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Abstract: Paralytic shellfish toxins (PST) and tetrodotoxin (TTX) are naturally-occurring toxins that may contaminate the food chain, inducing similar neurological symptoms in humans. They are co-extracted under the same conditions and thus their combined detection is desirable. Whilst PST are regulated and officially monitored in Europe, more data on TTX occurrence in bivalves and gastropods are needed before meaningful regulations can be established. In this study, we used three separate analytical methods - pre-column oxidation with liquid chromatography and fluorescence detection, ultrahigh performance hydrophilic interaction liquid chromatography (HILIC) tandem mass spectrometry (MS/MS) and HILIC high resolution (HR) MS/MS - to investigate the presence of PST and TTX in seawater and shellfish (mussels, clams) collected in spring summer 2015 to 2017 in the Mediterranean Sea. Samples were collected at 10 sites in the Syracuse Bay (Sicily, Italy) in concomitance with a mixed bloom of Alexandrium minutum and A. pacificum. For the first time a significant PST contamination in mussels emerged, with maximum total concentration of 10851 ug saxitoxin equivalents per kg of shellfish tissue measured in 2016. In addition, for the first time TTX was detected in most of the analysed samples in the range 0.8-6.4 ug TTX eq/kg. The recurring blooms of PST-producing species over the 3-year period, the high PST levels and the first finding of TTX in Italian mussels, suggest that human health concerns exist and that monitoring programmes of PST and TTX in seafood should be activated in this geographical area.

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Napoli, July 28th 2018

Dear Prof. de Boer,

here attached, please find the electronic version of the manuscript entitled "*First detection of Paralytic Shellfish Poisoning Toxins and Tetrodotoxin in shellfish from Sicily (Italy): a multi-platform chromatographic approach*" by Carmela Dell'Aversano*, Luciana Tartaglione, Giuseppe Polito, Karl Dean, Mariagrazia Giacobbe, Silvia Casabianca, Samuela Capellacci, Antonella Penna, and Andrew D. Turner, submitted for publication as *Research Article* on *Chemosphere*.

All authors that have participated to the research, have read the manuscript, agree the work is ready for submission to journal, and accept responsibility for the manuscript's contents. The authors declare that there are no actual or potential conflicts of interest.

The manuscript is an original work, it has not been previously published whole or in part and is not under consideration for publication elsewhere.

The manuscript is within the 6500 word limit of a Research Article in containing 5650 words in the text (excluding references), 4 figures and 3 tables. We believe that both figures and tables are highly functional to understanding the rational basis of the study, however, we are available to meet your suggestions and reduce further the length of the paper, if needed.

Rationale for consideration by Chemosphere

Paralytic shellfish toxins (PST) and tetrodotoxin (TTX) are highly potent marine toxins that may contaminate the food chain resulting in severe neurological syndromes. PST are regulated and officially monitored in Europe which significantly decreases risks for shellfish consumers, while TTX is listed among the emerging toxins and more data on its occurrence in bivalves and gastropods are needed before meaningful regulations can be established. In Italy, TTX distribution in seafood has not been investigated so far, while the presence of PST-producing species is reported as an occasional phenomenon so far limited to some specific sites.

In this study, we used three separate analytical methods (PreCOX-HPLC-FLD, UHPLC-HILIC-MS/MS and HILIC-HRMS/MS) to investigate the presence of both PST and TTX in the environment (concentrated seawater) and in shellfish (mussels and clams) collected over a 3-year period in the Syracuse Bay, to the aim of determining the likelihood of risk from PST and TTXs in bivalve mollusks from the Mediterranean area. As a result for the first time a significant PST contamination in shellfish from Sicily emerged and for the first time TTX was detected in Italian shellfish. In a methodological perspective, all the 3 approaches turned out to be applicable to the high throughput testing of shellfish tissues in a monitoring framework, although formal validation of the HILIC-HRMS method is still needed before the platform can be used routinely.

We wish to suggest the following outstanding scientists as potential reviewers of the manuscript:

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We are looking forward to hearing from you,

Yours Sincerely

Carmela Dell'Aversano

*Title Page

First detection of Paralytic Shellfish Poisoning Toxins and Tetrodotoxin in shellfish from Sicily (Italy): a multi-platform chromatographic approach

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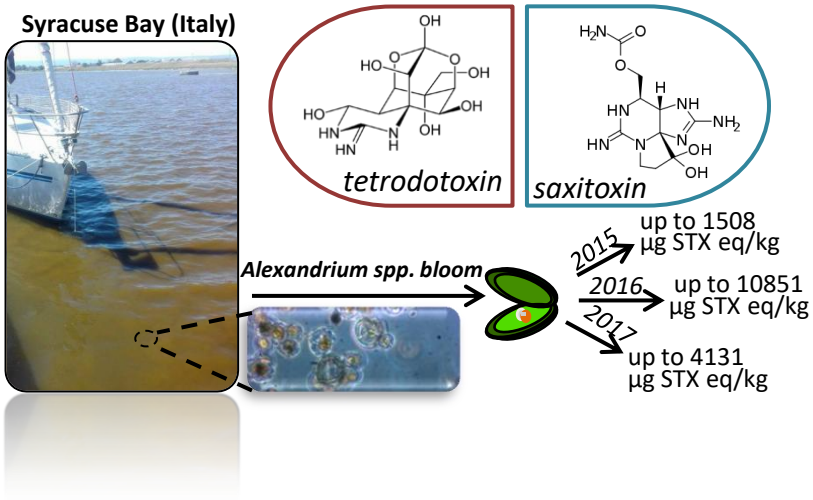
***Highlights (3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

Highlights

- Recurring blooms of *Alexandrium minutum* and *Alexandrium pacificum* in Syracuse bay
- Toxin profile and content of seawater and shellfish collected in 2015, 2016, 2017
- Paralytic shellfish toxins contaminating mussels at levels up to 10851 μg STX eq/Kg
- First finding in Italy of tetrodotoxin in mussels at levels up to 6.4 μg TTX eq/Kg
- Comparison among PreCOX-HPLC-FLD, UHPLC-HILIC-MS/MS, and HPLC-HILIC-HRMS results

Graphical Abstract



First detection of Paralytic Shellfish Poisoning Toxins and Tetrodotoxin in shellfish from Sicily (Italy): a multi-platform chromatographic approach

Carmela Dell'Aversano[1,2*], Luciana Tartaglione[1,2], Giuseppe Polito[1], Karl Dean[3], Mariagrazia Giacobbe[4], Silvia Casabianca[5], Samuela Capellacci[5], Antonella Penna[5], Andrew D. Turner[3]

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Abstract

Paralytic shellfish toxins (PST) and tetrodotoxin (TTX) are naturally-occurring toxins that may contaminate the food chain, inducing similar neurological symptoms in humans. They are co-extracted under the same conditions and thus their combined detection is desirable. Whilst PST are regulated and officially monitored in Europe, more data on TTX occurrence in bivalves and gastropods are needed before meaningful regulations can be established. In this study, we used three separate analytical methods - pre-column oxidation with liquid chromatography and fluorescence detection, ultrahigh performance hydrophilic interaction liquid chromatography (HILIC) tandem mass spectrometry (MS/MS) and HILIC high resolution (HR) MS/MS - to investigate the presence of PST and TTX in seawater and shellfish (mussels, clams) collected in spring summer 2015 to 2017 in the Mediterranean Sea. Samples were collected at 10 sites in the Syracuse Bay (Sicily, Italy) in concomitance with a mixed bloom of *Alexandrium minutum* and *A. pacificum*. For the first time a significant PST contamination in mussels emerged, with maximum total concentration of 10851 μg saxitoxin equivalents per kg of shellfish tissue measured in 2016. In addition, for the first time TTX was detected in most of the analysed samples in the range 0.8-6.4 μg TTX eq/kg. The recurring blooms of PST-producing species over the 3-year period, the high PST levels and the first finding of TTX in Italian mussels, suggest that human health concerns exist and that monitoring programmes of PST and TTX in seafood should be activated in this geographical area.

Keywords: *Alexandrium* spp., Paralytic Shellfish Toxins, Tetrodotoxin, UHPLC-HILIC-MS/MS, HPLC-HILIC-HRMS/MS, HPLC-FLD.

1. Introduction

Paralytic shellfish toxins (PST) are a group of more than 50 structurally-related tetrahydropurine derivatives, with saxitoxin (STX) being the parent compound (Wiese et al., 2010) (Fig. S1). They are produced by specific phytoplankton species- belonging to the genus *Alexandrium*, *Gymnodinium*, and *Pyrodinium* - and accumulate in filter-feeding shellfish (Hallegraeff, 2003), thus inducing a paralytic shellfish poisoning (PSP) syndrome in humans (Llewellyn, 2006). PST target voltage-gated sensitive sodium channels (VSSC) and the human illness following the consumption of contaminated shellfish is characterized by a wide variety of neurological symptoms. Consequently, in the European Union (EU) there is a statutory requirement for member states to carry out official monitoring of this toxin class in shellfish (Anon, 2004).

Tetrodotoxin (TTX) is also a naturally-occurring toxin, well known in Japan to cause lethal food poisonings following ingestion of contaminated puffer fish (*fugu*) (Isbister and Kiernan, 2005). Unlike PST, the primary source of TTX in the environment is thought to be marine bacteria (Yasumoto *et al.*, 1988; Wu *et al.*, 2005; Wang *et al.*, 2008; Chau *et al.,* 2011). The toxin can then accumulate in various edible marine species including fish, gastropods, and bivalve molluscs (Kodama *et al.*, 1993; McNabb *et al.*, 2014) where it may be bio-transformed in a number of analogues (Fig. S1) (Bane *et al.* 2014). Despite showing different structural features, TTX acts on the same molecular target as PST and induces similar effects in humans (Alcaraz *et al.*, 1999; Noguchi *et al.*, 2011). However, TTX and its analogues are not monitored on a regular basis in the EU and no maximum permitted level (MPL) has been defined, although a concentration lower than 44 μg of TTX eq/kg of shellfish meat is expected not to harm humans (EFSA, 2017). To date, TTXs have been reported only in mussels and Pacific

69 oysters from UK (Turner *et al.*, 2015a, 2015b), in mussels from Greece (Vlamis *et al.*, 2015) and
70 Netherlands (Gerssen, 2016, personal communication).

71 Besides exerting similar toxic effects, PST and TTX are co-extracted under then same
72 conditions, thus availability of a methodological approach for their combined detection is a
73 prerequisite. The EU's reference method for detecting and quantifying PST in bivalve molluscs is the
74 mouse bioassay (MBA) (Anon, 2005a, 2005b). An alternative method (Official method AOAC 2005.06)
75 commonly referred as the "Lawrence method" (Lawrence *et al*., 2005), is also incorporated into EU
76 legislation (Anon, 2006). It uses pre-column oxidation derivatisation (Pre-COX) followed by high
77 performance liquid chromatography with fluorescence detection (HPLC-FLD) (Anon, 2005c). Since
78 2006 the Pre-COX HPLC-FLD method has been refined and incorporated into official control
79 monitoring programmes around the world (Harwood *et al*., 2013; Turner *et al*., 2009, 2010, 2011;
80 Turner and Hatfield, 2012). However, neither Pre-COX HPLC-FLD nor subsequently developed
81 alternative methods (Van de Riet *et al*., 2011; van Dolah *et al*., 2012) allow combined detection of PST
82 and TTX.

83 Over the last 16 years, the development of assorted mass spectrometry (MS) based methods
84 for PST detection and quantitation has been reported. Dell'Aversano *et al*. (2005) first reported the
85 use of hydrophilic interaction liquid chromatography with tandem MS (HILIC-MS/MS), a specific and
86 sensitive method that facilitates the separation of PST within 30 min, including PST analogues formed
87 in mussels (M-toxins), which are not amenable to FLD detection (Dell'Aversano *et al*., 2008). Various
88 other authors have reported the development of LC-MS/MS for PST (Diener *et al*., 2007; Turrell *et al*.,
89 2008; Sayfritz *et al*., 2008; Zhuo *et al*., 2013), although quantitation was affected by problems
90 including in-source fragmentation, salt-related ionisation suppression, poor analyte recovery and
91 irreproducible retention times. More recently, however, Boundy *et al*. (2015) described the

development of a novel ultrahigh performance liquid chromatography (UHPLC) HILIC-MS/MS method which compared well with the pre-COX HPLC-FLD method and was subjected to a full single laboratory validation (SLV) by Turner *et al*. (2015c), testing it for twelve species of bivalve molluscs, including a variety of mussels, oysters, cockles, scallops and clams. Another advantage of this UHPLC-HILIC-MS/MS approach is its ability to detect and quantify TTX (Boundy *et al*., 2015) and, thus, it was subjected to a full SLV for detection of TTX in mussels and oysters from the UK (Turner *et al*., 2017).

The Pre-COX HPLC-FLD and UHPLC-HILIC-MS/MS methods both directly quantify toxin concentrations against external toxin calibrants, with sample toxicity subsequently calculated as total STX equivalents through application of Toxicity Equivalence Factors (TEFs). The HPLC-FLD method provides a good estimation of toxicity, although there are some perceived issues, including the inability of Pre-COX to separate PST epimeric pairs (DeGrasse *et al*., 2011; Turner *et al*., 2014). The complexity of the quantitation method is also known to be a factor in slowing the move away from reliance on the MBA, with the method incorporating multiple clean-up and pre-column oxidation stages. The UHPLC-HILIC-MS/MS method facilitates the analysis of a wider range of toxin analogues with full separation of PST epimers and TTX, using a simple and rapid extraction method, all within one single analytical run of 11 min. An additional analytical run of 8 min using the same column, MS source conditions and mobile phases can be conducted to detect a larger number of TTX analogues (Turner *et al.*, 2017).

In Italy, a systematic study on TTX distribution in seafood has not been performed so far while the presence of PST-producing species such as *A. minutum, A. pacificum*, and *G. catenatum* is a phenomenon so far limited to some specific sites (Ciminiello *et al*., 2014; Penna *et al*., 2015). The first report dates back to 1994 when *A. minutum* was identified along the Emilia-Romagna coast (NW Adriatic Sea) (Pistocchi *et al.*, 2012) and mussels from the same geographical area were found to be

contaminated by PST above the EU regulatory limit, although no human poisoning was reported concomitantly (Poletti, 1998). In the following years, PST contamination of shellfish was reported again in the same area (EU-NRL, 1995) and in Friuli Venezia Giulia (EU-NRL, 2001) (NE Adriatic Sea) at levels <400 μg STX eq/kg, as well as along the Sardinia coasts (Tyrrhenian Sea) where a maximum contamination level of 503 μg STX eq/kg was detected in mussels, in concomitance to the presence of *A. pacificum* and *A. minutum* in seawater (EU-NRL, 2002; Bazzoni *et al*., 2016). Just a few records of PST-contaminated bivalve molluscs from Southern Italy, including Sicily (Giacobbe *et al*, 2006) have been reported so far. Both *A. minutum* and *A. pacificum* were detected in the Ionian and the Tyrrhenian Sea (Penna et al. 2005; 2008; 2015; Casabianca et al., 2012; Lugliè et al., 2017). Based on the *sxtA1* gene quantification in the seawater samples, qPCR assay proved to quantify the potential STX-producing cell abundance during bloom events, while HILIC coupled to high-resolution tandem mass spectrometry (HRMS/MS) proved to detect PST in microalgal cultures and environmental samples (Penna *et al*., 2015).

In the period 2015-2017, *Alexandrium* spp. were again detected every spring along the Ionian coasts of Sicily, with a consequent risk of toxin accumulation in bivalve molluscs. Therefore, the aim of this study was to measure toxin levels in both seawater and shellfish samples collected during algal blooms from the inshore marine waters at Syracuse Bay (Ionian Sea). Molecular qPCR assay was applied to both estimate toxic *Alexandrium* cell abundance and *sxtA1* gene copy number in seawater samples during the bloom (Penna et al., 2015). PST detection was conducted in two different laboratories using the same extraction and clean-up procedure and three separate chemical detection methods, pre-COX HPLC-FLD, UHPLC-HILIC-MS/MS, and HPLC-HILIC-HRMS/MS. The UHPLC-HILIC-MS/MS method was also used for the analysis of TTXs in shellfish and seawater. Evidence from the

analysis would then be used to determine the likelihood of risk from PST and TTXs in bivalve molluscs from the Mediterranean Sea.

2. Materials and methods

2.1. Reagents and chemicals

Sample preparation and SPE reagents as well as Pre-COX HPLC-FLD reagents were HPLC grade. Water, acetonitrile and methanol (HPLC grade), glacial acetic acid, formic acid (Laboratory grade) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Solvents and additives for UHPLC-HILIC-MS/MS and HPLC-HILIC-HRMS analysis were LC-MS grade (Fisher Optima, ThermoFisher, UK). Certified reference material (CRM) of STX, GTX1-6, NEO, dcSTX, C1&2, dcNEO and dcGTX2&3 and non-certified but well characterised reference material of C3&4 and dcGTX1&4 were all obtained from the Institute of Biotoxin Metrology, National Research Council of Canada (NRCC, Halifax, Canada). TTX CRM was obtained from Cifga (Lugo, Spain). A reference standard for deoxydecarbamoylsaxitoxin and a freeze-dried naturally contaminated tissue of the sea slug *Pleurobranchaea maculata* containing extremely high concentrations of TTX and its analogues was purchased from Cawthron Natural Compounds (CNC; Nelson, New Zealand). Preparation of toxin calibration standards for PST analysis was conducted for UHPLC-HILIC-MS/MS (Turner et al., 2015c), PreCOX HPLC-FLD (Anon, 2005c), and for TTXs following Turner et al. (2017).

2.2. Seawater and shellfish samples

Subsurface seawater samples were collected at 10 stations of the Syracuse bay (Ionian Sea) in 2015, 2016 and 2017 from April to July (Table S1). Samples were taken between the dock of Cantiere Nautico (Stations #1-6) and Sanità Marittima (Stations #7-9) (Fig. S2) by using a bucket. A 500 mL

160 aliquot of each sample was immediately fixed with 1% Lugol solution in amber glass bottles and used
161 for microscopy analyses. In 2017, 50 mL fixed subsamples were also used for molecular quantification
162 of harmful *Alexandrium* species and *sxtA1* gene copy number. Additional 5-10 L unfixed seawater
163 samples and/or net hauls obtained by towing a phytoplankton net were taken at the above stations
164 and concentrated through a 10 μm filter up to 50-150 mL. Samples were then centrifuged twice (at
165 7000 rpm for 5 min and at 12000 rpm for 10 min) and the supernatant removed to obtain a final 2-3
166 mL concentrated sample or a pellet containing natural assemblages of *Alexandrium* spp. These
167 samples were stored at -20°C for toxin analyses.
168 Samples of mussels (*Mytilus galloprovincialis*) and clams (*Venerupis decussata*) were collected
169 in the same period (Table S2) by means of a small landing net from the dock of Cantiere Nautico and
170 Sanità Marittima (Fig. S2) where they were found attached at -1 m depth, colonizing all the area.
171 Mussels were also taken from floating buoys with ropes (Stations #6 and 8) and from Cooperativa
172 Onda Blu (Station #10). Aliquots (20 g) of mussel tissue were frozen at -20°C and used for chemical
173 analysis.
174 Reference shellfish samples were utilised for Quality Controls (QC) during the analysis as
175 described previously (Turner *et al*., 2015c, 2017). These materials facilitated the analysis of the TTX
176 analogues 4-epi-TTX, 5,6,11-trideoxy TTX, 11-nor TTX-6-ol, 5-deoxy TTX, and 4,9-anhydro TTX.
177
178 ***2.3. Phytoplankton detection and enumeration***
179 Fixed seawater samples were kept in 10-50 mL Utermohl settling chambers and examined
180 under an Axiovert 200 microscope equipped with Axiocam and a Zeiss fluorescence UV-filter for cell
181 counts and identification. Thecal plates of *Alexandrium* specimens were stained at 2-3% with

182 Calcofluor White M2R, a fluorescent dye binding to cellulose and other β-linked glucans of the cell
183 wall (Fritz & Triemer, 1985).
184

185 ***2.4. Molecular qPCR assay of Alexandrium* species and *sxtA1* gene quantification**

186 Molecular qPCR analyses were performed on 50 mL aliquots of fixed Lugol seawater samples C1 and
187 C3 collected in 2017 for genus *Alexandrium* and *A. minutum* and *A. pacificum* quantification based on
188 the *sxtA1* gene and 5.8S-ITS ribosomal gene, respectively. Briefly, the seawater samples were
189 centrifuged at 4000 rpm for 15 min and the pellets frozen at −20 °C until analyses. Cell pellets were
190 used for DNA extraction. Genomic DNA was extracted and purified using the PowerSoil DNA Isolation
191 Kit (MoBio Laboratories, Carlsbad, CA, USA). The quantification of *A. minutum* and *A. pacificum*
192 abundance expressed as cells/L in the seawater samples was done using the qPCR assay based on the
193 5.8S-ITS rDNA fragment amplification as described in Galluzzi et al. (2004) and Perini et al. (2018). The
194 quantification of total *sxtA1* gene copy number/L in seawater samples was carried out following the
195 protocol of Penna et al. (2015).

196

197 ***2.5. Extraction and clean-up***

198 Concentrated pellet samples (Table S1) were filtered through a 0.2 µm filter paper. The filter
199 was allowed to dry, before being extracted with 500 µL of 0.1M acetic acid. Each sample was then
200 sonicated for 10 min (0.5 cycle, 70% amplitude) before centrifugation for 10 min at 8,000 rpm, with
201 the resulting supernatant taken. The extraction procedure was repeated with a second aliquot of 500
202 µL 0.1 M acetic acid, and the two supernatants combined.

Aliquots of filtered seawater were subsequently subjected to clean-up using Supelclean ENVI-Carb 250mg/3mL SPE cartridges according to Boundy *et al*. (2015) and Turner *et al*. (2015c). 400 µL of each sample were loaded onto pre-conditioned SPE tubes and subjected to clean-up to remove salt-based interferences.

Mussels and clams (Table S2) were subjected to a single dispersive extraction using 1% acetic acid, following the method exactly as validated by Turner *et al*. (2015c). Reverse-phase (C18) and ion-exchange SPE clean-up of acetic acid extracts was performed prior to Pre-COX HPLC-FLD analysis as detailed by Turner *et al*. (2009). SPE clean-up of crude extracts prior to UHPLC-HILIC-MS/MS and HPLC-HILIC-HRMS/MS was performed with the same method as used for seawater samples according to Turner *et al*. (2015c).

2.6. Ultrahigh performance hydrophilic interaction liquid chromatography-tandem mass spectrometry (UHPLC-HILIC-MS/MS)

UHPLC-HILIC-MS/MS analyses of both PST and TTXs were carried out using a Waters (Manchester, UK) Xevo TQ-S tandem quadrupole mass spectrometer coupled to a Waters Acquity UPLC I-Class. Mass spectrometric parameters were exactly those described by Turner et al. (2015c) with MRM transitions summarised in Table S3.

HILIC separation of PST analogues and TTX was achieved using a 1.7 µm, 2.1x150 mm Waters Acquity Glycan UPLC column in conjunction with a Waters VanGuard Glycan guard cartridge. The column was held at +60^{o}C, with samples held in the autosampler at +4^{o}C. The sample injection volume was 2 or 5 µL. Mobile phase A1 consisted of water + 0.015% formic acid + 0.06% of 25% ammonia. Mobile phase B1 comprised 70% MeCN + 0.01% formic acid. The UPLC gradient (Table S4) was a refined version of the method detailed in Boundy et al. (2015). A separate HILIC method was

conducted for the full detection and quantitation of TTXs (Table S4), using the same analytical column and mobile phase reagents as described by Turner et al. (2017).

The UHPLC-HILIC-MS/MS method for PST involves the direct quantitation of fifteen STX analogues and TTX against toxin standards available as CRM. Four additional analogues (C3, C4 and dcGTX1, dcGTX4) were incorporated into the method, with quantitation performed using the calibrations generated from their nearest structural analogue, using experimentally determined relative response factors (RRF) (Boundy *et al*., 2015). Samples found to contain TTX were then subjected to analysis using the specific TTX method, in order to detect the full range of TTXs.

Toxicity equivalence factors (TEFs) for STX, NEO, dcSTX, dcNEO, dcGTX2&3, GTX1-6, C2 and C4 were taken from EFSA recommendations (EFSA, 2009). TEFs for other congeners C1, C3, dcGTX1&4, doSTX were taken from other published evidence (Oshima, 1995; Sullivan, 1983; Harwood *et al*., 2014) as summarised by Turner *et al.* (2015c). At the time of the study, no RM were available for the M-toxins, and no data was available describing TEFs. Semi-quantitation of M-toxins was therefore facilitated by using a RRF of 1.0 in comparison to the calibration response generated by the nearest structurally similar analogue. TEFs for M-toxins were also taken from the structurally-similar PST and rounded up to 1 significant figure (Turner *et al.*, 2015c) (Table S3). Individual toxin concentrations were calculated and total toxicity was summed from the individual concentration contributions from all quantified toxins and quoted in terms of µg STX eq/kg. Performance characteristics of the method, including limit of detection (LOD), limit of quantitation (LOQ) and limit of reporting (LOR) are described in Turner *et al*. (2015c).

TTXs were quantified against external TTX standards. Additional TTX analogues (4-epi-TTX, 5,6,11-trideoxy TTX; 11-nor TTX-6-ol; 4,9-anhydro TTX; 5-deoxy TTX/11-deoxy TTX) were incorporated into the method, with semi-quantitation performed using the TTX calibration, assuming an equimolar

response. No TEFs were applied to calculations of TTX analogues, given the current absence of any formal published recommendations. Performance characteristics of the method, including LOD, LOQ and LOR are described in Turner *et al*. (2017). For the quantitation of PST and TTX in shellfish, concentrations were quantified using matrix-matched standards so not adjusted for recovery.

2.7. Pre-column oxidation and liquid chromatography-fluorescence detection (Pre-COX LC-FLD)

An Agilent (Manchester, UK) fluorescence detector (1200 model FLD) was used for the detection of the PST oxidation products following pre-analysis derivatisation. Fluorescence excitation was set to 340 nm and emission to 395 nm. Mobile phase A comprised 0.1M ammonium formate, adjusted to pH6 +/- 0.1 with 0.1 M acetic acid, mobile phase B was prepared from 0.1 M ammonium formate with 5% acetonitrile, also adjusted to pH6 +/- 0.1 with 0.1 M acetic acid. The mobile phase was delivered by an Agilent 1200 series LC at a flow rate of 2 mL/min. Chromatographic separation was performed under gradient elution (Table S4) with a Kinetex C18 reversed-phase column (150 mm x 4.6 mm, 5 µm; Phenomenex, UK) together with a Kinetex C18 guard column (both set at 20 °C).

Samples were oxidised using both periodate and peroxide oxidation and run alongside instrumental calibrants. Chromatographic results from standards and samples were assessed to determine toxin oxidation peak retention times, peak area responses and calculated toxin concentrations. In the case of epimeric pairs, the highest TEF was used for each pair. TEFs were taken directly from EFSA recommendations (EFSA, 2009).

2.8. High performance hydrophilic interaction liquid chromatography-high resolution tandem mass spectrometry (HPLC HILIC-HRMS/MS)

HPLC-HILIC-HRMS/MS analyses of both PST and TTX were carried out using an Ultimate 3000 quaternary system coupled to a hybrid linear ion trap LTQ Orbitrap XLTM Fourier Transform MS (FTMS) equipped with an ESI ION MAX^{TM} source (Thermo-Fisher, San Jose, CA, USA). Separations were performed by using the same column and mobile phase described by Turner et al. (2017) and a slightly modified gradient (Table S4).

LC-HRMS^2 analyses were performed in positive ion mode, by using the following source settings: spray voltage= 4.8 kV, capillary temperature= 220 °C, capillary voltage= 49 V, sheath gas= 62 and auxiliary gas=16.5 (arbitrary units), tube lens voltage= 250 V. Collision induced dissociation (CID) were carried out using an activation Q of 0.250, an activation time of 30 ms, and collision energies in the range 22−28% as reported in Perini *et al.* (2014). In all the experiments, a resolving power of 60,000 was used (FWHM at *m/z* 400). Extracted ion chromatograms (XIC) were obtained from MS/MS spectra by selecting the most diagnostic fragment ions of each toxin (Perini *et al.* 2014).

3. Results and Discussion

3.1. Phytoplankton identification by microscopy

The microscopical analyses of seawater samples collected in Syracuse bay (Sicily) over a 3-year period (2015, 2016, 2017) from April to July showed the occurrence of toxic dinoflagellates and species producing high biomass and water discolorations. In spring 2015 and 2016 the abundance of *A. minutum* and *A. pacificum* in the water was in the range 10^3-10^4 cells L^{-1} whereas higher cell concentrations (10^6 cells L^{-1}) were measured in spring 2017 (Table S1). These PST-producing species

292 were found in most of the sampling points of the harbour between Cantiere Nautico and Sanità
293 Marittima (Station #2-8 in Fig. S2), often accompanied by non-PST-producing dinoflagellates, such as
294 *Prorocentrum* spp. and *Lingulodinium polyedra*. In late spring-summer 2015 and 2016, a small chain-
295 forming *Gymnodinium* sp. replaced completely *Alexandrium* spp. producing high biomasses.
296 Suspected *Alexandrium* cysts were also found in June 2015 in the waters. As for *Alexandrium*, also
297 the outbreak of *Gymnodinium* sp. was extended over most of the area Cantiere Nautico to Sanità
298 Marittima (Sample codes A1 and B3) with evident greenish-yellow water discolouration.
299
300 ***3.2. Abundance of* Alexandrium *species and sxtA1 gene copy number***
301 The identification and abundance of the two species, *A. minutum* and *A. pacificum*, were also
302 determined by qPCR assay in concomitance with the *sxtA1* gene content in the seawater. The
303 abundance of *A. pacificum* and *A. minutum* was in the range of $10^4 – 10^6$ cells/L in the samples C1 and
304 C3 (Table S5), while both *Alexandrium* species abundance ranged from 10^5 to 10^6 cells. This step was
305 performed to confirm the species-specificity of the bloom together with the prompt and accurate
306 quantification of the two species in relation to the toxin contribution during the bloom.
307 The *sxtA1* gene content was associated with PST toxin presence in the two seawater samples
308 C1 and C3. The total *sxtA1* gene copy number /L of both *Alexandrium* species was found in the range
309 of $10^4 – 10^6$ copies/L. It was relevant to notice that the amount of *sxtA1* gene and the PST content
310 varied in parallel in the same samples: the highest PST content and *sxtA1* gene copies/L were
311 measured in sample C3, while lower values of toxins and gene copies occurred in sample C1.
312 Therefore, it is possible to argue that the *sxtA1* gene content can provide an indication of the real PSP
313 risk during the bloom prior carrying out chemical analyses of PST (Penna *et al.*, 2015).
314

3.3. PST in seawater by UHPLC HILIC-MS/MS

UHPLC HILIC-MS/MS analysis of seawater samples revealed the presence of multiple PST analogues in several samples (Fig. S3). The total volume of seawater filtered (Table S1) was used to calculate toxin concentrations on a per liter basis. Table 1 summarises the concentrations of each toxin detected, including C1&2, C3&4, GTX1&4, GTX2&3, GTX5, GTX6, NEO, STX and dcSTX. Trace amounts of dcNEO and dcGTX2 were also detected. Concentrations of individual toxins ranged from sub pmol/L, for dcNEO and STX in sample B1 to 149.8 pmol/L for GTX5 in sample C3. Fig. 1a illustrates the mean proportions of individual PST analogues in terms of molarity in the seawater samples collected between 2015 and 2017 which were found to contain toxins. The mean profile was dominated by C1&2 and GTX5 accounting for more than 65% of the total toxin content. These were followed by GTX6, GTX1&4 and GTX2&3. Lower proportions of STX, NEO, C3&4 with trace amounts of dcNEO, dcGTX2 and dcSTX were observed, together accounting for 5% of the total toxin content. Profiles varied from year to year, with 2017 dominated by GTX5, whereas previous years when C1&2 showed the highest relative proportions. These profiles show similarities to those reported for *A. catenella* strains from other regions, such as South America (Krock *et al.* 2007). The profiles of the seawater samples reported here, however, show significant differences from those reported from environmental samples containing mixes of *A. pacificum* and *A. minutum* collected in Italy between 2012-2014 (Fig. 1b) (Penna *et al.*, 2015). Specifically, whilst C1&2 and GTX5 dominated the toxin profile of seawater samples collected in the 3-year period of this study, a clear dominance of GTX1&4 was observed in the previous years. No toxins were detected in sample A1 and B3, from 2015 and 2016 respectively, which were found by microscopy to contain a chain-forming *Gymnodinium*, as well as possible cysts of *Alexandrium* in sample A1 only. This may indicate that we were dealing with a

337 non-toxic *Gymnodinium* species, different from *G. catenatum*, although further morphological
338 analyses would be needed to confirm this hypothesis.
339
340 ***3.4. PST in shellfish by UHPLC HILIC-MS/MS***
341 Table 2 summarizes the PST concentrations quantified in mussels and clams harvested from
342 Syracuse bay during May-June 2015 and 2016 and April-May 2017. Representative MRM
343 chromatograms are shown in Figure 2. Samples AA to AI, collected during 2015, were found to
344 contain a maximum total PST content of 1508 µg STX eq/kg, nearly two times higher than the EU MPL
345 of 800 µg STX eq/kg. The highest concentrations were quantified in shellfish harvested during May
346 2015 (31 to 1508 µg STX eq/kg). Samples AA to AG were all sampled on the same day, 6th May 2015.
347 Whilst the clams showed no evidence for PST accumulation, the mussels were found to vary greatly in
348 total toxicity, depending on the geographical location. Mussels from Sanità Marittima (Sample AD),
349 exhibited total PST content at double the total PST measured in mussels harvested from Cantiere
350 Nautico (Sample AA, AB, AC). Mussels collected on the same day from Cooperativa Onda Blu (Sample
351 AE, AF, AG) showed only very low PST concentration, which may help and explain why the two
352 samples of clams (Sample AF, AG) harvested from Cooperativa Onda Blu did not contain any
353 detectable toxin level.
354 Shellfish harvested during May 2016 was shown to contain notably higher PST concentrations
355 in comparison to those from the same locations harvested a year earlier. Mussels from Cantiere
356 Nautico were found to contain the highest overall levels of PST, ranging from 6542 to 10851 µg STX
357 eq/kg. In comparison, mussels from Cooperativa Onda Blu contained total PST contents well below
358 the regulatory action limit, with a maximum total concentration of 188 µg STX eq/kg. Interestingly,
359 later in the year at Cantiere Nautico during July, mussels were not found to contain toxins above 60

360 µg STX eq/kg. No clams were sampled and analyzed during 2016 from any of the sites under
361 investigation. During April and May 2017, total PST was found to reach a maximum of 4131 µg STX
362 eq/kg again at Cantiere Nautico, evidencing continued accumulation of PST in mussels throughout the
363 three-year period of study.

364 Fig. 3 illustrates the mean toxin profile determined in both shellfish and phytoplankton
365 samples harvested during 2015-2017 in terms of saxitoxin equivalents. The phytoplankton profile is
366 clearly dominated by GTX4, C2, GTX1, GTX2, followed by lower proportions of GTX5, GTX6, NEO, STX
367 GTX3 and C1, whereas in shellfish the most abundant toxin present was GTX1, followed progressively
368 by GTX2, GTX3, STX, GTX4 and GTX5 with lower proportions of dcSTX, NEO, C1 and C2. The standard
369 deviation associated with the mean total profile from all samples is, however, large, indicating a large
370 inter-annual variability in the toxin profiles produced. During 2015, for example, GTX2 was the
371 dominant toxin in mussels, followed in turn by STX, GTX1 and GTX3. In direct contrast, one year later,
372 GTX1 was clearly the most prominent PST, with very low relative proportions of STX. In 2017,
373 however, higher relative proportions of STX were measured once again, with an overall toxin profile
374 similar to 2015. The comparison between profiles determined in phytoplankton and shellfish shows a
375 likely conversion in mussel tissue of C2 into gonyautoxins, most significantly GTX3. These results are in
376 agreement with the findings of Choi et al. (2003), who reported the conversion of C2 produced by
377 *Alexandrium* spp. to a mixture of other PST analogues in both mussel and scallop tissues, including
378 most notably GTX2, GTX3, dcGTX2&3 and GTX5.

379 A range of M toxins were also detected by UHPLC-HILIC-MS/MS: M1, M2, M3 and M4 were
380 detected in all the PST-positive shellfish samples containing a total PST content above 129 µg STX
381 eq/kg (Table S6). Overall, the proportions of M toxins present in the shellfish were low (< 5%;
382 median= 1.7%) in comparison to the total PST concentrations. An exception was noted for two mussel

383 samples harvested during 2016 (Sample BE and BF), which were found to contain low levels of total
384 PST concentrations (129-166 µg STX eq/kg) but higher relative proportions of M toxins, around 10%.
385
386 ***3.5. TTX in shellfish by UHPLC-HILIC-MS/MS***
387 The presence of TTX was ascertained by UHPLC-HILIC-MS/MS in 14 out of the 25 analysed
388 samples (Table 3). Only the parent TTX toxin was detected, with the exception of one sample (BC)
389 containing a small amount of 4-epi TTX (Fig. S4). As such for these samples, the joint PST and TTX
390 acquisition method would have been sufficient to identify and quantify TTX. Out of the 14 positive
391 samples, 10 contained TTX above the LOR of 2 µg/kg. Although TTX concentrations found in the
392 samples were low - with a maximum concentration of 6.4 µg/kg - in comparison to the higher
393 concentrations of PST, this is the first detection of TTX in bivalve shellfish from Italy. Whilst these
394 levels exhibit only a low toxicity risk, nevertheless, TTX has now been shown to represent a potential
395 hazard of concern in Italian mussels. Unfortunately, HPLC-HILIC-HRMS/MS was not able to confirm
396 TTX contamination.
397
398 ***3.6. PST in shellfish by Pre-COX HPLC-FLD***
399 Qualitatively, the Pre-COX HPLC-FLD chromatograms showed the same toxins as detected by
400 UHPLC-HILIC-MS/MS. By way of example, the chromatograms of sample BC (Fig. S5) show the clear
401 presence of a large range of toxins, including C1&2, dcSTX, GTX2&3, GTX5 and STX following peroxide
402 oxidation. Analysis of periodate oxidized fractions confirmed the presence of C1-4 (F1), GTX1&4 and
403 GTX6 (F2) and NEO (F3). No significant oxidation peaks were measured in the unoxidized
404 chromatogram, therefore indicating an absence of naturally-fluorescent matrix co-extractives.

405 Quantitation of each toxin was accomplished as described by AOAC 2005.06 and Table 2
406 summarizes PST concentrations for each of the 20 shellfish samples harvested in 2015-2017. Pre-COX
407 HPLC-FLD data confirms the notably higher toxicities present in the 2016 samples, with samples BA to
408 BD showing the highest overall toxicity levels. The most recurrent toxin was generally GTX1&4,
409 followed by GTX2&3, C1&2 and STX. Anyway, dcSTX, GTX5, GTX6, dcNEO, NEO and C3&4 were also
410 quantified.
411
412 ***3.7. PST in shellfish – UHPLC-HILIC-MS/MS vs Pre-COX HPLC-FLD***
413 In general a very good correlation between the toxin concentrations generated by Pre-COX
414 HPLC-FLD and UHPLC-HILIC-MS/MS was found (Table 2), as previously described by Turner *et al.*
415 (2017) for a range of shellfish samples obtained from the UK over a five-year period. This was
416 important to conduct in this study, as neither of the two methods have been validated for mussels
417 and clams from Italy. The use of two independent methods therefore provided greater confidence in
418 the quantitative results obtained. Figure 4 illustrates the comparisons in toxin concentrations
419 determined using both methods indicating an excellent agreement between the quantitation
420 performed, as shown by the results for the most prevalent toxins GTX2&3, STX and GTX5, as well as
421 for GTX6, dcSTX, dcGTX2&3 and dcNEO (Table 2). There is evidence for an expected over-estimation in
422 PST concentrations using LC-FLD, due to the use of the highest TEF for each epimeric pair (e.g.
423 GTX1&4, C1&2, GTX2&3) (Turner et al., 2009). As a consequence, HPLC-FLD concentrations for these
424 epimers are higher than the summed concentrations of the individual epimers quantified by UHPLC-
425 HILIC-MS/MS.
426
427

3.8. PST in shellfish by HPLC-HILIC-HRMS/MS

A first attempt to develop the UHPLC-HILIC-MS/MS method (Boundy et al., 2015) for use on the HPLC-HILIC LTQ Orbitrap XL HRMS system was done, with results revealing that the method was not suitable on this system, mostly due to the long scan times needed to acquire HRMS data. So, keeping the same mobile phases and HILIC column, a slower chromatographic method was developed that finally allowed the chromatographic separation of all the PST and TTX in a single 20 min analysis.

In order to investigate the comparison between the qualitative data generated by the two MS platforms, all mussel samples were analyzed by HPLC-HILIC-HRMS/MS. Results compared well with those generated by UHPLC-HILIC-MS/MS and Pre-COX HPLC-FLD (Table 2, S7 and Fig. 4). Mussels collected in 2015 were found to have a total PST content up to 1879 μg STX eq/kg (Sample AD), more than two times higher than the EU MPL. The highest PST accumulation levels (9224-19639 μg STX eq/kg) were again determined in samples collected in 2016, with the sample BC being the most toxic one (Fig. S6). Samples which were devoid of any toxicity by UHPLC-HILIC-MS/MS (Sample AF and AG,) were also confirmed not to contain toxins by HPLC-HILIC-HRMS/MS. The comparison between quantitative results obtained for the two MS methods revealed that toxins detected by UHPLC-MS/MS at levels < 30 μg STX eq/kg were generally not detected by the HPLC-HILIC-HRMS/MS system. This was expected considering that a full MS scan HRMS analysis for small molecules like PST would gain less sensitivity in comparison with a triple quadrupole instrument operating in MRM. Furthermore, a good correlation between quantitative results obtained on the two platforms was observed for C1&2, GTX1, GTX2, and GTX5. The more significant differences between the two sets of results generally related to the minor PST analogues present in the contaminated mussels and clams. Discrepancies were observed notably for the β-epimers of the gonyautoxins such as GTX3 (samples AH) and GTX4 (2017 samples), as well as STX (2015 samples) and dcSTX (all samples). The absence of

451 standards for C3&4, doSTX and GTX6 precluded the possibility to compare quantitation for these
452 toxins. The most surprising divergence occurred for NEO and dcNEO with LC-HRMS measured levels
453 being significantly higher than those obtained by UHPLC-HILIC-MS/MS. One possible explanation for
454 this over-estimation may relate to the presence of isobaric compounds and/or matrix interferences
455 which were chromatographically unresolved under HPLC-HILIC conditions prior to HRMS. In addition,
456 the HRMS method was unable to detect the low concentrations of dcSTX present in the study
457 samples. Overall, the results indicate that further work is required to better establish the relative
458 performances of the two MS platforms.
459
460 **4. Conclusions**
461 In this study, for the first time a significant PST contamination in shellfish from Sicily emerged.
462 The recurring blooms of PST-producing *Alexandrium* spp. in the Spring-Summer 2015-2017 and the
463 high contamination levels measured in mussels suggest that human health concerns exist. In addition,
464 for the first time TTX has been detected in Italy. Future work should monitor the presence of TTX in
465 both shellfish flesh and fish tissue, as well as the potential producing microorganisms such as *Vibrio*
466 spp. in the marine environment.
467 Three different chromatographic platforms were used for analysis of these samples. Each of these
468 may be applicable to the high throughput testing of shellfish tissues in a monitoring framework,
469 although currently only the Pre-COX HPLC-FLD method is allowable within EU law. Whilst the
470 qualitative results compared well between the three approaches, some significant differences
471 emerged, particularly in comparison with the HPLC-HILIC-HRMS/MS method. Consequently, further
472 work will be required to understand the reasons for such differences and to conduct formal validation

of the HRMS method before the platform can be used routinely. Ultimately, such development would be required prior to dissemination of the methods to monitoring agencies.

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482 **References**

483 Alcaraz, A., Whiplle, R. E., Gregg, H. R., Anderson, B. D., and Grant, P. M. (1999). Analysis of
484 Tetrodotoxin. *Forensic Science International, 99*, 35-45.

485 Anon. (2004). Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29th
486 April 2004 laying down specific rules for the organisation of official controls on products of
487 animal origin intended for human consumption. Official Journal of the European Union. L226,
488 83-127.

489 Anon. (2005a). AOAC Official Method 959.08. Paralytic Shellfish Poison. Biological method. Final
490 action. In: AOAC Official methods for analysis, 18th Edition Chapter 49: Natural toxins (chapter
491 ed. M.W. Truckses), pp. 79-80. Gaithersburg, MD, USA: AOAC International.

492 Anon. (2005b). COMMISSION REGULATION (EC) No 2074/2005 of 5th December 2005 laying down
493 implementing measures for certain products under Regulation (EC) No 853/2004 of the
494 European Parliament and of the Council and for the organisation of official controls under
495 Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation
496 (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation
497 (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations
498 (EC) No 853/2004 and (EC) No 854/2004. L 338:27-59

499 Anon. (2005c). AOAC Official method 2005.06 Quantitative determination of Paralytic Shellfish
500 Poisoning Toxins in shellfish using pre-chromatographic oxidation and liquid chromatography
501 with fluorescence detection. Gaithersburg, MD, USA: AOAC International.

502 Anon. (2006). Commission Regulation (EC) No 1664/2006 of 6th November 2006 amending Regulation
503 (EC) No 2074/2005 as regards implementing measures for certain products of animal origin

intended for human consumption and repealing certain implementing measures. Official Journal of the European Union. L320, 13-45.

Bane, V., Lehan, M., Dikshit, M., O'Riordan, A., and Furey, A. (2014). Tetrodotoxin: Chemistry, Toxicity, Source, Distribution and Detection. *Toxins*, *6*, 693-755.

Bazzoni, A. M., Mudadu, A. G., Lorenzoni, A., Vivaldi, B., Cicotelli, V., Sanna, G., Tedde, G., Ledda, S., Alesso, E., Marongiu, E., and Virgilo, S. (2016). Occurrence of harmful algal species and shellfish toxicity in Sardinia (Italy). *Italian Journal of Food Safety. 5*:60695. 194-199.

Boundy, M. J., Selwood, A. I., Harwood, D. T., McNabb, P. S., and Turner, A. D. (2015). Development of a sensitive and selective liquid chromatography-mass spectrometry method for high throughput analysis of paralytic shellfish toxins using graphitic carbon SPE. *Journal of Chromatography A. 1387,* 1-12.

Casabianca, S., Penna, A., Pecchioli, E., Jordi, A., Basterretxea, G., Vernesi, C. (2012). Population genetic structure and connectivity of the harmful dinoflagellate Alexandrium minutum in the Mediterranean Sea. *Proceedings of the Royal Society B Biological Sciences* 279. doi:10.1098/rspb.2011.0708.

Chau, R., Kalaitzis, J. A., and Neilan, B. A. (2011). On the origin and biosynthesis of Tetrodotoxin. *Aquatic Toxicology. 104*, 61-72.

Choi, M. C., Hsieh, D. P. H., Lam, P. K. S., and Wang, W-X. (2003). Field depuration and biotransformation of paralytic shellfish toxins in scallop *Chlamys noblis* and green-lipped mussel *Perna viridis. Marine Biology. 143*, 927-934.

Ciminiello, P., Dell'Aversano, C., Forino, M., and Tartaglione, L. (2014). Marine toxins in Italy: the more you look, the more you find. *European Journal of Organic Chemistry.* 1357-1369.

526 DeGrasse, S. L., Van de Riet, J., Hatfield, R. and Turner, A. D. (2011). Pre- versus post-column oxidation
527 liquid chromatography fluorescence detection of paralytic shellfish toxins. *Toxicon. 57*, 619-
528 624.

529 Dell'Aversano, C., Hess, P., and Quilliam, M. A. (2005). Hydrophilic interaction liquid chromatography-
530 mass spectrometry for the analysis of paralytic shellfish poisoning (PSP) toxins. *Journal of*
531 *Chromatography A.*, *1081*, 190-201.

532 Dell'Aversano, C., Walter, J. A., Burton, I. W., Stirling, D. J., Fattorusso, E., and Quilliam, M. A. (2008).
533 Isolation and structure elucidation of new and unusual saxitoxin analogues from mussels.
534 *Journal of Natural Products*. *71*, 1518-1523.

535 Diener, M., Erler, K., Christian, B., and Luckas, B. (2007). Application of a new zwitterionic hydrophilic
536 interaction chromatography column for determination of paralytic shellfish poisoning toxins.
537 *Journal of Separation Science*. *30*, 1821-1826.

538 EFSA. (2009). Scientific opinion of the panel on contaminants in the food chain on a request from the
539 European Commission on marine biotoxins in shellfish - saxitoxin group. *The EFSA Journal,*
540 1019, 1-76.

541 EFSA. (2017). Scientific opinion on the risks for public health related to the presence of tetrodotoxin
542 (TTX) and TTX analogues in marine bivalves and gastropods. The *EFSA Journal,* 15(4):4752, 1-
543 65.

544 EU-NRL **(**1995). State of the art NRLs on marine biotoxins. CRL Rep 01/95, European Community
545 Reference Laboratory on Marine Biotoxins (CRL). Vigo, Spain.

546 EU-NRL (2001). Minutes of the 4th meeting of EU National Reference Laboratories (EU-NRL) on marine
547 biotoxins. 29-31 October 2001. Vigo, Spain.

548 EU-NRL (2002). Minutes of 5th meeting of EU National Reference Laboratories (EU-NRL) on marine
549 biotoxins. 10-12 December 2002. Brussels, Belgium.

550 Fritz L. & Triemer R.E. (1985). A rapid simple technique utilizing Calcofluor White M2R for the
551 visualization of dinoflagellate thecal plates *Journal of Phycology*. *21(4)*, 662-664

552 Galluzzi, L., Penna, A., Bertozzini, E., Vila, M., Garcés, E., Magnani, M.(2004). Development of a Real-
553 Time PCR Assay for Rapid Detection and Quantification of Alexandrium minutum (a
554 Dinoflagellate). *Applied and Environmental Microbiology. 70 (2)*, 1199–1206.

555 Giacobbe, M. G**.**, Maso', M., Milandri, A., Penna, A., Poletti, R. (2006). Plankton toxicity and shellfish
556 contamination by phycotoxins in a new Mediterranean locality. *In* Henshilwood,K., Deegan, B.,
557 McMahon, T., Cusack, C., Keaveney, S., Silke J., O' Cinneide, M., Lyons, D., & P. Hess eds.
558 Proceedings of the 5th International Conference on Molluscan Shellfish Safety ICSS04 (14-18,
559 June 2004 Galway, Ireland), p.206-214.

560 Hallegraef, G. M. (2003). Harmful Algal Blooms: A Global Overview in *Manual on Harmful Marine*
561 *Microalgae,* G. M. Hallegraef, D.M. Anderson & A.D. Cembella (Eds), UNESCO, Paris, France, pp
562 25-49.

563 Harwood, D. T., Boundy, M., Selwood, A. I., Van Ginkel, R., MacKenzie, L. and McNabb, PS. (2013).
564 Refinement and implementation of the Lawrence method (AOAC 2005.06) in a commercial
565 laboratory: assay performance during an *Alexandrium catenella* bloom event. *Harmful Algae,*
566 *24*, 20-31.

567 Harwood, D. T., Selwood, A. I., Van Ginkel, R., Waugh, C., McNabb, P. S., Munday, R., Hay, B., Thomas,
568 K., Quilliam, M. A., Malhi, N., Dowsett, N, McLeod, C. (2014). Paralytic shellfish toxins,

569 including deoxydecarbamoyl-STX, in wild-caught Tasmanian abalone (*Halitotis rubra*). *Toxicon.*
570 *90*, 213-225.

571 Isbister, G. K., and Kiernan, M. C. (2005). Neurotoxic marine poisoning. *The Lancet Neurology*, *4(4)*,
572 219-228.

573 Kodama, M., Sato, S., Sakamoto, S. and Ogata, T. (1996). Occurrence of Tetrodotoxin in *Alexandirum*
574 *tamarense*, a causative dinoflagellate of paralytic shellfish poisoning. *Toxicon. 34*, 1101-1105.

575 Krock, B., Sequel, C. G., and Cembella, A. D. (2007). Toxin profile of *Alexandrium catenella* from the
576 Chilean coast as determined by liquid chromatography with fluorescence detection and liquid
577 chromatography coupled with tandem mass spectrometry. *Harmful Algae. 6*, 734-744.

578 Lawrence, J. F., Niedzwiadek, B., Menard, C. (2005). Quantitative determination of paralytic shellfish
579 poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography
580 with fluorescence detection: Collaborative study. *Journal of AOAC International*. *88 (6)*, 1714-
581 1732.

582 Llewellyn, L. E. (2006). Saxitoxin, a toxic marine natural product that targets a multitude of receptors.
583 *Natural Product Reports*. *23*, 200-222.

584

585 Lugliè, A., Giacobbe, M. G., Riccardi, E., Bruno, M., Pigozzi, S., Mariani, M. A., Satta, C. T., Stacca, D.,
586 Bazzoni, A. M., Caddeo, T., Farina, P., Padedda, B. M., Pulina, S., Sechi, N., and Milandri, A.
587 (2017). Paralytic shellfish toxins and cyanotoxins in the Mediterranean: new data from
588 Sardinia and Sicily (Italy). *Microorganisms*. *5*, 72; doi:10.3390/microorganisms5040072

589 McNabb, P. S., Taylor, D. I., Ogilvie, S. C., Wilkinson, L., Anderson, A., Hamon, D., Wood, S. A. and
590 Peake, B. M. (2014). First detection of Tetrodotoxin in the bivalve *Paphies autralis* by liquid

591 chromatography coupled to triple quadrupole mass spectrometry with and without pre-
592 column reaction. *Journal AOAC International. 97(2)*, 325-333.

593 Noguchi, T., Miyazawa, K., Daigo, K. and Arakawa, O. (2011). Paralytic shellfish poisoning (PSP_ toxin –
594 and/or Tetrodotoxin-contaminated crabs and food poisoning by them. *Toxin reviews. 30(4)*,
595 91-102.

596 Oshima, Y. (1995). Postcolumn derivatisation liquid chromatographic method for paralytic shellfish
597 toxins. *Journal AOAC International. 78*, 528-532.

598 Penna, A., Garcés, E., Vila, M., Giacobbe, M. G., Fraga, S., Lugliè, A., Bravo, I., Bertozzini, E., Vernesi, C.,
599 (2005). Alexandrium catenella (Dinophyceae), a toxic ribotype expanding in the NW
600 Mediterranean Sea. *Marine. Biology.* 148, 13–23. doi:10.1007/s00227-005-0067-5.

601 Penna, A., Fraga, S., Masó, M., Giacobbe, M. G., Bravo, I., Garcés, E., Vila, M., Bertozzini, E., Andreoni,
602 F., Lugliè, A., Vernesi, C., (2008). Phylogenetic relationships among the Mediterranean
603 Alexandrium (Dinophyceae) species based on sequences of 5.8S gene and Internal Transcript
604 Spacers of the rRNA operon. *Eurean Journalof Phycology* 43.
605 doi:10.1080/09670260701783730.

606 Penna, A., Perini, F., Dell'Aversano, C., Capellacci, S., Tartaglione, L., Giacobbe, M. G., Casabianca, S.,
607 Fraga, S., Ciminiello, P., and Scardi, M. (2015). The sxt gene and paralytic shellfish poisoning
608 toxins as markers for the monitoring of toxic *Alexandrium* blooms. *Environmental Science and*
609 *Technology. 49*, 14230-14238.

610 Perini, F., Bastianini, M., Capellacci, S., Pugliese, L., Dipoi, E., Cabrini, M., & Buratti, S. (2018).
611 Molecular methods for cost-efficient monitoring of HAB (harmful algal bloom) dinoflagellate

612 resting cysts. *Marine Pollution Bulletin*. (in press)
613 https://doi.org/10.1016/j.marpolbul.2018.06.013

614 Pistocchi, R., Guerrini, F., Pezzolesi, L., Riccardi, M., Vanucci, S., Ciminiello, P., Dell'Aversano, C.,
615 Forino, M., Fattorusso, E., Tartaglione, L., Milandri, A., Pompei, M., Cangini, M., Pigozzi, S. and
616 Riccardi, E. (2012). Toxin levels and profiles in microalgae from the north-western Adriatic Sea
617 – 15 years of studies on cultured species. *Marine Drugs. 10*, 140-162.

618 Poletti, R. (1998). Distribution of toxic dinoflagellates and their impact on shellfish along the
619 northwest Adriatic coast. *In* Reguera, B., Blanco, J., Fernandez, M. & T. Wyatt, eds. (1997).
620 *Harmful Algae, Proceedings of the VIII International Conference on Harmful Algae* (June 1999,
621 Vigo, Spain), pp. 88-90. Xunta de Galicia and IOC of UNESCO.

622 Sayfritz, S. J., Aasen, J. A. B., and Aune, T. (2008). Determination of paralytic shellfish poisoning toxins
623 in Norwegian shellfish by liquid chromatography with fluorescence and tandem mass
624 spectrometry detection. *Toxicon. 52*, 330-340.

625 Sullivan, J. (1983). Enzymatic transformation of PSP toxins in the Littleneck Clam (*Protothaca*
626 *staminea*). *Biochemical and Biophysical Research Communications*, *114(2)*, 465-472.

627 Turner, A. D., Norton, D. M., Hatfield, R. G., Morris, S., Reese, A. R., Algoet, M., and Lees D.N. (2009).
628 Single laboratory validation of the AOAC HPLC method (2005.06) for mussels: refinement and
629 extension of the method to additional toxins. *Journal AOAC International*. *92* (1), 190-207.

630 Turner, A. D., Norton, D. M., Hatfield, R. G., Rapkova-Dhanji, M., Algoet, M. and Lees, D.N. (2010).
631 Single laboratory validation of a refined AOAC LC method for oysters, cockles and clams in UK
632 shellfish. *Journal AOAC International*. *93(5)*, 1482-1493.

633 Turner, A. D., Hatfield, R. G., Rapkova, M., Higman, W., Algoet, M., Suarez-Isla, B. A., Cordova, M.,
634 Caceres, C., van de Riet, J., Gibbs, R., Thomas, K., Quilliam, M. and Lees, D. N. (2011).
635 Comparison of AOAC 2005.06 LC official method with other methodologies for the
636 quantitation of paralytic shellfish poisoning toxins in UK shellfish species. *Analytical*
637 *Bioanalitical Chem*istry *399*, 1257-1270.

638 Turner, A. D., and Hatfield, R. G. (2012). Refinement of AOAC Official Method 2005.06 Liquid
639 Chromatography-Fluorescence detection method to improve performance characteristics for
640 the determination of Paralytic Shellfish Toxins in King and Queen scallops. *Journal AOAC*
641 *International*. *95(1)*, 129-142.

642 Turner, A. D., Lewis, A. M., Routke, W. A., and Higman, W. A. (2014). Interlaboratory comparison of
643 two liquid chromatographic fluorescence detection methods for Paralytic Shellfish Toxin
644 analysis through characterisation of an oyster reference material. *Journal of AOAC*
645 *International*. *97(2)*, 380-390.

646 Turner, A. D., Powell, A., Schofield, A., Lees, D. N. and Baker-Austin, C. (2015a). Detection of the
647 pufferfish toxin Tetrodotoxin in European bivalves, England, 2013 to 2014. *Eurosurveillance.*
648 20(2): pii=21009 https://doi.org/10.2807/1560-7917.ES2015.20.2.21009

649 Turner, A. D., Higgins, C., Higman, W., and Hungerford, J. (2015b). Potential threats posed by
650 tetrodotoxins in UK waters: examination of detection methodology used in their control.
651 *Marine Drugs. 13*, 7357-7376.

652 Turner, A. D., McNabb, P. S., Harwood, T., Selwood, A. I. and Boundy, M. J. (2015c). Single-laboratory
653 validation of a multitoxin ultra-performance LC-Hydrophilic Interaction LC-MS/MS method for

quantitation of Paralytic Shellfish Toxins in bivalve shellfish. *Journal of AOAC International. 98(3)*, 609-621.

Turner, A. D., Boundy, M. J., Dhanji-Rapkova, M. (2017). Development and single laboratory validation of a liquid chroimatography mass spectrometry method for quantitation of Tetrodotoxin in mussels and oysters. *Journal of AOAC International*, *100(5)*, 1-14.

Turrell, E., Stobo, L., Lacaze, J. P., Piletsky, S. And Piletska, E. (2008). Optimisation of hydrophilic interaction liquid chromatography/mass spectrometry and development of solid-phase extraction for the determination of paralytic shellfish poisoning toxins. *Journal of AOAC International 91(6)*, 1372-1386.

Van de Riet, J. M. R. S., Gibbs, P. M., Muggah, W. A., Rourke, J. D., MacNeil, and M. A Quilliam. (2011). Liquid chromatographic post-column oxidation (PCOX) method for the determination of paralytic shellfish toxins in mussels, clams, oysters and scallops: collaborative study. *Journal of AOAC International. 94(4)*, 1154-1176.

Van Dolah, F. M., Fire, S. E., Leighfield, T. A., Mikulski, C. M., and Doucette, G. J. (2012). Determination of paralytic shellfish toxins by receptor binding assay: collaborative study. *Journal of AOAC International. 95(3)*, 795-812.

Vlamis, A., Katikou, P., Rodriguez, I., Rey, V., Alfonso, A., Papazacharious, A., Zacharaki, T., Botana, A. M. and 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.

Wiese, M., D'Agostino, P. M., Mihali, T. K., Moffitt, M. C. and Neilan, B. A. (2010). Neurotoxic alkaloids: saxitoxin and its analogs. *Marine Drugs 8*(7),2185–2211.

676 Wang, X. J., Yu, R. C., Luo, X., Zhou, M. J. and Lin, X-T. (2008). Toxin-screening and identification of
677 bacteria from highly toxic marine gastropod *Nassarius semiplicatus*. *Toxicon. 52*(1), 55-61.

678 Wu, Z., Xie, L., Xia, G., Zhang, J., Nie, Y., Hu, J., Wang, S. And Zhang, R. (2005). A new tetrodotoxin-
679 producing actinomycete, *Norcardiopsis dassonvillei*, isolated from the ovaries of puffer fish
680 *Fugu rubripes*. *Toxicon. 45*, 851-859.

681 Yasumoto, T., Endo, A., Yasumura, D., Nagai, H., Murata, M. and Yotsu, M. (1988). Bacterial
682 production of Tetrodotoxin and its derivatives. *Toxicon. 26*(1), 50.

683 Zhuo, L., Yin, Y., Fu, W., Qiu, B., Lin, Z., Yang, Y., Zheng, L., Li, J. And Chen, G. (2013). Determination of
684 paralytic shellfish poisoning toxins by UHPLC-HILIC-MS/MS coupled with dispersive solid phase
685 extraction. *Food Chemistry. 137*, 115-121.

Table

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Table 1. PST and TTX toxin concentrations (pmol/L) determined by UHPLC-HILIC-MS/MS in seawater samples collected in Syracuse bay in 2015, 2016 and 2017.

Sample ID	C1	C2	C3	C4	dcGTX2	GTX2	GTX3	GTX1	GTX4	GTX5	GTX6	dcNEO	dcSTX	STX	NEO	Total PST	TTX
A1		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A2	2.12	6.89	-	-	-	1.41	-	-	0.30	3.51	-	-	-	0.20	-	14.4	-
A3	3.51	5.67	-	-	-	2.13	-	0.36	0.72	4.04	-	-	-	0.21	-	16.6	-
B1	5.28	14.49	0.47	0.32	-	0.22	0.49	3.03	8.36	5.46	5.38	0.09	-	0.09	0.72	44.4	-
B2	4.71	8.66	0.32	0.64	0.32	0.47	0.38	2.23	4.39	5.01	3.47	-	-	0.23	0.68	31.5	-
B3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C1	1.09	0.27	-	-	-	0.36	0.09	0.24		0.47	-	-	-	0.41	-	2.9	-
C2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C3	13.03	46.20	-	-	-	3.54	17.26	0.65	1.61	149.8	3.74	-	1.70	29.7	0.86	267.5	-

Table 2. PST concentrations (µg STX di-HCl eq/kg) quantified by UHPLC-HILIC-MS/MS (in black) and by Pre-COX HPLC-FLD (in red)in mussels and clams from Sicily during 2015 to 2017.[a]

Sample ID	C1	C2	C3	C4	dcGTX2	dcGTX3	GTX2	GTX3	GTX1	GTX4	GTX5	GTX6	doSTX	dcSTX	dcNEO	STX	NEO	Total
AA	4.6	23.8	-	-	1.5	2.4	209.6	116.8	161.7	30.6	46.1	1.1	-	4.9	-	84.8	6.1	694
	55.9		-		3.9		317.3		265.0		52.9	1.7	-	5.1	-	85.7	-	787.5
AB	4.2	26.1	-	-	1.8	2.4	238.6	159.7	189.7	45.8	43.5	1.5	-	5.5	-	127.0	6.2	852
	54.2		-		1.2		363.3		347.0		43.2	1.3		4.5	-	125.0	6.8	946.6
AC	1.6	8.7	-	-	1.0	-	83.7	53.0	95.8	24.8	20.7	0.9	-	3.7	-	78.4	6.5	379
	20.5		-		4.1		164.8		140.2		26.3	1.2		1.5	-	82.2	-	440.9
AD	6.3	31.2	-	-	1.7	3.3	315.2	205.3	435.0	99.7	66.2	2.8	-	9.6	-	311.5	19.8	1508
	81.1		0.2		4.1		505.2		785.2		72.6	3.7		9.1	-	337.0	17.3	1815
AE	-	-	-	-	-	-	4.5	2.4	16.8	4.8	-	-	-	-	-	2.2	-	31
	-		0.1		-		9.2		36.4		2.1	-		-	-	9.1	-	56.9
AF	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-		-		-		-		-		1.4	-		0.9	-	12.0	-	13.2
AG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	-	-
	-		-		-		-		-		-	-		-	-	-	-	-
AH	7.6	33.9	-	-	6.0	6.7	140.2	102.6	27.2	8.4	34.5	0.7	-	20.0	-	114.5	3.6	506
	93.0		0.3		4.7		215.3		109.2		32.2	0.7		14.6	-	99.0	15.3	584.3
AH d	9.2	46.1	-	-	8.1	10.5	162.0	100.5	31.9	10.2	40.3	0.7	-	22.7	-	140.6	5.2	588
	141.1		-		9.5		265.8		159.1		36.5	0.9		18.5	-	128.7	-	760.0
AI	-	-	-	-	-	-	-	-	-	-	0.7	-	-	-	-	1.1	-	2
	2.4		0.2		0.1		5.0		-		4.6	-		2.3	-	5.7	-	20.4
AI d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.7	-	1
	1.5		-		-		5.1		-		2.1	-		1.6	-	2.4	-	12.7
BA	42.4	185.6	3.3	2.2	15.6	16.2	1018.2	485.5	4040.9	912.6	204.2	29.5	-	21.7	2.8	142.7	63.6	7187
	509.3		3.0		33.2		1781		6509		172.3	35.6		19.7	7.9	149.5	130.8	9312.3
BB	53.6	180.7	2.4	1.5	20.0	15.6	1563.4	652.8	2988.5	590	235.2	19.8	-	25.6	1.6	157.6	34.2	6542
	567.1		2.5		38.1		2684		4338		192.1	19.7		24.2	-	170.7	81.7	8095.6
BC	141.1	614.5	8.9	7.3	47.8	47.0	1815.6	1033.2	4473.2	1147.5	830.6	118.4	0.3	89.5	11.2	357.5	107.6	10851
	1327		10.4		80.7		2522		8796		558.1	164.5		73.3	13.4	310.7	100.4	13782

BD	92.5	358.0	7.2	5.6	38.0	28.3	874.4	471.0	4320.7	1032.8	492.8	104.3	0.1	63.3	9.6	228.6	102.7	8230
	1011		9.7		56.2		1463		7346		402.7	106.0		54.1	10.8	243.5	62.5	10649
BE	0.5	2.8	-	-	-	-	11.9	6.1	59.2	26.4	20.1	4.5	-	2.3	0.5	9.3	6.2	150
	11.3		-		-		22.7		105.9		28.4	4.7		1.5	-	27.2	-	196.9
BF	0.5	3.3	-	-	-	-	8.9	6.5	47.6	16.3	14.8	2.7	-	1.9	-	8.5	5.1	116
	10.9		0.1		-		23.5		80.5		19.3	4.0		-	-	30.6	-	164.8
BG	0.9	3.7	-	-	-	-	22.9	14.0	66.8	18.7	33.4	4.3	-	3.2	0.6	15.5	4.5	188
	11.6		-		-		42.5		107.1		62.1	5.2		1.1	-	31.3	-	255.8
BH	0.9	2.6	-	-	1.0	1.3	10.6	3.6	4.2	-	1.9	-	-	5.6	-	25.6	1.5	59
	11.9		-		-		24.4		29.4		5.1	-		4.3	-	30.9	-	105.9
BI	1.0	3.0	-	-	1.7	1.2	8.4	4.1	4.5	-	2.0	-	-	4.3	-	21.3	2.1	54
	8.7		-		-		13.7		-		6.5	-		2.1	-	19.5	-	50.5
CA	9.8	37.9	0.2	0.2	3.6	2.6	409.7	311.7	459.9	102.4	104.0	4.5	-	30.0	0.6	525.0	58.2	2060
	124.5		-		-		898.6		862.1		208.3	6.6		45.7	-	973.7	51.0	3171
CB	20.6	150.6	0.2	0.2	9.8	12.2	602.8	664.8	265.4	79.7	428.1	6.9	0.1	115.8	1.7	885.2	44.0	3288
	271.2		-		8.2		1036		704.6		496.2	10.9		100.3	-	1040	13.0	3680
CC	49.5	287.2	1.0	0.5	17.3	18.4	928.8	773.5	841.5	214.0	316.1	17.4	0.2	83.5	3.0	528.4	51.1	4131
	751.2		5.2		46.6		1884		1767		570.7	25.0		100.5	-	846.8	37.8	6035
CD	35.1	263.4	1.0	0.7	12.4	16.9	738.7	810.1	728.7	206.7	372.1	19.0	0.2	88.0	3.6	617.3	60.6	3974
	501.4		3.1		36.0		1406		1240		514.2	23.1		82.6	-	780.5	39.6	4626
CE	20.9	87.3	0.4	0.4	9.1	6.1	752.7	555.2	1081.9	234.7	163.2	9.5	-	40.1	2.0	659.5	75.7	3699
	230.0		1.0		6.6		1308		1355		264.9	10.2		47.0	-	974.8	52.0	4250

[a] d = duplicate sample "-" = not detected

Table 3. TTXs quantified in shellfish (µg/kg) by UHPLC-HILIC-MS/MS collected in the period 2015-2017. None of the other TTX analogues; 5,6,11-trideoxy TTX, 11-nor TTX-6-ol, Mono deoxy TTX, 4,9-anhydro TTX, and 11-oxo TTX were detected.[a]

Sample ID	TTX	4-Epi TTX	Total TTXs
AA	3.6	-	3.6
AB	-	-	-
AC	-	-	-
AD	-	-	-
AE	-	-	-
AF	-	-	-
AG	-	-	-
AH	2.0	-	2.0
AH d	2.4	-	2.4
AI	-	-	-
AI d	-	-	-
BA	2.0	-	2.0
BB	1.6	-	1.6
BC	6.0	0.4	6.4
BD	-	-	-
BE	-	-	-
BF	-	-	-
BG	0.8	-	0.8
BH	4.8	-	4.8
BI	1.6	-	1.6
CA	5.1	-	5.1
CB	1.7	-	1.7
CC	2.3	-	2.3
CD	2.0	-	2.0
CE	5.1	-	5.1

[a]d = duplicate sample; Underscored results are those for toxins detected at concentrations >LOD but <LOR (2 µg TTX); "-" = not detected

Figure

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Figure 1.

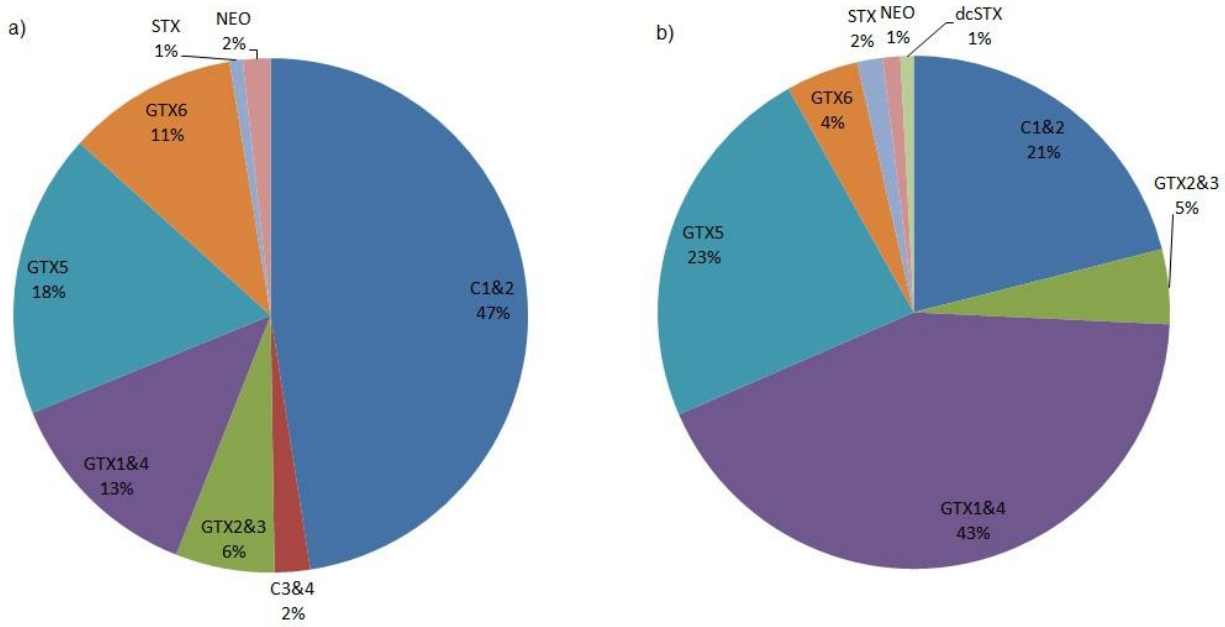

Figure 2.

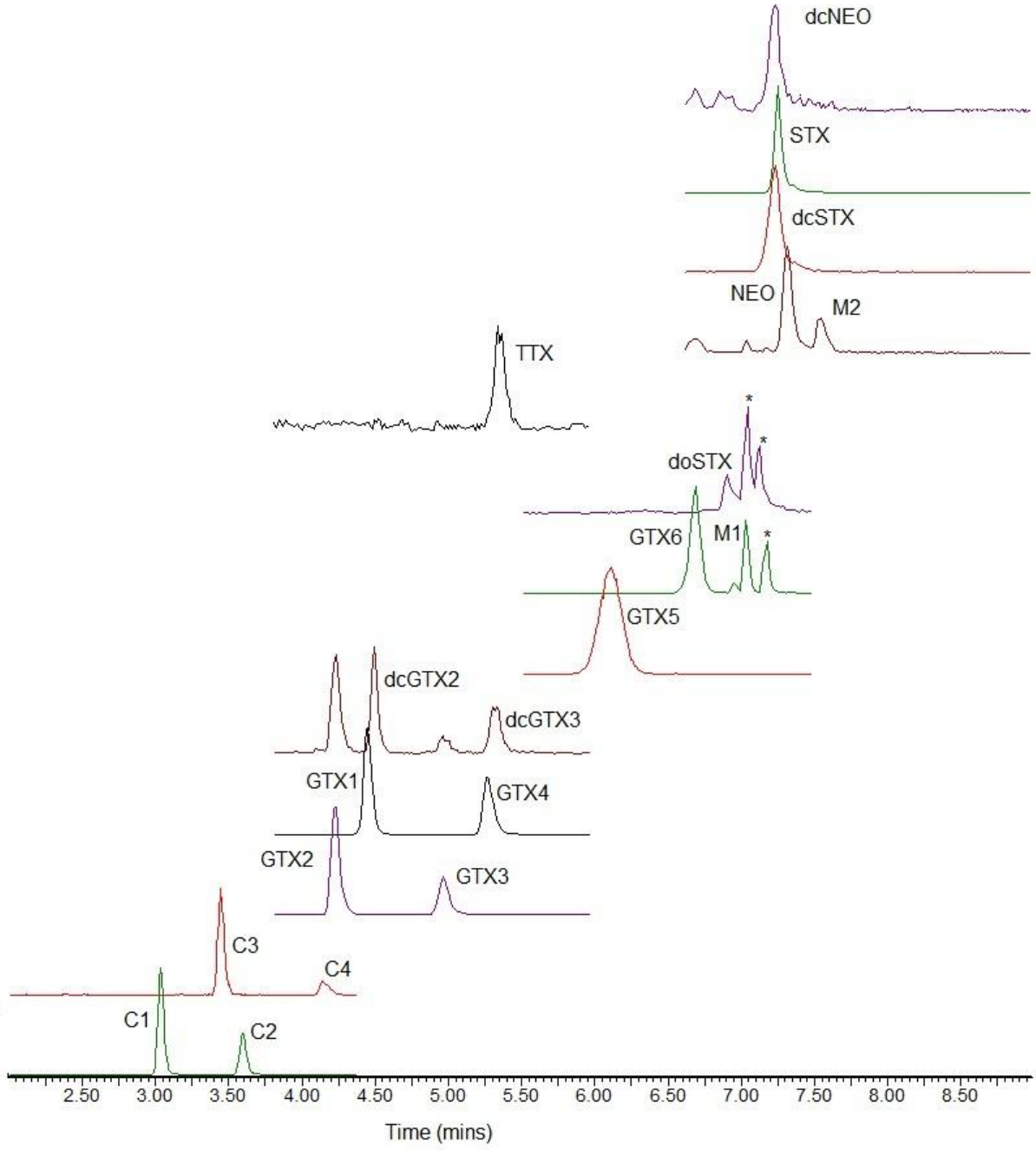

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Figure 3.

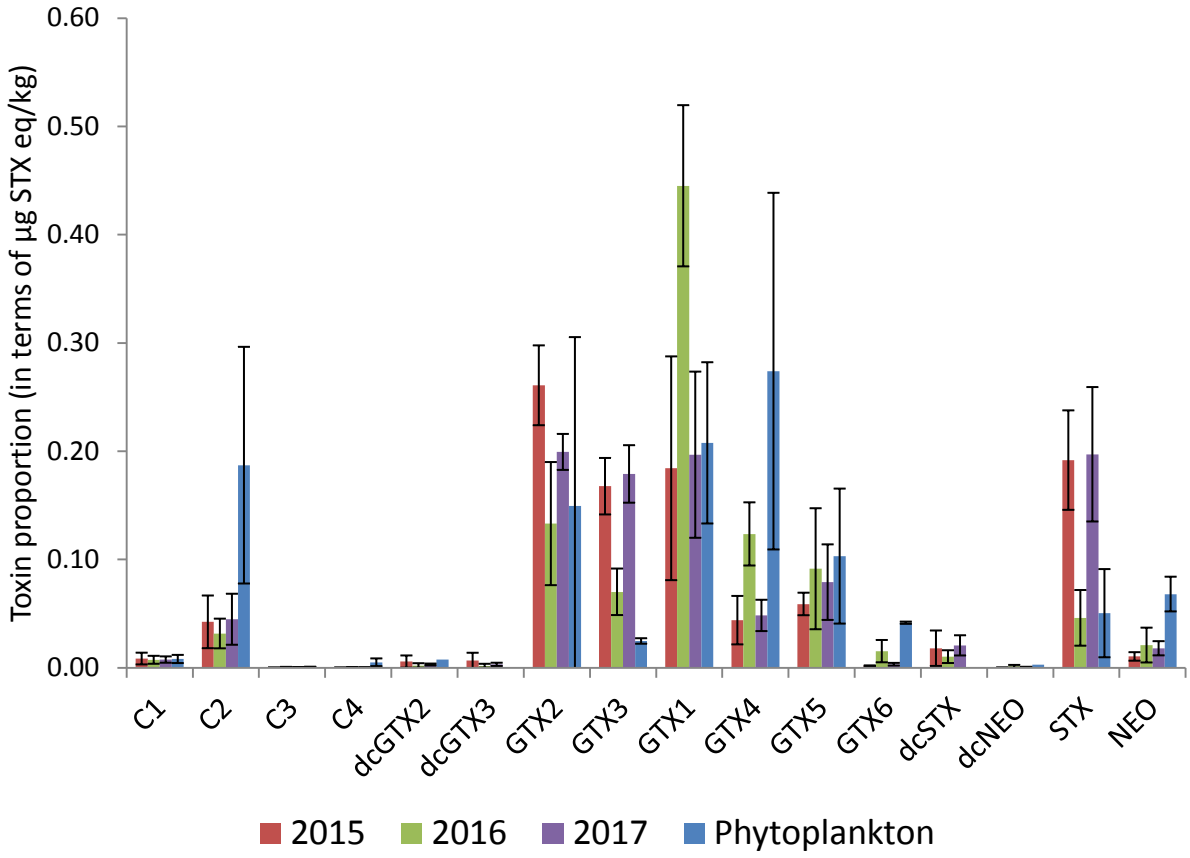
Toxin proportion (in terms of µg STX eq/kg)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
C1
C2
C3
C4
dcGTX2
dcGTX3
GTX2
GTX3
GTX1
GTX4
GTX5
GTX6
dcSTX
dcNEO
STX
NEO
2015
2016
2017
Phytoplankton

Figure 4.

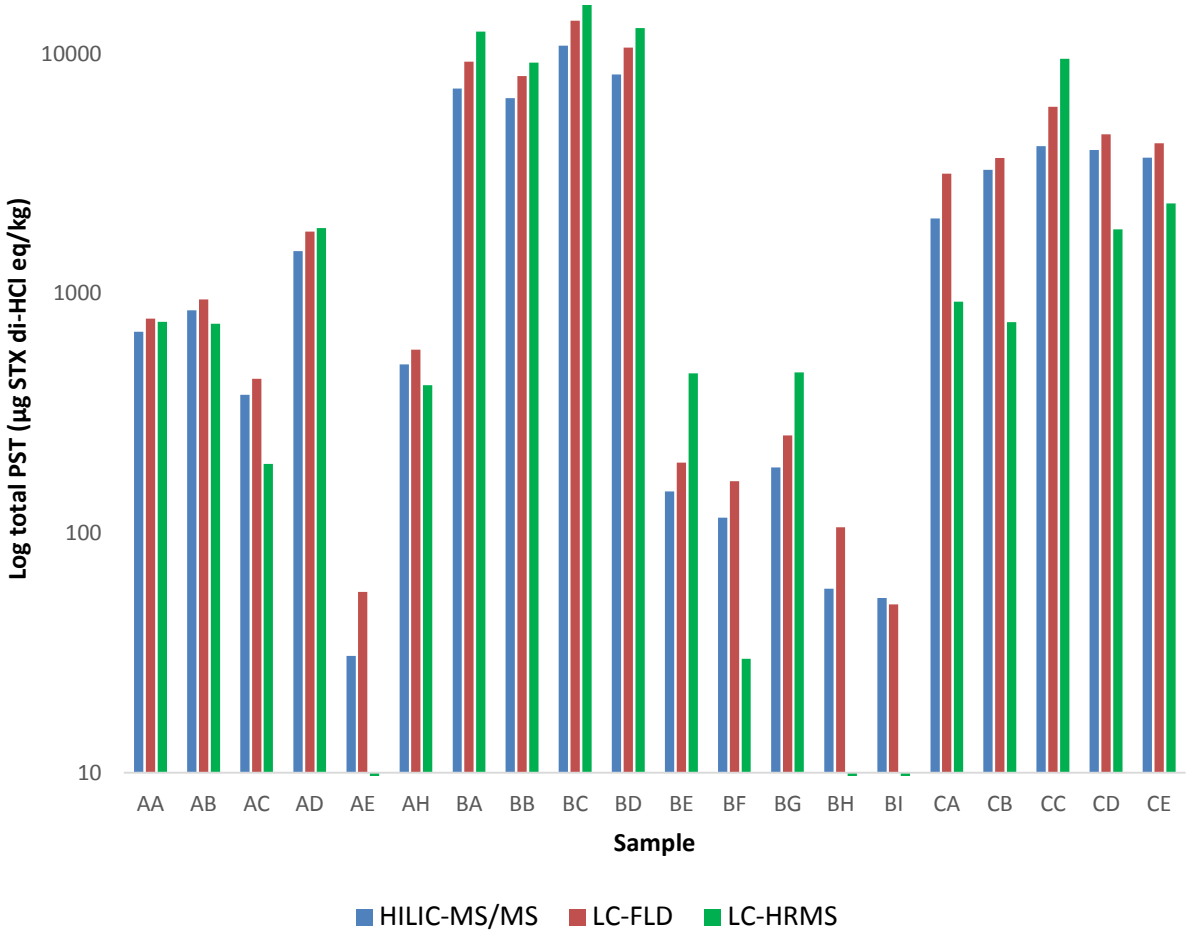
10000
1000
100
10
Log total PST (µg STX di-HCl eq/kg)
AA
AB
AC
AD
AE
AH
BA
BB
BC
BD
BE
BF
BG
BH
BI
CA
CB
CC
CD
CE
Sample
HILIC-MS/MS
LC-FLD
LC-HRMS

Figure Captions

Figure 1. Mean relative molar abundance of PST analogues quantified using UHPLC HILIC-MS/MS in toxin-producing *Alexandrium* species collected from Syracuse bay during a) 2015 to 2017 and b) 2012 to 2014 (Penna *et al.*, 2015).

Figure 2. Example of dynamic MRM transitions following HILIC-MS/MS for PST and TTX quantified in mussel sample harvested during 2016 (sample BC, Cantiere Nautico, Sicily, May 2016).

Figure 3. Mean proportions of PST (± sd), determined by HILIC-MS/MS, in terms of µg STX equivalents (M toxins not included) in shellfish (µg STX eq/kg) and in phytoplankton(pmol STX eq/L) harvested between 2015 and 2017.

Figure 4. Comparison of total PST toxins quantified (log of µg STX di-HCl eq/kg) in Sicily samples using HILIC-MS/MS, LC-FLD and HILIC-HRMS/MS.

Supplementary Material

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Data Statement

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