Science of the Total Environment 885 (2023) 163905

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Sea temperature influences accumulation of tetrodotoxin in British bivalve shellfish

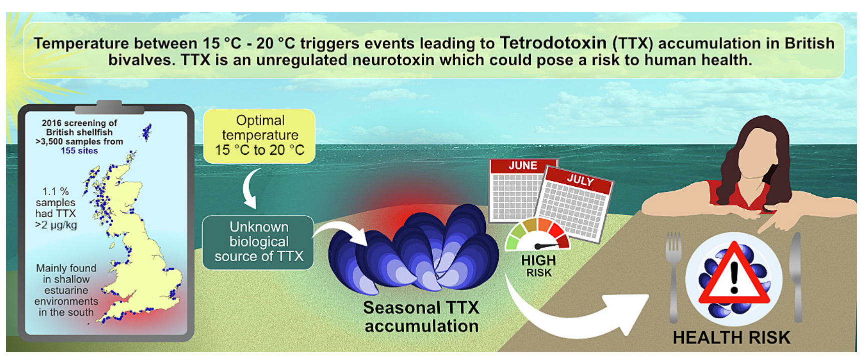
Monika Dhanji-Rapkova [a,b,*], Mickael Teixeira Alves [a], Joaquin A. Triñanes [c,d,e], Jaime Martinez-Urtaza [f], David Haverson [g], Kirsty Bradley [g], Craig Baker-Austin [a], Jim F. Huggett [h], Graham Stewart [b], Jennifer M. Ritchie [b,*], Andrew D. Turner [a]

[a] Centre for Environment, Fisheries and Aquaculture Science (Cefas), The Nothe, Barrack Road, Weymouth DT4 8UB, United Kingdom
[b] Faculty of Health and Medical Sciences, University of Surrey, Guildford GU2 7XH, United Kingdom
[c] Laboratory of Systems, Department of Electronics and Computer Sciences, Universidade de Santiago de Compostela, Campus Universitario Sur, Santiago de Compostela, Spain
[d] National Oceanic and Atmospheric Administration, Atlantic Oceanographic and Meteorological Laboratory, 4301 Rickenbacker Causeway, Miami, FL 33149, USA
[e] Rosenstiel School of Marine and Atmospheric Science, University of Miami, Cooperative Institute for Marine and Atmospheric Studies, 4600 Rickenbacker Causeway, Miami, FL 33149, USA
[f] Department of Genetics and Microbiology, Facultat de Biociències, Universitat Autònoma de Barcelona, Barcelona, Spain
[g] Centre for Environment, Fisheries and Aquaculture Science (Cefas), Pakefield Road, Lowestoft, NR33 0HT, United Kingdom
[h] National Measurement Laboratory, LGC, Teddington TW11 0LY, United Kingdom

HIGHLIGHTS

- Large field study testing >3500 shellfish samples for neurotoxin tetrodotoxin (TTX).
- Low prevalence and seasonality of TTX in British bivalve shellfish.
- TTX-positive bivalves were mainly found in southern England coastal areas.
- *In situ* and satellite-derived data imply sea temperature affects TTX accumulation.
- Shellfish sites with greater sea temperature variance may be at more risk from TTX.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Daniel Wunderlin

Keywords:
Tetrodotoxin
Bivalve shellfish
Sea temperature
Great Britain

ABSTRACT

Tetrodotoxin (TTX), a potent neurotoxin mostly associated with pufferfish poisoning, is also found in bivalve shellfish. Recent studies into this emerging food safety threat reported TTX in a few, mainly estuarine, shellfish production areas in some European countries, including the United Kingdom. A pattern in occurrences has started to emerge, however the role of temperature on TTX has not been investigated in detail. Therefore, we conducted a large systematic TTX screening study, encompassing over 3500 bivalve samples collected throughout 2016 from 155 shellfish monitoring sites along the coast of Great Britain. Overall, we found that only 1.1 % of tested samples contained TTX above the reporting limit of 2 µg/kg whole shellfish flesh and these samples all originated from ten shellfish production sites in southern England. Subsequent continuous monitoring of selected areas over a five-year period showed a potential seasonal TTX accumulation in bivalves, starting in June when water temperatures reached around 15 °C. For the first time, satellite-derived data were also applied to investigate temperature differences between sites with and without confirmed presence of TTX in 2016. Although average annual temperatures were similar in both groups, daily mean values were higher in summer and lower in winter at sites where TTX was found. Here, temperature also increased significantly faster during late spring and early summer, the critical period for TTX. Our study supports the hypothesis that temperature is one of the key triggers of events leading to TTX accumulation in European bivalves.

* Corresponding authors.
E-mail addresses: monika.dhanjirapkova@cefas.gov.uk (M. Dhanji-Rapkova), j.ritchie@surrey.ac.uk (J.M. Ritchie).

http://dx.doi.org/10.1016/j.scitotenv.2023.163905
Received 20 January 2023; Received in revised form 21 April 2023; Accepted 28 April 2023
Available online 3 May 2023

However, other factors are also likely to play an important role, including the presence or absence of a *de novo* biological source, which remains elusive.

1. Introduction

Tetrodotoxin (TTX) is a potent neurotoxin found in several phylogenetically distant animal species, including starfish, marine worms as well as terrestrial amphibians (reviewed in (Bane et al., 2014; Chau et al., 2011; Noguchi et al., 2011)). TTX in edible marine animals, like pufferfish (family *Tetraodontidae*), sea snails (predominantly from family *Nassaridae*) and arthropods (*Carcinoscorpius rotundicauda*), account for 97 % of human intoxication cases, including fatalities (Guardone et al., 2019; Katikou et al., 2022; Noguchi and Arakawa, 2008). The poisoning risk is especially high in southeast and east Asia due to consumption of locally sourced contaminated seafood. In contrast, 13 out of 14 TTX intoxication cases in Europe were caused by consumption of fish imports (Guardone et al., 2019; Katikou et al., 2022). However, there is now growing evidence for TTX presence in edible marine species in subtropical and temperate waters around Europe. Partly, this is due to the rapid establishment of a TTX-bearing invasive pufferfish *Lagocephalus sceleratus* (*Tetraodontidae*) in the Mediterranean following its discovery off the Turkish coast in 2003 (Akyol et al., 2005). In 2007, a TTX-poisoning case was recorded in Spain after consumption of a Portuguese-sourced gastropod *Charonia lampas* (Fernández-Ortega et al., 2010; Rodriguez et al., 2008). More concerns about seafood safety were raised in 2015, when TTX in bivalve shellfish were reported for the first time in Europe (Turner et al., 2015; Vlamis et al., 2015). To safeguard human health, a complete ban or restrictions on trading pufferfish have been adopted in several countries worldwide, including within the European Union (EU) (Anon, 2019). However, there is currently no legislative requirement to monitor TTX levels in bivalves, except in the Netherlands. The maximum permitted level (MPL) of 44 μg TTX eq./kg of bivalve flesh was adopted by the Dutch national biotoxin monitoring programme (Gerssen et al., 2018) following publication of the scientific opinion on TTX by the European Food Safety Authority (EFSA) (EFSA et al., 2017).

The first findings of TTX in European shellfish prompted two large-scale (~1000 sample) screening studies (Gerssen et al., 2018; Turner et al., 2017b) and several smaller-scale investigations. TTX has now been reported in bivalve shellfish of several European countries, although only a low proportion of all examined samples were found to contain TTX (reviewed in (Biessy et al., 2019a; Katikou et al., 2022). Notably, most TTX occurrences appear confined to specific locations within each country: Eastern Scheldt in Netherlands, (Gerssen et al., 2018), several estuarine sites and bays in the southern England (Turner et al., 2017b), northern France (Réveillon et al., 2021) and north Aegean Sea in Greece (Vlamis et al., 2015), as well in the Mediterranean lagoons Marano in Italy (Bordin et al., 2021) and Ingril lagoon in France (Hort et al., 2020). One common feature of these marine environments seems to be their relatively shallow water depth and low water refreshment rate. Additionally, TTX accumulation in European bivalves appears seasonal, with the main period lasting several weeks in the early summer months (Gerssen et al., 2018; Hort et al., 2020; Réveillon et al., 2021; Turner et al., 2017b; Vlamis et al., 2015). The observed seasonality has prompted discussion about the link between sea surface temperature (SST) and TTX occurrence, however the lack of continuous SST monitoring data and appropriate statistical analyses has limited such assessment. To date, the environmental, biological or physical conditions that promote TTX production and subsequent accumulation in bivalves remain undefined.

Although a pattern in geographical and seasonal distribution of TTX in European shellfish has started to emerge, this has not been systematically investigated. In this study, we used large TTX and SST datasets to test the hypothesis that TTX accumulation in British bivalves is affected by temperature. Firstly, TTX analysis was conducted on >3500 shellfish samples collected from around the coast of Britain in 2016, enabling assessment of TTX prevalence and geographical distribution. Then, temporal distribution was explored by collating data from four shellfish production areas with a history of TTX over a five-and-a-half-year period. Finally, continuous temperature records were obtained from 2016 for locations with and without TTX-positive shellfish and compared, to better understand how the sea temperature profile influences TTX accumulation in British bivalve shellfish. The findings from this study thus serve as a basis for uncovering one of the key variables leading to TTX accumulation in bivalves and contributes to the wider discussion about associated risk for the shellfish consumer and the industry.

2. Material and methods

2.1. Bivalve samples

Samples received through the Official Control (OC) testing of bivalves for the presence of regulated marine biotoxins were used in this study with the permission of relevant authorities. Live animals were collected by either dedicated shellfish sampling officers, or by Local Authorities from 155 Representative Monitoring Sites (RMSs) within classified shellfish productions areas in England, Wales and Scotland (Fig. 1). Sites targeted for further TTX investigations were anonymised to protect the shellfish producers. Species collected included common mussels (*Mytilus spp.*), Pacific oysters (*Crassostrea gigas*), native oysters (*Ostrea edulis*), hard clams (*Mercenaria mercenaria*), manila clams (*Ruditapes philippinarum*), cockles (*Cerastoderma edule*), razors (*Ensis spp.*) and surf clams (*Spisula solida*). In addition, king scallops (*Pecten maximus*) were received through the OC monitoring programme for verification of wild *Pectenidae*. The samples were utilised as follows:

1. To assess the overall prevalence of TTX in British bivalve shellfish, systematic TTX screening was conducted on archived OC samples collected in 2016. This year was selected due to a higher-than-usual number of samples collected as part of a regulated biotoxin risk assessment exercise in English and Welsh RMSs (~25 % increase compared to previous or following years). In total, 4194 samples were received over the year, with 3514 undergoing TTX analysis. This data set includes 154 samples from seven production areas in England, which were analysed in a previous targeted TTX screen (Turner et al., 2017b). Notably, all but 22 samples collected between May and September, the period with higher risk of TTX accumulation, were subject to TTX analysis. Testing was not performed on these samples due to the lack of an archived sample extract or flesh homogenate.
2. To examine temporal distribution of TTX, a targeted screening was conducted on samples from areas with TTX over a period of five and a half years. Based on the results from 2016 screen, seven RMSs contained TTX above LOR in shellfish samples on at least two different days, and were therefore identified as suitable for a multi-year analysis. However, one RMS did not have SST measurements taken at the time of sampling, while another two RMSs had OC sampling suspended for spring and summer months in each year since 2018. Thus, these three sites could not be included in our dataset. In total, 371 samples from four RMSs were analysed between November 2016 and December 2020, and the results from the same areas were combined with previously published data collated prior to November 2016 (Turner et al., 2017b).
3. The effect of SST was assessed by comparing the sea temperature profiles of RMSs found to yield TTX-positive and TTX-negative bivalve samples. All six RMSs, where TTX was found above LOR in more than two shellfish samples during 2016, and for which satellite-derived SST

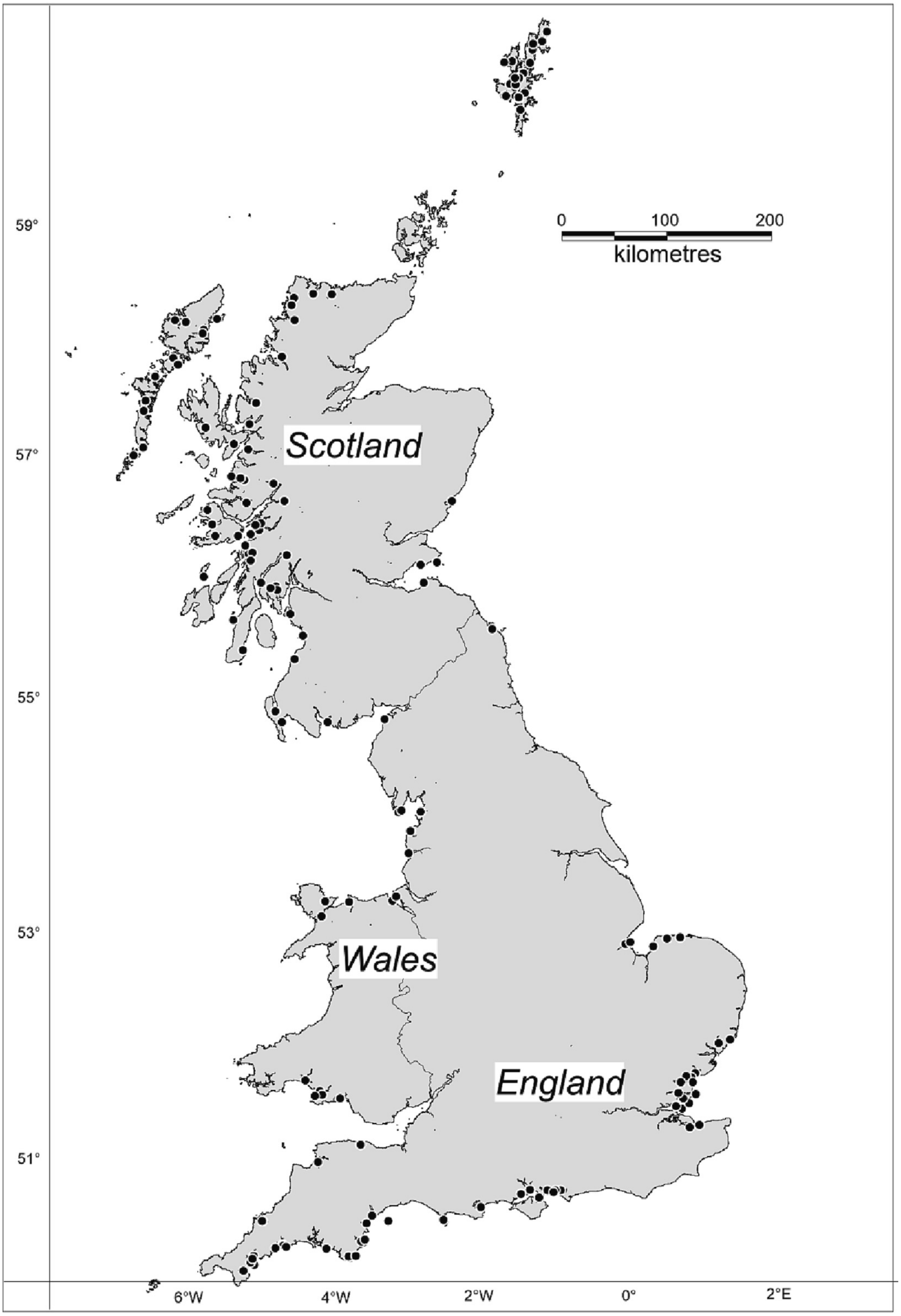


Fig. 1. Map of Great Britain showing shellfish representative monitoring sites (RMSs).
RMSs from classified production areas active during the study period. The location of sites associated with TTX presence could not be visualised due to commercial sensitivities and conditions upon which permission to use shellfish samples for this study was granted. The map was created using licenced Mapinfo software.

were available, were included in the TTX-positive group, while RMSs forming the TTX-negative group (sites where TTX had not been detected) were 'matched' as closely as possible in terms of water depth, location in southern England, frequency of sampling and bivalve species. Mussels (*Mytilus spp.*), Pacific oysters (*Crassostrea gigas*), and native oysters (*Ostrea edulis*) were found in both site groups (with and without TTX), while hard clams (*Mercenaria mercenaria*) were present in one group. We endeavoured to assess the effect of salinity on TTX occurrences, however salinity data for these sites lacked sufficient resolution for use in the analyses.

2.2. Tetrodotoxin analysis

Each bivalve sample represented a minimum of 10 live animals, which were shucked, pooled, and homogenised generally within 48 h of collection. Whole flesh homogenates were prepared for statutory biotoxin analyses, including Paralytic Shellfish Toxins (PSTs). Acidic extracts obtained through a two-step procedure used for PST analysis (Turner et al., 2014) were found to be comparable to dispersive extraction as described in Boundy et al. (2015) and suitable for TTX analysis in bivalves (Turner et al., 2017a). Therefore, archived PST extracts (stored at < −15 °C) from OC testing were subjected to Solid Phase Extraction (SPE) carbon clean-up as described by Turner et al. (2017a). Repeated analysis of TTX-positive PST extracts kept at < −15 °C for nearly three years indicated that TTX was stable in sample extracts stored under these conditions. In instances when PST extracts were not available, archived shellfish homogenates (stored at < −15 °C) were extracted using dispersive extraction (Turner et al., 2017a). This extraction procedure was applied to 587 samples (16.7 %) out of 3514 tested samples. The SPE cleaned extracts were then diluted with acetonitrile (1:3 ratio) and subject to TTX analysis by Liquid Chromatography—Tandem Mass Spectrometry as described below (Section 2.2.2).

2.2.1. Reagents and chemicals

Reagents for the sample extraction and subsequent SPE sample clean-up were HPLC grade or equivalent. Reagents for mobile phases and instrument washes such as water, acetonitrile, formic acid and 25–31 % ammonium hydroxide were of LC-MS grade. TTX certified reference material (CRM) was purchased from Cifga (Lugo, Spain) to prepare a TTX stock solution. The content of one Cifga TTX CRM ampoule was diluted 1:10 with 0.25 % acetic acid in deionised water and stored at < −15 °C. The stock was diluted ten-fold using 0.25 % acetic acid in 80 % acetonitrile to prepare TTX calibration solutions over six concentration levels (0.04–64.75 ng/mL). Once prepared, the calibration solutions were kept in the refrigerated autosampler (< 10 °C) and used within one week. In the absence of certified control materials in shellfish matrix, several samples have been utilised as TTX-positive controls for various stages of TTX-analysis. These include a non-certified mussel reference material (National Research Council Canada, Halifax, Canada), a Retention Time Marker (Cawthron Natural Compounds, Nelson, New Zealand) and a Laboratory Reference Material prepared in-house, all containing TTX and a range of TTX analogues as described before (Dhanji-Rapkova et al., 2021; Turner et al., 2020, Turner et al., 2017a).

2.2.2. Liquid chromatography—tandem mass spectrometry

An Agilent (Manchester, UK) 1290 Ultra High-Performance Liquid Chromatography system coupled to an Agilent 6495B tandem quadrupole mass spectrometer (MS/MS) was used for TTX analysis. Hydrophilic Interaction Liquid Chromatography (HILIC) column (Acquity BEH Amide, 1.7 μm, 2.1 × 150 mm) was utilised in conjunction with a VanGuard BEH Amide guard cartridge (Waters Ltd., UK). Mobile phase composition, mobile phase gradients, injection volume, autosampler and column temperature settings were as described by Turner et al. (2017a, 2018), with slight modifications of the gradient to suit the Agilent instrument. MS/MS conditions of the ESI interface were as follows: gas temperature 150 °C, gas flow 15 L/min, nebulizer gas 50 psi, sheath gas temperature 400 °C, sheath gas flow 12 L/min, Capillary voltage 2500 V. Multiple Reaction Monitoring (MRM) transitions for TTX and 4-epi-TTX (320.1 > 302.1, 162.1) were acquired in the positive ionisation mode. MRMs for several TTX analogues were acquired in the same analytical run alongside, as described elsewhere (Dhanji-Rapkova et al., 2021; Turner et al., 2018; Turner et al., 2017a).

2.2.3. Tetrodotoxin data analysis

TTX data were analysed using Agilent Technologies MassHunter Workstation software (version V.B.10.00). TTX was quantified against Cifga calibration solutions and weighting 1/X was applied for all calibration curves. The presence of TTX was confirmed by comparing retention times of MRM peaks and their associated ion ratios against those established in the calibration solutions and the TTX-positive control materials as specified in Reagents and chemicals section. Even though multiple TTX analogues were measured, only a few of them were found in bivalves and these were always in low proportion in relation to TTX. Where TTX analogues were reported, no toxicity equivalent factors were applied. TTX, as the predominant toxin, was used to assess overall TTX prevalence in 2016, interannual variability in selected RMSs between 2015 and 2020, or to categorise RMSs as "TTX-positive" or "TTX-negative" for subsequent comparison of SST profiles between these two types of sites. The concentration data were not adjusted for recovery, however recoveries for sample processing and TTX analysis using this method were previously reported to be between 66 and 84 % in whole flesh of mussel and oyster matrices (Dhanji-Rapkova et al., 2021; Turner et al., 2017a). For these matrices, the Limit of Detection (LOD) and the Limit of Quantitation (LOQ) were approximately 0.2 μg/kg and 0.8 μg/kg, respectively (Dhanji-Rapkova et al., 2021; Turner et al., 2018, Turner et al., 2017a). In order to align TTX reporting with our previous studies, while taking into account possible LOQ variation for other shellfish matrices, a Limit of Reporting (LOR) of 2 μg/kg was adopted (Turner et al., 2017a, 2017b).

2.3. Tide-corrected sea depth

Bathymetric elevations at the shellfish sampling locations were extracted from the Environment Agency's 2019 Surfzone Digital Elevation Model (Environment Agency, 2022), a combination of LIDAR and near-shore multibeam SONAR bathymetry elevation data. The vertical datum is with respect to Ordnance Datum Newlyn (ODN).

2.4. In situ and satellite-derived temperature data

In situ temperature data were recorded at the time of shellfish collection by dedicated sampling officers through OC monitoring programme. Various methods are used throughout the UK for measuring temperature, depending on the geographical area and available resources, with the majority utilising handheld, calibrated electronic temperature monitoring probes. When the shellfish were collected from water, the temperature of the surrounding seawater was measured and recorded. If the shellfish were not submerged in water, for instances of inter-tidal shellfish which are sampled dry, the temperature of the bagged shellfish was taken and recorded instead. However, temperature of shellfish was not used in any of our *in-situ* SST comaprisons.

Satellite-derived SST data were obtained from the Group for High Resolution Sea Surface Temperature (GHRSST) using the Multiscale Ultrahigh Resolution (MUR) analysis based on 1 km resolution for selected shellfish production sites. The "SST Difference" dataset in 2016 was created by subtracting the overall lowest daily mean SST value from each daily mean SST data.

2.5. Statistical analysis of satellite-derived temperature data

Temperature profiles of sites with or without detectable TTX in bivalves were compared using a *t*-test. A two-way analysis of variance (ANOVA) was also performed on SST data to test the interaction between TTX status and time. The rate of temperature change between TTX groups was estimated and between group differences in linear regression slope coefficients were compared for each month. Statistical analyses of temperature data were performed using the statistical software R (R Core Team, 2022). The level of significance was set at $p \leq 0.05$ for all statistical tests.

3. Results

3.1. Prevalence and spatial distribution of tetrodotoxin in British bivalves during 2016

In total, 3514 archived bivalve samples, collected in 2016 from 155 sites around Great Britain, were tested for TTX. This constituted 84 % of all received OC samples during 2016, and 97 % - 100 % of samples received each month between May and September 2016, the historical high-risk season (Table 1).

Out of all samples analysed for TTX, only 41 (1.1 %) samples contained TTX above the LOR (2 μg/kg), with a further 42 samples containing TTX below this level but above LOD (Table 1). All 41 shellfish samples with TTX above LOR originated from England, and more specifically from ten production areas in southern England. All sites with TTX above LOD and LOR are listed in Table A.1, however information about their exact location or species tested could not be included due to commercial sensitivities. TTX prevalence in England was 3.6 %, while no TTX was detected in Wales and only two samples with TTX below LOR were recorded in Scotland. Overall TTX prevalence was low, suggesting a low risk of TTX in shellfish from Scotland, Wales, and from the majority of sites in England.

3.2. Temporal distribution and concentrations of tetrodotoxin in bivalves from selected sites

Analysis of samples from 2016 revealed that the highest number of samples with TTX, and the highest concentrations, were observed in June and July (Table 1). To better understand temporal distribution of the toxin, four shellfish sites were monitored over a five-and-a half-year period (Fig. 2). The accumulation of TTX in bivalves was found to be potentially seasonal in each year, with increased concentrations measured between June and August, reaching a peak generally by the end of June or beginning of July. Concentrations below LOR were measured on occasion during colder months but spikes in TTX above this level were rare. The highest TTX concentrations were consistently recorded at Site 1, indicating this location had more favourable conditions for TTX accumulation in bivalves compared to the other monitored sites (Fig. 2). TTX maxima at Site 1 exceeded 93 μg/kg in each year between 2015 and 2018, while in the next two years, TTX stayed below 30 μg/kg. The highest TTX level of 202 μg/kg was recorded on 26th June 2018. Multiple TTX analogues were also present in small proportions in this sample, amounting to total TTXs of 270 μg/kg (quantified using TTX due to absence of certified reference materials for TTX analogues). The most abundant analogue was 6,11-dideoxy TTX, followed by 5,6,11-trideoxy TTX, while low (< 5 μg/kg) levels were observed for 5-deoxy TTX, 11-deoxy TTX and 4,9-anhydro TTX. Overall, our findings show considerable inter-annual differences in TTX accumulation within and between locations.

3.3. Effect of sea surface temperature on tetrodotoxin accumulation

3.3.1. In situ sea surface temperature at selected sites with tetrodotoxin

In the four sites selected for more extensive monitoring, TTX concentrations peaked at the end of June/early July, coinciding with SST increasing above 15 °C (Fig. 2). Further increases in SST in July and August did not lead to further increases in TTX concentration. On the contrary, by the time SST reached its peak in July and August, TTX concentrations had decreased (Fig. 2). Thus, the relationship between these two variables was not linear ($R^2 = 0.026$) and nearly 76 % of samples with TTX concentrations > LOR fell into a temperature category between 15 °C and 20 °C (Table 2). Twelve samples with TTX > LOR were associated with SST < 15 °C and these were investigated further. Seven of these samples were collected between September and April outside of the main TTX accumulation period, with all but one (Site 2: 19.9 μg/kg) containing relatively low TTX levels (< 4.5 μg/kg) (Table 3). Five other samples had TTX concentrations in the range of 6–115 μg/kg and were collected either in June or in July (Table 3). Although *in situ* SSTs were below 15 °C at Sites 3 and 4, satellite-derived SSTs on the same day (12th July) were recorded as above this temperature threshold (Table 3), as were previous weekly *in situ* SST measurements (Fig. 2C, D). Three samples at Site 1 also contained TTX > LOR but were collected when SSTs <15 °C in 2017 and 2018. A lack of continuous *in situ* or satellite-derived SST data for Site 1 prohibited further investigation, however it was noted that the samples and temperature measurements were taken early in the morning (before 9:30 am) (Table 3), most likely before SSTs had recovered from exposure to lower overnight air temperatures. Thus, in summary our data show strong evidence that increasing SST at the start of the summer period provides conditions conducive for TTX accumulation in bivalves at certain sites.

Table 1
Summary of tetrodotoxin (TTX) screen results following analysis of bivalve tissues collected around Great Britain (England, Wales and Scotland) during 2016.
The table summarises the overall number of samples received through Official Control biotoxin monitoring programme, the number of samples tested for TTX and TTX-positive bivalve samples, maximum TTX concentration (μg/kg) in each month and in 2016.

	Number of samples	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec	2016
England	received	75	90	89	87	100	121	112	129	114	105	92	53	1167
	tested for TTX	64	90	89	87	100	120	110	127	113	105	92	53	1150
	tested for TTX/received (%)	85 %	100 %	100 %	100 %	100 %	99 %	98 %	98 %	99 %	100 %	100 %	100 %	99 %
	TTX > LOD	3	3	7	3	4	12	20	8	2	3	7	9	81
	TTX > LOR	1	1	1	0	0	7	17	7	2	1	2	2	41
	Maximum TTX conc. [μg/kg]	20	4.8	4.6	<LOR	<LOR	93	78	11	7.7	10.6	5.0	3.2	93
Wales	received	7	7	8	12	14	14	12	15	14	10	8	9	130
	tested for TTX	7	7	8	12	14	12	11	15	14	10	8	9	127
	tested for TTX/received (%)	100 %	100 %	100 %	100 %	100 %	86 %	92 %	100 %	100 %	100 %	100 %	100 %	98 %
	TTX > LOD	0	0	0	0	0	0	0	0	0	0	0	0	0
	TTX > LOR	0	0	0	0	0	0	0	0	0	0	0	0	0
	Maximum TTX conc. [μg/kg]													
Scotland	received	97	120	293	272	293	289	270	331	257	282	272	121	2897
	tested for TTX	61	120	120	196	293	288	263	330	253	162	85	66	2237
	tested for TTX/received (%)	63 %	100 %	41 %	72 %	100 %	100 %	97 %	100 %	98 %	57 %	31 %	55 %	77 %
	TTX > LOD	0	0	0	0	1	1	0	0	0	0	0	0	2
	TTX > LOR	0	0	0	0	0	0	0	0	0	0	0	0	0
	Maximum TTX conc. [μg/kg]					<LOR	<LOR							<LOR
Great Britain	received	179	217	390	371	407	424	394	475	385	397	372	183	4194
	tested for TTX	132	217	217	295	407	420	384	472	380	277	185	128	3514
	tested for TTX/received (%)	74 %	100 %	56 %	80 %	100 %	99 %	97 %	99 %	99 %	70 %	50 %	70 %	84 %
	TTX > LOD	3	3	7	3	5	13	20	8	2	3	7	9	83
	TTX > LOR	1	1	1	0	0	7	17	7	2	1	2	2	41
	Maximum TTX conc. [μg/kg]	20	4.8	4.6	<LOR	<LOR	93	78	11	7.7	10.6	5.0	3.2	93

LOD = Limit of detection (0.2 μg/kg); LOR = Limit of reporting (2 μg/kg).

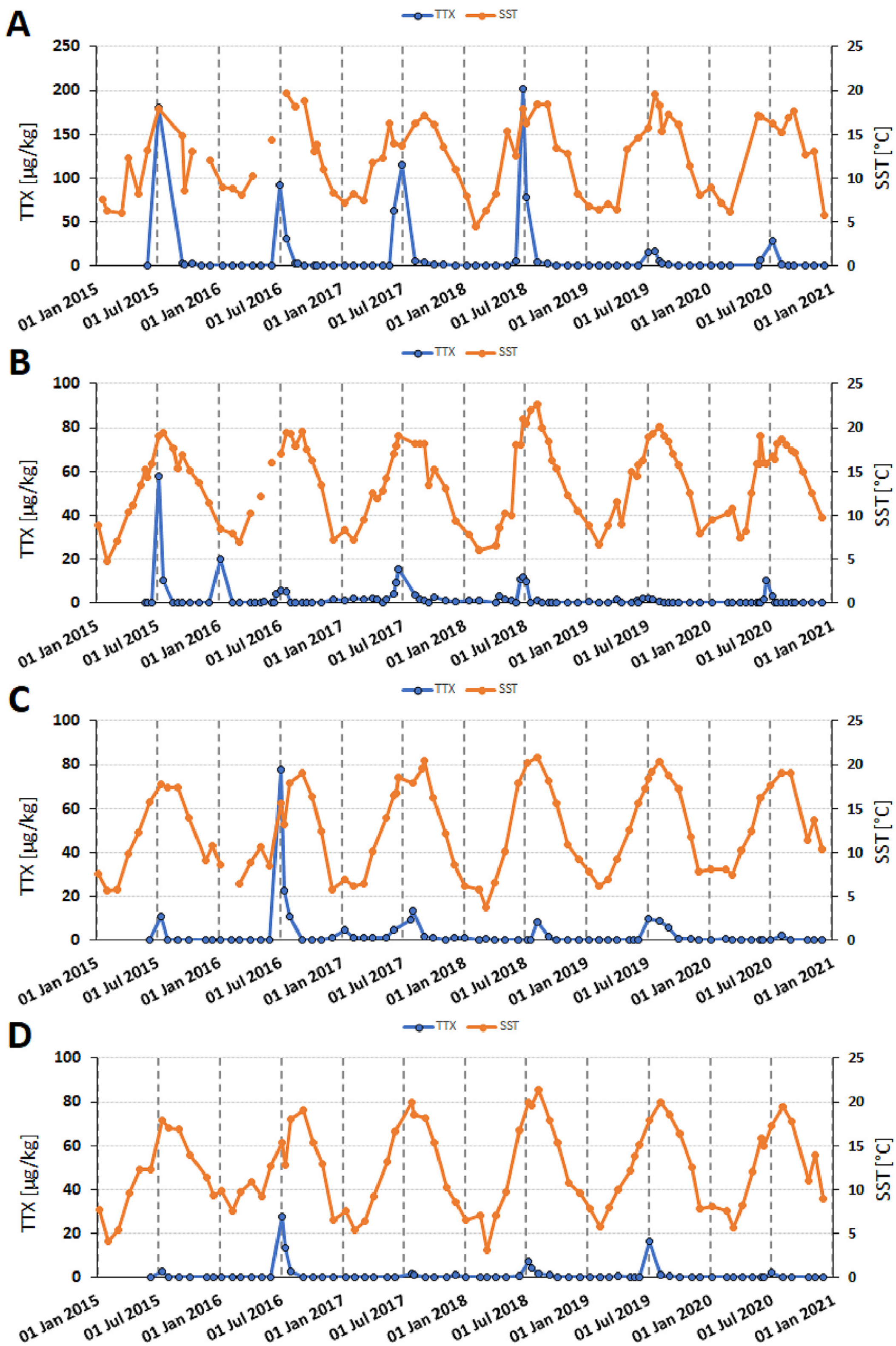


Fig. 2. TTX concentrations in bivalves and sea-surface temperature (SST) over multiple years.
The data were collected from selected shellfish representative monitoring sites: (A) Site 1, (B) Site 2, (C) Site 3 and (D) Site 4 and include previously published TTX results between May 2015 and November 2016 (Turner et al., 2017b). SST was measured at the time of sample collection.

3.3.2. Satellite-derived sea surface temperature profiles at sites with and without tetrodotoxin in 2016

To better understand which aspects of SST correspond to TTX accumulation, satellite-derived daily mean SST data of sites with TTX-positive and TTX-negative bivalves in 2016 were compared. All locations, where TTX was found in bivalves above LOR level on more than two different days, were included in the TTX-positive group (Table A.1). TTX-negative sites were matched for location, water depth, frequency of sampling and bivalve species as much as possible. The nature of TTX-positive Site 4 (20 m deep) reflected its situation in a channel in proximity to an estuary. Relevant

Table 2
Number of British bivalve samples from four shellfish representative monitoring sites (RMSs) categorised based on sea-surface temperature and TTX concentration. TTX concentrations were measured in bivalves collected from four RMSs (Site 1–4) between May 2016 and December 2020. The sea-surface temperatures were *in situ* one-point measurements taken by the sampling officers at the time of shellfish collection.

	0–10 °C	>10–15 °C	>15–20 °C	> 20 °C	Total
Total	94	81	127	10	312
TTX > LOD < LOR	24	15	21	5	65
TTX > LOR	4	8	47	3	62

LOD = Limit of Detection (0.2 μg/kg); LOR = Limit of Reporting (2 μg/kg).

characteristics for each location, including mean SSTs are summarised in Table 4. While the mean annual SSTs in 2016 did not differ statistically between sites with TTX-positive and TTX-negative bivalve samples (mean ± standard deviation: 12.69 ± 3.98 °C and 12.85 ± 2.83 °C, respectively, *t*-test, $p = 0.12$), a larger variance in SST was observed at TTX-positive sites (Table 4, Fig. 3A). By taking into account the lowest SST at each site and instead calculating the "SST Difference" value, significantly larger temperature differences were found at sites with TTX-positive shellfish compared to those lacking TTX (mean sea temperature difference ± standard deviation: 5.97 ± 3.98 °C and 4.22 ± 2.83 °C, respectively; *t*-test, $p \leq 0.001$, Table 4, Fig. 3B). Tukey's post-hoc multiple comparison test revealed that within each group, not all pairwise comparisons of sites met the statistical threshold (Table A.2), perhaps suggesting additional impacts of other factors (*e.g.* salinity) at these locations.

Since larger annual variations in sea temperature seemed to be indicative of shellfish areas where TTX was detected, time-series analysis was used to examine SST profile changes throughout the year. SSTs were generally lower in winter and higher in summer at sites with TTX-positive shellfish (Fig. 4A). Plotting "SST Difference" values also showed the greater temperature change for sites where TTX-positive samples were found (Fig. 4B). Consistent with this, statistically significant interactions for SST between time and TTX status were found, regardless of which SST data set was used (daily mean SST or "SST Difference") ($p = 1.66 \times 10^{-4}$ and $p = 1.89 \times 10^{-4}$, respectively). Temperature increased significantly faster in spring and decreased significantly faster in autumn at sites with TTX-positive bivalve samples (Table A.3). During winter, specifically in February and in December, no statistical difference in temperature changes

Table 3
Collection details of bivalve samples with TTX concentrations > LOR (2 μg/kg) and sea temperatures <15 °C.
TTX concentrations were measured in bivalves collected from four representative monitoring sites (Sites 1–4) between May 2016 and December 2020. *In situ* sea temperature measurements were taken by the sampling officers at the time of shellfish collection. The satellite-derived daily mean sea temperature data were GHRSST MUR with 1 km resolution.

Site	Date	Time of collection	TTX [μg/kg]	Temperature (*in situ*) [°C]	Temperature (satellite) [°C]
1	15/09/2015	09:03	2.4	14.8	NA
	13/10/2015	09:08	3.0	13.0	NA
	06/06/2017	08:15	62.9	14.0	NA
	28/06/2017	08:52	115	13.7	NA
	05/06/2018	09:10	6.0	12.6	NA
2	05/01/2016	11:55	19.9	8.4	10.9
	07/02/2017	10:55	2.0	7.2	7.96
	04/04/2017	12:00	2.1	12.6	10.3
	16/04/2018	08:00	3.0	8.6	8.34
3	12/07/2016	12:15	22.5	13.2	15.4
	09/01/2017	13:15	4.5	6.9	8.75
4	12/07/2016	09:00	13.4	12.8	15.6

NA = Not Available.

between sites with and without TTX were found ($p = 0.10$ and $p = 0.66$, respectively). Fig. 5 provides more detailed view of temperature during the period critical for TTX accumulation (May – July). Throughout this period, the sites with confirmed TTX presence showed a steady increase of SST, reaching around 15 °C several days before the first recording of TTX above the LOR on 21st June. For the next two weeks, SST continued to increase and TTX concentrations peaked on the 4th July. After this date, however, further increases in SST did not lead to a further increase in TTX. Importantly, the SST trajectory at sites with TTX-negative bivalves was different, especially between mid-May and mid-July. Even though similar temperatures (or around 15 °C) were observed in the 2nd week of June, they were not sustained at TTX-negative sites. Instead, SSTs of this group dropped on several occasions, around 23rd May, 13th - 20th June and 27th – 4th July, which might have interfered with TTX accumulation.

4. Discussion

4.1. Tetrodotoxin occurrences and risk assessment considerations

We have performed the largest and most comprehensive TTX screen of bivalve shellfish reported to date. The screening of >3500 samples collected in 2016 from around the British coast revealed that only a small fraction (1.1 %) contained TTX levels above the LOR (2 μg/kg). The detection of TTX-positive bivalves at just a few locations in southern England is in line with previous observations (Turner et al., 2017b) and is consistent with wider reporting that shallow, semi-enclosed estuarine environments are more likely to favor TTX accumulation (Bordin et al., 2021; Gerssen et al., 2018; Hort et al., 2020; Réveillon et al., 2021). Alongside prevalence and distribution, toxin concentration is another important aspect for TTX risk assessment. In our study, only five samples from 2016 contained TTX above 44 μg/kg of whole shellfish flesh, the MPL proposed by EFSA (EFSA et al., 2017) as unlikely to be detrimental to human health. However, the highest TTX concentration in 2016 was 93 μg/kg, more than twice the proposed MPL, with even higher levels, up to 202 μg/kg (270 μg/kg when TTX analogues were included), found in subsequent years. The range of quantified values are comparable to those reported from shellfish species studied elsewhere (reviewed in (Antonelli et al. (2021), Katikou et al. (2022)).

Currently, there is no Europe-wide legislative requirement to monitor TTX in seafood, negated in part by a complete ban on the import of pufferfish and other TTX-bearing fish being in place (Anon, 2019). The single exception involves the Netherlands, which implement the EFSA-proposed MPL for TTX in bivalves (Gerssen et al., 2018). As more data on TTX toxicity emerge, what comprises a suitable MPL in bivalves for legislative purposes remains an open question (Antonelli et al., 2021; Boente-juncal et al., 2020; Finch et al., 2018; Guardone et al., 2019). Thus far, no human TTX intoxication cases have been reported associated with bivalve shellfish consumption, although poisonings caused by TTX-bearing marine gastropods have been reported (reviewed in (Guardone et al., 2019)). Setting TTX regulatory concentration in bivalves will require a wide range of discussions, not only about appropriate threshold levels, but also about contribution of various TTX analogues to overall toxicity (while toxicity equivalent factors remain to be established), the possible incorporation of TTX into the existing Paralytic Shellfish Toxin group and the choice of suitable reference methods. Undoubtedly, comprehensive understanding of TTX as a food safety threat would benefit from knowledge of the *de novo* biological source and/or carrier. While this remains uncertain despite years of study, investigating parameters correlated to TTX accumulation in bivalves is important and offers an alternative approach to assessing risk.

4.2. Temporal distribution of tetrodotoxin

One of the noticeable findings from the TTX screening was a potential seasonal accumulation of TTX in bivalves. We found that TTX concentrations increased in early summer, reaching maxima in late June and early July, in each of six seasons monitored in our study (Fig. 2). Similar temporal

Table 4
TTX status of bivalves, testing frequency in 2016 and site characteristics of shellfish representative monitoring sites used for the temperature profile analyses. SST Mean ± s.d. values for each TTX status group were calculated using the complete 2016 SST data sets, rather than mean annual SST. TTX testing frequency represents number of samples tested for TTX in each month. Species tested included mussels (*Mytilus* spp.), Pacific oysters (*Crassostrea gigas*), native oysters (*Ostrea edulis*) and hard clams (*Mercenaria mercenaria*).

Site	TTX status	Mean Annual Temperature in 2016 [°C]	Mean Annual Temperature Difference in 2016 [°C]	Water depth [m ODN]	Environment	TTX testing frequency in 2016		
						May	June	July
1[a]	YES	NA	NA	−0.418	E	1	2	1
2	YES	12.83	5.27	−3.757	E	2	3	2
3	YES	12.62	6.06	0.000	E	1	1	2
4	YES	12.71	5.91	−20.022	E	1	1	2
5	YES	12.69	5.97	−2.205	E	1	4	3
6	YES	12.67	5.99	−1.360	E	1	3	3
7	YES	12.64	6.62	−1.668	E	1	1	1
8	NO	12.91	3.80	−2.619	E	5	4	4
9	NO	12.61	4.95	−2.143	E	2	3	3
10	NO	12.94	3.77	−3.150	E	5	4	4
11	NO	12.85	4.03	−0.559	E	4	5	3
12	NO	12.90	4.17	−2.188	R	4	5	4
13	NO	12.84	4.26	−1.492	R	2	3	2
14	NO	12.90	4.59	−0.893	E	1	3	1
Mean ± s.d.	YES	12.69 ± 3.98	5.97 ± 3.98					
Mean ± s.d.	NO	12.85 ± 2.83	4.22 ± 2.83					

NA = Not Available, ODN = Ordnance Datum Newlyn, E = Estuarine, R = River, s.d. = standard deviation.
[a] Site 1 was included in the inter-annual seasonal study, but satellite-derived data were not available.

patterns in bivalves have been described elsewhere in Europe (Gerssen et al., 2018; Hort et al., 2020; Réveillon et al., 2021; Turner et al., 2017b, Turner et al., 2015), although the timing of the peak TTX accumulation was observed a few weeks earlier (end of May to early June) in the Mediterranean region (Bacchiocchi et al., 2023; Bordin et al., 2021, Bordin et al., 2019; Hort et al., 2020; Vlamis et al., 2015). Similarly, the duration of TTX accumulation appears variable, ranging from two weeks in Northern France (Réveillon et al., 2021), four to five weeks in Rodopi in Greece (Vlamis et al., 2015) and in the Marano lagoon in Italy (Bordin et al., 2019), to 6 weeks in the Netherlands (Gerssen et al., 2018). It is clear that future research would benefit from frequent (weekly) sample collections over multiple seasons to enable better tracking and description of TTX dynamics in shellfish.

Data from our study, and that of others, seem to suggest that European bivalves accumulate and depurate TTX relatively quickly. This contrasts with findings in New Zealand's endemic "Pipi" clam (*Paphies australis*), in which TTX levels were sustained throughout one year (Biessy et al., 2020; Boundy et al., 2020). Moreover, TTX depurated very slowly from captive "Pipi" clams, suggesting the toxin may be retained for a specific ecological role in this species (Biessy et al., 2019b). TTX dynamics in Japanese scallops, *Mizuhopecten yessoensis* and *Azumapecten farrei* subsp. Akazara, appear to exhibit an intermediate phenotype, with elevated levels maintained in

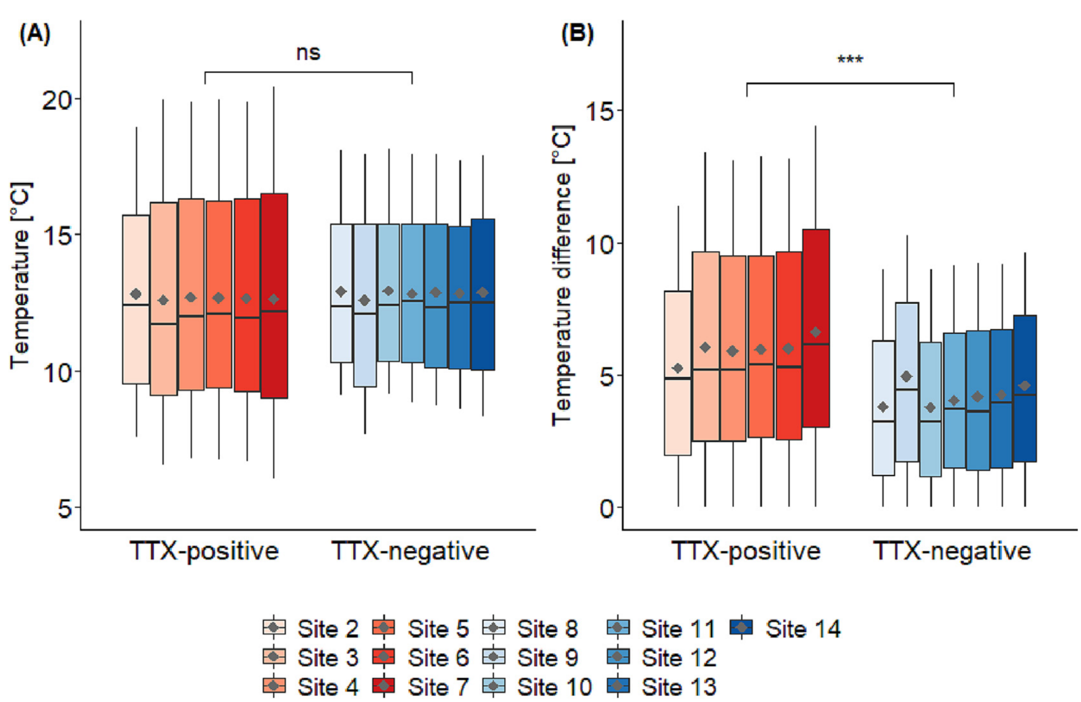


Fig. 3. Sea-surface temperature characteristics of selected shellfish representative monitoring sites where TTX-positive or TTX-negative bivalves were recovered in 2016. Box and whisker plots showing median (horizontal line), mean (diamond) and 1st and 3rd quartiles for satellite-derived SST from 2016, based on (A) daily mean SST and (B) "SST Difference" data sets. "SST Difference" values were determined by subtracting the lowest daily mean SST value from the daily mean SST values at each site. Data were analysed using *t*-test (ns = not significant, *** $p \leq 0.001$).

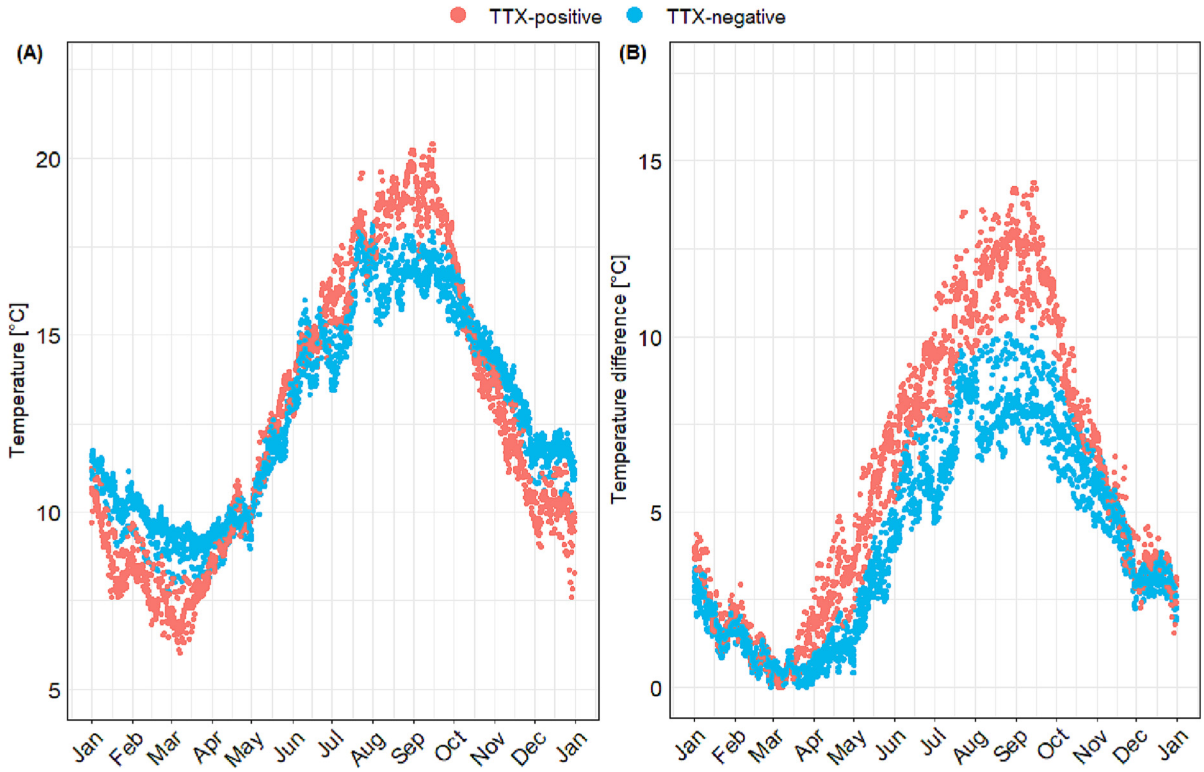


Fig. 4. Daily continuous sea-surface temperature changes at selected shellfish representative monitoring sites where TTX-positive or TTX-negative bivalves were recovered in 2016.
Satellite-derived SST from 2016 using (A) daily mean SST and (B) "SST Difference" data sets. "SST Difference" values were determined by subtracting the lowest daily mean SST value from the daily mean SST values at each site. Significant interactions were found between time and TTX status for both data sets (two-way ANOVA, $p = 1.66 \times 10^{-4}$ and $p = 1.89 \times 10^{-4}$, respectively).

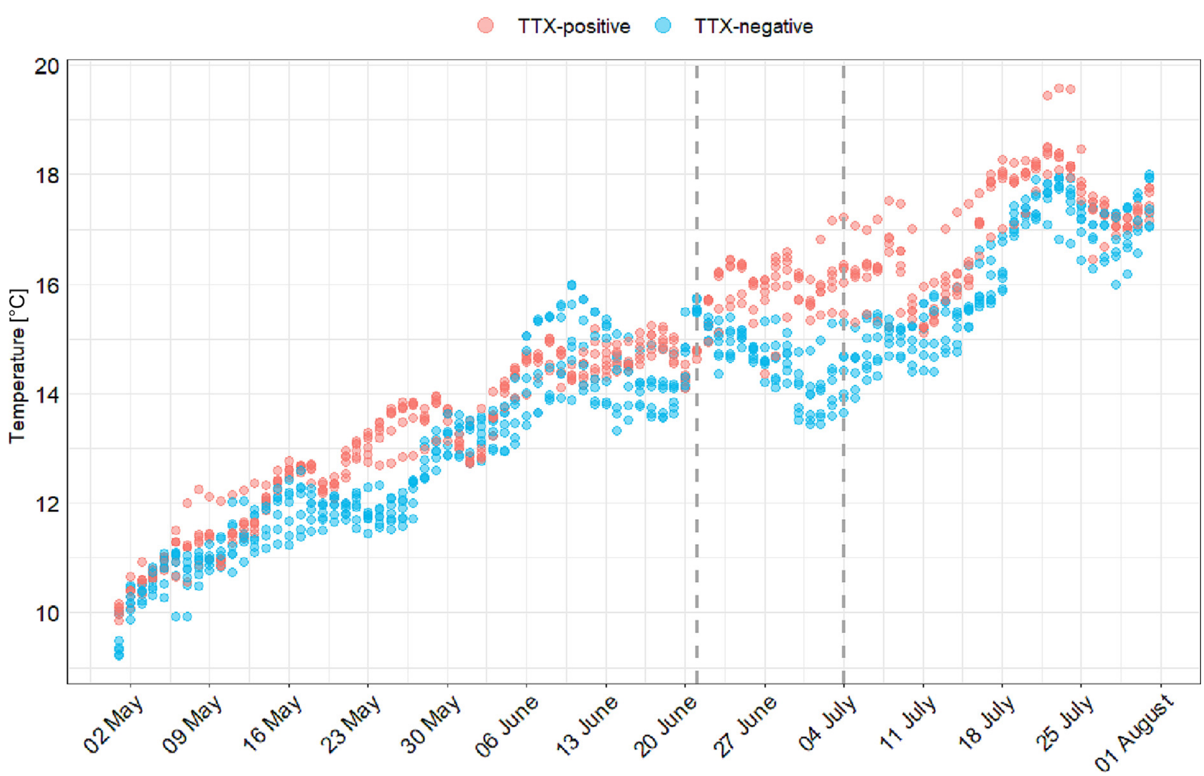


Fig. 5. Magnified view of period corresponding to TTX accumulation in bivalves at selected shellfish representative monitoring sites in 2016.
Plot shows satellite-derived daily mean SST values between May and July 2016 at sites with TTX (red dots) and without TTX (blue dots). The dashed vertical lines represent the onset of TTX accumulation on 21st June and when maximum TTX concentrations were recorded (4th July).

their digestive glands over several consecutive months but with seasonal variation also apparent (Numano et al., 2019; Okabe et al., 2021). In these species, lowest TTX levels were recorded in June and July, with higher levels occurring from late summer (August) until late autumn (November). It is possible that in some species TTX seasonality may be masked by their differential ability to retain toxins, as has been was reported for domoic acid, another hydrophilic toxin (Blanco et al., 2006, Blanco et al., 2002; Bresnan et al., 2017). Comparing TTX occurrences between countries and sites remains challenging without deeper knowledge of inter-species differences, as well as without concurrent continuous monitoring of TTX alongside key physical environmental parameters such as sea temperature.

4.3. Effect of sea surface temperature on tetrodotoxin accumulation

Using *in situ* sea temperature monitoring at the time of shellfish sampling, we found that TTX accumulated in bivalves generally after sea temperatures warmed to ~15 °C, with the majority of TTX-positive bivalves recovered when SSTs were within the 15–20 °C temperature range. These findings extend our earlier observations using a smaller set of samples (Turner et al., 2017b) and are consistent with studies reported from the Mediterranean (Bordin et al., 2021; Hort et al., 2020). However, the use of time-of-sampling one-point temperature measurements often fails to fully capture temperature evolution at individual sites. For example, Hort and colleagues reported that sea water temperatures were only 11.9 °C when TTX-positive mussels were collected from a French coastal lagoon, however, all three temperature readings in the preceding three weeks at this location were > 15 °C (Hort et al., 2020). Because of the varied methodology used for *in situ* time-of-sampling temperature measurements in our study and the inherent limitations of single-point measurements, we employed satellite-derived data to further examine the link between TTX accumulation and SST.

In our study, continuous satellite-derived sea temperature data were used for the first time to statistically evaluate temperature changes at shellfish production locations where TTX-positive or TTX-negative bivalves were found. Sites were otherwise matched as closely as possible for location, sea depth and sampling frequency. While no difference in the mean annual sea temperature of these sites was found, we uncovered other aspects of SST that appear important in TTX accumulation. First, we noted that TTX-positive bivalves were associated with sites with a larger temperature range (Fig. 4). Water depth can impact temperature, with relatively shallow intertidal areas such as those used in shellfish production, being more susceptible to temperature change (*e.g.* higher temperatures in summer and lower temperatures in winter). Yet, we found that tide-corrected water depth did not differ at TTX-positive and TTX-negative sites. Second, analyses of the period prior to the first detection of TTX revealed some differences in SST. Although the proposed "trigger" temperature of ~15 °C was reached at sites regardless of whether TTX-positive bivalves were found, subtle differences in the evolution of SST were apparent. For example, SST at sites with TTX-positive bivalves tended to show steady increases in SST, whereas those without TTX showed a less consistent upward temperature trajectory. Thus, it is possible that the evolution of SST, whether it raises steadily or dips occasionally, and how long it is sustained above a certain level, may be an important aspect related to TTX accumulation. More frequent and systematic time-series analysis in localities susceptible to TTX accumulation in bivalves would help to understand and model this relationship better. However, temperature is unlikely to be the only factor affecting TTX prevalence. For instance, SSTs in the Mediterranean exceeds 15 °C more widely than in the north-east Atlantic, however TTX occurrences in the former, are also rare and localized (Bacchiocchi et al., 2023, Bacchiocchi et al., 2021; Bordin et al., 2021; Dell'Aversano et al., 2019; Hort et al., 2020; Vlamis et al., 2015). Because of the apparent increased incidence of TTX in bivalves at shallow estuarine environments with a low water refreshment rate, a role for decreased salinity and high solar radiation has also been proposed (Gerssen et al., 2018; Turner et al., 2017b).

4.4. Temperature and biological source of tetrodotoxin in bivalves

Temperature is a key abiotic factor that governs many biological processes. The findings of this study support the hypothesis that temperature influences seasonal accumulation of TTX in European bivalves, however the middle link, the biological source of TTX, remains unknown. TTX is thought to be produced by bacteria due to its detection in phylogenetically diverse organisms. Indeed, several bacterial genera have now been linked to TTX production including *Vibrio*, *Bacillus*, *Pseudomonas, Alteromonas*, *Streptomyces*, *Roseobacter* and most recently cyanobacteria (reviewed in (Magarlamov et al., 2017; Biessy et al., 2020)). Interestingly, *Vibrio* species proliferate in shellfish and estuarine waters when temperatures exceed 15 °C (Parveen et al., 2008; Vezzulli et al., 2015). Moreover, TTX has been detected in cultures of several strains of *Vibrio parahaemolyticus* and one strain of *V. cholerae* isolated from TTX-positive Pacific oysters and mussels in the UK (Turner et al., 2015). Thus far, this represents the only report of TTX in bacterial strains isolated from bivalves, despite subsequent efforts (Bacchiocchi et al., 2021; Réveillon et al., 2021). As yet, sustained TTX production in *Vibrio* and other non-*Vibrio* bacterial cultures has not been achieved, hindering discovery of the TTX biosynthetic pathway as well as confirmation of these species as definitive *de novo* sources of the toxin.

Free-living bacteria smaller than 1 μm are unlikely to be actively retained by filter-feeding shellfish, unless adhered to detritus or larger organisms like nanoplankton and microplankton (Møhlenberg and Riisgård, 1978). Earlier hypotheses that phytoplankton, specifically *Alexandrium tamarense* and *Prorocentrum cordatum*, were TTX producers or carriers (Kodama et al., 1996; Rodríguez et al., 2017; Vlamis et al., 2015), have not been substantiated (Hort et al., 2020; Numano et al., 2019; Turner et al., 2017b). Most recently, the larvae of a TTX-bearing flatworm (*Planocera multitentaculata*) was proposed to be a source of TTX in Japanese Akazara scallops (*Azumapecten farrei* subsp. *akazara*), and were able to successfully intoxicate mussels (*Mytilus galloprovincialis*) when supplied by filter-feeding under laboratory conditions (Okabe et al., 2021). While this worm species spawns when SSTs are around 20 °C (Yamada et al., 2017), its presence has not been documented in European seas. Regardless, such findings reaffirm the multiple ways by which TTX accumulation in bivalves may occur, and how SSTs could trigger a biological event.

5. Conclusions

Collectively, data from our large study suggest that TTX accumulation in British bivalves predominantly occurs when seawater temperatures warm to around 15 °C and at production areas that experience larger and more rapid shifts in temperature. As such, our findings reinforce the need to explore the relationship between temperature and TTX in greater detail, especially in the geographic areas already identified in previous screening studies. It is important to note however, that not all biological and abiotic aspects of these complex environmental niches are known or controllable, thus it is likely that TTX accumulation is directly, and indirectly affected by other factors. Regardless, the increased understanding of the relationship between temperature and TTX accumulation in bivalves provides a useful variable that could feed-in to risk assessment analyses. Identification of the *de novo* biological source of TTX in bivalves remains a high priority, not only to map the process(es) leading to TTX accumulation, but also to answer wider questions about this emerging food safety threat in Europe.

CRediT authorship contribution statement

Monika Dhanji-Rapkova: Conceptualization, Methodology, Formal analysis, Writing - original draft.
Mickael Teixeira Alves: Formal analysis (statistics), Writing - review & editing.
Joaquin A. Triñanes: Temperature data curation.
Jaime Martinez-Urtaza: Temperature data.
David Haverson: Bathymetry data curation.
Kirsty Bradley: Visualization (graphical abstract).

Craig Baker-Austin: Conceptualisation, Supervision, Funding acquisition, Writing - review & editing.

Jim Huggett: Supervision.

Graham Stewart: Supervision, Writing - review & editing.

Jennifer M. Ritchie: Conceptualisation, Supervision, Funding acquisition, Writing - review & editing.

Andrew D. Turner: Conceptualisation, Supervision, Methodology, Funding acquisition, Writing - review & editing.

Funding

This research was funded by Cefas Seedcorn grant (DP901M) with additional support from Interreg Alertox-Net EAPA-317-2016 (Atlantic Area Program) and a Faculty Studentship Award (Faculty of Health and Medical Sciences, University of Surrey).

Data availability

Data will be made available on request.

Declaration of competing interest

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

Acknowledgements

All bivalve shellfish samples analysed in this study were acquired as a part of OC biotoxin monitoring programmes funded by the Food Standard Agency (FSA) and Food Standards Scotland (FSS). We would like to thank the Biotoxin team at Cefas for processing these samples and FSA and FSS for allowing us to utilise the archived extracts for the purpose of this study. The permission to use bivalve samples from OC programme was granted on condition of preserving anonymity of shellfish production areas found to be associated with TTX. We would like to thank Cefas colleague Lewis Coates for creating the map for the Fig. 1 and Ben Maskrey for the internal review. We appreciate comments from three anonymous reviewers, thus helping to improve the quality of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.163905.

References

Akyol, O., Ünal, V., Ceyhan, T., Bilecenoglu, M., 2005. First confirmed record of *Lagocephalus sceleratus* (Gmelin, 1789) in the Mediterranean Sea. J. Fish Biol. 66, 1183–1186. https://doi.org/10.1111/j.0022-1112.2005.00667.x.

Anon, 2019. Commision Implementing Regulation (EU) 2019/627 of 15 March 2019 laying down uniform practical arrangements for the performance of official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of. Off. J. Eur. Union L 131, 51–100.

Antonelli, P., Salerno, B., Bordin, P., Peruzzo, A., Orsini, M., Arcangeli, G., Barco, L., Losasso, C., 2021. Tetrodotoxin in live bivalve mollusks from Europe: is it to be considered an emerging concern for food safety? Compr. Rev. Food Sci. Food Saf. 1–19. https://doi.org/10.1111/1541-4337.12881.

Bacchiocchi, S., Campacci, D., Siracusa, M., Dubbini, A., Leoni, F., Tavoloni, T., Accoroni, S., Gorbi, S., Giuliani, M.E., Stramenga, A., Piersanti, A., 2021. Tetrodotoxins (TTXs) and *Vibrio alginolyticus* in mussels from Central Adriatic Sea (Italy): are they closely related? Mar. Drugs 304.

Bacchiocchi, S., Campacci, D., Siracusa, M., Dubbini, A., Accoroni, S., Romagnoli, T., Campanelli, A., Griffoni, F., Tavoloni, T., Gorbi, S., Totti, C., Piersanti, A., 2023. A hotspot of TTX contamination in the Adriatic Sea: study on the origin and causative factors. Mar. Drugs 21, 8.

Bane, V., Lehane, M., Dikshit, M., O'Riordan, A., Furey, A., 2014. Tetrodotoxin: chemistry, toxicity, source, distribution and detection. Toxins 6, 693–755. https://doi.org/10.3390/toxins6020693 Basel.

Biessy, L., Boundy, M.J., Smith, K.F., Harwood, D.T., Hawes, I., Wood, S.A., 2019a. Tetrodotoxin in marine bivalves and edible gastropods: a mini-review. Chemosphere 236, 124404. https://doi.org/10.1016/j.chemosphere.2019.124404.

Biessy, L., Smith, K.F., Harwood, D.T., Boundy, M.J., Hawes, I., Wood, S.A., 2019b. Spatial variability and depuration of tetrodotoxin in the bivalve Paphies australis from New Zealand. Toxicon X 2, 100008. https://doi.org/10.1016/j.toxcx.2019.100008.

Biessy, L., Pearman, J.K., Smith, K.F., Hawes, I., Wood, S.A., 2020. Seasonal and spatial variations in bacterial communities from tetrodotoxin-bearing and non-tetrodotoxin-bearing clams. Front. Microbiol. 11, 1860. https://doi.org/10.3389/fmicb.2020.01860.

Blanco, J., Acosta, C.P., Bermúdez de la Puente, M., Salgado, C., 2002. Depuration and anatomical distribution of the amnesic shellfish poisoning (ASP) toxin domoic acid in the king scallop *Pecten maximus*. Aquat. Toxicol. 60, 111–121. https://doi.org/10.1016/S0166-445X(01)00274-0.

Blanco, J., Acosta, C.P., Mariño, C., Muñiz, S., Martín, H., Moroño, Á., Correa, J., Arévalo, F., Salgado, C., 2006. Depuration of domoic acid from different body compartments of the king scallop *Pecten maximus* grown in raft culture and natural bed. Aquat. Living Resour. 19, 257–265. https://doi.org/10.1051/alr:2006026.

Boente-juncal, A., Otero, P., Rodríguez, I., Camiña, M., Rodriguez-vieytes, M., Vale, C., Botana, L.M., 2020. Oral chronic toxicity of the safe tetrodotoxin dose proposed by the European Food Safety Authority and its additive effect with saxitoxin, pp. 1–18 https://doi.org/10.3390/toxins12050312.

Bordin, P., Antonelli, P., Peruzzo, A., Longo, A., Milandri, A., Dall'Ara, S., Cangini, M., Orlandi, C., Guiatti, D., Zanolin, B., Cainero, M., Zentilin, A., Dalla Pozza, M., Losasso, C., Arcangeli, G., Barco, L., 2019. Developments on the presence of tetrodotoxins in Mediterranean shellfish from the Marano lagoon in the northern Adriatic sea - Italy. Book of Abstracts, 2nd Meeting on "Natural Toxins", Parma, Italy, 18-19th September, p. 21 https://doi.org/10.1016/j.protcy.2014.10.179.

Bordin, P., Dall'Ara, S., Tartaglione, L., Antonelli, P., Calfapietra, A., Varriale, F., Guiatti, D., Milandri, A., Dell'Aversano, C., Arcangeli, G., Barco, L., 2021. First occurrence of tetrodotoxins in bivalve mollusks from Northern Adriatic Sea (Italy). Food Control 120, 107510. https://doi.org/10.1016/j.foodcont.2020.107510.

Boundy, M.J., Selwood, A.I., Harwood, D.T., McNabb, P.S., Turner, A.D., 2015. Development of a sensitive and selective liquid chromatography-mass spectrometry method for high throughput analysis of paralytic shellfish toxins using graphitised carbon solid phase extraction. J. Chromatogr. A 1387, 1–12. https://doi.org/10.1016/j.chroma.2015.01.086.

Boundy, M.J., Biessy, L., Roughan, B., Nicolas, J., Harwood, D.T., 2020. Survey of tetrodotoxin in New Zealand bivalve molluscan shellfish over a 16-month period. Toxins 12, 512 Basel.

Bresnan, E., Fryer, R.J., Fraser, S., Smith, N., Stobo, L., Brown, N., Turrell, E., 2017. The relationship between Pseudo-nitzschia (Peragallo) and domoic acid in Scottish shellfish. Harmful Algae 63, 193–202. https://doi.org/10.1016/j.hal.2017.01.004.

Chau, R., Kalaitzis, J.A., Neilan, B.A., 2011. On the origins and biosynthesis of tetrodotoxin. Aquat. Toxicol. 104, 61–72. https://doi.org/10.1016/j.aquatox.2011.04.001.

Dell'Aversano, C., Tartaglione, L., Polito, G., Dean, K., Giacobbe, M., Casabianca, S., Capellacci, S., Penna, A., Turner, A.D., 2019. First detection of tetrodotoxin and high levels of paralytic shellfish poisoning toxins in shellfish from Sicily (Italy) by three different analytical methods. Chemosphere 215, 881–892. https://doi.org/10.1016/j.chemosphere.2018.10.081.

Dhanji-Rapkova, M., Turner, A.D., Baker-Austin, C., Huggett, J.F., Ritchie, J.M., 2021. Distribution of tetrodotoxin in Pacific oysters (*Crassostrea gigas*). Mar. Drugs 19, 84. https://doi.org/10.3390/md19020084.

EFSA, Knutsen, H.K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Edler, L., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L.(Ron), Nebbia, C.S., Oswald, I.P., Rose, M., Roudot, A., Schwerdtle, T., Vleminckx, C., Vollmer, G., Wallace, H., Arnich, N., Benford, D., Botana, L., Viviani, B., Arcella, D., Binaglia, M., Horvath, Z., Steinkellner, H., van Manen, M., Petersen, A., 2017. Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. EFSA J. 15, 4752. https://doi.org/10.2903/j.efsa.2017.4752.

Environment Agency, 2022. SurfZone Digital Elevation Model 2019 [WWW Document]. https://www.data.gov.uk/dataset/fe455db0-5ce5-4d63-8b38-d74612eb43d5/surfzone-digital-elevation-model-2019 accessed 10.27.22.

Fernández-Ortega, J.F., Morales'de los Santos, J., Herrera-Gutiérrez, M.E., Fernández-Sánchez, V., Loureo, P.R., Rancaño, A.A., Téllez-Andrade, A., 2010. Seafood intoxication by tetrodotoxin: first case in europe. J. Emerg. Med. 39, 612–617. https://doi.org/10.1016/j.jemermed.2008.09.024.

Finch, S.C., Boundy, M.J., Harwood, D.T., 2018. The acute toxicity of tetrodotoxin and tetrodotoxin-saxitoxin mixtures to mice by various routes of administration. Toxins 10, 423. https://doi.org/10.3390/toxins10110423 Basel.

Gerssen, A., Bovee, T.H.F., Klijnstra, M.D., Poelman, M., Portier, L., Hoogenboom, R.L.A.P., 2018. First report on the occurrence of tetrodotoxins in bivalve mollusks in the Netherlands. Toxins 10, 450. https://doi.org/10.3390/toxins10110450 Basel.

Guardone, L., Maneschi, A., Meucci, V., Gasperetti, L., Nucera, D., Armani, A., 2019. A global retrospective study on human cases of tetrodotoxin (TTX) poisoning after seafood consumption. Food Rev. Int. 36, 645–667. https://doi.org/10.1080/87559129.2019.1669162.

Hort, V., Arnich, N., Guérin, T., Lavison-Bompard, G., Nicolas, M., 2020. First detection of tetrodotoxin in bivalves and gastropods from the French mainland coasts. Toxins 12, 1–17. https://doi.org/10.3390/toxins12090599 Basel.

Katikou, P., Gokbulut, C., Kosker, A.R., Campàs, M., Ozogul, F., 2022. An updated review of tetrodotoxin and its peculiarities. Mar. Drugs 20, 47. https://doi.org/10.3390/md20010047.

Kodama, M., Sato, S., Sakamoto, S., Ogata, T., 1996. Occurrence of tetrodotoxin in *Alexandrium tamarense*, a causative dinoflagellate of paralytic shellfish poisoning. Toxicon 34, 1101–1105. https://doi.org/10.1016/0041-0101(96)00117-1.

Magarlamov, T.Y., Melnikova, D.I., Chernyshev, A.V., 2017. Tetrodotoxin-producing bacteria: detection, distribution and migration of the toxin in aquatic systems. Toxins 9, 166. https://doi.org/10.3390/toxins9050166 Basel.

Møhlenberg, F., Riisgård, H.U., 1978. Efficiency of particle retention in 13 species of suspension feeding bivalves. Ophelia 17, 239–246.

Noguchi, T., Arakawa, O., 2008. Tetrodotoxin - distribution and accumulation in aquatic organisms, and cases of human intoxication. Mar. Drugs 6, 220–242. https://doi.org/10.3390/md20080011.

Noguchi, T., Onuki, K., Arakawa, O., 2011. Tetrodotoxin poisoning due to pufferfish and gastropods, and their intoxication mechanism. Int. Sch. Res. Netw. ISRN Toxicol. 2011, 1–10. https://doi.org/10.5402/2011/276939.

Numano, S., Kudo, Y., Cho, Y., Konoki, K., Yotsu-Yamashita, M., 2019. Temporal variation of the profile and concentrations of paralytic shellfish toxins and tetrodotoxin in the scallop, Patinopectedn yessoensis, cultured in a bay of East Japan. Mar. Drugs 17, 653. https://doi.org/10.3390/md17120653.

Okabe, T., Saito, R., Yamamoto, K., Watanabe, R., Kaneko, Y., Yanaoka, M., Furukoshi, S., Yasukawa, S., Ito, M., Oyama, H., Suo, R., Suzuki, M., Takatani, T., Arakawa, O., Sugita, H., Itoi, S., 2021. The role of toxic planocerid flatworm larvae on tetrodotoxin accumulation in marine bivalves. Aquat. Toxicol. 237, 105908. https://doi.org/10.1016/j.aquatox.2021.105908.

Parveen, S., Hettiarachchi, K.A., Bowers, J.C., Jones, J.L., Tamplin, M.L., McKay, R., Beatty, W., Brohawn, K., DaSilva, L.V., DePaola, A., 2008. Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. Int. J. Food Microbiol. 128, 354–361. https://doi.org/10.1016/j.ijfoodmicro.2008.09.019.

R Core Team, 2022. R: A Language And Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria http://www.R-project.org/ [WWW Document].

Réveillon, D., Savar, V., Schaefer, E., Chevé, J., Halm-Lemeille, M.-P., Hervio-Heath, D., Travers, M.-A., Abadie, E., Rolland, J.-L., Hess, P., 2021. Tetrodotoxins in French bivalve mollusks - analyticalmethodology, environmental dynamics and screening of bacterial strain collections. Toxins 13, 740 Basel.

Rodriguez, P., Alfonso, A., Vale, C., Alfonso, C., Vale, P., Tellez, A., Botana, L.M., 2008. First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell *Charonia lampas* lampas in Europe. Anal. Chem. 80, 5622–5629. https://doi.org/10.1021/ac800769e.

Rodríguez, I., Alfonso, A., Alonso, E., Rubiolo, J.A., Roel, M., Vlamis, A., Katikou, P., Jackson, S.A., Menon, M.L., Dobson, A., Botana, L.M., 2017. The association of bacterial C 9-based TTX-like compounds with *Prorocentrum* minimum opens new uncertainties about shellfish seafood safety. Sci. Rep. 7, 1–12. https://doi.org/10.1038/srep40880.

Turner, A.D., Stubbs, B., Coates, L., Dhanji-Rapkova, M., Hatfield, R.G., Lewis, A.M., Rowland-Pilgrim, S., O'Neil, A., Stubbs, P., Ross, S., Baker, C., Algoet, M., 2014. Variability of paralytic shellfish toxin occurrence and profiles in bivalve molluscs from Great Britain from official control monitoring as determined by pre-column oxidation liquid chromatography and implications for applying immunochemical tests. Harmful Algae 31, 87–99. https://doi.org/10.1016/j.hal.2013.10.014.

Turner, A.D., Powell, A., Schofield, A., Lees, D.N., Baker-Austin, C., 2015. Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014. Eurosurveillance 20, 21009. https://doi.org/10.2807/1560-7917.ES2015.20.2.21009.

Turner, A.D., Boundy, M.J., Dhanji-Rapkova, M., 2017a. Development and single-laboratory validation of a liquid chromatography tandem mass spectrometry method for quantitation of tetrodotoxin in mussels and oysters. J. AOAC Int. 100, 1469–1482. https://doi.org/10.5740/jaoacint.17-0017.

Turner, A.D., Dhanji-Rapkova, M., Coates, L., Bickerstaff, L., Milligan, S., O'Neill, A., Faulkner, D., McEneny, H., Baker-Austin, C., Lees, D.N., Algoet, M., 2017b. Detection of tetrodotoxin shellfish poisoning (TSP) toxins and causative factors in bivalve molluscs from the UK. Mar. Drugs 15, 277. https://doi.org/10.3390/md15090277.

Turner, A.D., Fenwick, D., Powell, A., Dhanji-Rapkova, M., Ford, C., Hatfield, R.G., Santos, A., Martinez-Urtaza, J., Bean, T.P., Baker-Austin, C., Stebbing, P., 2018. New invasive nemertean species (Cephalothrix simula) in England with high levels of tetrodotoxin and a microbiome linked to toxin metabolism. Mar. Drugs 16, 452. https://doi.org/10.3390/md16110452.

Turner, A.D., Dhanji-Rapkova, M., Fong, S.Y.T., Hungerford, J., McNabb, P.S., Boundy, M.J., Harwood, D.T., 2020. Ultrahigh-performance hydrophilic interaction liquid chromatography with tandem mass spectrometry method for the determination of paralytic shellfish toxins and tetrodotoxin in mussels, oysters, clams, cockles, and scallops: collaborative study. J. AOAC Int. 103, 533–562.

Vezzulli, L., Pezzati, E., Brettar, I., Höfle, M., Pruzzo, C., 2015. Effects of global warming on *Vibrio* ecology. Microbiol. Spectr. 3. https://doi.org/10.1128/microbiolspec.ve-0004-2014.

Vlamis, A., Katikou, P., Rodriguez, I., Rey, V., Alfonso, A., Papazachariou, A., Zacharaki, T., Botana, A.M., Botana, L.M., 2015. First detection of tetrodotoxin in greek shellfish by UPLC-MS/MS potentially linked to the presence of the dinoflagellate *Prorocentrum* minimum. Toxins 7, 1779–1807. https://doi.org/10.3390/toxins7051779 Basel.

Yamada, R., Tsunashima, T., Takei, M., Sato, T., Wajima, Y., Kawase, M., Oshikiri, S., Kajitani, Y., Kosoba, K., Ueda, H., Abe, K., Itoi, S., Sugita, H., 2017. Seasonal changes in the tetrodotoxin content of the flatworm *Planocera multitentaculata*. Mar. Drugs 15, 1–10. https://doi.org/10.3390/md15030056.