

Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014

A D Turner (andrew.turner@cefas.co.uk)¹, A Powell¹, A Schofield^{1,2}, D N Lees¹, C Baker-Austin¹

1. Food Safety Group, Centre for Environment Fisheries and Aquaculture Science, Weymouth, Dorset, United Kingdom

2. Department of Chemistry, University of Hull, Hull, United Kingdom

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We report the first detection of tetrodotoxins (TTX) in European bivalve shellfish. We demonstrate that TTX is present within the temperate waters of the United Kingdom, along the English Channel, and can accumulate in filter-feeding molluscs. The toxin is heat-stable and thus it cannot be eliminated during cooking. While quantified concentrations were low in comparison to published minimum lethal doses for humans, the results demonstrate that the risk to shellfish consumers should not be discarded.

Background

Tetrodotoxin (TTX) is the causative agent responsible for pufferfish/fugu poisoning, a fatal marine poisoning found predominantly in tropical regions. It is found mainly in the organs of fish from the *Tetraodontidae* family, as well as other marine species such as the blue-ringed octopus and gastropods [1]. The toxin and its structural analogues are thought to originate from a variety of marine bacteria, including *Vibrio* spp. [2].

Clinical effects include a range of neuromuscular symptoms such as paraesthesia of lips and tongue, dizziness and headache, together with gastrointestinal symptoms such as nausea, abdominal pain, diarrhoea and vomiting. Higher degree symptoms include ataxia, incoordination, cardiac arrhythmias, seizures and respiratory failure, leading to death [3]. To date, the only reported occurrences of TTX in bivalve molluscs (clams, cockles, mussels, oysters, scallops and others) have been in New Zealand clams [4] and in Japanese scallops [5]. In European seafood, the only reported occurrence was in 2007. It was detected in the course of a non-fatal human intoxication following consumption of the contaminated sea snail *Charonia lampas lampas* (a gastropod) harvested in Spain [6]. There has been no evidence for the accumulation of tetrodotoxin in bivalve molluscs grown within European waters to date, and the threat from this toxin is deemed negligible within the European Union. However, with *Vibrio* spp., reported to be associated with TTX production, detected in United Kingdom shellfish in 2010 [7], and evidence for increasing sea temperatures [8], we aimed

to assess the potential for this toxin to accumulate in bivalves grown on the south coast of England, along the Channel.

Testing of bivalve shellfish samples

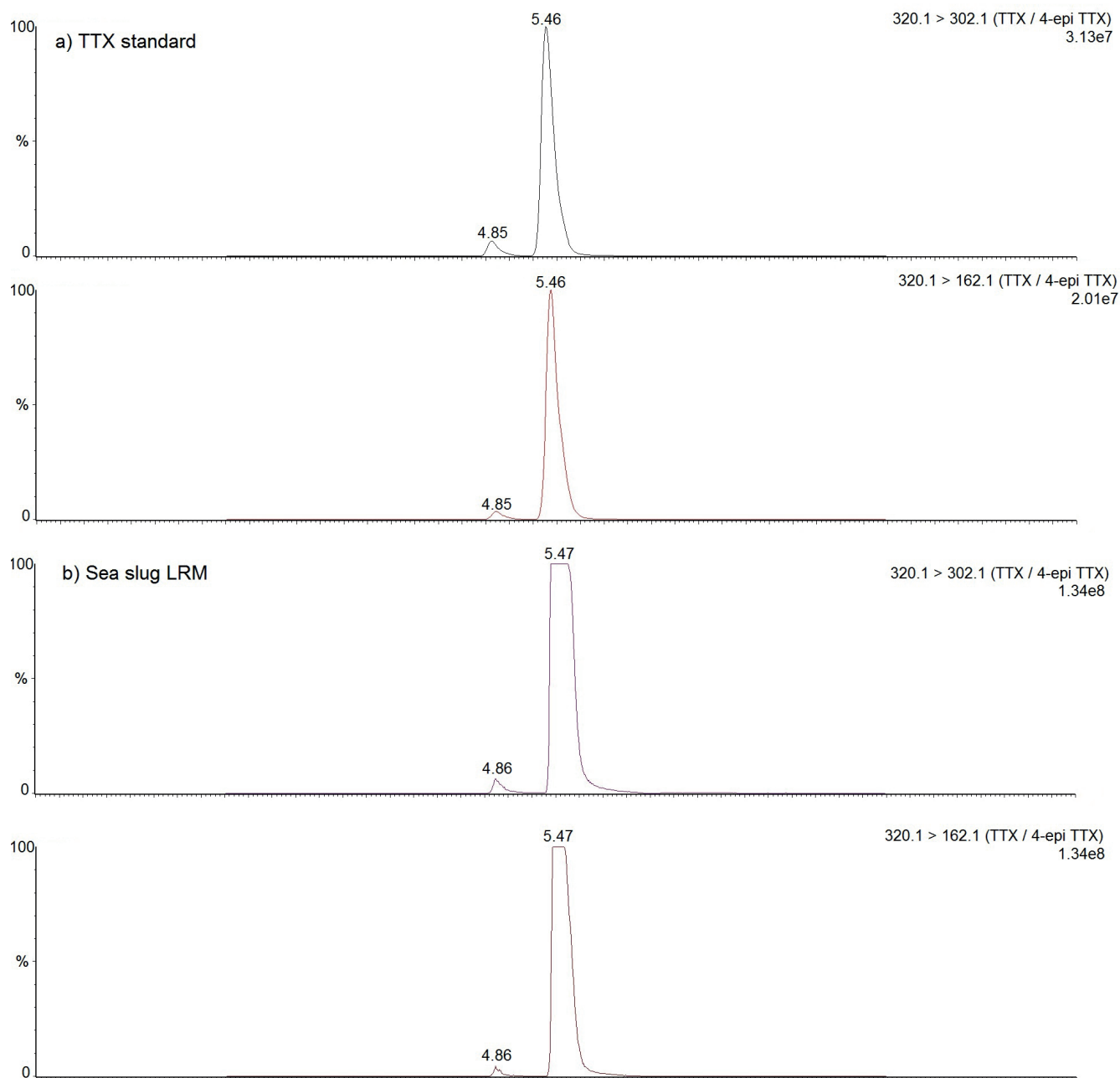
Twenty-nine shellfish samples (*Mytilus Edulis* and *Crassostrea gigas*), each comprising a minimum of 20 live animals, were harvested between February 2013 and October 2014 from two marine sites on the south coast of England. After shucking, shellfish tissue was prepared for bacterial pathogen detection as previously described [9] and the remainder frozen in storage before chemical analysis. TTXs were analysed in thawed, homogenised shellfish tissues following the methods described by McNabb et al. [4], with a modified shellfish extraction procedure based on [10] and incorporating additional TTX analogues taken from [11]. Hydrophilic interaction chromatography (HILIC) using an ultra performance liquid chromatograph (UPLC) with electrospray ionisation tandem quadrupole mass spectrometry (MS/MS) was used for detection of TTXs. The TTX standard was sourced from Enzo Life Sciences (Exeter, UK).

Two selected reaction monitoring (SRM) transitions were optimised for each of the seven tested toxins, enabling the quantification of toxin concentrations against an external TTX calibration. A Waters Acquity UPLC and Xevo TQ-S MS/MS were optimised for detection of TTX and six TTX congeners (4-epi TTX; 5,6,11-trideoxy TTX; 4,9-anhydro TTX; 11-nor TTX-6-ol; monodeoxy TTX; 11-oxo TTX) based on previous studies [4,11]. Semiquantitation of TTX analogues was conducted assuming a relative response factor of 1 to the parent TTX. A second HILIC-MS/MS method based on the detection of TTX dehydration products (C₉ base 2-amino-6-(hydroxymethyl)quinazolin-8-ol) following alkaline derivatisation, was used for additional confirmation [4].

Additionally, *Vibrio parahaemolyticus* isolated from six of the shellfish samples, and confirmed by PCR targeting species specific markers [9] were cultured in the

FIGURE A

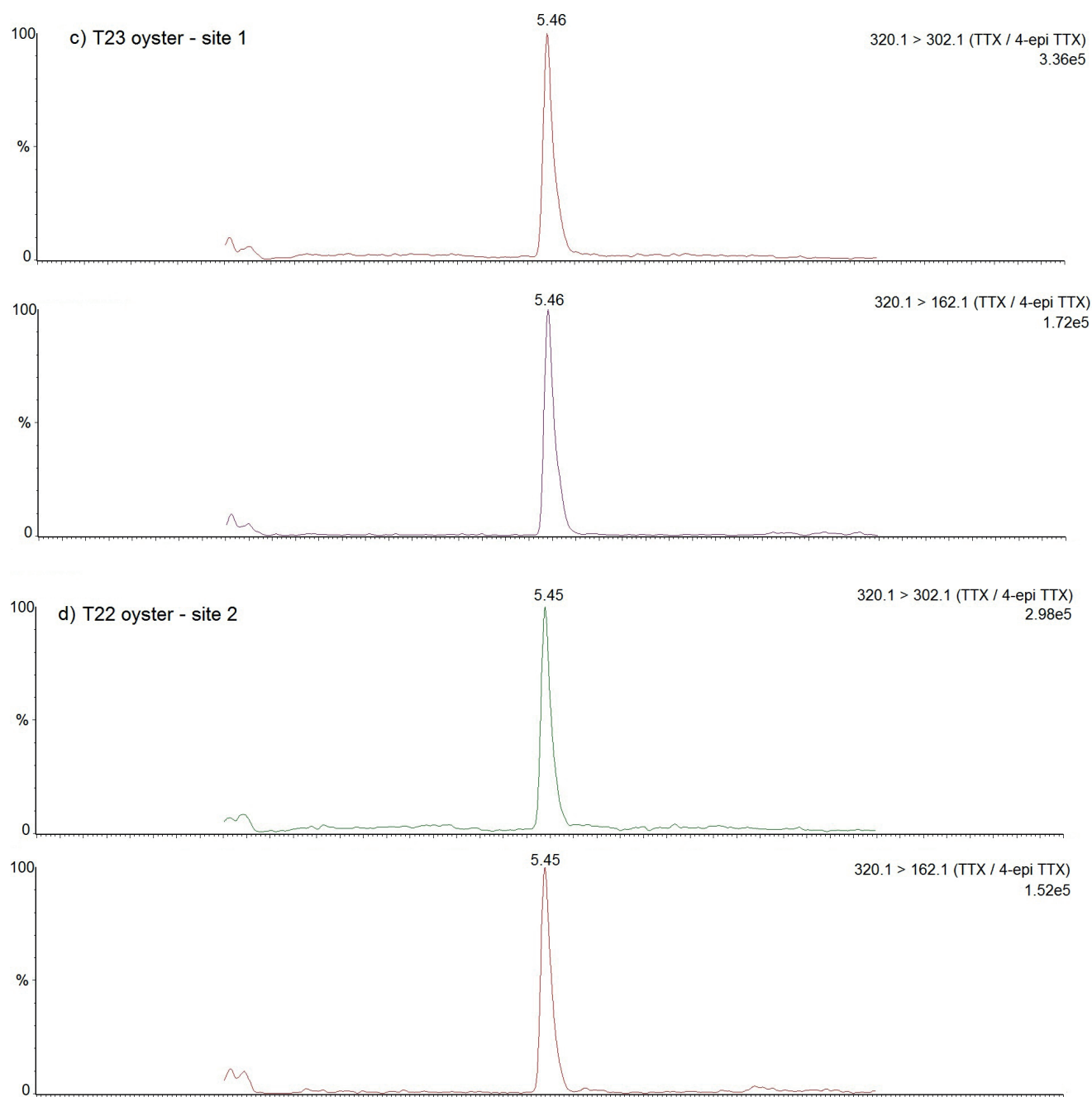
Selected reaction monitoring chromatograms obtained following the analysis of tetrodotoxin (TTX) in TTX calibration standard (a), laboratory reference material (b), T23 oyster (c), T22 oyster (d), culture APC6 (e)



LRM: laboratory reference material; TTX: tetrodotoxin.

FIGURE B

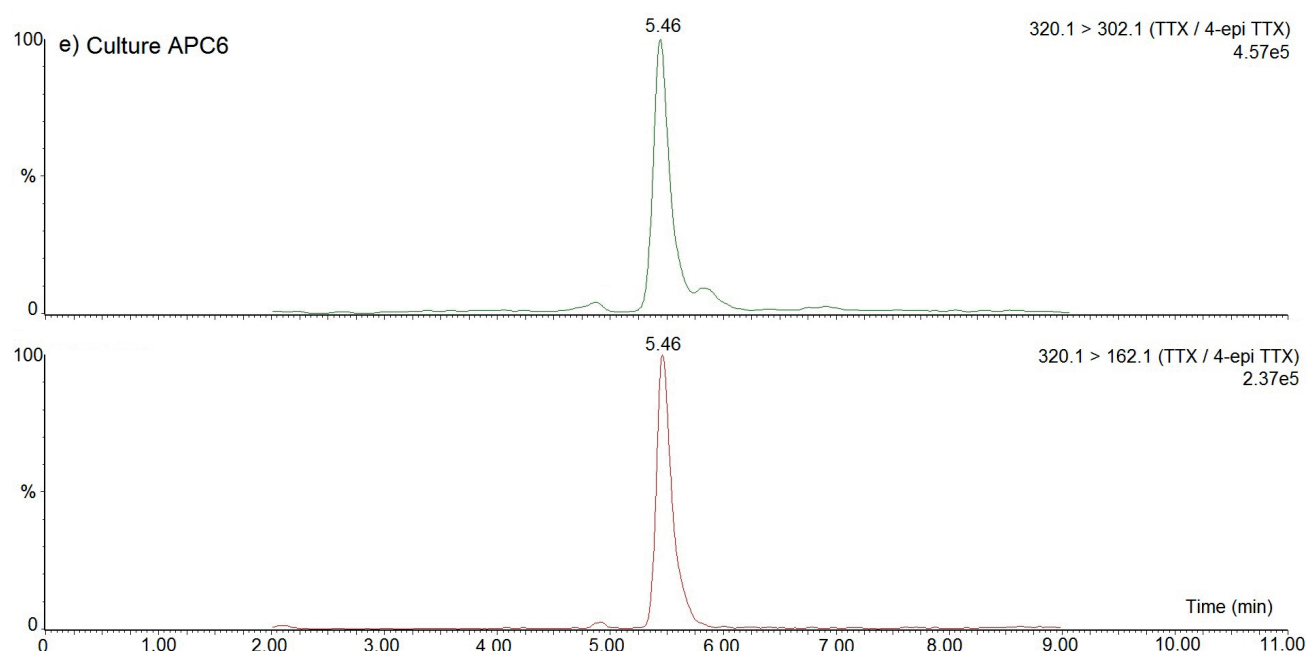
Selected reaction monitoring chromatograms obtained following the analysis of tetrodotoxin (TTX) in TTX calibration standard (a), laboratory reference material (b), T23 oyster (c), T22 oyster (d), culture APC6 (e)



LRM: laboratory reference material; TTX: tetrodotoxin.

FIGURE C

Selected reaction monitoring chromatograms obtained following the analysis of tetrodotoxin (TTX) in TTX calibration standard (a), laboratory reference material (b), T23 oyster (c), T22 oyster (d), culture APC6 (e)



LRM: laboratory reference material; TTX: tetrodotoxin.

laboratory and tested for TTXs. Cultures were centrifuged and the bacterial pellets extracted in 1% acetic acid before HILIC-MS/MS analysis. Analysis of all unknown samples was conducted alongside two sets of six-level calibration standards and a highly TTX-positive laboratory reference material (LRM) extract prepared from New Zealand Sea Slugs (*Pleurobranchaea maculata*) [4].

Results

Bivalve shellfish samples

Eleven of 29 shellfish samples were found to contain *V. parahaemolyticus* in the shellfish tissue, with one additional sample found to be positive for *V. cholerae*. TTX was detected in 14 of 29 samples, with detection confirmed through the presence of chromatographic peaks for both the primary (quantifier) and secondary (qualifier) SRM transitions, at the same retention time as the TTX standard calibrants and in the LRM (Figures A, B).

The mean primary to secondary SRM peak ratios were 1.87 ± 0.13 (7%) for the TTX standards and 1.83 ± 0.26 (14%) for the average of all TTX-positive samples.

4-epi TTX was identified in five out of 29 samples, notably those containing the highest TTX concentrations. 5,6,11-trideoxy TTX and 4,9-anhydro TTX were detected in 13 and one sample respectively, with

detection confirmed with SRM peaks at the same retention time as those present in the LRM. Detection and semi-quantitation of the C₉ base product provided a further level of TTX confirmation in the five samples containing the highest concentrations of toxin. The absence of the C₉ base product in samples containing lower concentrations of TTX is thought to relate to differences in method sensitivity. TTX concentrations ranged from approximately the limit of quantitation (3 µg TTX/kg shellfish tissue) to a maximum of 120 µg/kg. TTX analogues were quantified at lower levels, typically 10–15% of the total TTX content (Table 1). The maximum summed concentration quantified of all TTX analogues was 137 µg TTX/kg in sample T23.

Tetrodotoxins in bacterial cultures

Eleven bacterial isolates were obtained from six different TTX-contaminated bivalve samples. These were cultured for two days, before being processed for TTX analysis. Ten of the cultures were *V. parahaemolyticus*, with the other isolate *V. cholerae*. TTX was detected in ten of the cultures (Figure C), at concentrations between 42 and 718 ng TTX/L of culture (Table 2), with TTX the only analogue detectable in any of the cultured samples.

Discussion

Our study reveals, to our best knowledge, the first detection of the causative agent of pufferfish/fugo

TABLE 1

Analysis of bivalve molluscs for *Vibrio parahaemolyticus* and tetrodotoxins, England, 2013–2014

Sample	Date of collection	Site info	Species	Vibrio	TTX ^a	4-epi TTX ^a	5,6,11-trideoxy TTX ^a	4,9-anhydro TTX ^a	C ₉ base of TTX ^a
T1	30 Oct 2013	Site 1	PO	ND	ND	ND	5.1	ND	ND
T2	17 Dec 2013	Site 1	PO	ND	ND	ND	2.8	ND	ND
T3	17 Dec 2013	Site 2	PO	ND	11	ND	ND	ND	ND
T4	26 Feb 2014	Site 1	PO	ND	ND	ND	4.4	ND	ND
T5	26 Nov 2014	Site 2	PO	ND	5.6	ND	ND	ND	ND
T6	29 Oct 2013	Site 2	PO	ND	4.4	ND	ND	ND	ND
T7	29 Jan 2014	Site 2	M	Y	3.0	ND	ND	ND	ND
T8	29 Jan 2014	Site 2	PO	Y	ND	ND	ND	ND	ND
T9	26 Feb 2014	Site 2	PO	ND	ND	ND	ND	ND	ND
T10	26 Feb 2014	Site 2	M	ND	ND	ND	ND	ND	ND
T11	17 Dec 2014	Site 2	PO	ND	ND	ND	ND	ND	ND
T12	29 Jan 2014	Site 1	PO	ND	ND	ND	2.4	ND	ND
T13	27 Aug 2013	Site 1	PO	ND	7.6	ND	ND	ND	ND
T14	25 Nov 2013	Site 1	PO	ND	ND	ND	2.8	ND	ND
T15	29 Feb 2013	Site 1	PO	Y	52	2.0	3.4	ND	37
T16	27 Aug 2013	Site 2	PO	Y	14	ND	4.3	ND	ND
T17	26 Feb 2014	Site 2	PO	Y	15	ND	1.3	ND	ND
T18	26 Feb 2014	Site 2	M	ND	ND	ND	ND	ND	ND
T19	31 Oct 2013	Site 2	PO	ND	ND	ND	ND	ND	ND
T20	29 Jul 2013	Site 2	PO	Y	14	0.4	3.1	ND	ND
T21	27 Aug 2013	Site 2	PO	ND	2.7	ND	ND	ND	ND
T22	17 Jun 2014	Site 1	PO	Y†	89	2.8	6.5	ND	76
T23	17 Jun 2014	Site 2	PO	Y	120	3.9	11	1.8	121
T24	17 Jun 2014	Site 2	M	Y	39	1.2	3.8	ND	28
T25	25 Nov 2013	Site 2	M	ND	ND	ND	ND	ND	ND
T26	29 Jul 2013	Site 2	PO	Y	15	ND	1.9	ND	22
APF1	15 Sep 2014	Site 2	PO	Y	ND	ND	ND	ND	ND
APF2	15 Sep 2014	Site 2	M	Y	ND	ND	ND	ND	ND
APF3	16 Sep 2014	Site 1	PO	ND	ND	ND	ND	ND	ND

M: mussels; ND: not detected; PO: Pacific oyster; TTX: Tetrodotoxin; Y: *Vibrio* spp. detected; Y†: *Vibrio cholera* detected.^a µg per kg shellfish tissue.

poisoning, TTX in bivalve molluscs, mussels and Pacific oysters harvested in Europe. It is also the first detection of TTX in any form within the marine waters of the UK. TTXs are not monitored routinely anywhere in the world for their presence in bivalves, given the absence of published data demonstrating a risk of TTX intoxication from bivalves. The findings reported here are notable given the established assumption that TTXs are associated either with pufferfish or with marine bacteria found exclusively in tropical and sub-tropical oceans and seas [3,6]. Here we provide new evidence for the presence of TTX in the temperate waters of the English Channel, thereby extending the range of known occurrences of these important toxins.

TTX was quantified against known standards, with confirmation in positive samples coming from the acquisition of two SRMs, toxin retention time checks and determination of SRM ion ratios. Further confirmation was achieved through detection of TTX C₉ base products. Toxin profiles in the bivalve shellfish were dominated by the parent toxin. With *Vibrio* cultures containing only TTX, the analogues may result from metabolism by shellfish, as opposed to direct bacterial products. The overall concentrations of TTX were lower than those quantified previously in a sample of the New Zealand bivalve *Paphies australis* [4]. Interestingly, here also the parent TTX was the only analogue detected in the bacterial culture samples.

TABLE 2

Analysis of bacterial cultures for tetrodotoxins, England, 2013–2014

Culture sample	Known pathogen	TTX ng/L in culture	Associated shellfish sample
APC 1	<i>Vibrio parahaemolyticus</i>	59	T23
APC 2	<i>V. parahaemolyticus</i>	67	T24
APC 3	<i>V. parahaemolyticus</i>	42	T26
APC 4	<i>V. cholerae</i>	84	T22
APC 5	<i>V. parahaemolyticus</i>	117	T17
APC 6	<i>V. parahaemolyticus</i>	718	T24
APC 7	<i>V. parahaemolyticus</i>	62	T23
APC10	<i>V. parahaemolyticus</i>	ND	T23
APC11	<i>V. parahaemolyticus</i>	103	T24
APC13	<i>V. parahaemolyticus</i>	84	T23
APC14	<i>V. parahaemolyticus</i>	116	T24

ND: not detected; TTX: tetrodotoxin.

The detection of TTX in all but one of the *V. parahaemolyticus* cultures isolated from bivalve molluscs may be significant, providing additional compelling evidence for the production of TTX by *Vibrio* spp. The detection of quantifiable levels of TTX in the bivalves in tandem with the detection of *Vibrio* spp., strengthens the possibility that the bacteria provide the source of the toxin detected in bivalve molluscs, however, further work in this area is clearly necessary. Interestingly, not all TTX-positive bivalves were found to contain *Vibrio* species, while three of the *Vibrio*-positive bivalve samples showed no TTX above the limit of detection. However, in the absence of quantitative data for *Vibrio*, these differences may relate to differences in method sensitivities.

Given the absence of any formal regulatory guidance of TTX in shellfish, the maximum concentration of 137 µg/kg TTX quantified here, equates to 17% of the maximum permitted level of saxitoxin (STX) equivalents (800 µg STX equivalents/kg shellfish tissue), noting the similarity in biological activity between the two toxin groups. 137 µg/kg would also equate to a low level dose of toxin in comparison to the proposed minimum lethal dose (MLD) for TTX of between 0.5 to 2 mg [3]. Consumption of 500g of shellfish contaminated with 137 µg/kg of TTXs would equate to the intake of ca 70 µg TTX, ca 14% of the proposed MLD if taken as 0.5 mg TTX for a 60 kg human [12]. However, this calculation does not incorporate any additional safety factors as applied by the European Food Standards Agency (EFSA) in their risk assessment methods, taking into account measurement or toxicity-related uncertainties [13], and/or the likely high variability of toxin content in bulk samples of shellfish across harvesting areas.

Consequently, while the human health risk determined from the samples analysed in this study is shown to be low, there is the potential for health impacts, particularly if the levels of TTX were significantly higher at other times or in other areas associated with shellfish harvesting. It is important to note that while bacterial pathogens may be eliminated in shellfish products following effective cooking, TTXs are heat stable and will thus not be destroyed in the food preparation process.

Given the evidence presented here for TTX occurrence in European bivalve molluscs, and the traditional occurrence of these toxins in warm tropical waters, an important question is whether this is linked to increasing sea surface temperatures. The frequency of extreme hot days has increased significantly in the last decade along the margins of the east Atlantic, most notably in the North Sea and English Channel. The frequency of extreme cold periods has also gone down and annual warming is seen to occur earlier in the year on average [8].

Conclusions

We reveal the presence, for the first time, of the neurotoxin tetrodotoxin in bivalve mollusc shellfish grown at two marine sites along the south coast of England. These toxins have previously been assumed not to occur in bivalve molluscs, particularly in temperate waters. Further, we found an association between the occurrence of TTX and marine *Vibrio* species both in bivalve molluscs and in bacterial cultures. Given the increasingly favourable conditions for *Vibrio* proliferation in European waters as sea surface temperatures will possibly rise in the coming decades, we suggest that the potential for occurrence of autochthonous marine bacteria such as *Vibrio* and TTXs in seafood grown in temperate areas should be more widely investigated.

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Conflicts of interest

None declared.

Authors' contributions

AT and AP designed the study. AP performed the sample preparation and bacterial analysis. CBA performed molecular confirmation of *Vibrio* strain. AS and AT extracted and SPE-cleaned the shellfish. AT performed HILIC-MS/MS quantitation of TTXs in shellfish extracts and bacterial cultures. AT, AP, DL and CBA discussed the results and participated in the writing.

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