetrodotoxin and analogues in trumpet shell *Charonia lampas* (Linnaeus, 1758)

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Abstract

Tetrodotoxin (TTX) is a potent neurotoxin, first identified in a fish from the Tetraodontidae family. The first and only known case of human intoxication in the European Union (EU) occurred in Spain in 2007, following the consumption of a trumpet shell, *Charonia lampas*. In response to this incident and subsequent studies identifying TTX in bivalves and marine gastropods within the EU, the European Food Safety Authority (EFSA) recommended a safety limit of 0.04 mg TTX eq/kg. However, no regulation has yet been established in the EU. This study aims to evaluate the temporal variability of TTX and its analogues in trumpet shells and their primary prey, the starfish species *Astropecten aranciacus*. A total of 25 individuals from each species were captured between November 2021 and October 2022. The results indicated high concentrations of TTX and its analogues in the non-edible tissues of trumpet shells, exceeding the EFSA's recommended limit. In edible tissues, only the analogues dideoxyTTX3 and trideoxyTTX3 were present at quantifiable levels. For starfish tissues, nine samples surpassed the EFSA's recommended limit. These findings align with existing literature, which reports higher toxicity in non-edible tissues and organisms from higher trophic levels, supporting the theory of TTX bioaccumulation. No statistically significant temporal variability of TTX was observed, implying that the time of year does not influence TTX accumulation in the studied species. These results support the need for implementing TTX monitoring programs in Europe and increasing public awareness to prevent intoxication episodes.

Keywords: emerging toxins; TTX; gastropods; seafood safety; LC-HRMS

Resumo

A tetrodotoxina (TTX) é uma neurotoxina potente, que é responsável por inúmeros casos de intoxicação alimentar, principalmente em países asiáticos. O seu nome provém do primeiro organismo a partir do qual foi isolada e identificada, um peixe da família Tetraodontidae. A TTX é conhecida por bloquear os canais de sódio que, por sua vez, são responsáveis pela excitabilidade dos nervos e músculos de mamíferos. Alguns dos sintomas consistem na paralisia corporal, vómitos, diarreia, e em casos mais graves, pode levar à morte. A TTX existe como uma mistura de análogos, sendo conhecidos pelo menos 30 análogos. A sua origem ainda gera controvérsia, mas sabe-se que é produzida por algumas bactérias marinhas dos géneros *Vibrio*, *Alteromonas*, *Shewanella*, *Pseudomonas*, *Bacillus* e *Aeromonas*. No entanto, a sua presença em organismos marinhos está ainda por decifrar, havendo duas teorias principais: *i*) produção endógena (produção por bactérias simbióticas que habitam no sistema digestivo dos organismos) e *ii*) bioacumulação/produção exógena (produção pelas bactérias marinhas no meio ambiente, seguida de acumulação da TTX pela cadeia alimentar). Até ao início de século XXI, pensava-se que a presença de TTX nos organismos marinhos estava confinada às águas quentes dos países asiáticos, nunca tendo sido considerada um perigo para a saúde pública na Europa. No entanto, em 2007, foi reportado em Espanha o primeiro, e de momento o único, caso de intoxicação alimentar num homem de 49 anos que cozinhou uma espécie de gastrópode, a buzina (*Charonia lampas*), e consumiu todo o organismo (músculo, fração edível, e vísceras, fração não-edível). O que restou do organismo foi analisado, e foi encontrada uma concentração de TTX de 315.000 mg de TTX equivalente/kg e uma concentração do análogo 5,6,11-trideoxyTTX de 1.004 mg de TTX equivalente/kg nas vísceras. Suspeita-se que esta buzina tenha sido capturada na costa algarvia (Portugal). A buzina é endémica das águas mediterrâneas e atlânticas, não sendo uma espécie alvo dos pescadores, é capturada na sua grande maioria como pesca acessória. No entanto, é uma iguaria local (Algarve). A alimentação deste gastrópode consiste principalmente em estrelas-do-mar. TTX e bactérias conhecidas como produtoras desta toxina foram já reportadas em estrelas-do-mar na literatura. Após o incidente em Espanha, e estudos que se seguiram que registaram a presença de TTX e alguns análogos em gastrópodes e bivalves colhidos em vários locais da Europa, em 2017 a Autoridade Europeia para a Segurança Alimentar (EFSA) emitiu um parecer científico sobre o potencial da TTX como uma toxina emergente e o risco relacionado com a sua presença e dos seus análogos em bivalves e gastrópodes marinhos. A EFSA estabeleceu que concentrações inferiores a 0.04 mg/kg seriam consideradas não causadoras de efeitos adversos, porém, os dados de consumo e ocorrência existentes na Europa não eram suficientes para identificar o risco desta toxina para a saúde pública. O presente estudo teve por objetivo avaliar a variabilidade temporal da acumulação de TTX e dos seus análogos (TTXs) na buzina da costa algarvia e na sua principal presa, a estrela-do-mar *Astropecten aranciacus*. Os resultados obtidos fornecem dados de ocorrência de TTX em Portugal que serão relevantes para a tomada de decisões sobre a regulamentação e monitorização de TTX na União Europeia.

No total foram capturados 25 indivíduos de cada espécie (*C. lampas* e *A. aranciacus*), através de redes de emalhar e tresmalho, entre novembro de 2021 e outubro de 2022. No momento de captura foram registadas as coordenadas e a profundidade. Após a chegada ao laboratório, todos os indivíduos foram medidos, pesados, e dissecados em tecidos não edíveis e edíveis para as buzinas e em glândula digestiva e estômago (incluindo o conteúdo estomacal) para as estrelas-do-mar, com posterior congelamento a -20ºC. A extração e deteção da TTX foi realizada de acordo com o Procedimento Operacional Padrão para a determinação de TTX do Laboratório de Referência da União Europeia para Biotoxinas Marinhas. As análises foram realizadas através de Cromatografia Líquida acoplada com Espectrometria de Massa de Alta Resolução (LC-HRMS). Os dados resultantes da análise LC-HRMS foram tratados no programa Xcalibur 4.1, procedendo-se a análise estatística dos mesmos, utilizando o RStudio (versão 4.3.1).

No que diz respeito às buzinas e à fração não edível, mais de metade das amostras analisadas continham valores de toxicidade muito elevados e acima do limite recomendado pela EFSA. O valor máximo de toxicidade neste estudo foi de 113.707 mg TTX eq/kg. Os análogos mais comuns foram a TTX, anhydrotrideoxyTTXs e trideoxyTTXs. Pelo contrário, na fração edível, o análogo 4,9-anhydroTTX foi apenas detetado em duas amostras com valores de toxicidade inferiores ao limite de quantificação (LOQ). Os análogos dideoxyTTX3 e trideoxyTTX3 foram os únicos análogos quantificáveis nestas amostras; com valores de toxicidade abaixo do limite recomendado. Os resultados apresentados são consistentes com estudos anteriores que revelaram uma maior toxicidade em tecidos não-edíveis em buzinas. Estes níveis elevados podem também sugerir a acumulação de TTX através da cadeia alimentar, *i.e.*, uma possível origem exógena da TTX nas buzinas.

Considerando as frações das estrelas-do-mar, apenas 9 amostras (6 amostras da glândula digestiva e 3 do estômago) apresentaram valores de toxicidade superiores ao recomendado pela EFSA, sendo os mais comuns os anhydrotrideoxyTTXs, trideoxyTTXs, dideoxyTTXs e a TTX. A toxicidade nas estrelas-do-mar é menor que a toxicidade nas buzinas; o que está de acordo com a teoria de origem exógena de TTX; ou seja, a toxicidade é mais elevada quanto mais alto for o nível trófico. No entanto, são necessários mais estudos para confirmar esta via de bioacumulação de TTX nas buzinas e estrelas-do-mar e também determinar a presença de bactérias produtoras de TTX nestes organismos.

No que se refere à análise estatística, no caso das buzinas, todas as variáveis analisadas (peso, dimensões dos organismos e profundidade) não apresentaram correlações estatisticamente significativas com a toxicidade. No caso da glândula digestiva das estrelas-do-mar, a variável peso e raio do disco (r) não apresentaram correlações estatisticamente significativas com nenhuma das toxicidades dos análogos, porém, a variável comprimento do braço (R) apresentou correlações estatisticamente significativas (moderadas) para com as toxicidades dos análogos dideoxyTTX3, trideoxyTTX1 e 2, e anhydrotridoexyTTX1 e 2, não apresentando correlações apenas com a toxicidade dos análogos que possuem material de referência certificado e a toxicidade do análogo trideoxyTTX3. A variável profundidade não apresentou correlações estatisticamente significativas para com a toxicidade dos análogos que possuem material de referência certificado e para com a toxicidade do análogo trideoxyTTX3, porém, apresentou correlações estatisticamente significativas (moderadas) para com a toxicidade dos análogos dideoxyTTX3, trideoxyTTX1 e 2, e anhydrotridoexyTTX1 e 2. No que diz respeito ao estômago (incluindo conteúdo estomacal), o peso, r e R não apresentaram correlações estatisticamente significativas com os valores de toxicidade. A profundidade não apresentou correlações estatisticamente significativas com os valores de toxicidade dos análogos trideoxyTTX3 e anhydrotrideoxyTTX1 e 2. No entanto, apresentou correlações estatisticamente significativas com os valores de toxicidade do análogo dideoxyTTX3. Estes resultados podem sugerir que, apesar das exceções apresentadas, as variáveis testadas não influenciam a acumulação de TTXs pelas espécies estudadas.

Na análise da variação temporal, os resultados obtidos não apresentam diferenças significativas entre as várias amostras, sugerindo que não existe variação ao longo do ano na acumulação de TTX nos organismos estudados. Apesar disso, a monitorização da temperatura parece ser um fator importante a considerar. No presente estudo, foram comparados os valores de toxicidade com a temperatura registada no fundo do mar, visto que as espécies estudadas são de profundidade, e foi observado que, apesar de algumas exceções, as amostras com mais toxicidade foram capturadas a temperaturas acima de 15ºC, o que pode sugerir uma influência da temperatura na toxicidade.

Tal como em estudos anteriores, os resultados do presente trabalho mostram um perfil semelhante de toxinas, mas uma elevada variabilidade na toxicidade entre indivíduos. Isto pode indicar que os

indivíduos estão expostos a fontes semelhantes de TTX, mas apresentam diferentes capacidades de acumulação das toxinas. Outros fatores como as diferentes localizações geográficas onde os indivíduos foram capturados, a dieta antes da amostragem, e a capacidade para se moverem e afastarem da fonte de toxinas podem também contribuir para essa variabilidade.

Os resultados do presente estudo sugerem que devem ser implementados programas de monitorização de TTX na Europa, principalmente em regiões mais afetadas pelo aquecimento global, onde as temperaturas possam ocorrer com frequência acima de 15°C. Adicionalmente, a sensibilização dos agentes de saúde pública e a sensibilização da população são essenciais para a prevenção de casos de intoxicação alimentar, nomeadamente através da aprendizagem de uma correta evisceração dos indivíduos antes do seu consumo.

Palavras-chave: toxinas emergentes; TTX; gastrópodes; segurança alimentar; LC-HRMS

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Protonated molecular ion	$[M+H]^+$
Accurate mass-extracted ion chromatogram	AM-XIC
Azaspiracids	AZAs
β-N-methylamino-L-alanine	BMAA
Bottom seawater temperature	BST
Certified reference material	CRM
Ciguatoxins	CTXs
Domoic acid	DA
European Commission	EC
European Food Safety Authority	EFSA
Enzyme-linked immunosorbent assay	ELISA
Electrospray ionization	ESI
Europe Union	EU
Gas chromatography-mass spectrometry	GC-MS
Higher-energy collisional dissociation	HCD
Heated electrospray ionization source	HESI-II
Hydrophilic interaction chromatography	HILIC
High-performance liquid chromatography	HPLC
Liquid chromatography high resolution mass spectrometry	LC-HRMS
Liquid chromatography with tandem mass spectrometry	LC-MS/MS
Limits of detection	LOD
Limits of quantification	LOQ
Mouse bioassay	MBA
Matrix effect	ME
Mouse neuro-2A neuroblastoma	N2A
Nuclear magnetic resonance	NMR
Okadaic acid	OA
Palytoxin	PLTX
Paralytic shellfish poisoning	PSP
Relative potency	RP
Length of the arm	R
Radius of the disk	r
Sea surface temperature	SST
Standard addition method	SAM
Standard deviations	SD
Standard operating procedure	SOP
Solid-phase extraction	SPE
Surface plasmon resonance	SPR
Saxitoxin	STX
Toxicity equivalency factors	TEFs
Thin layer chromatography	TLC
Tetrodotoxin	TTX
Tetrodotoxins (TTX and analogues)	TTXs
Yessotoxins	YTXs
Significance level	α

1. Introduction

1.1. Marine Biotoxins

Marine biotoxins are natural contaminants, produced by algae and/or bacteria (Gerssen *et al*., 2018), that accumulate in marine organisms, such as bivalve molluscs and gastropods (Biessy *et al*., 2019). Given that these organisms are an important source of animal protein for humans, their contamination poses a potential risk to consumers (Estevez *et al*., 2019; Hamli *et al*., 2013; Visciano *et al*., 2016). Hence, to protect public health, legislation, monitoring protocols, and regulatory limits of marine biotoxins in seafood have been established in the European Union (EU). The biotoxins included in European legislation are the yessotoxins (YTXs); azaspiracids (AZAs); okadaic acid (OA) and respective analogues; saxitoxin (STX), domoic acid (DA) and respective analogues (Estevez *et al*., 2019; Otero and Silva, 2022). Until 2013, the mouse bioassay (MBA) was the most common of the detection methods, used to determine the regulatory limits for human consumption of shellfish (Knutsen *et al*., 2017; Otero and Silva, 2022). However, this caused ethical and technical concerns, and so this method was replaced by chromatographic techniques, and the legislation was updated. Therefore, these analytical methods are encouraged to be used as the reference method, following the indications agreed upon by the National Reference Laboratories Network (Commission Regulation (EU) No 15/2011). Once toxins' concentrations in the contaminated organisms (*i.e.* bivalve molluscs, echinoderms, tunicates and marine gastropods) are higher than the regulatory limits, the governmental authorities close harvesting in the respective production areas, either from natural banks or from aquaculture. This closure results in extreme economic losses for both industries. If these closures are not obeyed, it may result in human intoxication episodes (Berdalet *et al*., 2015). After this closure, the production area is only reopened for harvest after two consecutive results below regulatory limits separated by 48 hours (Commission Implementing Regulation (EU) 2019/627).

In recent years, several marine biotoxin's producers/vectors are expanding and migrating to geographical areas where they had never been reported before, causing concern on their impact on seafood contamination, and consequently, on public health. These toxins are designated as emerging toxins, and they can be divided in three groups: *i*) toxins that appear in waters and seafood where they had not been previously detected (*e.g.* tetrodotoxins - TTXs, and ciguatoxins - CTXs), *ii*) non-regulated known toxins, which are considered to be of concern but for which additional toxicological evidence is needed before establishing further regulations (*e.g.* palytoxin - PLTX), and *iii)* recently discovered toxins (*e.g.* β-N-methylamino-L-alanine - BMAA) (EFSA , 2010; Farabegoli *et al*., 2018; Knutsen *et al*., 2017; Lance *et al*., 2018). This expansion is possibly due to a combination of several factors such as climate change, the introduction of non-indigenous species through ballast water and other vectors, increased anthropogenic nutrient input into coastal systems, and other man mediated environmental changes (Gobler, 2020; Hallegraeff *et al*., 2021; Karlson *et al*., 2021; Otero and Silva, 2022). On the other hand, the advances in the optimization of analytical methods, as well as the increase in scientific interest may also be a crucial factor in the detection of these toxins in other areas (Gerssen and Gago-Martínez, 2019; Gobler, 2020; Hallegraeff *et al*., 2021). Although the number of human intoxication cases related to these emergent toxins appears to be increasing in Europe, they may represent only a small fraction of the real number of cases, because health professionals are not aware of their existence and symptomology (Otero & Silva, 2022). Consequently, in 2006, the European Food Safety Authority (EFSA), by request of the European Commission (EC), produced scientific recommendations concerning marine biotoxins, including toxin groups already regulated in the EU, and emerging toxins (Estevez *et al*., 2019).

1.2. Tetrodotoxin

As one of the emerging toxins that have been alarming the scientific community in the EU, tetrodotoxin (TTX) is described as one of the most toxic forms responsible for marine seafood borne intoxications (Biessy *et al*., 2019). TTX is a very potent hydrophilic toxin with neurotoxicity (Blanco *et al*., 2019; Bordin *et al*., 2021; Costa *et al*., 2021), commonly known as the pufferfish toxin (Ahasan *et al*., 2004; Bordin *et al*., 2021). TTX was first isolated from a fish of the Tetraodontidae family, from where the toxin's name originated (Bordin *et al*., 2021; Costa *et al*., 2021). These fishes are a traditional food in Japan and considered as a delicacy (Ahasan *et al*., 2004.; How *et al*., 2003; Hwang & Noguchi, 2007).

1.2.1. TTX chemistry

TTX is a non-protein low-molecular-weight (319.2 g/mol) molecule (Hort *et al*., 2020; Kodama *et al*., 1983; Miyazawa and Noguchi, 2001), with a highly unusual structure. It is a heterocyclic compound containing a guanidinium moiety linked to an oxygenated backbone possessing a 2,4-dioxo-adamantane structure with five hydroxyl groups, with a chemical formula $C_{11}H_{17}N_3O_8$ (Chau *et al*., 2011; Hort *et al*., 2020), see Table 1.1. Although its structure has been confirmed (Table 1.1), its biosynthetic pathway remains unknown (Biessy *et al*., 2019).

TTX is recognized to block voltage-gated sodium channels, that are responsible for mammalian nerve and muscle (tissues) excitability, consequently inhibiting the extrusion of monovalent cations through the cell membrane (How *et al*., 2003; Narahashi and Moore, 1964; Saoudi *et al*., 2007; Walker *et al*., 2012). After the ingestion of the contaminated individual, doesn't take long for the symptoms to start (Biessy *et al*., 2019). This includes tongue and lips numbness; face and limb paralysis; head and abdominal pain; nausea and vomiting, diarrhea, hypotension, and bradycardia are some of the symptoms caused by TTX poisoning. In extreme cases, can lead to ataxia; cardiac arrhythmias; convulsions, and respiratory and heart failure, causing death (How *et al*., 2003; Knutsen *et al*., 2017; Noguchi and Ebesu, 2001). These are symptoms similar to STX intoxication, a Paralytic Shellfish Poisoning (PSP) (Knutsen *et al*., 2017), also found in organisms contaminated with TTX. Although both toxin groups have effects on the interaction with voltage-gated sodium channels, their structures are different (Biessy *et al*., 2019). Usually, TTX exists as a mixture of its analogues, and it is known that TTX has 30 structural analogues, usually mentioned as TTXs (Bane *et al*., 2014), which toxicity is influenced by its structure (Table 1.1), but it is known that analogues have a lower potency than TTX itself (Yotsu-Yamashita *et al*., 1999). TTX is a small, heat-stable and water-soluble molecule, that can dissolve in water and other polar solvents due to the presence of several polar functional groups (amino and hydroxyl groups) in its chemical structure (Ahasan *et al*., 2004; Antonelli *et al*., 2022; Saoudi *et al*., 2007).

Table 1.1. TTXs structures (adapted from Knutsen *et al*., 2017 and Turner *et al*., 2015).

	R1	R2	R3	R4	
TTX	H	OH	CH_2OH	OH	
11-oxoTTX	H	OH	$CH(OH)_2$	OH	
4-epiTTX	OH	H	CH_2OH	OH	
11-norTTX-6-ol (*R*- or *S*- form)	H	OH	OH/H	H/OH	
6,11-dideoxyTTX	H	OH	H	CH_3	
11-deoxyTTX	H	OH	OH	CH_3	
5,6,11-trideoxyTTX	H	OH	CH_3	H	
5-deoxyTTX	H	OH	CH_2OH	OH	
4,9-anhydroTTX	CH_2OH	OH	-	-	

1.2.2. TTX origin and bioaccumulation

TTX origin is still controversial, but it is known that marine bacteria from the genera *Vibrio*, *Alteromonas*, *Shewanella*, *Pseudomonas*, *Bacillus*, and *Aeromonas*, have been identified as TTX producers (Matsui *et al*., 1989; Noguchi *et al*., 1986; Wang and Fan, 2010; Wu *et al*., 2005; Yang *et al*., 2010; Yasumoto *et al*., 1986; Yotsu *et al*., 1967). A potential link between the phytoplankton species *Prorocentrum minutum* has also been suggested (Vlamis *et al*., 2015). Moreover, there are also uncertainties about the tetrodotoxin origin in marine organisms (Figure 1.1). However, there are two main theories: *i*) endogenous production, *i.e*., TTX is produced by symbiotic bacteria inhabiting the marine organism digestive system, and *ii*) bioaccumulation/ exogenous production, *i.e*., TTX is first produced by marine free-living bacteria, and then the organisms accumulate TTX through the food chain (Noguchi *et al*., 2006a; Noguchi and Arakawa, 2008).

To our knowledge, the endogenous production of TTX is limited to studies on the rough-skinned newt, *Taricha granulosa* (Skilton, 1849). It was demonstrated that captive *T. granulosa* showed increased TTX concentrations, even when fed with a TTX-free diet (Cardall *et al*., 2004; Hanifin *et al*., 2002). An answer to this could be explained by symbiotic TTX-producing bacteria, however, these bacteria were not detected in toxic tissues, so the origin of TTX in these species by bacteria is still to be proven (Lehman *et al*., 2004). Salvitti *et al.* (2015) detected high concentrations of TTX in the tissues of *Pleurobranchaea maculata* (Quoy & Gaimard, 1832) and *Stylochoplana* (Stimpson, 1857) species, and the presence of *Vibrio* sp. (a potential TTX producing bacteria). However, when this bacteria species was isolated and cultured in laboratory producing dense bacteria assembles, no TTX was detected. Thus, the endogenous TTX production theory does not seem to be supported. Moreover, the majority of investigations into TTX-producing bacteria have predominantly focused on detecting the presence of TTX without providing quantitative concentrations. Among those that did a quantitative analysis, TTX was observed at significantly lower levels compared to host individuals. This is consistent with the understanding that toxin producers, including algae and/or bacteria, typically produce smaller amounts than those accumulated by the organisms hosting the toxin (Salvitti *et al*., 2015). However, these results

in cultured bacteria may be due to the lack of required specific inducers derived from the hosts to produce TTX (Chau *et al*., 2011).

On the other hand, there are several studies with evidence to support an exogenous origin of TTX, through the diet. It was demonstrated that pufferfish *Takifugu rubripes* (Temminck & Schlegel, 1850) when raised in captivity and fed with toxic tissues of other pufferfish species, accumulate TTX in their skin, liver and ovaries (Noguchi *et al*., 2006b). Also, the starfish *Astropecten polyacanthus* (Müller & Troschel, 1842) tissues were detected in the stomach of a trumpet shell contaminated with TTX (Noguchi *et al*., 1982). Salvitti *et al.* (2015) found high TTX concentrations in a species of platyhelminthes (*Stylochoplana* sp.), suggested to be a dietary source of TTX for *Pleurobranchaea maculata*, in New Zealand. However, these authors suggest other sources of TTX besides their diet, due to the variability among specimens, coupled with the absence of *Stylochoplana* sp. at sites with TTX contaminated *P. maculata*, and considering the expected life spans and TTX concentration within *P. maculata* adults (Salvitti *et al*., 2015). Moreover, *P. maculata* when maintained in populations contaminated with TTX, was shown that it can sequester low concentrations of TTX but it is unclear if this is through diet or symbiotic bacteria (Salvitti *et al*., 2017).

Regarding marine bivalves and gastropods TTX intoxication, limited progress has been achieved. However it is suspected that their initial source of TTX is marine bacteria (Hwang and Noguchi, 2007; Magarlamov *et al*., 2017), and the main route of TTX accumulation appears to be through the diet (Hwang and Noguchi, 2007; Noguchi and Arakawa, 2008).
Within marine gastropods, the tissue where is generally found higher TTX concentrations is the digestive gland, also suggesting the TTX accumulation via dietary sources, from TTX-bearing food, like starfish, the main prey of trumpet shells, that has been already associated with TTX (Miyazawa et al., 1985).

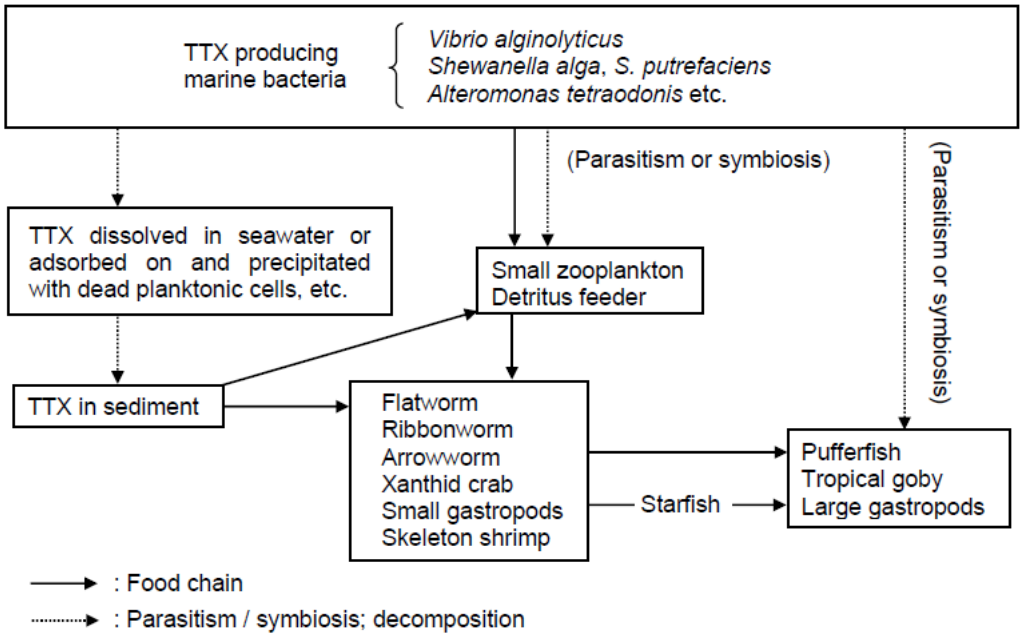

Figure 1.1. Proposed mechanism of TTX accumulation in marine animals (Noguchi & Arakawa, 2008).

1.2.3. TTX history

The food contamination caused by TTX is well known in East Asian countries (Noguchi *et al*., 2011), where it was first identified as a threat to human health, and where most of the TTX food poisoning cases are reported, specifically in Japan, through the consumption of pufferfish. However, since 1964,

TTX has been detected in newts, which represented the first report of TTX detection in other animal than pufferfish (Mosher *et al*., 1964). The first TTX detection and intoxication case regarding marine gastropods occurred in 1979, and since then, incidents with contaminated gastropods have been reported (Miyazawa and Noguchi, 2001; Narita *et al*., 1981). TTX accumulation in marine organisms was thought to be confined to the warm waters of Asian countries, and for Europe, this toxin was never considered a potential threat (Hort *et al*., 2020). However, in the last decade, different marine taxa from European waters have been reported to accumulate TTX in their tissues (besides the Tetraodontidae fish family), including Nemertinea, Anellidae, Crustacea, Gastropoda, and Cephalopoda (Antonelli *et al*., 2022; Biessy *et al*., 2019; Blanco *et al*., 2019). This variability of TTX contaminated species supports the exogenous origin of tetrodotoxin (Bordin *et al*., 2021) since it is highly questionable that these organisms, of distinct phyla, have the same gene that codes for TTX production (Noguchi *et al*., 2006a; Noguchi and Arakawa, 2008). However, we cannot exclude the hypothesis of the same TTX-producing bacteria species in a symbiotic association with organisms of distinct phyla.

1.2.4. Iberian case

In the year 2007, in Spain, the trumpet shell species *Charonia lampas* (Linnaeus, 1978), was the first marine gastropod contaminated with TTX recorded in Europe, and its consumption resulted in the first and only (known) report of TTX intoxication in Europe (Fernández-Ortega *et al*., 2010; Rodriguez *et al*., 2008).

Briefly, a 49-year-old man bought the specimen, known to be caught off the southern coast of Portugal during September 2007, for consumption. This man boiled the specimen 45min, and besides the white muscle, *i.e.*, the edible portion, the viscera, *i.e.*, the non-edible portion, was also consumed. A few minutes after the consumption of the ventral portion, the symptoms started with perioral numbness, evolving to both arms, followed by abdominal pain, nausea, and vomiting. When admitted to the Emergency Department of the hospital, endotracheal intubation and mechanical ventilation were required. This man recovered 72 hours after entering the hospital (Fernández-Ortega *et al*., 2010). The remains of the trumpet shell were stored at -20ºC and the digestive gland and remaining tissues were dissected. The TTX concentration in the non-edible tissue of that specimen was 315.0 mg/kg, and the 5,6,11-trideoxyTTX analogue concentration was 100.4 mg/kg (Rodriguez *et al*., 2008). After this episode, the presence of TTX and some analogues were reported in Portuguese samples of gastropods (Lage *et al*., 2022; Nzoughet *et al*., 2013; Silva *et al*., 2012, 2019).

This trumpet shell species belongs to the Ranellidae family and is typical of the Atlantic and Mediterranean waters (Rodriguez *et al*., 2008). It has generally low commercial importance and it is not a target species for fishermen, being caught mostly as bycatch. However in the Algarve, south of Portugal, this is offered as a delicacy in some local restaurants (Costa *et al*., 2021). This species feeds mainly on starfish, but also sea urchins, and holothurians (Morton, 2012). Some species of starfish were previously shown to contain TTX and bacteria known to produce this toxin were isolated from them (Lin and Hwang, 2001; Narita *et al*., 1981, 1987; Noguchi *et al*., 1982).

1.2.5. Detection of TTX in organisms from Europe

Other than the case reported above, in Europe, TTX has also been detected in gastropods from France and Spain, and bivalve mollusks from the United Kingdom, France, Greece, Italy, Spain and The Netherlands (Antonelli *et al*., 2022; Knutsen *et al*., 2017).

The warmer waters due to the climate change and the opening of the Suez Canal has already been reported as possible causes of the migration of TTX fish vectors (pufferfish) from the Red Sea to the

Mediterranean Sea (Lessepsian migration phenomenon) which now can be found in species of bivalves and marine gastropods from more temperate regions (Lasram et al., 2009; Silva et al., 2012). Additionally, the temperature appears to play a role in the TTX uptake process (Matsumoto *et al*. 2007). The study of Matsumoto *et al*. (2007) showed that *in vitro T. rubripes* TTX uptake rate was higher at 20ºC than at 5ºC.This adding to the Iberian case, triggered some studies investigating other potential TTX vectors in European waters (Blanco *et al*., 2019; Bordin *et al*., 2021). The reports regarding the detection of TTX in both marine bivalves and edible marine gastropods in Europe are summarized in Tables 1.2 and 1.3, with the indication of the maximum TTX concentrations detected in the reported species.

In Europe, the presence of TTX in bivalve mollusks was first reported in English waters (The United Kingdom), in *Mytilus edulis* (Linnaeus, 1758) and *Crassostrea sp.* (Sacco, 1897), between 2013 and 2014 (Turner *et al*., 2015). In France, the studies were carried along the French mainland coasts: English Channel, Atlantic Ocean and Mediterranean Sea (Hort *et al*., 2020; Réveillon *et al*., 2021). In Spain, the studies were carried in the Galician Rias, in the Atlantic west coast of Spain (Leão *et al*., 2018), and along the Spanish coast: Galicia; Catalonia, and Valencia, see Table 1.2 (Blanco *et al*., 2019).

Besides the first report of TTXs presence in marine gastropods in Spain, TTX has already been reported in 9 gastropod species from eleven countries, see Table 1.3 (Antonelli *et al*., 2022).

Table 1.2. Reports of Tetrodotoxin presence in marine bivalve mollusks from Europe. * TTX maximum concentration in mg/kg (adapted from Antonelli *et al*., 2022).

Countries	Sampling Year	Species	TTX concentration (mg/kg)*	Reference
The United Kingdom	2013	*Crassostrea gigas*	0.05	Turner *et al*., 2015
	2014	*Mytilus edulis*	0.04	
		Crassostrea gigas	0.1	
	2014-2016	*Crassostrea gigas*	0.3	Turner *et al*., 2017
		Ostrea edulis	0.08	
		Mytilus edulis	0.07	
		Mercenaria mercenaria	0.2	
	2019	*Crassostrea gigas*	0.2	Dhanji-Rapkova *et al*., 2021
France	2018	*Mytilus edulis / Mytilus galloprovincialis*	0.01	Hort *et al*., 2020
	2018	*Crassostrea gigas*	0.01	Réveillon *et al*., 2021
	2019	*Ruditapes philippinarum*	0.008	
		Crassostrea gigas	0.03	
Greece	2006	*Mytilus galloprovincialis*	0.07	Vlamis *et al*., 2015
	2008	*Mytilus galloprovincialis*	0.07	
	2008	*Venus verrucosa*	0.2	
	2009	*Mytilus galloprovincialis*	0.05	
	2012	*Mytilus galloprovincialis*	0.2	
	2014	*Mytilus galloprovincialis*	0.04	

Table 1.2. Continuation

Countries	Sampling Year	Species	TTX Concentration (mg/kg)*	Reference
Italy	2016	*Mytilus galloprovincialis*	0.006	Dell'Aversano *et al*., 2019
	2017	*Mytilus galloprovincialis*	0.5	
	2018	*Mytilus galloprovincialis*	0.01	Bordin *et al*., 2021
	2019	*Mytilus galloprovincialis*	0.08	Bacchiocchi *et al*., 2021
Spain	2017	Cockles (sp. Unknown)	0.002	Leão *et al*., 2018
		Oysters (sp. Unknown)	0.001	
	2018-2019	*Ruditapes decussatus*	<0.01	Blanco *et al*., 2019
		Mytilus galloprovincialis		
		Crassostrea gigas		
The Netherlands	2015	*Mytilus edulis*	0.03	Gerssen *et al*., 2018
		Crassostrea gigas	0.1	
	2016	*Mytilus edulis*	0.04	
		Crassostrea gigas	0. 3	
	2017	*Mytilus edulis*	<0.02	
		Crassostrea gigas	0.05	

Table 1.3. Reports of Tetrodotoxin presence in edible marine gastropods from Europe. * TTX maximum concentration in mg/kg (adapted from Antonelli *et al*., 2022).

Countries	Sampling Year	Species	TTX concentration (mg/kg)*	Reference
France	2017-2018	*Buccinum undatum*	0.007	Hort *et al*., 2020
Portugal	2007	*Charonia lampas*	315.0	Rodriguez *et al*., 2008
	2010	*Charonia lampas*	0.07	Nzoughet *et al*., 2013
	2009-2010	*Steromphala umbilicalis*	0.06	Silva *et al*., 2012
		Phorcus lineatus	0.09	
		Charonia lampas	0.006	
	2011	*Phorcus lineatus*	<0.005	Silva *et al*., 2019
		Charonia lampas		
		Nucella lapillus		
		Gibbula umbilicalis		
	2017	*Charonia lampas*	56.3	Costa *et al*., 2021
	2022	*Charonia lampas*	7.2	Lage *et al*., 2022
		Charonia lampas	3.3	
	2021-2022	*Charonia lampas*	113.7	This study
Spain	2018-2019	*Calliostoma* sp.	<0.01	Blanco *et al.* 2019
		Patella sp.		
		Bolinus brandaris		

1.2.6. European Food Safety Authority report and TTX regulation in Europe

In 2017, EFSA stated a scientific opinion on the TTX potential as an emerging toxin and the risk related to its presence and its analogues in marine bivalve mollusks and gastropods. It was established that in a large portion of 400g of shellfish consumed by a 70kg person, TTX concentrations lower than 0.04 mg TTX eq/kg were not a cause of critical (adverse) effects (Knutsen *et al*., 2017). This value is currently applied by the Netherlands, which is the only EU country monitoring the presence of TTX in shellfish, for decision measures on when to close production areas (Aquaculture Advisory Council and the Market Advisory Council, 2018).

As recommended by EFSA, the studies that followed the first reports tried to obtain more data on TTX contamination in bivalves and gastropods to perform a proper risk assessment. Iberian studies showed that, in bivalves, TTX concentration does not exceed the maximum limit recommended by EFSA (Blanco *et al*., 2019; Leão *et al*., 2018). On the other side, some gastropod species, including the non-edible tissues of trumpet shells, were identified as potential TTX vectors (Blanco *et al*., 2019; Costa *et al*., 2021).

This toxin is not regulated in Europe, there is only legislation prohibiting its place in the market of fish of the families Tetraodontidae, Molidae, Diodontidae, and Canthigasteridae or derivatives (Corrigendum to Regulation (EC) No 854/2004). Therefore, a complete risk characterization of TTX is, for now, not possible, and therefore this toxin is not included in regular monitoring programs. There is also a lack of occurrence and consumption data for marine gastropods, which may pose a high risk to public health. For this purpose, more data on TTX occurrence in bivalves and gastropods is crucial for establishing regulations (Blanco *et al*., 2019, Knutsen *et al*., 2017).

1.2.7. TTX regulation worldwide

Japan is the country with the most articulate legislation regarding TTX, with a regulatory limit of 2.00 mg TTX eq/kg. Since 1958, it has been established that pufferfish-based dishes can be only cooked by a licensed and certificated pufferfish chef (Cong and Tuan, 2006). Besides this, since 1983, the body tissues and fishing areas of the edible species have a strict regulation (Noguchi and Arakawa, 2008). In Thailand, because of the fatal cases of TTX intoxication, since 2002, the selling of pufferfish was forbidden, besides the continuous illegal landing and selling in markets (Chulanetra *et al*., 2011). In the case of Taiwan, only two species of pufferfish are allowed to be sold in fish markets, *Lagocephalus gloveri* (Abe & Tabeta, 1983) and *Lagocephalus wheeleri* (Abe, Tabeta & Kitahama, 1984) (Huang *et al*., 2014). In Vietnam, pufferfish is considered a delicacy in some rural regions, and are occasionally ingested, mainly by rural fishermen, despite the pufferfish trade being forbidden (Cong and Tuan, 2006). In USA, only a specific Japanese importer certified by the Japanese Ministry for Health and Welfare can import legally pufferfish, in order to ensure that the fish is proper to human consumption (Cohen *et al*., 2009). In New Zealand, the importation of Korean pufferfish is allowed, as long as it comes with a certificate with the identification of the species, a guarantee that the product was well gutted and prepared by a certified person and that it is, therefore, considered proper for human consumption (Dansted, 2019).

1.2.8. TTX detection methods

Through the years, many TTX detection methods and techniques have been approached. The first method, and most common for TTX detection in seafood is the MBA, based on the intraperitoneal injection and consequent toxic effects shown by mice (Estevez *et al*., 2019). This method has been used for many years, despite the existence of ethical concerns. An additional problem with this method is that

it is not specific for TTX, since the presence of STX toxins can give positive samples. Both toxins display the same symptoms in mice, so some cases have been incorrectly assigned (Biessy *et al*., 2019; Costa *et al*., 2021).

Surface plasmon resonance (SPR) and Enzyme-Linked Immunosorbent Assay (ELISA) are methods based on immunological approaches and are very useful for qualitative identification. However, due to the lack of sensibility for all the TTX analogues and the possibility of not identifying all of them, these methods aren't suitable for routine screening (Estevez *et al*., 2019). An alternative method is the mouse neuro-2A neuroblastoma (N2A) which doesn't need multiple reference standards and is very sensitive. It is known for giving monitoring results below the EFSA's limit, being able to detect the toxin at 0.0200 mg/kg (Biessy *et al*., 2019; Gerssen *et al*., 2018).

The analytical chemical methods to detect marine toxins have progressed, and several methods have been developed and adjusted for TTX detection (Biessy *et al*., 2019; Costa *et al*., 2021). The early chemical detection was based on chemical conversion with alkali treatment of TTX and its analogues to fluorescent 2-amino-quinazoline derivatives, with detection in a fluorescent spectrophotometer. This method was developed on a high-performance liquid chromatography (HPLC) system, that splits the TTX analogues with exchange or ion pairing chromatography and performs the derivatisation to the fluorescent C9-base post-chromatographic separation continuously in line with the detector. The analysis of the C9-base isn't specific to TTX, since toxicity may be overestimated because less toxic analogues can be converted to the C9-base and lead to false positives. The gas chromatography-mass spectrometry (GC-MS) is similar to the derivatisation procedure, but the analysis needs an additional derivatisation process to get a GC-suitable product for analysis. The problems with the TTX specificity were surpassed with additional techniques like thin layer chromatography (TLC), electrophoresis, and nuclear magnetic resonance (NMR), used to confirm the presence of TTX (Biessy *et al*., 2019).

Nowadays, the method that is demonstrated to be robust for the analysis of TTX and analogues is Liquid chromatography with tandem mass spectrometry (LC-MS/MS), also recommended by EFSA as the most suitable method for the identification and quantification (Knutsen *et al*., 2017). This method gained better resolution and sensitivity with the development of the method based on hydrophilic interaction chromatography (HILIC) (Knutsen *et al*., 2017). LC-MS monitors TTX and analogues with a high level of specificity or may be derivatised and monitored as the C9-base (Biessy *et al*., 2019). The high sensitivity and specificity ensure explicit identification and quantification. However, this sensitivity does not guarantee the total elimination of endogenous compounds that can cause interferences, and this can affect the use of electrospray ionization (ESI) for quantitative analysis, caused by matrix compounds (Costa *et al*., 2022). This phenomenon is called "matrix effect" (ME) and can lead to erroneous quantitation. These effects are dependent on the sample characteristics, preparation, chromatographic separation, and ionization conditions, so an evaluation for each matrix, sample preparation method, and analytical conditions is required. The most common procedure to evaluate ME consists of, at the same concentration, comparing the signal of the target compound spiked in an analyte-free sample extract with the signal obtained from a standard solution (Costa *et al*., 2022).

The ME is needed for quantitation by LC-MS to be evaluated and corrected. This phenomenon (ME) is related to compounds co-eluting with the analytes, so it is important to know the main components of the matrix, in order to evaluate and predict them. Yet, the sample composition of living organisms depends on several parameters, including environment, age, species, life cycle, and processing. Thus, all samples need analytical information so that this evaluation can be made. Matrix effects are evaluated by comparing the full scan spectra of the target sample and the sample with known ME (Costa *et al*., 2022).

1.2.9. TTX: a potential public health risk

Since TTX can interfere with the normal functioning of nerve cells, it brings a hazard to public health. Besides that, TTX is resistant to heat, acid, and other food preparation methods (Hwang and Noguchi, 2007), meaning that cooking does not eliminate this toxin. As a result, consuming contaminated seafood or other contaminated animals can lead to TTX poisoning. The solubility of TTX in water can be dangerous too (Choi *et al*., 2006). When cooking food that is contaminated by TTX, if there is no correct evisceration, the toxin can quickly spread to edible tissues, that are less likely to be contaminated. It is also important to emphasize that there is no antidote for TTX (Hwang and Noguchi, 2007), and the only alternative is to wait for it to be eliminated from the body while caring to manage the symptoms (Arakawa *et al*., 2010).

1.3. Objectives and contribution of the study

The main goal of the present study is to evaluate the temporal variability of TTX accumulation and its analogues in trumpet shells. As the source of TTX contamination of trumpet shells in Europe is still unknown, the presence and potential temporal variations of TTXs in one of its main preys, the starfishes, were also explored. Furthermore, it was evaluated how individual morphological characteristics (weight and length), and sampling site characterization (capture depth).

The data generated in this thesis will be relevant for decision-making on TTX regulation and monitoring in Portugal. This is Europe´s first study to regularly analyse the TTXs levels throughout time in this marine gastropod.

2. Materials and methods

2.1. Harvest and preparation of the samples for TTX extraction

Between November 2021 and October 2022, a total of 25 trumpet shells, *Charonia lampas* (Linnaeus, 1758), and 25 starfishes, *Astropecten aranciacus* (Linnaeus, 1758) (Figure 2.1) were caught by fishermen as by-catch, using gillnets (220 mm) and trammel nets (120 mm). In 2021, samples were only collected in November. In 2022, samples were collected every month, starting in March (Table 2.1). The number of samples collected in each month is indicated in Table 2.1. The specimens were brought to the laboratory, where all the individuals were measured and weighed. Then, they were dissected, on the same day of capture, in edible (cerebral ganglia; foot muscle; mantle; mouth/proboscis, and salivary glands), and non-edible tissues for trumpet shell (anus/rectum; digestive gland; gill; heart; intestine; kidney, and stomach) (Table 2.2. and Figure 2.1), and in the digestive gland and stomach plus stomach content (both cardiac and pyloric stomach) for the starfish (Table 2.2. and Figure 2.1). All the tissues were frozen at -20ºC before TTX extraction.

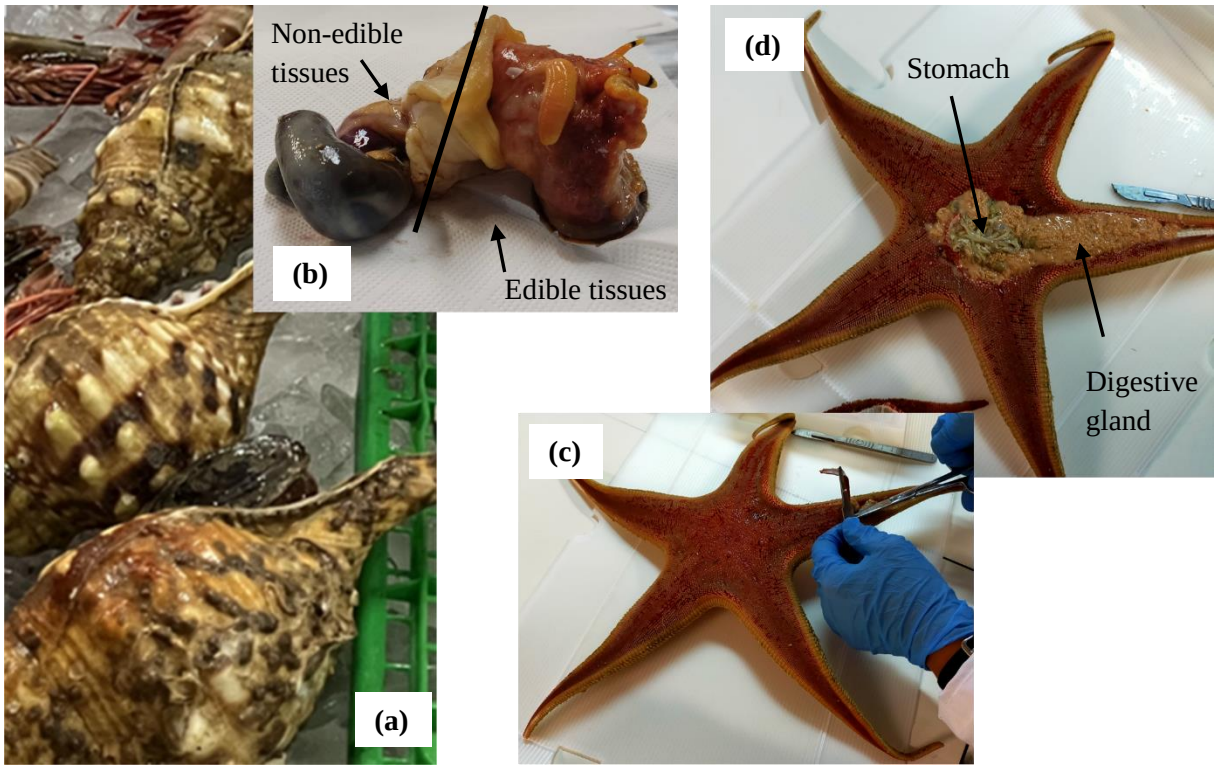

Figure 2.1. Trumpet shells *Charonia lampas* (a) whole body with shell (b) whole body without shell, with the identification of the edible and non-edible tissues. Starfish, *Astropecten aranciacus* (c) whole body upper side (d) whole body with shown digestive gland and stomach.

Table 2.1. Number of samples (each sample corresponds to one organism) of trumpet shell and starfish collected in each month, and the respective year.

	2021	**2022**							
	November	**March**	**April**	**May**	**June**	**July**	**August**	**September**	**October**
Trumpet shell	3	1	3	4	2	2	2	4	4
Starfish	4	1	2	3	1	4	1	2	6

Table 2.2. Trumpet shell and starfish dissected tissues.

Dissected tissues		
Trumpet shell		**Starfish**
Edible	**Non-edible**	Digestive Gland
cerebral ganglia; foot muscle; mantle; mouth/proboscis, and salivary glands	anus/rectum; digestive gland; gill; heart; intestine; kidney, and stomach	Stomach + stomach content

2.2. Sampling site characterization

The geographic coordinates of the capture site (Figure 2.2), and depth were recorded. All the samples were collected from the Algarve offshore coast, at the south coast of Portugal. The depth registered ranged between 77.0 meters and 115 meters deep for the trumpet shell specimens, and between 76.0 meters and 173 meters deep for the starfish specimens. The bottom seawater temperature (BST) was provided by Tunipex (https://www.tunipex.eu/pt/information_oceanic.php).

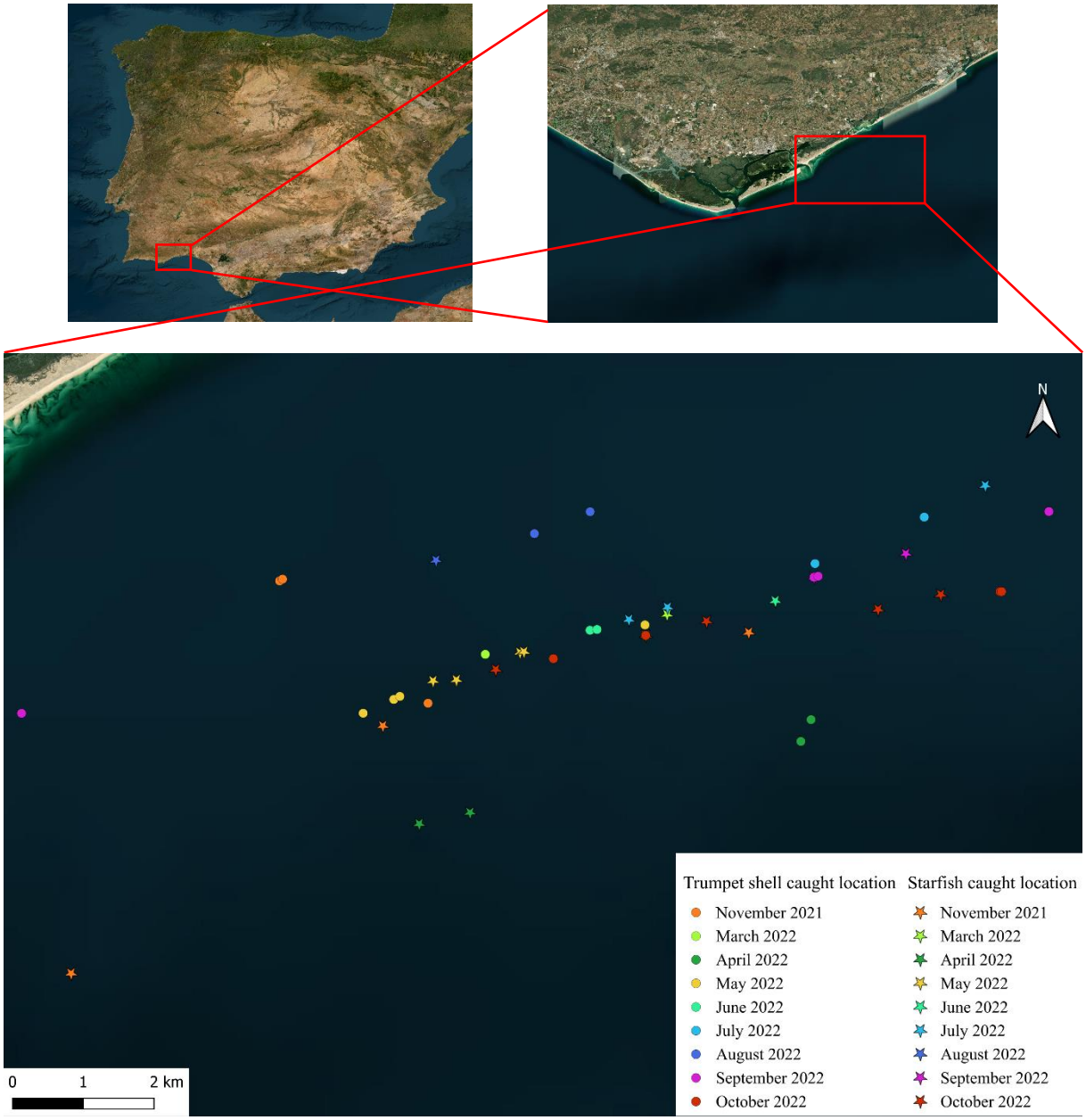

Figure 2.2. Map indicating where the different specimens were collected. Different colours indicate different sampling months and years. Circles represent each trumpet shell caught and stars represent each starfish caught.

2.3. Materials and reagents

Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS) grade solvents: acetonitrile (LC-MS grade, Merck, Darmstadt, Germany), water (LC-MS grade, J.T. Baker, Center Valley, PA, USA), ammonium hydroxide (LC-MS grade, Fluka Analytical, Steinheim, Germany), formic acid (LC-MS grade, Fluka Analytical, Steinheim, Germany), methanol (LC-MS grade) and acetic acid (LC-MS grade, Fluka Analytical, Steinheim, Germany).

A certified reference standard (CRM) in aqueous acetic acid (1mM), pH 3.91 containing certified concentrations of tetrodotoxin, 0.02 ± 0.001 mg/g; 4,9-anhydroTTX, 0.005 ± 0.0004 mg/g; and 4-epiTTX, 0.002 ± 0.0002 mg/g, purchased from CIFGA Laboratorio S.A. (Spain) was used. Certified TTX and 4,9-anhydroTTX material was purchased from CIFGA Laboratorio S.A. (Lugo, Spain) for LC-MS/MS analysis.

2.4. TTX and analogues extraction

The toxin extraction was performed according to the Standard Operating Procedure (SOP) of the European Union Reference Laboratory for Marine Biotoxins for the determination of TTX (EURLMB, 2017). Briefly, 5g of tissue samples were homogenized in an Ultra-Turrax (T 25 easy clean digital, IKA-Werke GmbH & Co. KG, Germany) with 5 mL of 1% acetic acid (CH_3COOH). Samples were vortexed for 3 min, boiled in water for 5 min., cooled to room temperature for a couple of minutes, vortexed again for 3 min, and centrifuged for 10 min at 4500g and 15°C (Mega Star 600 R, VWR, Avantor, USA). Then 1 mL of the supernatant was moved for another Eppendorf and 5 µL of 25% v/v of ammonia (NH_3) was added.

For the solid-phase extraction (SPE) clean-up step, the ENVI-Carb cartridge (Supelclean, Supelco, Sigma-Aldrich, Germany) was conditioned with 3 mL of at 20% v/v acetonitrile (CH3CN) + 1% v/v of acetic acid (CH3COOH) and 5 mL of 0.025% v/v ammonia (NH_3). After the conditioning, 500 µL of sample was added to the cartridge and then washed with 700 µl of Milli-Q water. Toxin elution was carried out through the addition of 2 mL 20% v/v acetonitrile (CH_3CN) + 0.25% v/v acetic acid (CH_3COOH). This extract was diluted by transferring 200 µL to a vial and adding 600 µL of acetonitrile (CH_3CN) for LC-HRMS analysis, and it was done according to the protocol from Lage *et al*., (2022). In order to summarize the methodology, a diagram is presented in Figure 2.3.

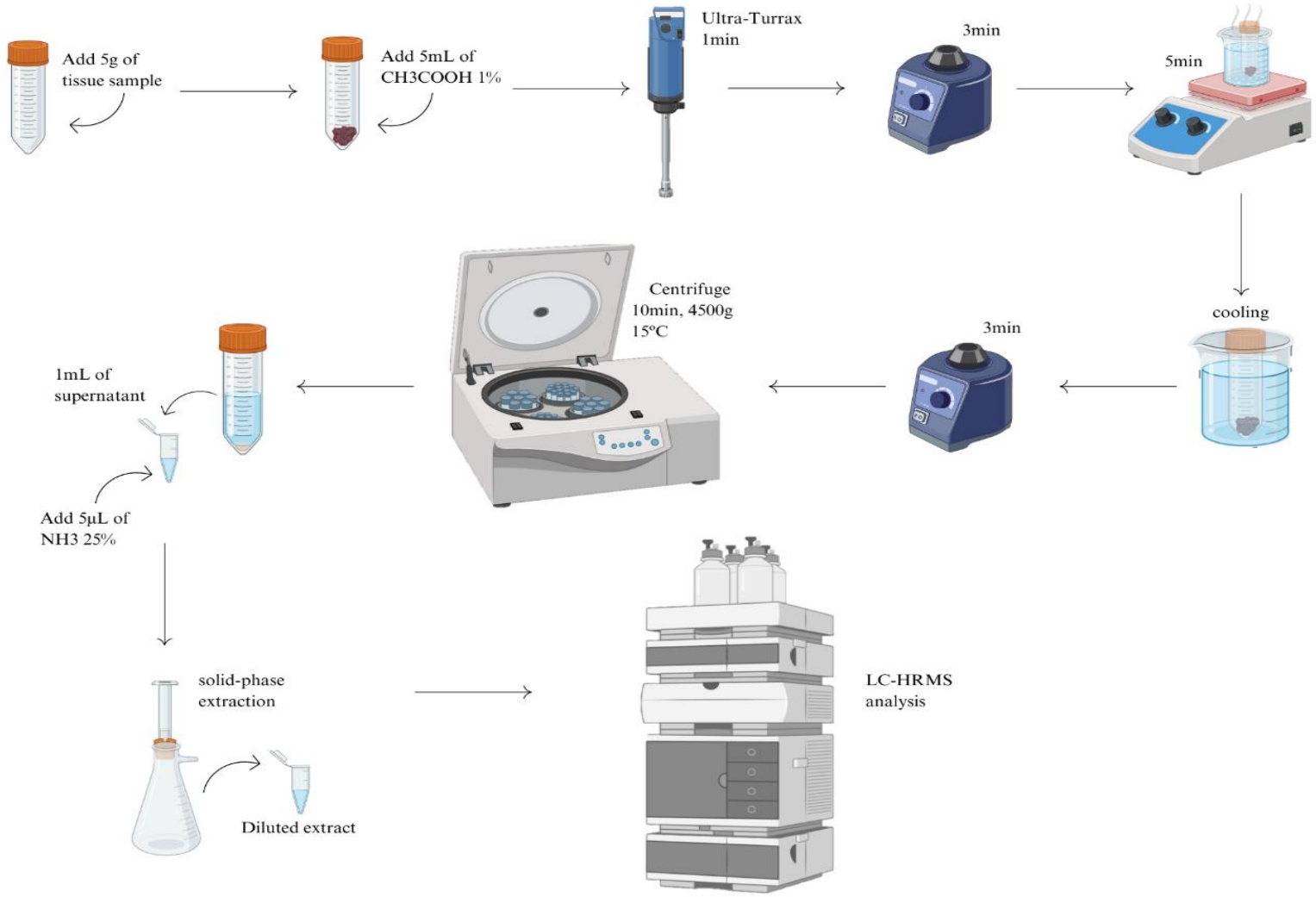

Figure 2.3. Diagram illustrating the sample preparation method of TTXs extraction.

2.5. TTX and analogues LC-HRMS analysis

An UltiMate 3000 UHPLC coupled to an Orbitrap Elite mass spectrometer (Thermo Scientific, Germany) equipped with a heated electrospray ionization source (HESI-II) was used for the chromatographic separation. The separation of toxins was carried out 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 containing 0.1% formic acid and 10mM ammonium formate (A), as well as acetonitrile containing 0.1% formic acid and 2% 10mM ammonium formate solution (B). A linear gradient (in v/v%) was started with 5% of B and increased to 95% in 11 min. This composition was maintained for 1 min before a return to 5% of B in 1 min. this composition was maintained for 2 min before the next run (Rodríguez *et al*., 2018 in Lage *et al*., 2022). The flow rate was set at 0.3 mL/min, and the injection volume was 5 mL. Source conditions were optimized to achieve the greatest sensitivity for TTX. 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 spectra represent averages obtained for the corresponding chromatographic peak.

The identification of TTXs from the full-scan profiles was carried out by generating accurate mass-extracted ion chromatograms (AM-XIC) (Annex 1), based on the exact masses of their protonated molecular ions $[M+H]^+$ *m/z* (Table 2.3) (Lage *et al*., 2022), with a mass extraction window of ± 5ppm, their retention times and on their fragmentation spectra obtained by higher-energy collisional dissociation (HCD) (Annex 2). The concentrations of TTX analogues obtained are estimates, as a similar MS response was assumed for TTX standard and all metabolites. Other general MS parameters were adjusted to ensure an optimum signal for the TTX standard.

Table 2.3. TTX and analogues exact masses of their protonated molecular ions $[M+H]^+$ *m/z* (Lage *et al*., 2022).

TTX and analogues	$[M+H]^+$
TTX	320.109
4-epiTTX	320.109
deoxyTTXs	304.114
dideoxyTTXs	288.119
trideoxyTTXs	272.124
4,9-anhydroTTX	302.098
anhydrotrideoxyTTXs	254.114

A working solution containing approximately 2 µM of TTX, 0.55 µM of 4,9-anhydroTTX, and 0.16 µM of 4-epiTTX was prepared. The different tissue matrices, as well as the solvent (water) matrix, were spiked with 1, 2, 5, 10, 20, and 40 µL of the working solution per 200 µL of the matrix. The quantitation of TTX, 4,9-anhydroTTX and other related toxins were obtained from calibration curves prepared by following a standard addition method (SAM), and they were obtained for all matrices and samples. For TTX and 4,9-anhydroTTX quantitation, a molar toxicity equivalence factor of 1.0 and 0.02, respectively, in mg TTX eq/kg, was used. LC-HRMS data analysis was performed using Xcalibur 4.1 (Thermo Scientific, Bremen, Germany).

The limits of detection (LOD) and quantification (LOQ) were calculated based on the standard deviations (SD) obtained after five injections of each blank matrix spiked with the second-lowest concentration (3 × SD and 10 × SD, respectively). The matrix effect (ME) was determined after three injections of the third-lowest concentration standard solution and of each non-contaminated blank matrix spiked with this concentration and calculated using the equation ME (%) = B/A × 100, where A is the average peak area of the standard solution and B represents the average peak area in the extract spiked with the same concentration.

In order to have a value of toxicity that can be comparable with the results of previous studies, the TTX concentration results were transformed in mg of TTX equivalent/kg. To accomplish this, it is imperative to ascertain the relative toxicity of all analogues. This was achieved through the utilization of Toxicity Equivalency Factors (TEFs), which represent a toxicity ratio of a given compound in comparison to a reference compound belonging to the same chemical group and exhibiting a similar mode of action (Boundy and Harwood, 2020). Table 2.4 provides a comprehensive listing of the TEFs for each analyzed analogue, along with their corresponding citations. There isn't a published relative potency (RP) for anhydrotrideoxyTTX, however, because amhydrotrideoxyTTX is chemically equivalent with trideoxyTTX, we used RP of trideoxyTTX also for anhydrotrideoxyTTX (we are aware that the true RP for anhydrotrideoxyTTX can be different from what we are using, but there is no information published regarding this).

Table 2.4. Toxicity Equivalency Factors (TEFs) of TTX and analogues used in the present study.

Compounds	TEFs	Citation
TTX	1	Adapted from Knutsen *et al*., 2017
4-epiTTX	0.16	Adapted from Knutsen *et al*., 2017
4-9anhydroTTX	0.02	Adapted from Knutsen *et al*., 2017
deoxyTTX	0.01	Adapted from Knutsen *et al*., 2017
dideoxyTTX	0.02	Adapted from Knutsen *et al*., 2017
trideoxyTTX	0.011	Boundy and Harwood, 2020
anhydrotrideoxyTTX	0.011	Boundy and Harwood, 2020

2.6. Statistical analysis

Statistical analysis was performed with RStudio 4.3.1 version (RStudio team, 2023), in order to evaluate if there are any negative or positive correlations between TTXs toxicity and weight, length, and depth. For that, and to choose the more appropriate test, we performed a normality test, to verify if the variables followed a normal distribution with Shapiro-Wilk test (Shapiro and Wilk 1965), and if one of the variables did not follow a normal distribution, we performed non-parametric tests. The null hypothesis was that the data is normally distributed. As the data did not follow normality, since the *p*-values < 0.05 (results not shown), the null hypothesis was rejected, and the non-parametric Kendall test (Kendall, 1938) was performed. The existence of correlation was given by the *p*-value, and was measured with the tau correlation coefficient, that varies between -1 (100% negative association, or perfectly inversions) and 1 (100% positive association, or perfect agreement), and in between it can be highly correlated (tau between 0.7 and 1), moderately correlated (tau between 0.3 and 0.7), and weakly correlated (tau between 0 and 0.3).

To check if there were significant differences between the toxicity through the months and seasons, first we grouped seasons into Autumn (November 2021, September and October 2022), Spring (March, April

and May 2022), and Summer (June, July, and August 2022). In the statistical analysis of the months, those that did not contain triplicates were not counted, as they have no statistical significance (for the test used). A Shapiro-Wilk test was performed to check for data normality. Since the data did not follow a normal distribution (p-values < 0.05, results not shown), the non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) was used to access differences between TTXs toxicities with time. For all the tests, a significance level of α=0.05 was used.

3. Results

3.1. Weight and measurements of the captured specimens

The captured trumpet shell weighed between 70.0 and 675 grams without shell, and their shell length ranged between 5.00 and 30.0 centimetres. For samples 5, 6, 7, 8, and 11, the shell was not measured as it was removed onboard by fishermen. The captured starfish weighed between 100 and 700 grams. The length of the arm (R) ranged between 13.0 and 26.0 centimetres, and the radius of the disk (r) ranged between 2.00 and 6.00 centimetres.

3.2. LOD, LOQ, ME, and calibration curves

LOD and LOQ, ME, and calibration curves of TTX were estimated by AM-XIC for the four tissue extracts (Table 3.1 and Table 3.2). The LOD and LOQ were below the EFSA's maximum limit (0.04 mg TTX eq/kg) in all the matrices. Regarding the ME, ion suppression (ME below 100%) was observed in all matrices. Therefore, to account for the ME, four calibration curves were prepared using each matrix. The standard curves had a correlation coefficient (r) ≥ 0.99 (Figure 3.1).

Table 3.1. Limits of detection and quantification (LOD and LOQ, mg/kg), and matrix effects with corresponding relative standard deviation (ME ± RSD, %) of TTX for the edible and non-edible tissue extracts of trumpet shell, and starfish. The stomach includes stomach content.

Organism	Tissue Type	LOD (mg/kg)	LOQ (mg/kg)	ME±RSD (mg/kg)
Trumpet Shell	Non-edible	0.009	0.03	0.03±0.006
	Edible	0.01	0.03	0.04±0.006
Starfish	Digestive Gland	0.008	0.03	0.08±0.03
	Stomach	0.009	0.03	0.09±0.02

Table 3.2. Calibration curve parameters obtained for TTX following AM-XIC Full-scan on trumpet shell and starfish tissue extracts. The stomach includes stomach content.

Organism	Tissue Type	Slope	Intercept	r
Trumpet Shell	Non-edible	2×10^6	-0.150	0.99
	Edible	5×10^6	-0.009	0.99
Starfish	Digestive Gland	6×10^6	-0.019	0.99
	Stomach	8×10^6	-0.011	0.99

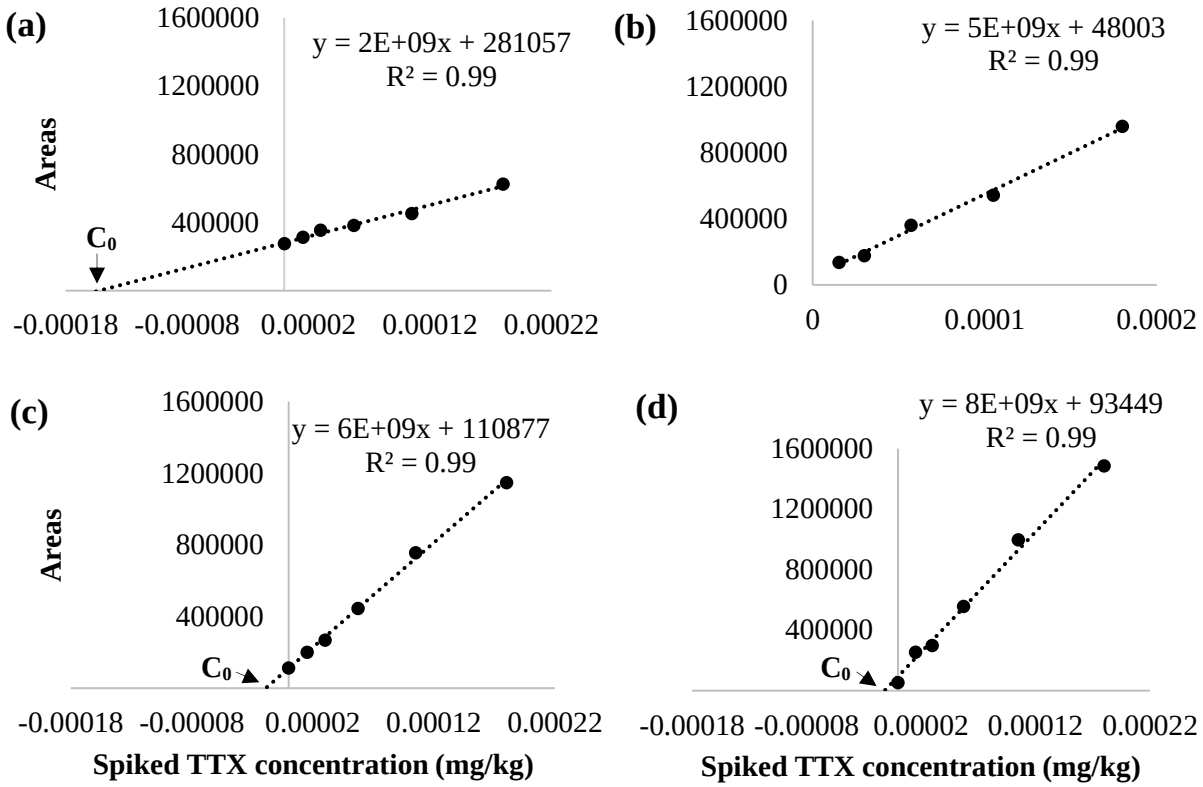

Figure 3.1. Calibration curves of TTX in contaminated tissue matrices of trumpet shell: (a) non-edible fraction and (b) edible fraction, and of starfish: (c) digestive gland and (d) stomach (including stomach content). TTX concentration (mg/kg) in contaminated samples (C_0) is indicated at the x-axis intercept of the linear regression.

3.3. Quantitation of Toxicity

The TTX compounds present in trumpet shell and starfish samples were quantified against the TTX-CRM, which contained TTX, 4-epiTTX and 4,9-anydroTTX. In this case, toxicity means the sum of TTX, 4-epiTTX and 4,9-anhydroTTX concentrations multiplied by the respective TEFs, because these analogues are the only ones with CRM and have TEFs determined by EFSA. Toxicity considering all quantified analogues were also calculated using TEFs suggested by several studies (Boundy and Harwood, 2020; Knutsen *et al*., 2017). When comparing these two values we verify that the additional analogues add a value of ~32%, 100%, ~88%, and ~94% increase of toxicity (in trumpet shell non-edible tissues, trumpet shell edible tissues starfish digestive gland, and starfish stomach, respectively) (Annex 3).

Regarding the non-edible tissues of the trumpet shell *C. lampas*, only three samples were negative for toxicity, and only two of the positive ones were below EFSA's recommendation limit (0.04 mg TTX equivalent / kg). The remaining samples were much higher than the limit recommended by EFSA (Figure 3.2). In the edible portion of *C. lampas*, no TTX, 4-epiTTX and 4,9-anhydroTTX analogues were detected.

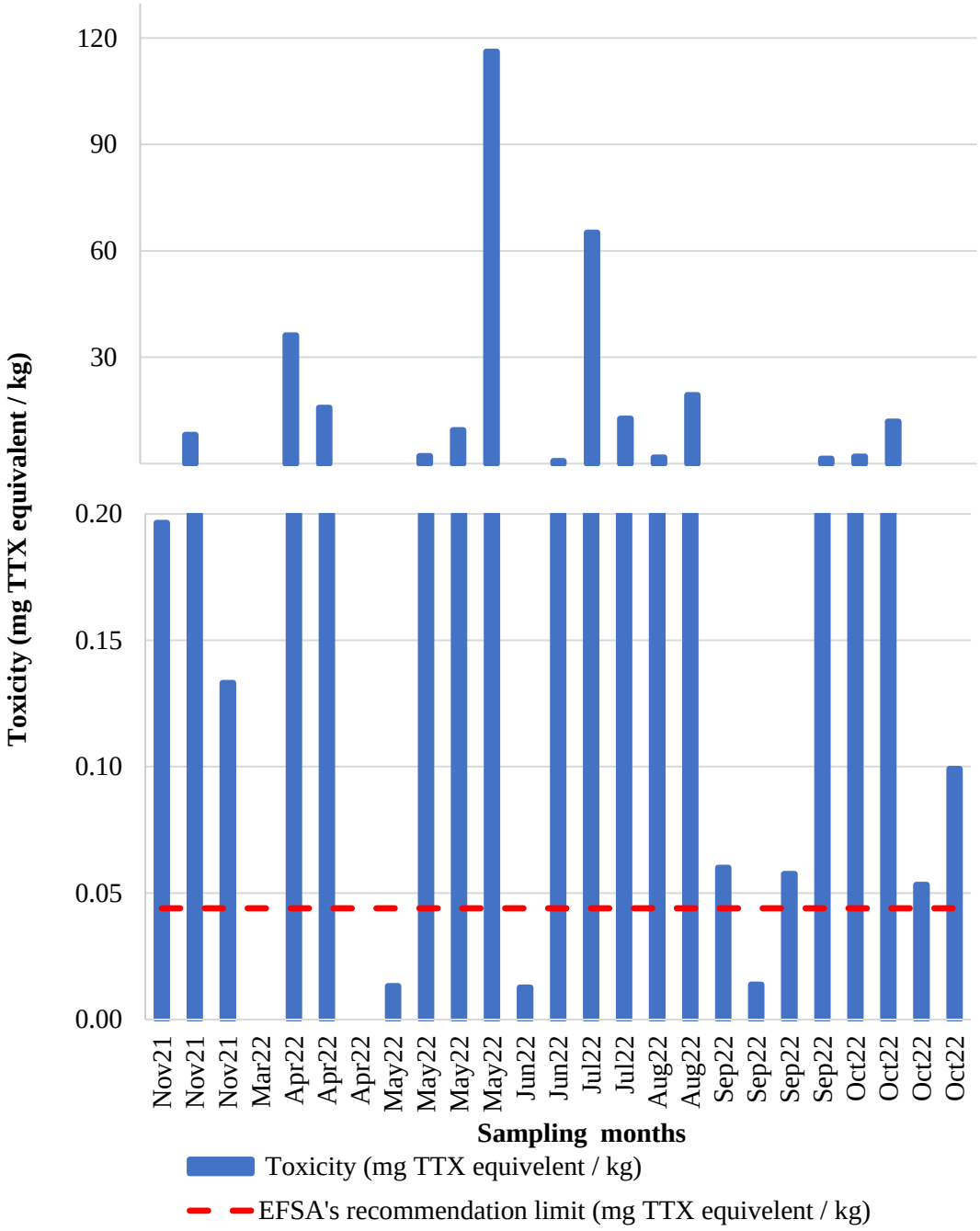

Figure 3.2. Toxicity in mg TTX equivalent / kg, in the non-edible tissues of trumpet shell.

Results for the digestive gland of starfish showed only five samples (of a total of 25 samples) quantifiable for toxicity, and only one above EFSA's recommended limit (Figure 3.3). For the stomach, only two samples were quantifiable for toxicity, and both were below EFSA's recommended limit (Figure 3.4).

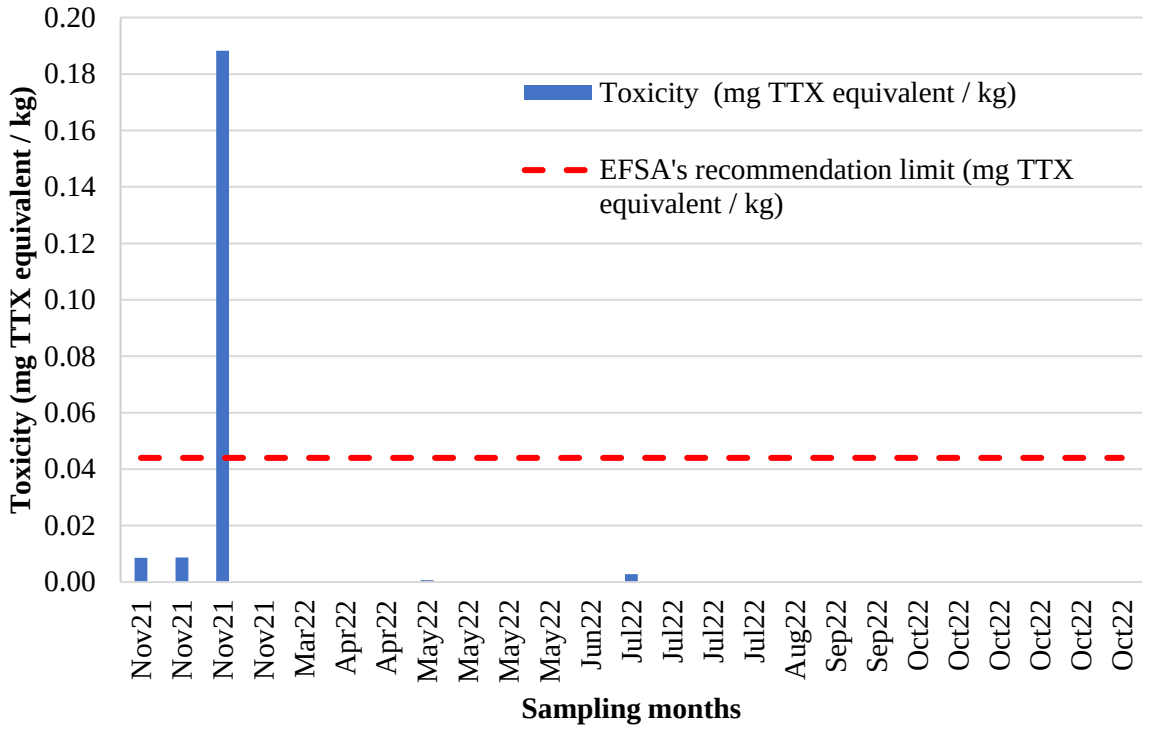

Figure 3.3. Toxicity in mg TTX equivalent / kg, in the digestive gland of starfish. It represents samples 1, 2, 3, 8 and 13, respectively.

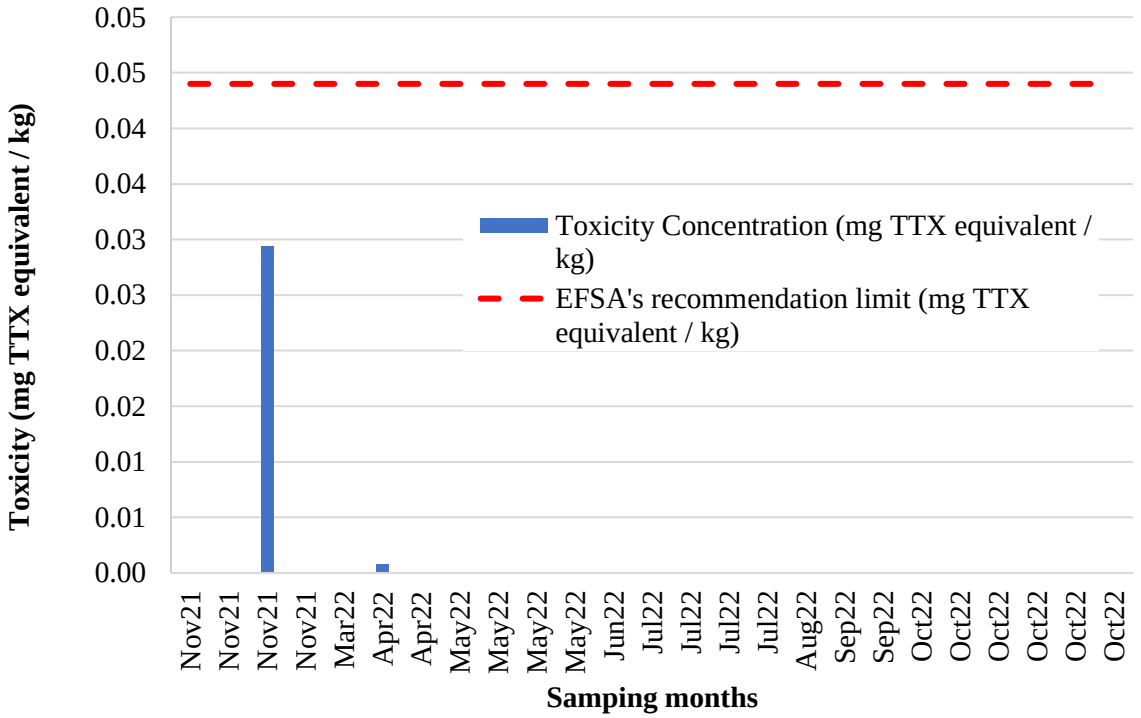

Figure 3.4. Toxicity in mg TTX equivalent / kg, in the stomach of starfish (including stomach content). It represents samples 3 and 6, respectively.

The results showed a high variability among specimens (Figures 3.2; 3.3 and 3.4). The highest toxicity was found in non-edible tissues from trumpet shell, with 116.359 mg TTX equivalent / kg.

3.4. Quantitation of analogues

For the non-edible tissues of trumpet shell all the analogues were found (but not in all the samples), and only three samples contained all the analogues studied. Of all the positive samples, ~60% were above the limit recommended by EFSA, and ~40% were below that limit (Figure 3.5). The three most common analogues were TTX (~66%), anhydrotrideoxyTTXs (1 and 2) (~21%), and trideoxyTTXs (~10%). Regarding the edible portion of these individuals, only dideoxyTTX3 and trideoxyTTX3 were found (~87% and ~13%, respectively), and were restricted to the months of April, May, June and August (data not shown), but all samples below the EFSA's recommended limit. Considering analogues distribution per month (in the non-edible tissues), the most common was TTX, with almost 90% of occurrence in September, followed by anhydrotrideoxyTTX1 and anhydrotrideoxyTTX2. However, March samples only exhibit dideoxyTTX3 and trideoxyTTX2 (Figure 3.6).

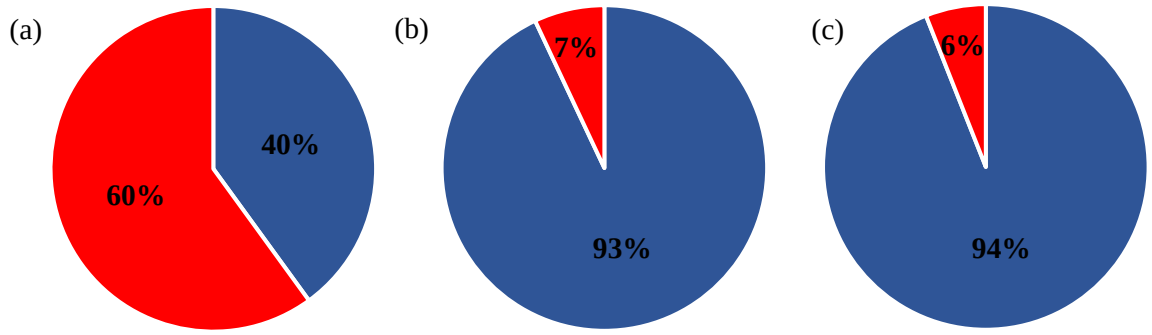

Figure 3.6. Percentage of all positive samples below (dark blue) and above (red) EFSA's recommended limit. (a) non-edible tissues of trumpet shell, (b) digestive gland of starfish and (c) stomach of starfish.

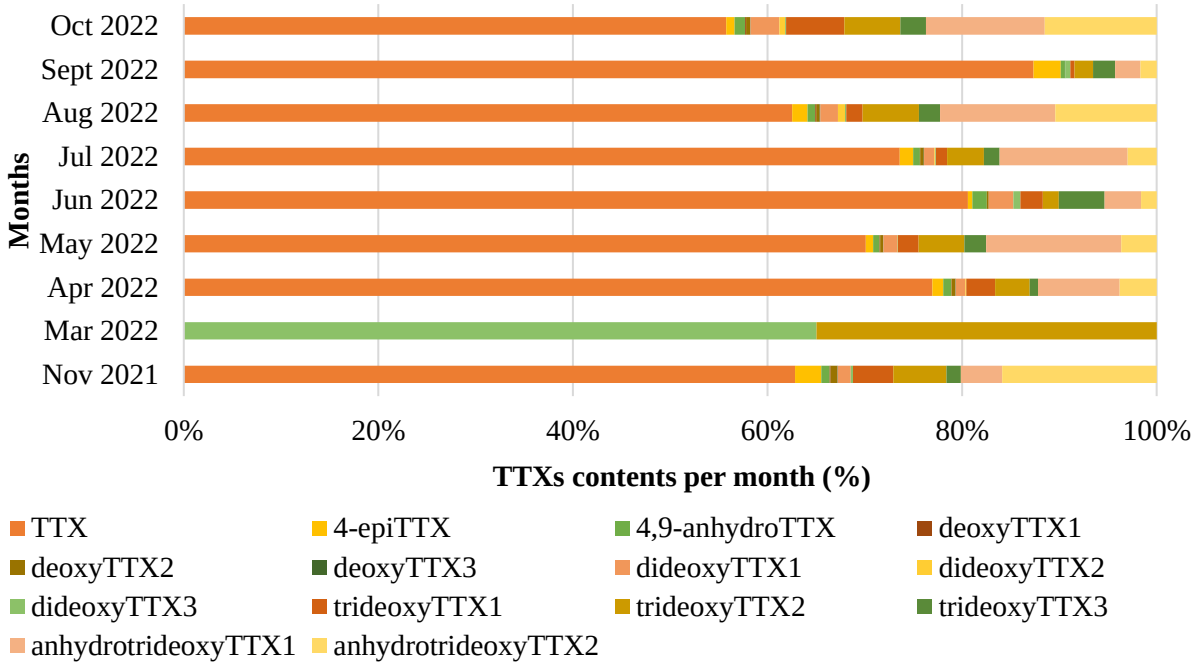

Figure 3.5. Monthly distribution of TTXs (%) in non-edible tissues of trumpet shell. This was determined using the mean of the toxicity from all of the samples from each month.

In the starfish digestive gland and stomach, ~93% and ~94%, respectively, of all the positive samples were below EFSA's recommended limit and only ~7% and ~6%, respectively, were above (Figure 3.5). Regarding the digestive gland, the three most common analogues were anhydrotrideoxyTTXs (~53%), trideoxyTTXs (~20%), and TTX (~12%). In the stomach, anhydrotrideoxyTTXs was the most common (~76%), followed by dideoxyTTX1 (~15%), and TTX (~6%).

During the year, the distribution of the TTX analogues in the digestive gland was dominated by trideoxyTTX3 and anhydrotrideoxyTTX1. TTX was only present in November 2021 (Figure 3.7). In the stomach, the most frequent during the study period was anhydrotrideoxyTTX1, followed by anhydrotrideoxyTTX2. In July, the only compound found was TTX (Figure 3.8).

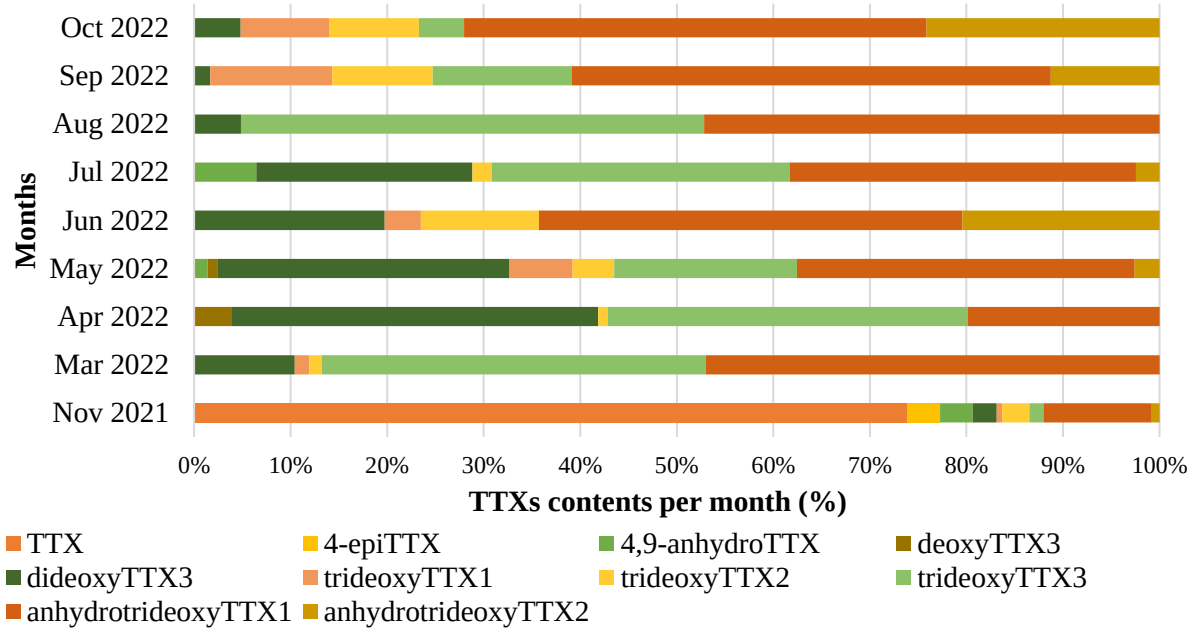

Figure 3.7. Monthly distribution of TTXs (%) in the digestive gland of starfish. This was determined using the mean of the toxicity from all of the samples from each month.

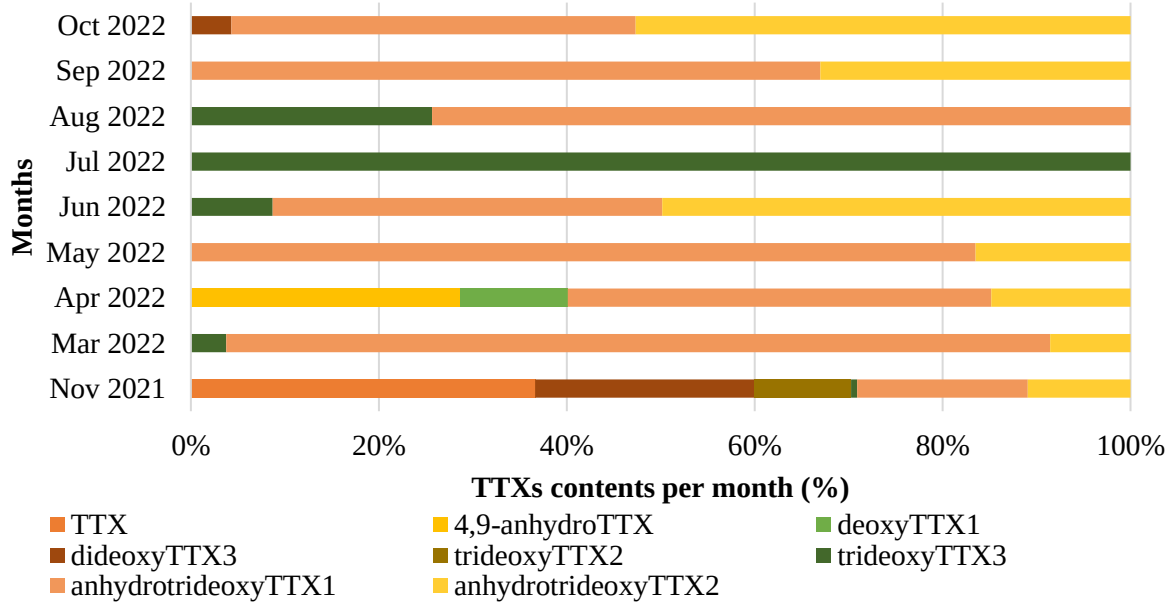

Figure 3.8. Monthly distribution of TTXs (%) in the stomach (including stomach content) of starfish. This was determined using the mean of the toxicity from all of the samples from each month.

3.5. Statistical analysis

The edible tissues of the trumpet shell were not toxicity positive for TTX or analogues, and thus were not considered for statistical analysis. To guarantee a minimum of three replicates for statistical analysis, those compounds that were not present in at least three individuals (in the same tissue) were not considered.

3.5.1. Correlation tests – Kendall test

Regarding non-edible tissues of trumpet shells, all the factors evaluated (weight, length, and depth), showed no statistically significant correlation with TTXs toxicities (*p*-values > 0.05) (Annex 4). The variables studied seem to not affect the accumulation of TTXs in trumpet shell.

For the digestive gland of starfish, the statistical test between weight, radius of the disk (r), and toxicity showed no statistically significant correlation (*p*-values > 0.05) (Annex 5). The statistical test on length of the arm (R) showed no statistically significant correlation (*p*-value > 0.05) for the analogues dideoxyTT3, trideoxyTTX1, trideoxyTTX2, anhydrotrideoxyTTX1 and anhydrotrideoxyTTX2, except for toxicity regarding the sum of the three compounds with CRM, with a positive moderate correlation (*p*-value = 0.02, tau = 0.4), and for the analogue trideoxyTTX3, with a negative moderate correlation (*p*-value = 0.03, tau = -0.3) (Annex 5). For the water depth statistical test, the analogues dideoxyTT3, trideoxyTTX1, trideoxyTTX2, anhydrotrideoxyTTX1 and anhydrotrideoxyTTX2 showed no statistically significant correlation (*p*-value > 0.05), except for toxicity regarding the sum of the three compounds with CRM, with a positive moderate correlation (*p*-value = 0.03, tau = 0.4), and for the analogue trideoxyTTX3, with a negative moderate correlation (*p*-value = 0.02, tau = -0.3) (Annex 5). Regarding the stomach (including content), the statistical test on weight, R, and r showed no statistically significant correlation (*p*-values > 0.05) (Annex 6). The statistical test on depth showed no statistically significant correlation (*p*-values > 0.05) for the analogues trideoxyTTX3, anhydrotrideoxyTTX1 and anhydrotrideoxyTTX2, and a statistically significant positive moderate correlation (*p*-value = 0.005, tau= 0.5) for the analogue dideoxyTTX3 (Annex 6).

3.5.2. Kruskal-Wallis tests – TTX with sampling months and seasons

For both species, the Kruskal-Wallis tests between sampling months and seasons, and TTXs toxicities showed no significant differences (*p*-values >0.05) (Annex 7).

4. Discussion

4.1. Temporal variability

Trumpet shells and starfishes from the Algarve coast tested positive for TTX during the study period (2021-22). Excluding edible tissues from trumpet shells where no TTXs could be detected, in both species, the different tissues presented a similar pattern, regarding correlation statistical tests with weight, measurements of the organisms, and sampling depth. In general, the studied morphological and environmental variables showed no statistically significant correlation with TTXs (except for the three cases mentioned before). This suggests that weight, measurements, and sampling depth do not influence the accumulation of TTXs in the studied species of trumpet shells and starfishes. According to the statistical results, there was no significant variability of TTXs with sampling months and seasons, which suggests that the toxicity found in the individuals were not influenced by the sampling month or the season.

Since there are no other studies regarding temporal variability in gastropods and starfish from Europe, we can only compare our results with results from other TTX-bearing groups. In the literature, there is a discussion regarding the influence of Sea Surface Temperature (SST) in TTXs concentration, but there is still a lack of continuous SST monitoring data and appropriate statistical analysis (Dhanji-Rapkova *et al*., 2023). These authors found that, in bivalves, TTX concentration increased in early summer, reaching a maximum in late June / early July. Also, they reported that at sampling time, when *in situ* SST was at a range between 15ºC and 20ºC, TTX was higher in the bivalves. In the present work, the species studied live in the sea bottom, so we examined the BST, measured by Tunipex. The data available does not mention the depth at which the BST was measured. It seems that, with some exceptions, the samples of trumpet shell and starfish caught with temperatures above 15ºC have higher toxicity, in agreement with Dhanji-Rapkova *et al.* (2023). However, further work is needed to better investigate this hypothesis. The impact of SST on European bivalves may be attributed to the sessile nature of these organisms, making them more susceptible to temperature influences. In contrast, trumpet shells and starfish, being more mobile, can actively seek optimal conditions, potentially reducing their vulnerability to temporal fluctuations. This could explain the outcomes observed in the present study. Nevertheless, further investigations are needed with improved sampling plans and the incorporation of SST and BST monitoring to enhance our understanding of these dynamics.

Wood *et al.* (2012) studied temporal, spatial and individual variability of TTX concentrations in *Pleurobranchea maculata*, a grey slide-gilled sea slug, an opportunistic carnivore that scavenge on a range of invertebrate organisms, confined in easily accessible shallow subtidal areas. The results obtained showed TTX concentrations declining from June (peak between June-July) to December, with significant differences. Also, studies concerning gastropod species *Tanea lineata* (Röding, 1798) (mentioned as *Natica lineata*) and *Nassarius glans* (Linnaeus, 1758), showed no clear temporal trends (Chen and Chou, 1998; Taniyama *et al*., 2013). Wood *et al*. (2012) considered the possibility of the TTX concentration be related to the size of the individual, which could partially explain the variability among individuals found, suggesting that if the toxin was produced endogenously, it would be expected that larger organisms produce more TTX, and consequently have higher TTX concentrations. However, their results showed no correlation between weight and TTX concentrations (Wood *et al*., 2012). The present study also showed no temporal variability, and no correlation between weight and toxicity, and similar results were showed with starfish species *A. polyacanthus* and *A. scoparius* (Müller & Troschel, 1842) (Asakawa *et al*., 2003), and ribbon worm *Cephalothrix* sp. (Örsted, 1843) (Miyazawa *et al*., 1985). To explain these results, it was suggested that genetic variation among organisms could also be a crucial factor affecting their ability to produce/sequester TTX (Asakawa *et al*., 2003; Miyazawa *et al*., 1985).

An important factor to take into account in future research could be maturity and spawning stages of trumpet shell, since it was already suggested the association of sexual maturity stages with temporal changes in TTXs concentrations, and it was found that the highest toxicity levels were found during spawning stage of maturity of the silver-cheeked toadfish *Lagocephalus sceleratus* (Gmelin, 1789) (Sabrah *et al*., 2006). As some studies suggest, regarding pufferfish, the highest toxicity levels appear to happen in the spawning season, *i.e.*, between March and June, which can indicate sexual differences between organisms toxicity, and that maturation can affect the accumulation in organisms (Noguchi *et al*., 2011).

Regarding starfishes, our results agree with the literature. It has already been reported that the TTX concentration of *A. polyacanthus* and *A. scoparius* did no correlate with toxicity and body weight or the season of collection of starfish (Miyazawa *et al.* 1985). A study in São Miguel Island, Azores, showed three positive samples of starfish species *Ophidiaster ophidianus* (Lamarck, 1816) for a TTX analogue (Silva *et al*., 2019). These authors suggest the SST increase as a factor of influence in their findings,

since it has already been proved that TTX production by bacteria is higher with higher water temperatures, in pufferfish species *Takifugu niphobles* (Jordan & Snyder, 1901) (registered as *Fugu niphobles*) (Sugita *et al*., 1989). Contrary to our results, where no temporal variability of toxicity on *A. aranciacus* was observed, a previous study found a temporal variation in starfish species *A. scoparius*, with higher values between September and November, coinciding with the mature period (Lin and Hwang, 2001).

Thus, the understanding of the sex and maturity status of the species studied appears to be essential for a comprehensive analysis of temporal variations in toxicity levels.

4.2. Toxicity in trumpet shells and starfishes

To the best of our knowledge, after the intoxication episode in Spain, the present study has the second highest toxicity detected in marine gastropods, with a maximum of 113.7 mg TTX eq/kg, in trumpet shells (Figure 3.1). Previous work by Rodriguez *et al.* (2008) detected a toxicity of 315.0 mg TTX eq/kg, also in non-edible tissues (Table 1.3). Furthermore, of all the analysed samples, only three were negative for toxicity. This confirms the digestive tissues as the most toxic in trumpet shells, as already reported in other studies (Costa *et al*., 2021; Lage *et al*., 2022; Narita *et al*., 1981; Rodriguez *et al*., 2008).

Our study reveals the presence of toxicity levels above the EFSA recommended maximum limit in almost half of the positive samples of non-edible tissues from trumpet shell (approximately 40%) (Figure 3.5), in agreement with previous studies from the Algarve coast (Costa *et al*., 2021; Fernández-Ortega *et al*., 2010; Lage *et al*., 2022; Rodriguez *et al*., 2008). This reinforces the need for a systematic assessment of the public health risks associated with this toxin in trumpet shell, and other organisms suspected to contain TTXs. The majority of samples from edible tissues presented negative results, and the positive ones were below LOQ and the EFSA recommended maximum limit, also consistent with the studies mentioned above.

The high toxicity found in the non-edible tissues of trumpet shells supports the TTX accumulation by dietary route, *i.e.*, the exogenous origin of TTX. This is consistent with previous studies regarding the detection and the higher values in these tissues (Costa *et al*., 2021; Lage *et al*., 2022; Narita *et al*., 1981; Noguchi *et al*., 2011; Rodriguez *et al*., 2008). Narita *et al.* (1981) showed toxicity was almost exclusively present in the digestive gland of trumpet shell, and Costa *et al.* (2021) also found higher values of TTXs in the viscera. Rodriguez *et al.* (2008) found TTX and 5,6,11-trideoxyTTX analogue in the trumpet shell digestive gland, supporting the exogenous accumulation of TTXs in gastropods. Thus, the quantification of TTXs in starfish, the most common prey of trumpet shells, is an important way to better understand the TTXs accumulation route given the association of some starfish species with this toxin and the toxin producing bacteria (Lin and Hwang, 2001; Narita *et al*., 1987, 1981; Noguchi *et al*., 1982). On the other hand, the differences observed among toxicity and the different tissues can suggest toxin distribution and biotransformation processes in gastropod tissues, as suggested by Lage *et al.* (2022). However, other factors could impact this, such as the different methods used for TTXs detection (Lage *et al*., 2022).

Most of the samples of starfishes were toxicity negative, and the positive ones ranged between 0.03 and 0.2 mg TTX eq/kg, lower than those found in trumpet shells (Figures 3.3 and 3.4). Also, the tissues analysed presented only 6% and 7% (digestive gland and stomach, respectively) of positive samples above EFSA's recommended limit, see Figure 3.5. This could potentially support the exogenous origin of TTX, as the bioaccumulation of TTXs in higher trophic levels suggests that the intake of starfish by trumpet shells appears to be the source of TTX in these individuals, aligning with the findings of Noguchi & Arakawa's (2008) review. However, and to our knowledge, in addition to the present study

only one more study found starfishes containing TTXs in European waters, in São Miguel Island (Azores), a starfish species found to possess TTX, 4-epiTTX, deoxyTTX, 11-deoxyTTX and norTTX (Silva *et al*., 2019).

TTX origin in starfishes continues to be uncertain. It is not clear if TTX is being produced endogenously by the starfishes, or if it comes from other sources, but some studies suggested the TTX accumulation through the food chain (Lin and Hwang, 2001; Maruyama *et al*., 1985; Noguchi *et al*., 1982). More studies are needed to try to understand if starfishes can produce TTX (Salvitti *et al*., 2017), as well as on the analysis of stomach content. Noguchi *et al.* (1982) identified in gastropod species *C. sauliae* fragments of starfish species *A. polyacanthus*, containing TTX (Noguchi *et al*., 1982). They also concluded that non-toxic *C. sauliae* fed with toxic starfishes became TTX-positive, so it can be assumed that TTX came from the food chain, supporting the exogenous origin of TTX. Also starfish species *A. scoparius* was found to contain TTX (Maruyama *et al*., 1985), and since they are carnivorous, TTX is estimated to come from the food chain. (Lin and Hwang, 2001).

Some studies have explored TTX analogues, and Silva *et al.* (2019) reported trideoxyTTX as the most common analogue along the continental coast of Portugal. In the research by Lage *et al.* (2022), anhydrotrideoxyTTX was identified as the most abundant analogue, followed by trideoxyTTXs and dideoxyTTXs analogues, and in Li *et al.* (2008) study, trideoxyTTXs emerged as the most prevalent analogue. In comparison with the present study, trumpet shell non-edible tissues predominantly contain TTX, followed by anhydrotrideoxyTTX. Notably, the sample from March exclusively exhibits dideoxyTTX3 and trideoxyTTX2 (Figure 3.6). In edible tissues, the identified analogues are limited to dideoxyTTXs and trideoxyTTXs (data not shown). Conversely, starfish predominantly exhibit anhydrotrideoxyTTXs, followed by trideoxyTTX3, with TTX being the primary analogue in the digestive gland in November (Figure 3.7 and Figure 3.8). These findings seem to align with previous studies, indicating consistency across different investigations.

The lack of commercially available standards presented a difficulty in precisely measuring and describing TTX analogues levels. This obstacle might hold back the establishment of a consistent baseline for comparisons, thereby impacting the precision of our results. It highlights the necessity for progress in standardization within the scientific community, emphasizing the importance of easily obtainable and reliable reference materials to improve the consistency and reproducibility of TTX studies. Closing this gap could substantially enhance the strength of future research works in this area.

The present study highlights a high variability in the toxicity among individuals, already observed in other studies (Costa *et al*., 2021; Lage *et al*., 2022; Silva *et al*., 2012; Wood *et al*., 2012). As already suggested, this variability can indicate exposure to the same/similar sources of TTX, but differences in sequestration capabilities among individuals, and could be influenced by factors such as geographic location, diet before sampling, and ability to move away from toxin sources (Salvitti *et al*., 2015; Shumway, 1995).

The geographic spread and the diversification of TTX-bearing organisms raise public health concerns, with suggestions that global warming may be a critical factor influencing and catalyzing this phenomenon (Noguchi *et al*., 2011). Tetrodotoxin has already been identified in the South of Europe and is extending towards the North (Karlson *et al*., 2021). Hence, it can be anticipated that there is a risk that TTX will further expand and establish its presence from the South of Portugal further North.

5. Conclusions

The current study indicates an absence of temporal variability in TTXs within *C. lampas* and *A. aranciacus*. The characterization of the risk of the presence of emerging marine biotoxins in European coastal areas and the establishment of monitoring programs for TTX needs to be a priority concern for the EU Health Authorities. This is particularly important in areas experiencing warming due to climate change. Additional surveys should be addressed, not only in the species studied here but also in other susceptible animals, such as marine worms and flatworms, known to reside inside organisms like shellfish, that may contain high levels of TTX. This would be crucial to evaluate temporal influences on TTXs levels, along with constant monitoring of the sea temperature to try to predict the potential presence of contaminated individuals in the northern regions of the country. This approach also aims to enhance the risk assessment of TTX in marine bivalves and gastropods, aligning with EFSA's recommendation.

The findings of our study show higher toxicity in non-edible tissues of *C. lampas*, including structures related to digestive and excretory functions. This leads us to suggest that a right consumption of the white muscle of *C. lampas* does not seem to be a risk to consumers. However, an incorrect evisceration of the individual, adding the high toxicity observed, can lead to an intoxication event. Furthermore, given that *C. lampas* is considered a delicacy in certain regions of the South of Portugal, raising public awareness seems to be crucial for preventing poisoning incidents. Moreover, the hospitals and medical centers should be aware and consider patients exhibiting typical symptoms of TTX poisoning, mainly if the patients consumed the organism. Further studies are essential to gain a deeper understanding of this process, and regular monitoring of this toxin and its association with environmental factors is crucial for effective management and prevention. As early as the recognition and consequent support measures, safer it will be for the consumer.

Additionally, the considerable variability in toxicity among individuals of the two studied carnivorous species and the higher TTXs levels in non-edible tissues of *C. lampas* leads us to support the accumulation of TTXs through the food chain in both species, aligning with the exogenous origin theory of TTX. However, according to the results reached in the current study, we cannot assure if TTX contamination of *C. lampas* is provided only by TTX contaminated starfishes, *i.e.*, through the diet. Moreover, the results of TTX in *A. aranciacus* are inconclusive regarding the original source of TTX. Potentially other approaches should be accessed with the aim of understanding better the origin of TTX, such as molecular methods, since the enzymes used in the biosynthetic routes of toxins can probably be paralleled in TTX assembly. Moreover, it is important to investigate the role of bacteria in TTX production, aligned with bacterial studies in *C. lampas* and *A. aranciacus* tissues, and further investigation of unculturable bacteria.

6. References

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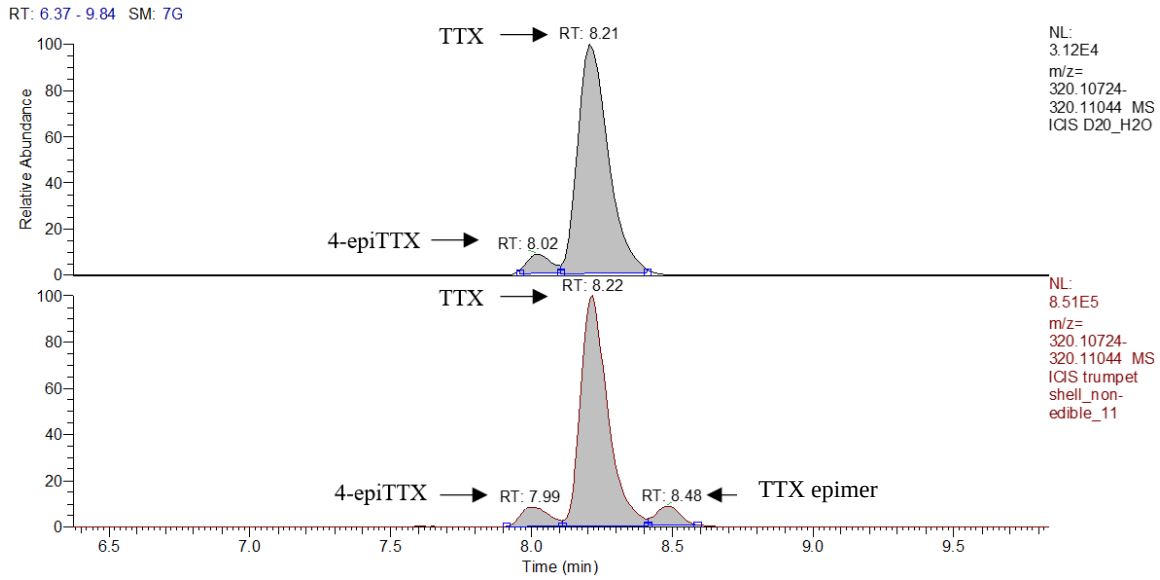
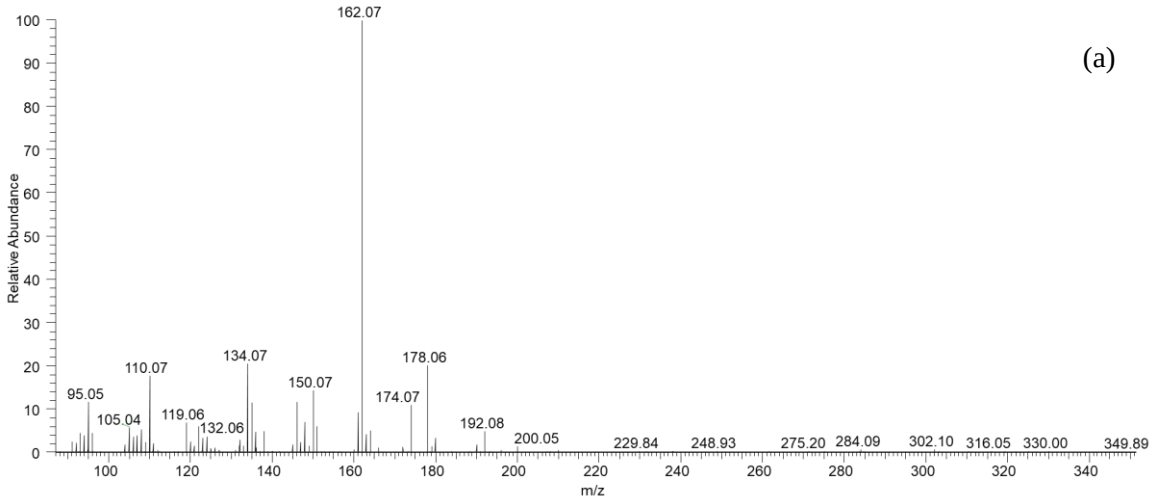
References using Zotero software, following Chemosphere citation style.

Annexes

Annex 1. Full scan chromatogram of m/z 320.109 in standard and in trumpet shell (sample eleven) non-edible tissues.



Annex 2. TTX analogues fragmentation spectra obtained by HCD. (a) HCD MS^2 spectrum of *m/z* 320 (TTX epimer) in non-edible tissues sample of *Charonia lampas*. Energy: 90 arbitrary units. (b) HCD MS^2 spectrum of *m/z* 304 (deoxyTTX 1) in non-edible tissues sample of *Charonia lampas*. Energy: 45 arbitrary units. (c) HCD MS^2 spectrum of *m/z* 304 (deoxyTTX 2) in non-edible tissues sample of *Charonia lampas*. Energy: 45 arbitrary units. (d) HCD MS^2 spectrum of *m/z* 304 (deoxyTTX 3) in non-edible tissues sample of *Charonia lampas*. Energy: 45 arbitrary units. (e) HCD MS^2 spectrum of *m/z* 288 (dideoxyTTX 1) in in non-edible tissues sample of *Charonia lampas*. Energy: 90 arbitrary units. (f) HCD MS^2 spectrum of *m/z* 288 (dideoxyTTX 2) in non-edible tissues sample of *Charonia lampas*. Energy: 90 arbitrary units. (g) HCD MS^2 spectrum of *m/z* 288 (dideoxyTTX 3) in non-edible tissues sample of *Charonia lampas*. Energy: 90 arbitrary units. (h) HCD MS^2 spectrum of *m/z* 272 (trideoxyTTX 1) in non-edible tissues sample of *Charonia lampas*. Energy: 70 arbitrary units. (i) HCD MS^2 spectrum of *m/z* 272 (trideoxyTTX 2) in non-edible tissues sample of *Charonia lampas*. Energy: 90 arbitrary units. (j) HCD MS^2 spectrum of *m/z* 272 (trideoxyTTX 3) in non-edible tissues sample of *Charonia lampas*. Energy: 90 arbitrary units. (k) HCD MS^2 spectrum of *m/z* 254 (anhydrotrideoxyTTX 1) in non-edible tissues sample of *Charonia lampas*. Energy: 70 arbitrary units. (l) HCD MS^2 spectrum of *m/z* 254 (anhydrotrideoxyTTX 2) in non-edible tissues sample of *Charonia lampas*. Energy: 70 arbitrary units.



Annex 2. Continuation.

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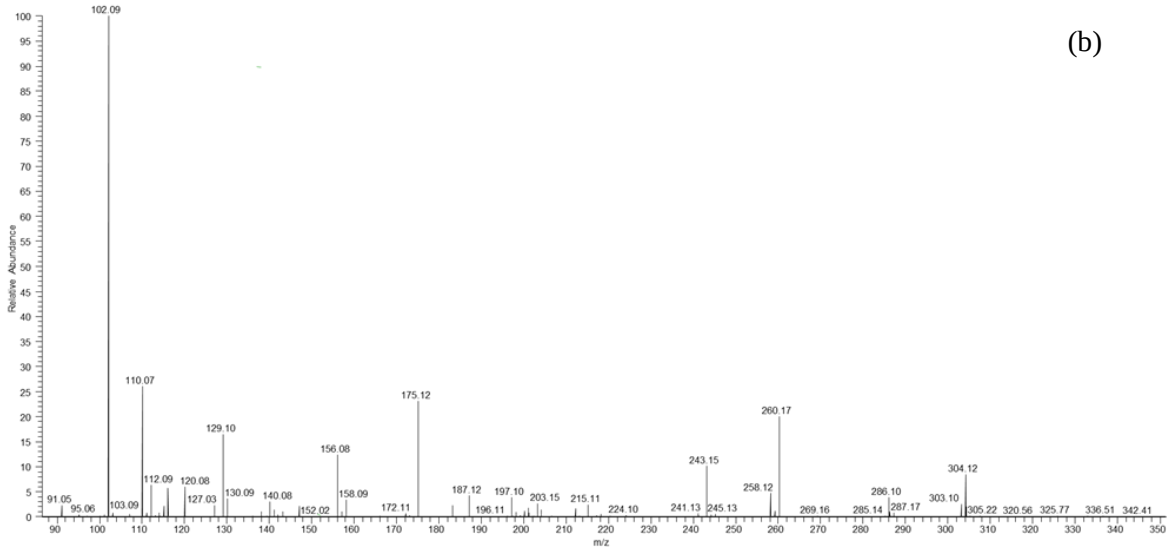

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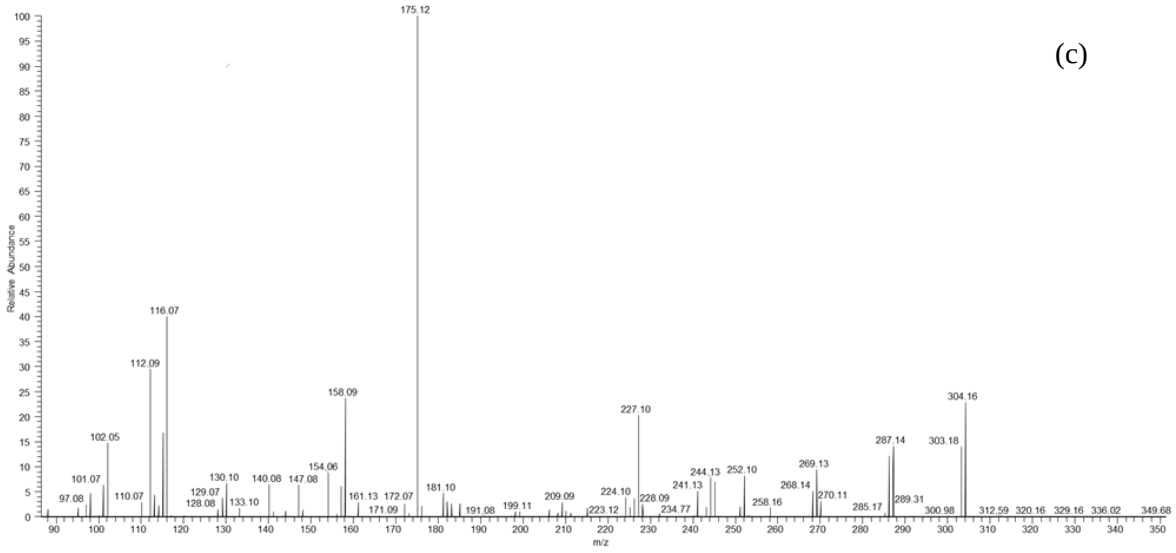

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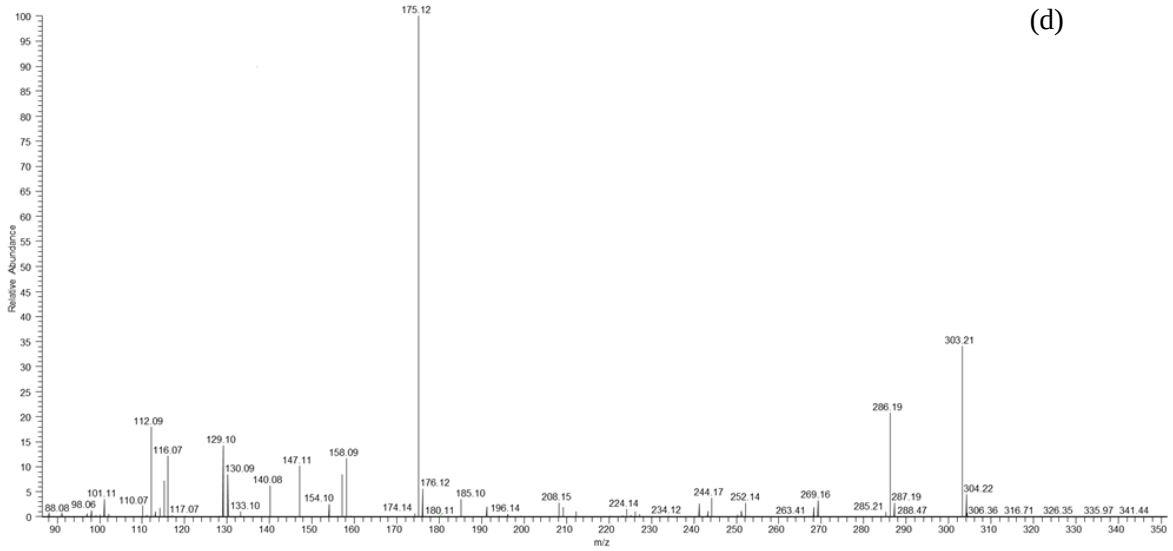

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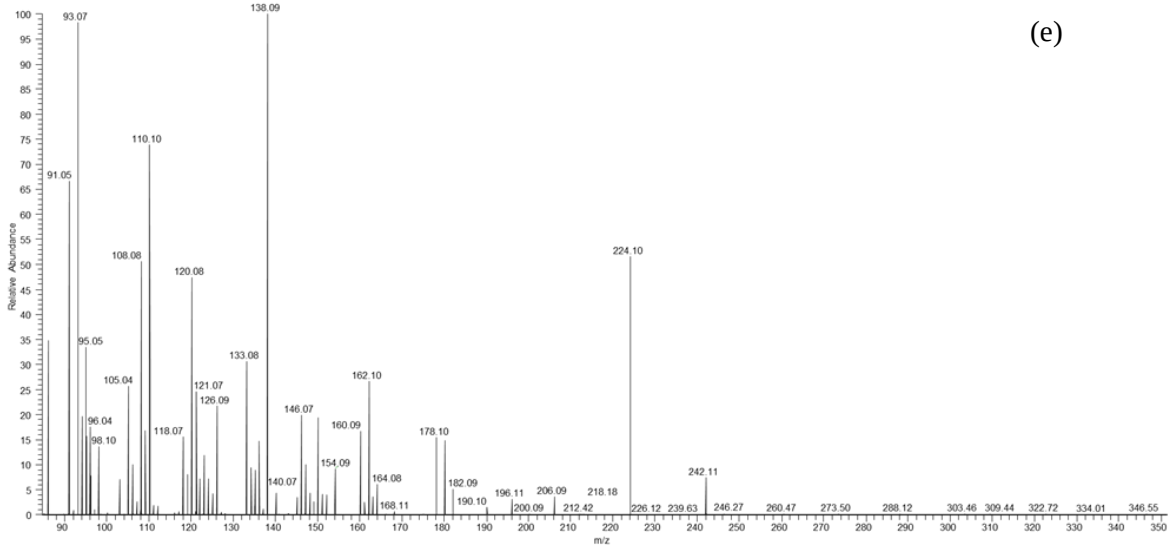

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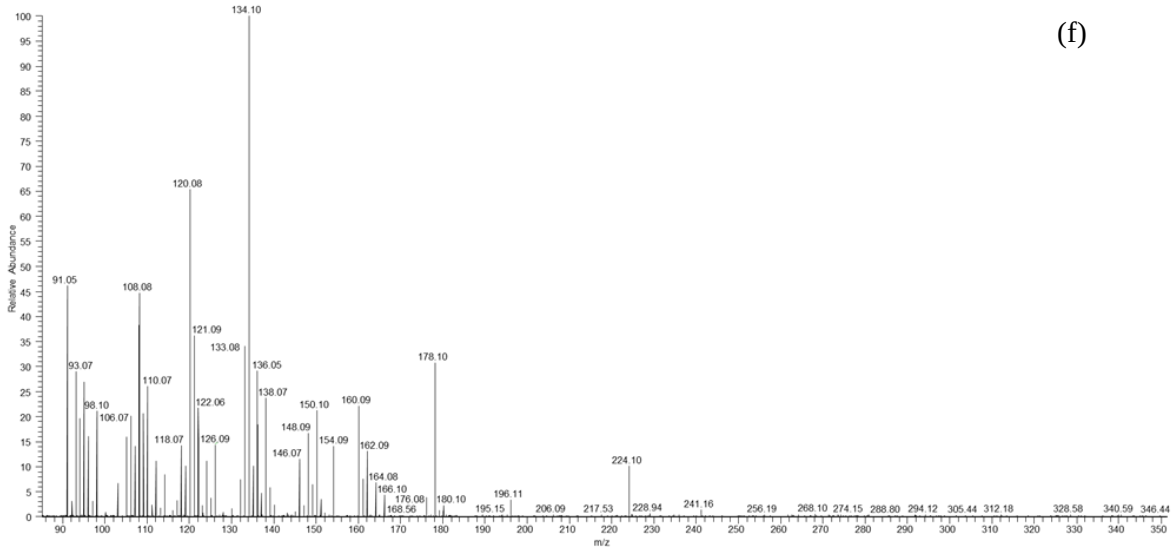

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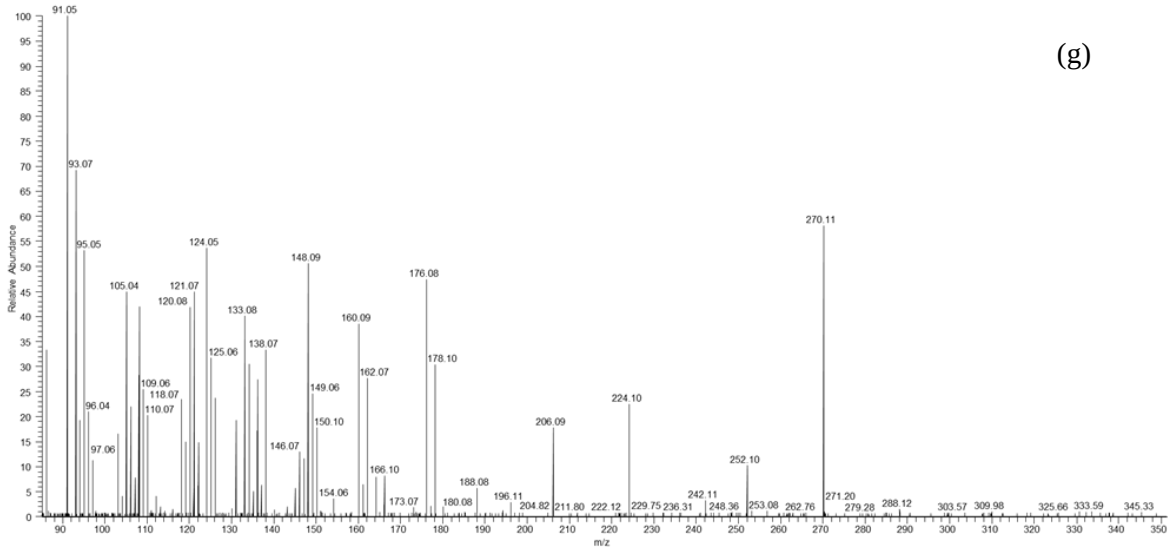

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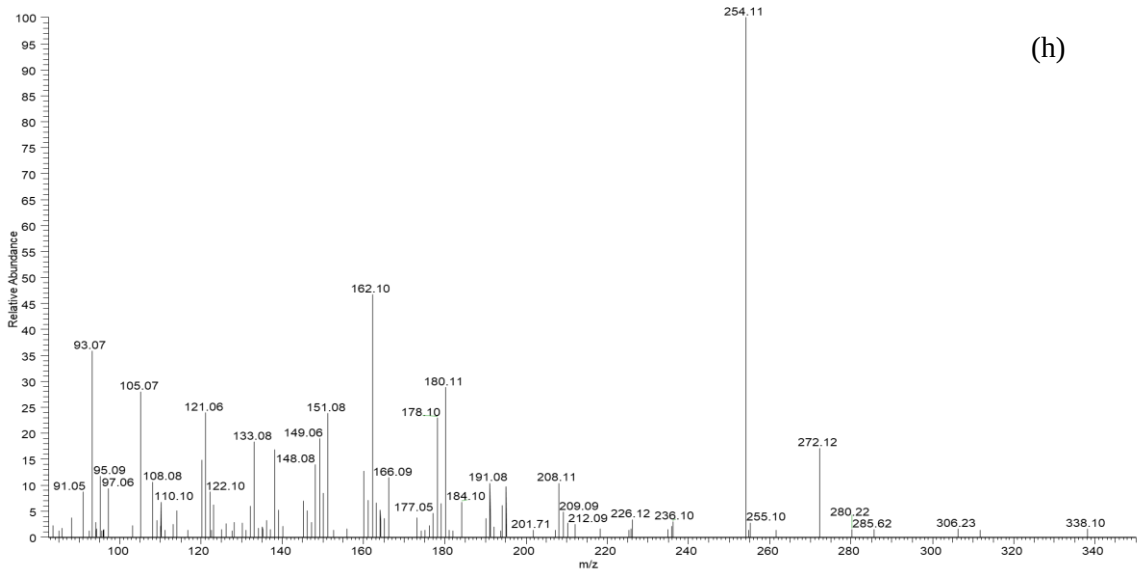
(h)



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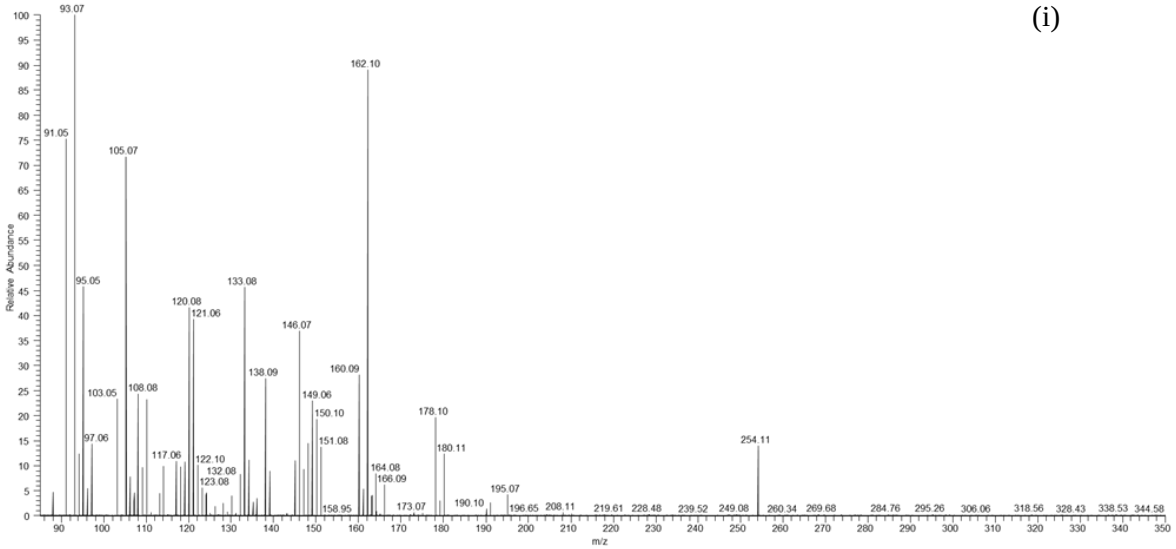

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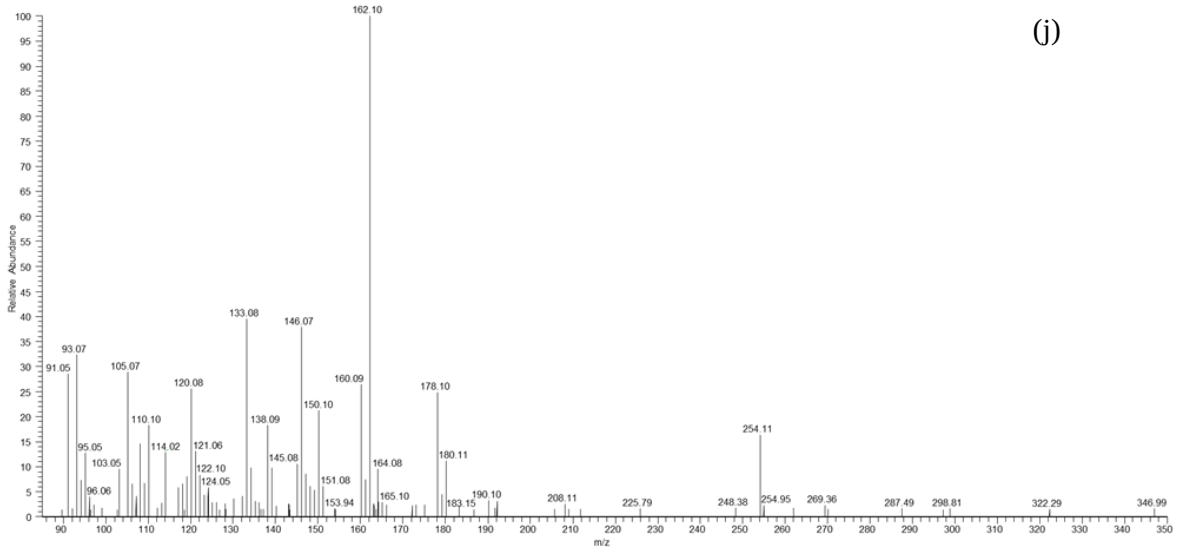

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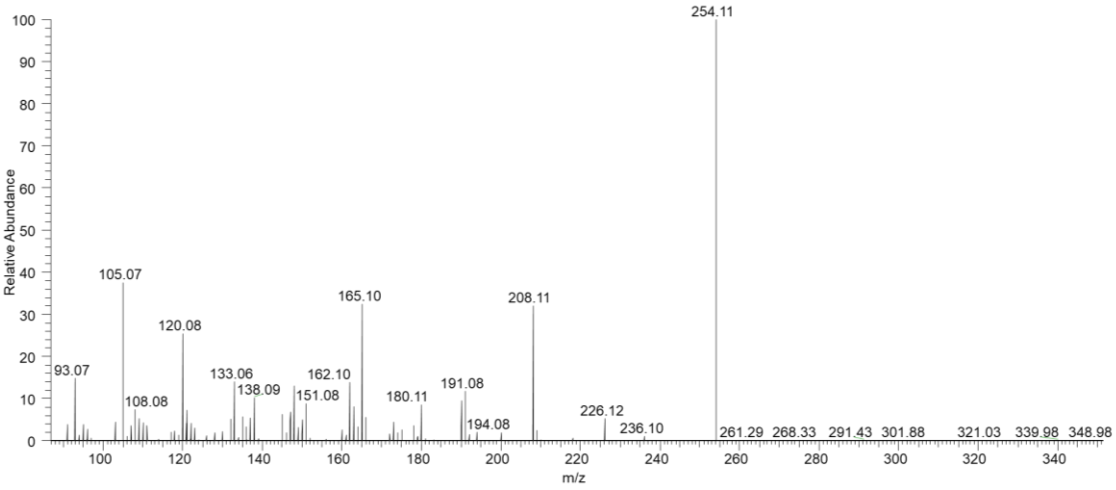

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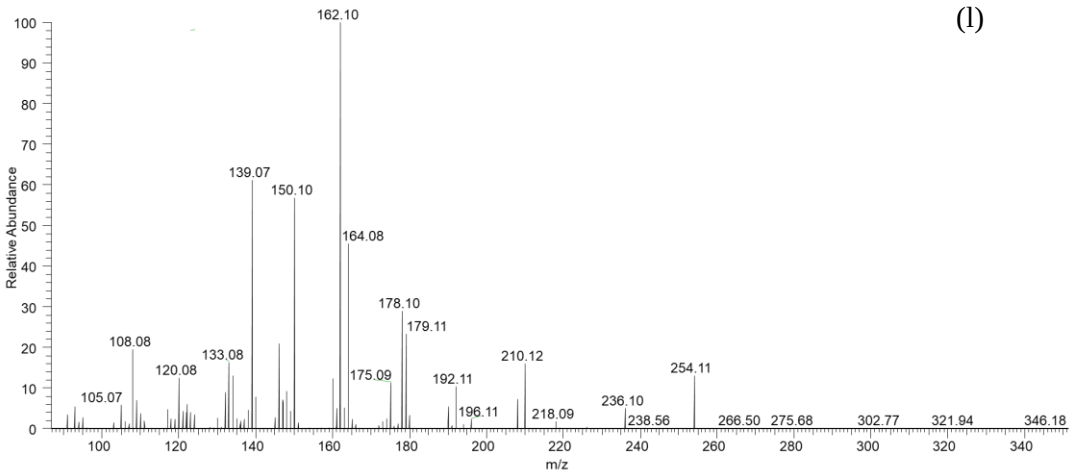
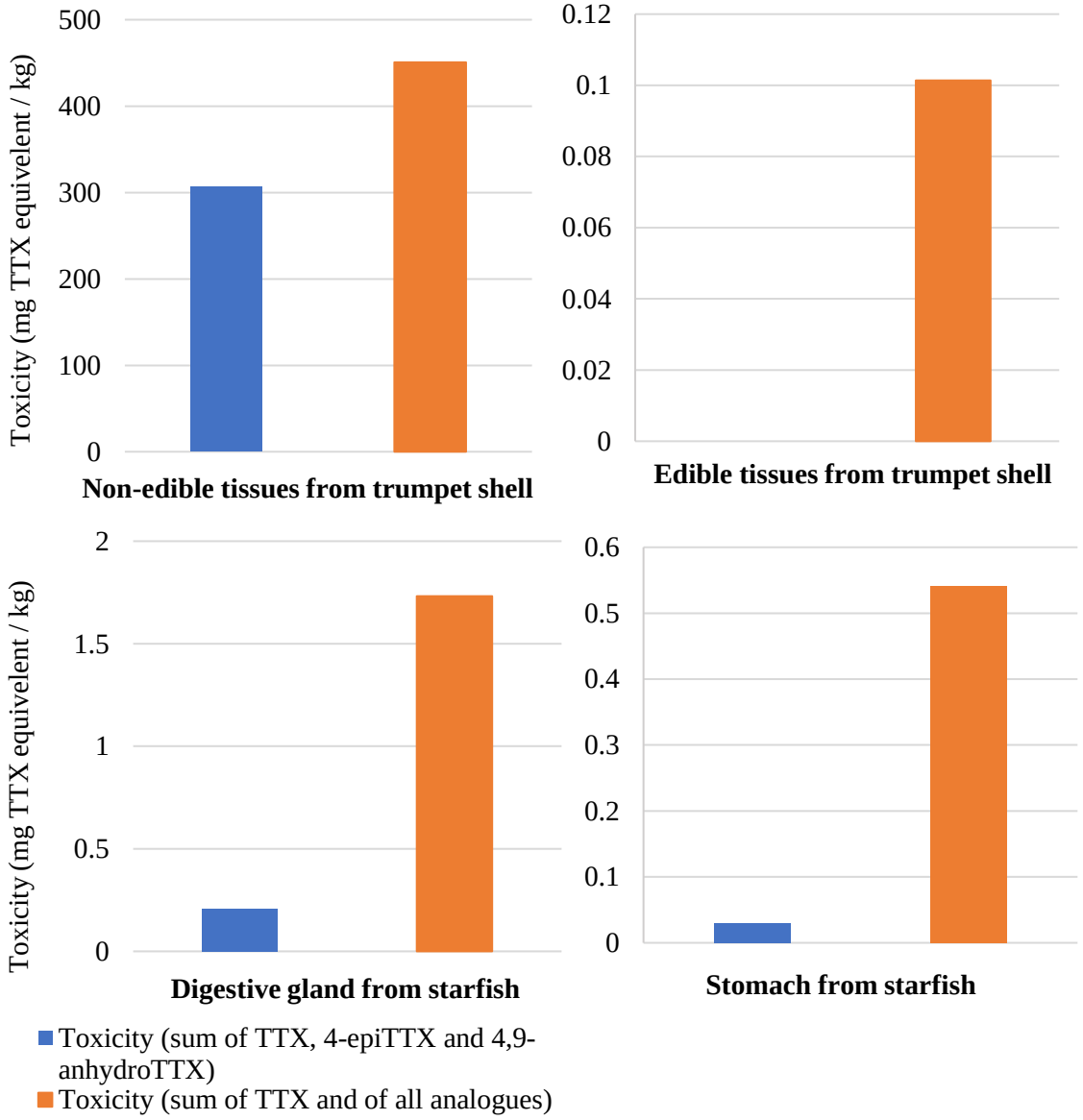


Annex 3. Comparison of the toxicity containing only the certified reference materials (the sum of TTX, 4-epiTTX and 4,9-anhydroTTX) and the toxicity containing the TTX and all of the other quantifiable analogues.



Annex 4. TTX and analogues *p*-values and tau correlation coefficient for *C. lampas* non-edible tissues. W – weight in g; L – length in cm, and D – depth in cm. Toxicity means the sum of compounds with certified reference material (TTX, 4-epiTTX, and 4,9-anhydroTTX), and the rest of the results correspond to the analysis using the toxicity of each analogue individually.

	TTX		**4-epiTTX**		**4,9-anhydroTTX**		**Toxicity**		**deoxyTTX1**	
	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**
W	0.3	-	0.6	-	0.3	-	0.5	-	0.5	-
L	0.3	-	0.5	-	0.4	-	0.4	-	0.7	-
D	0.9	-	0.8	-	0.8	-	0.9	-	0.7	-
	deoxyTTX2		**deoxyTTX3**		**dideoxyTTX1**		**dideoxyTTX2**		**dideoxyTTX3**	
	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**
W	0.3	-	0.4	-	0.2	-	0.9	-	0.3	-
L	0.3	-	0.9	-	0.3	-	0.7	-	0.7	-
D	0.8	-	0.3	-	0.6	-	0.5	-	0.3	-
	trideoxyTTX1		**trideoxyTTX2**		**trideoxyTTX3**		**anhydrotrideoxyTTX1**		**anhydrotrideoxyTTX2**	
	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**
W	0.1	-	0.1	-	0.2	-	0.5	-	0.1	-
L	0.3	-	0.2	-	0.4	-	0.4	-	0.3	-
D	0.9	-	0.7	-	0.8	-	0.9	-	0.6	-

Annex 5. TTX and analogues *p*-values and tau correlation coefficient for *A. aranciacus* digestive gland. W – weight in g; R – length of the arm as measured from the center of the animal to the tip of the arm (cm); r –radius of the disk measured from the center of the animal to the nutch between the arms (cm), and D – depth in cm. The underlined *p*-values represent the existence of a significant correlation, with the respective tau. Toxicity means the sum of compounds with certified reference material (TTX, 4-epiTTX, and 4,9-anhydroTTX), and the rest of the results correspond to the analysis using the toxicity of each analogue individually.

	Toxicity		**dideoxyTTX3**		**trideoxyTTX1**		**trideoxyTTX2**	
	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**
W	0.1	-	0.9	-	0.9	-	0.8	-
R	<u>0.02</u>	0.4	0.9	-	0.5	-	0.3	-
r	0.2	-	0.4	-	0.6	-	0.2	-
D	<u>0.03</u>	0.4	0.2	-	0.8	-	0.6	-
	trideoxyTTX3		**anhydrotrideoxyTTX1**		**anhydrotrideoxyTTX2**			
	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**		
W	0.8	-	0.7	-	0.5	-		
R	<u>0.03</u>	-0.3	0.2	-	0.9	-		
r	0.2	-	0.4	-	0.9	-		
D	<u>0.02</u>	-0.3	0.4	-	0.9	-		

Annex 6. TTX and analogues *p*-values and tau correlation coefficient for *A. aranciacus* stomach (including stomach content). W – weight in g; R – length of the arm as measured from the center of the animal to the tip of the arm (cm); r –radius of the disk measured from the center of the animal to the nutch between the arms (cm), and D – depth in cm. The underlined *p*-values represent the existence of a significant correlation, with the respective tau.

	dideoxyTTX3		**trideoxyTTX3**		**anhydrotrideoxyTTX1**		**anhydrotrideoxyTTX2**	
	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**	***p*-value**	**tau**
W	0.9	-	0.6	-	0.3	-	0.6	-
R	0.2	-	0.5	-	0.3	-	0.8	-
r	0.2	-	0.2	-	0.7	-	0.9	-
D	<u>0.005</u>	0.5	0.5	-	0.6	-	0.9	-

Annex 7. TTX and analogues *p*-values for Kruskal-Wallis non-parametric test for months and seasons of both species studied. Blank spaces represent compounds non-detected or <LOQ in that tissue. Toxicity means the sum of compounds with certified reference material (TTX, 4-epiTTX, and 4,9-anhydroTTX), and the rest of the results correspond to the analysis using the toxicity of each analogue individually.

Compounds	**Tissues**					
	Non-edible (*C.lampas*)		Digestive gland (*A. aranciacus*)		Stomach (*A. aranciacus*)	
	Months	**Seasons**	**Months**	**Seasons**	**Months**	**Seasons**
TTX	0.6	0.4	-	-	-	-
4-epiTTX	0.4	0.4	-	-	-	-
4,9-anhydroTTX	0.5	0.3	-	-	-	-
Toxicity	0.5	0.4	0.7	0.4	-	-
deoxyTTX1	0.3	0.3	-	-	-	-
deoxyTTX2	0.4	0.3	-	-	-	-
deoxyTTX3	0.5	0.4	-	-	-	-
dideoxyTTX1	0.5	0.3	-	-	-	-
dideoxyTTX2	0.3	0.4	-	-	-	-
dideoxyTTX3	0.3	0.4	0.5	0.4	0.9	0.9
trideoxyTTX1	0.5	0.4	0.6	0.5	-	-
trideoxyTTX2	0.7	0.5	0.5	0.5	-	-
trideoxyTTX3	0.2	0.4	0.2	0.4	0.4	0.7
anhydrotrideoxyTTX1	0.5	0.5	0.5	0.5	0.3	0.3
anhydrotrideoxyTTX2	0.4	0.5	0.2	0.3	0.2	0.2