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Reducing the Impact of Paralytic Shellfish Toxins on Australian Shellfish Industries

Final Report

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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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Abbreviations

CRM	certified reference material
C1&2	*N*-sulfocarbamoyl-gonyautoxin-2&3
C3&4	*N*-sulfocarbamoyl-gonyautoxin-1&4
dcNEO	decarbamoylneosaxitoxin
dcSTX	decarbamoylsaxitoxin
dcGTX2&3	decarbamoylgonyautoxin-2&3
GTX1&4	gonyautoxin-1&4
GTX2&3	gonyautoxin-2&3
GTX5	gonyautoxin-5 (B1)
GTX6	gonyautoxin-6 (B2)
i.p.	intraperitoneal
LC-MS	liquid chromatography-mass spectrometry
LD_{50}	median lethal dose
MBA	mouse bioassay
NEO	neosaxitoxin
NOAEL	No Observable Adverse Effect Level
NRC	National Research Council of Canada
Pre-COX	pre-column oxidation method with fluorescence detection
PCOX	post-column oxidation method with fluorescence detection
PSTs	paralytic shellfish toxins
STX	saxitoxin
TEF	toxicity equivalence factor

Executive Summary

The Cawthron Institute and AgResearch Ltd have successfully generated acute oral toxicity data for the marine toxin C3&4, allowing an updated toxic equivalence factor to be determined. C3&4 is an analogue of saxitoxin, which itself is a potent neurotoxin. These toxins are produced by certain marine micro-algae species and can accumulate in filter feeding shellfish such as mussels and oysters, and together are referred to as paralytic shellfish toxins. C3&4 can be regarded as being of low relative toxicity, 36 times less potent than saxitoxin when administered orally to mice.

A regulatory limit exists for paralytic shellfish toxins in seafood and analytical tests methods are increasingly being used for routine regulatory monitoring. These methods allow the concentration of toxin in shellfish to be determined. Together with knowledge of the relative toxicity of those toxins the overall toxicity of the sample can be calculated, enabling assessment of the potential risk to human health. The relative toxicity information typically used has been estimated by intraperitoneal injection of purified toxin using a mouse bioassay. This approach is flawed, as the mouse bioassay assumes that the dose death-time relationship for all paralytic shellfish toxins are the same as that for saxitoxin, which has since been demonstrated to be incorrect. There is a need for robust relative toxicity information for paralytic shellfish toxins based on relative acute oral toxicities using approved toxicological methods. The aim of this project was to generate acute oral toxicity data for C3&4, a paralytic shellfish toxin commonly found in Tasmanian shellfish during blooms of toxic micro-algae.

The objectives of this project were to generate a sufficient quantity of starting material containing C3&4 from contaminated shellfish or a suitable algal species; isolate sufficiently characterised C3&4 material to enable acute oral toxicity studies to be completed; and determine the acute oral toxicity of the purified C3&4 material using approved toxicological test methods, and based on this data derive a new relative toxicity information. All of these objectives have been achieved:

- Bulk micro-algal cells (*Alexandrium catenella*) cells were harvested and used for toxin isolation.
- Milligram quantities of C3&4 material were isolated and purified.
- Isolated toxin was determined to be of sufficient purity and quantity for toxicological evaluation.
- The median lethal dose (LD_{50}) of C3&4 by oral administration was found to be 42700 nmol/kg, with a 95% confidence interval between 40000-50000 nmol/kg.
- The relative oral toxicity of C3&4 when compared to saxitoxin was 0.028, which is similar to the value obtained in this study using the mouse bioassay (0.033).
- This relative oral toxicity value is slightly lower than the value typically used for C3&4 when using analytical test methods. Adoption of the orally-derived value would result in a decrease of the contribution of C3&4 to the calculated toxicity of a sample, should it be present.

This project completes a large body of research that has determined the oral toxicity of many regulated saxitoxin analogues, including STX; NEO; dcSTX; GTX1&4; GTX2&3; GTX5; GTX6; dcGTX2&3, dcNEO; C1&2 and now C3&4. Data for some of these toxins has already been presented and a manuscript detailing the remainder, including C3&4, is in the final stages of preparation. It is the firm opinion of the authors of this report that the relative oral toxicity values for paralytic shellfish toxins be adopted into routine regulatory test methods to afford a more appropriate assessment of potential risk to shellfish consumers.

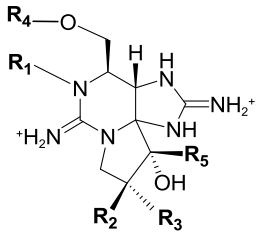
Keywords

Paralytic shellfish poisoning; Paralytic shellfish toxin; C3&4; Acute toxicity; Toxicity equivalence factors; Toxicology

Introduction

Elevated levels of paralytic shellfish toxins (PSTs) cause regular and sometimes prolonged closures of commercial shellfish harvesting, and more recently rock lobster wild harvest fisheries, in many parts of the world including various Tasmanian growing areas. These events represent a serious human health hazard and also impact seafood industries who are unable to harvest and sell product.

PSTs are a group of neurotoxins produced by some marine micro-algal species that naturally accumulate in filter feeding shellfish, such as mussels, scallops and oysters. The toxic micro-algal species *Gymnodinium catenatum* regularly blooms in Tasmania. It is a well-known PST-producer with a toxin profile typically dominated by low toxicity C-toxins. PSTs have a potentially severe impact on humans if contaminated seafood is consumed. More than 50 known PST analogues exist with variable toxicities observed among the analogues (Figure 1) (Wiese et al., 2010). Saxitoxin (STX) and neosaxitoxin (NEO) are regarded as the most toxic and the *N*-sulfocarbamoyl toxins (C-toxins) among the least. A maximum permissible level of 0.8 mg STX equivalents/kg shellfish has been established, and this has traditionally been enforced internationally using an outdated mouse bioassay (MBA) to evaluate the safety of shellfish for human consumption (Anon, 2005).



STX = Saxitoxin
NEO = Neosaxitoxin
GTX = Gonyautoxins
dc = Decarbamoyl

	R_1	R_2	R_3	R_4	R_5
STX	H	H	H	$CONH_2$	OH
GTX2	H	H	OSO_3^-	$CONH_2$	OH
GTX3	H	OSO_3^-	H	$CONH_2$	OH
NEO	OH	H	H	$CONH_2$	OH
GTX1	OH	H	OSO_3^-	$CONH_2$	OH
GTX4	OH	OSO_3^-	H	$CONH_2$	OH
GTX5 (B1)	H	H	H	$CONHSO_3^-$	OH
C1	H	H	OSO_3^-	$CONHSO_3^-$	OH
C2	H	OSO_3^-	H	$CONHSO_3^-$	OH
GTX6 (B2)	OH	H	H	$CONHSO_3^-$	OH
C3	OH	H	OSO_3^-	$CONHSO_3^-$	OH
C4	OH	OSO_3^-	H	$CONHSO_3^-$	OH
dcSTX	H	H	H	H	OH
dcGTX2	H	H	OSO_3^-	H	OH
dcGTX3	H	OSO_3^-	H	H	OH
dcNEO	OH	H	H	H	OH
dcGTX1	OH	H	OSO_3^-	H	OH
dcGTX4	OH	OSO_3^-	H	H	OH

Figure 1. Molecular structure of regulated PSTs commonly found in shellfish. C3 and C4 are epimers.

Analytical methods based on high performance liquid chromatography with fluorescence detection have been developed as alternatives to the MBA. Two such methods have been through a full collaborative inter-laboratory validation process and are internationally accepted. These are AOAC method 2005.06 (pre-column oxidation with fluorescence detection; Pre-COX) and AOAC method 2011.02 (post-column oxidation with fluorescence detection; PCOX).

Advanced Analytical Australia is a commercial laboratory based in Sydney responsible for the majority of routine regulatory monitoring of marine biotoxins in Australian shellfish. At present they use the Pre-COX method to monitor PST in bivalve molluscan shellfish. Both analytical approaches described above permit the assessment of the concentration of PSTs in shellfish and this, together with knowledge of the relative toxicity of the various compounds, allows the overall toxicity of the sample to be determined, enabling assessment of the potential risk to human health.

The relative toxicities are expressed as "Toxicity Equivalence Factors" (TEFs), which define the toxicities of these substances as a ratio to that of STX. An assay for just STX was developed by Sommer and Meyer in the 1930's (Sommer and Meyer, 1937), and is based on the relationship between the dose of pure STX administered to mice by intraperitoneal injection and the time to death of the animals. The amount of STX in the sample injected, expressed as "Mouse Units", can then be determined from the table of death-times established by these authors. Although validated only for STX, this MBA has more recently been applied to various PSTs, and TEFs have been estimated from this data (EFSA, 2009).

The validity of this approach is questionable as the TEFs derived from this method do not correlate with median lethal doses determined by approved toxicological methods (Munday et al., 2013). The use of the MBA assumes that the dose death-time relationship for PSTs are the same as that for STX, which has been demonstrated to be incorrect (Munday et al., 2013). The inadequacy of the present TEFs for risk assessment was implied in the Scientific Opinion of the European Food Safety Authority Panel on Contaminants in the Food Chain, which indicated the need for establishing robust TEFs based on the relative oral toxicities of PSTs (EFSA, 2009). The oral potency of PSTs has also been identified as a key research priority by the Director of Population Health at the Tasmanian DHHS. This is driven, in part, by the surprising lack of human illness recorded in Tasmania in relation to the consumption of PST-contaminated seafood.

Some recent work has been undertaken to address the paucity of oral toxicity information that exists for regulated PSTs. Published information has been generated for NEO, dcSTX, GTX1&4 and GTX2&3 (Munday et al., 2013). Due to recent efforts, the list of PST analogues has been extended to include GTX5, GTX6, dcGTX2&3, dcNEO, and C1&2: a manuscript describing these results is currently in preparation.

The aim of this project was to generate acute oral toxicity data from a purified epimeric mixture of *N*-sulfocarbamoyl gonyautoxin 1&4 (C3&4) to allow a more valid TEF to be generated and applied. In practical terms, this project aims to reduce the impact of PSTs on the Australian shellfish industry without compromising the safety of shellfish consumers or the integrity of the Tasmanian shellfish brand.

Objectives

To be able to generate purified material for toxicological evaluation, three objectives were created with each being dependent on the other.

Objective 1

Generate a sufficient quantity of starting material that contains C3&4 from contaminated shellfish or a suitable algal species.

Objective 2

Isolate sufficiently characterised C3&4 material to enable acute oral toxicity studies to be completed.

Objective 3

Determine the acute oral toxicity of the purified C3&4 material using OECD Test Guideline 425 and based on this data derive a new TEF to be used for regulatory testing.

Method

The methodology employed for each of the three objectives is detailed below:

1. Generate a sufficient quantity of starting material that contains C3&4 from contaminated shellfish or a suitable algal species.

All of our attempts to generate starting material were from various PST-producing marine micro-algal species. Initially our efforts were focused on *Gymnodinium catenatum*, a known C3&4 producing micro-algal species that blooms regularly in Tasmanian waters and results in closures to shellfish harvest areas. Cawthron scientist Craig Waugh travelled to Hobart during a documented *G. catenatum* bloom event (Jun 2014) and attempted to harvest cells for toxin isolation. Unfortunately this approach proved unsuccessful. This was due to several reasons, which included both technical and logistical challenges and lower than expected cell densities.

As an alternative source of toxin material our efforts were then focused on *Alexandrium catenella*. Over the past four summers this toxic dinoflagellate species has bloomed in an isolated bay of the Marlborough Sounds, which is located at the top of the South Island of New Zealand. *A. catenella* cells were harvested from this wild bloom event using a pump and phytoplankton net (20 µm) by Cawthron scientists in March 2013 (Photo, left). Settled harvested cells were stored frozen at Cawthron until required (Photo, right).

Photo. Cawthron scientist Craig Waugh harvesting *Alexandrium catenella* cells in Opua Bay, Marlborough Sounds, New Zealand (left) and resulting cell concentrate in 1 L plastic bottle (right).

The toxin content and profile of the harvested cells were determined using a recently described liquid chromatography tandem mass spectrometry (LC-MS) method (Boundy et al., 2015). As C3&4 calibration material was not available at the time of analysis, levels of C3&4 in the cell extracts was

quantified by calibrating the C3&4 response off the C1&2 calibration standard. Based on this data, the C3&4 content of the cells was thought to be relatively low, and represents the reason why *A. catenella* cells were not originally considered as the best option for toxin isolation purposes.

2. Isolate sufficiently characterised C3&4 material to enable acute oral toxicity studies to be completed.

Briefly, for toxin isolation, bulk *A. catenella* cells were extracted by heating in dilute acid. Cell debris was removed using centrifugation and filtration. The C-toxins, and co-extracted salts, were removed from the supernatant by precipitation with organic solvent and then recovered using various chromatographic techniques. The final purification step required C3&4 to be chromatographically separated from any C1&2 that was present in the extract. The chromatography employed used proprietary isolation methods developed at Cawthron over the past 20 years and included activated carbon chromatography, gel filtration chromatography and ion-exchange chromatography.

Detailed notes of the chromatographic separation and isolation of C3&4 are stored electronically at Cawthron in the following location (O:\MethodDevelopment\Labbooks\R&D\CNC\PSP\C3&4).

Quantitation of the material generated was undertaken using published methods. These were the post-column oxidation method with fluorescence detection (PCOX)(van de Riet et al., 2011), or by a recently described liquid chromatography tandem mass spectrometry method (LC-MS)(Boundy et al., 2015).

Due to the initial unavailability of reference material for direct quantitation, the C3&4 material that had been isolated was acid hydrolysed to form GTX1&4 using a described procedure (Hall et al., 1984). The GTX1&4 formed was then quantified by LC-MS and the PCOX method against certified GTX1&4 reference material.

Purity was assessed by LC-MS analysis using negative ion scan (m/z 50-600). It was assumed that all compounds measured had an equimolar response.

3. Determine the acute oral toxicity of the purified C3&4 material and based on this data derive a new TEF to be used for regulatory testing.

The toxicology assessment on the purified material was undertaken by Dr Rex Munday (AgResearch, New Zealand). Female Swiss albino mice, bred at Ruakura, were employed in all experiments. Except where indicated, the initial body weights of the mice were between 18 and 22 g. They were housed in solid-bottomed cages containing bedding of softwood shavings. The animals were allowed unrestricted access to food (Rat and Mouse Cubes, Speciality Feeds Ltd, Glen Forrest, Western Australia) and tap water throughout the experimental period. All experiments were approved by the institutional Animal Ethics Committee.

To calibrate the mice for the MBA, a certified standard of STX hydrochloride at a concentration of 0.8497 mM in 3 mM HCl was employed. The calibration was conducted according to AOAC Official Method 959.08 (Anon, 2005). The mean body weight of the mice employed in this experiment was 20.0 ± 0.1 g. Weighed aliquots of the standard solution were diluted to 1 mL with 3 mM HCl and injected intraperitoneally.

For determination of the specific activity of C3&4 by the MBA, aliquots dissolved in 3 mM HCl, were diluted to 1 mL with the same solvent and injected intraperitoneally in mice according to the

protocol of AOAC Official Method 959.08 (Anon, 2005). Median death times were calculated, and MU/mL determined from Table 959.08A in the AOAC Method. Specific activities were calculated as MU/mmole.

To establish the relationship between dose and death time for C3&4, a logarithmic progression of doses were administered intraperitoneally to groups of 3–9 mice. The dose levels employed spanned those causing death within 4 min and those that caused no deaths in any of the group of mice.

Acute toxicities were determined according to the principles of OECD Guideline 425 (OECD, 2008). In this method, one animal is given a dose of the test material at a step below the level of the best estimate of the LD_{50}. If this animal survives, the dose for the next animal is increased by a logarithmic factor. If it dies, the dose for the next animal is decreased by the same factor. Dosing is continued until 4 reversals have been achieved, a reversal being a situation in which death is observed at a particular dose but not at the next lowest dose, or a situation in which an animal survives at a particular dose but dies at the next highest one. This technique, which has been validated against other methods of determining acute toxicities, has the advantage of minimising the number of animals (no more than 15) required to give a robust estimate of the median lethal dose, and at the same time providing an estimate of the confidence intervals of this estimate, using the computer program associated with this Guideline.

Mice were weighed immediately before dosing, and the C3&4 was administered on a nmole/kg bodyweight basis. Aliquots of the test materials were diluted in 3 mM HCl. The volume administered for i.p. injection was 1 mL, while for gavage 200 µL was used (gavage is defined as ‘introduction of the toxin into the mouse stomach by means of a tube’). In order to minimise potential diurnal effects on toxicity, dosing by all routes of administration was conducted between 8.00 and 9.30 a.m. The mice were monitored intensively during the day of dosing. Those dying during the course of the experiment were necropsied, while survivors were examined each day for 14 days after dosing, after which time they were killed and necropsied.

For the determination of No Observable Effect Level (NOAEL), mice were dosed by gavage with the C3&4 material at a dose below the LD_{50}. A logarithmic dose progression was employed, using the protocol of OECD Guideline 425, but with “toxic effect” rather than death as the parameter. The mice were observed intensively after dosing. Grip strength was measured at intervals using an MK-380S Grip Strength Monitor (Muromachi Kikai Co., Tokyo, Japan) and exploratory behaviour was assessed by transferring the mice to a new cage and observing their exploratory movements. Abdominal breathing and lethargy were assessed visually.

Results

Starting material

Starting material for the toxin isolation procedure was generated from harvesting *A. catenella* cells from a natural wild bloom event, which occurred in New Zealand in March 2013. Cell harvesting gave approximately 5 kg of cells (wet weight). Chemical analysis using the LC-MS method determined that the cells contained a complex suite of saxitoxin analogues, including C-toxins (Figure 2).

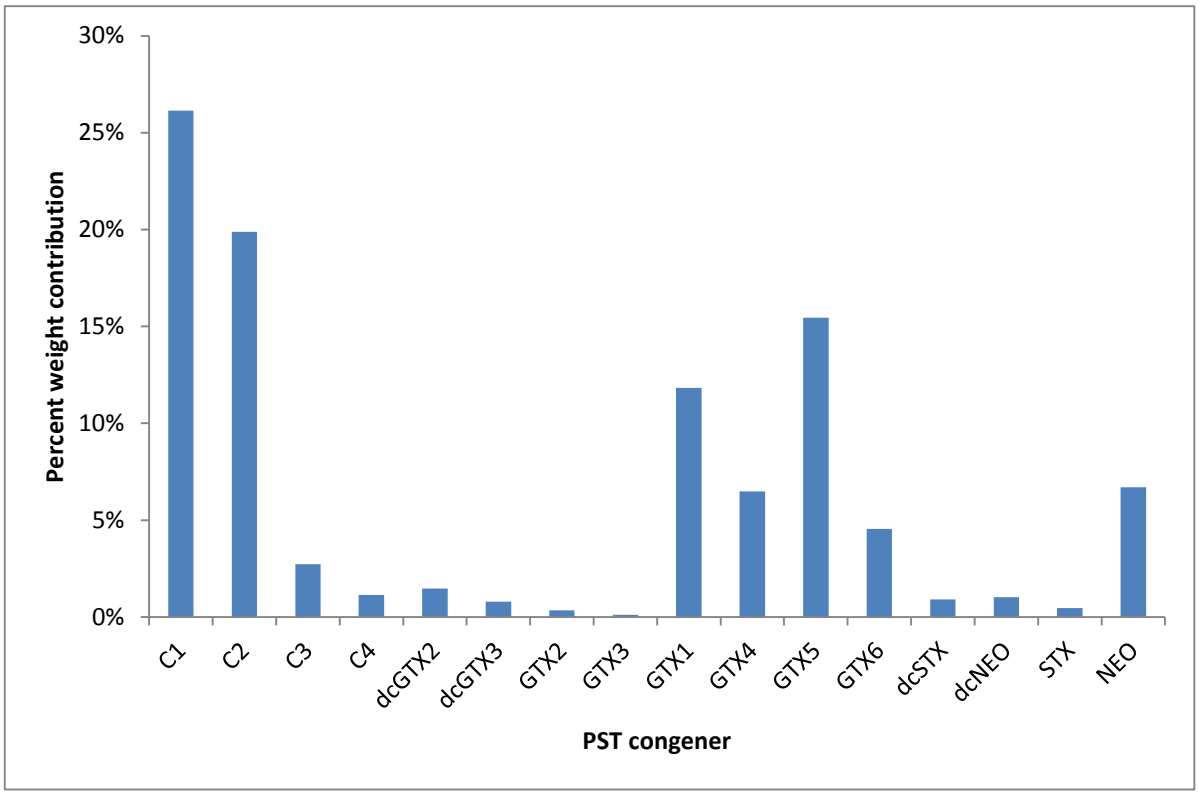


Figure 2. PST profile of wild harvested *Alexandrium catenella* cells used for the isolation of C3&4.

Purified C3&4 toxin

The isolation and purification of C3&4 involved various chromatographic steps that included activated carbon chromatography, gel filtration chromatography and ion-exchange chromatography. Analysis of the resulting purified fractions by the PCOX method showed that the isolation procedure was successful. The C3 and C4 epimers were the most abundant components (Figure 3), with C3 more abundant than C4. Trace levels of C1 and C2 were observed, which was not unexpected due to the difficulty of separating these structurally related congeners from C3&4 during the workup procedure.

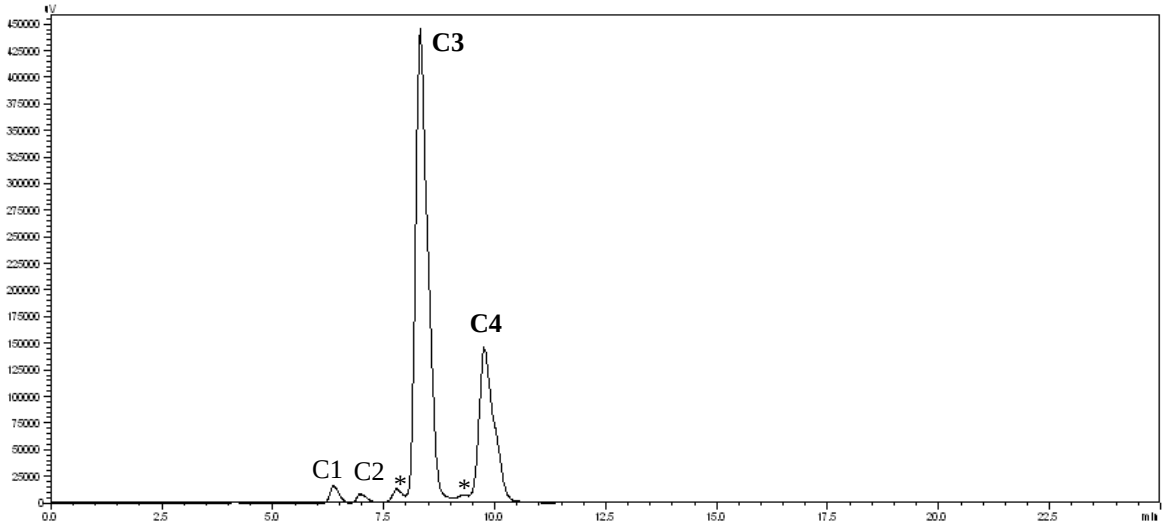


Figure 3. PCOX analysis of purified C3&4 material. *represent minor contaminants thought to be *N*-acetoxy analogues of C3&4 formed during the isolation procedure.

Due to the initial unavailability of reference material for direct quantitation, C3&4 was chemically converted into GTX1&4 using acid hydrolysis. The GTX1&4 was then quantified by both LCMS analysis and the PCOX method, using certified GTX1&4 reference material. There was very good agreement between the two methods (Table 1).

Table 1. Concentration of C3&C4 when hydrolysed to, and quantified as, GTX1&GTX4.

Compound	µg/mL		
	LCMS	**PCOX**	**Average**
C3	753	700	726
C4	299	287	293
Total	1051	987	1019

To ensure chemical conversion occurred as expected, a known quantity of a C1&2 CRM was added to the reaction mixture and its hydrolysis product GTX2,3 monitored. A conversion yield of 82% was observed, indicating there was near stoichiometric conversion and that limited degradation occurred. It was assumed that a similar yield applied for the conversion of C3&4 → GTX1&4.

After the hydrolysis experiments had been performed, a reference standard for C3&4 was sourced from the Institute of Marine Biosciences, National Research Council of Canada (NRC). This material is not commercially available. It was subsequently used to accurately calibrate the C3&4 material isolated. Reassuringly, the C3 and C4 concentrations assigned when using acid hydrolysis closely matched those determined when using NRC reference material (Table 2).

Table 2. Quantitation comparison when hydrolysed to GTX1&4 and using NRC reference material.

Compound	µg/mL		% difference
	Hydrolysed ave (LCMS&PCOX)	**NRC calibrated (PCOX)**	
C3	726	712	2
C4	293	305	4

From these analyses, we were able to accurately quantify the amount of C3&4 material generated. Purity was determined by LC-MS (Figure 4). Minor impurities were observed, including some C1&2, which was also observed by PCOX analysis. Other minor components were observed (*m/z* 532), and these were likely to be *N*-acetoxy analogues of C3&4 formed during the isolation procedure. Another minor impurity (<2%) was also observed (*m/z* 471). At this time it remains unclear what this mass represents.

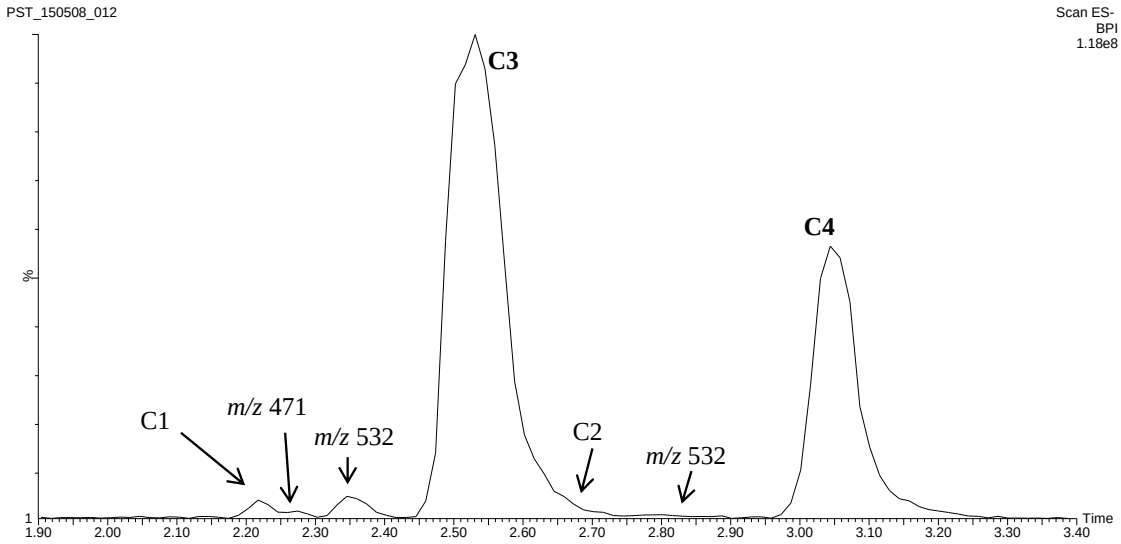


Figure 4. Negative ion LC-MS analysis of purified C3&4 material. Minor impurities were detected in addition to C1&2.

Based on the peak areas observed from the LC-MS analysis the C3&4 material was determined to be 94% pure, with respect to other PST-like components (Table 3).

Table 3. Percentage contribution (peak area) of PST congeners detected by HILIC LC-MS analysis.

Compound	**% contribution**
C3&4 $[M-H]^-$ *m/z* 490	94
$[M-H]^-$ *m/z 532*	3
C1&2 $[M-H]^-$ *m/z* 474	2
$[M-H]^-$ *m/z 471*	1

As a result of the isolation and purification efforts milligram quantities of C3&4 were generated from the *A. catenella* cells and this material was of sufficient purity to warrant toxicological evaluation. A certificate of analysis for the C3&4 material is attached as Appendix A.

Toxicological analysis

The C3&4 material generated was sent to Dr Rex Munday (AgResearch) for toxicological evaluation using described procedures:

1) specific activity by the MBA.
2) median lethal dose by intraperitoneal injection.
3) median lethal dose by gavage.

For the MBA, the median death times of mice injected with C3&4 determined a specific activity of 70 MU/µmol (SEM 1.2 MU/µmol). This was considerably lower than STX and slightly lower than that of C1&2 (Table 4). From these analyses updated TEFs were able to be generated as a ratio between the specific activity of C3&4 (and C1&2) and STX.

Table 4. MBA derived relative specific activities and associated TEFs.

Compound	Specific Activity (MU/µmol)	TEF
STX†	2090	1.0
C-3,4	**70**	**0.033**
C-1,2‡	367	0.18

†STX specific activity from Munday 2013 Toxicon article (Munday et al., 2013)
‡C1&2 specific activity from Munday (manuscript under preparation – see Appendix B)

The median lethal dose (LD_{50}) of C3&4 by i.p. injection was determined to be 480 nmol/kg with a 95% confidence interval between 450-500 nmol/kg (Table 5). Based on this information C3&4 is slightly less toxic than C1&2 but the difference is not statistically significantly due to the wide confidence interval with the latter compound.

Table 5. Acute toxicity of C3&4 and other PST analogues by i.p. injection and associated TEFs.

Compound	LD_{50} (nmol/kg)	TEF
STX†	27.8 (24.6-31.2)	1.0
C-3,4	**480 (472-500)**	**0.058**
C-1,2‡	400 (327-663)	0.070

†STX acute toxicity from Munday 2013 Toxicon article (Munday et al., 2013)
C1&2 acute toxicity from Munday (manuscript under preparation – see Appendix B)

The median lethal dose (LD_{50}) of C3&4 by oral administration was determined to be 42700 nmol/kg with a 95% confidence interval between 40000-50000 nmol/kg (Table 6). The TEF for C3&4 generated by oral administration (0.028) was very similar to that derived via the MBA (0.033).

Table 6. Acute toxicity of C3&4 and other PST analogues by gavage and associated TEFs.

Compound	LD_{50} (nmol/kg)	TEF
STX†	1190 (1019-1300)	1.0
C-3,4	**42700 (40000-50000)**	**0.028**
C-1,2‡	35000 (30600-46700)	0.034

†STX acute toxicity by gavage from Munday 2013 Toxicon article (Munday et al., 2013)
‡C1&2 acute toxicity by gavage from Munday (manuscript under preparation – see Appendix B)

The No Observable Adverse Effect Level (NOAEL) for C3&4 after oral administration was determined to be 25500 nmol/kg with a 95% confidence interval between 23800-30000 nmol/kg (Table 7). For STX, the NOAEL was 544 nmol/kg with a 95% confidence interval between 500-560 nmol/kg.

Table 7. No Observable Adverse Effect Level of C3&4 and other PST analogues by gavage.

Compound	NOAEL (nmol/kg)
STX†	544 (500-560)
C-3,4	**25500 (23800-30000)**
C-1,2‡	15000 (10500-19900)

†STX acute toxicity by gavage from Munday 2013 Toxicon article (Munday et al., 2013)
‡C1&2 NOAEL from Munday (manuscript under preparation – see Appendix B)

Discussion

Sourcing starting material represented a critical first step to the overall success of this project. Our initial preference was to obtain *G. catenatum* micro-algal material from a wild bloom event. Based on micro-algal monitoring data received in June 2014 it was decided to attempt a wild harvesting mission to southeast Tasmania. Cawthron scientist Craig Waugh travelled to Tasmania in June 2014. Alison Turnbull (PIRSA-SARDI); Jason Whitehead and Megan Burgoyne (TSQAP); Sam Ibbott (Marine Solutions) were also involved. Unfortunately, this mission proved largely unsuccessful from a harvesting perspective due to the low cell densities found, the patchiness of the bloom itself and the poor weather experienced during the week that harvesting was attempted.

Although this harvesting mission was unsuccessful an alternative micro-algal source was being investigated. Over the past 3 years Cawthron scientists have been isolating and purifying various saxitoxin analogues (C1&2; GTX5; GTX6; dcNEO and dcGTX2,3) from bulk *A. catenella* cells harvested during a wild bloom event. This project was being performed, as part of a funded research project in New Zealand, to determine the acute toxicity of various saxitoxin analogues to mice using various routes of administration. It represented a continuation of work already described (Munday et al., 2013) and excluded C3&4. Extracts generated from bulk *A. catenella* cells were shown to contain some C3&4 although initially it was regarded as not being abundant enough to warrant isolation. It is now apparent that the amount of C3&4 present in the algal cell extract was underestimated due to the unavailability of a reference standard for C3&4 and application of an incorrect relative response factor during analysis. Once this was known, bulk cells already in storage were used for C3&4 isolation and purification purposes. The C3&4 material generated had an equilibrated epimer ratio, the same as that found in contaminated shellfish, and was of sufficient quantity and purity to warrant toxicological evaluation. The separations employed used proprietary isolation methods developed at Cawthron over the past 20 years and included activated carbon chromatography, gel filtration chromatography and ion-exchange chromatography.

Based on toxicity data generated as part of this study C3&4 can still be regarded as a low toxicity saxitoxin analogue and the TEF values generated using the various mouse toxicity tests reflect this (see Table 8 for summary).

Table 8. Summary of TEFs for STX analogues derived using various mouse toxicity tests.

Compound	**TEF MBA**	**TEF LD_{50} by ip**	**TEF LD_{50} by gavage**
STX	1.0	1.0	1.0
C3&4	**0.033**	**0.058**	**0.028**
C1&2	0.18	0.070	0.034

The oral toxicity data shows that C3&4 is 36 times less potent than saxitoxin and has very similar toxicity to C1&2. In all cases C1&2 was slightly more toxic than C3&4, regardless of the toxicity test performed and route of administration. The toxicity of C3&4 and C1&2 was lower when administered by gavage than by i.p., a trend that has been observed previously for other PSTs (Munday et al., 2013). Somewhat surprisingly, the TEF value derived by oral administration is remarkably similar to that generated using the MBA. This does not validate the use of the MBA-generated value, but does give confidence that the TEF value currently used for analysis is fit-for-purpose. Also, the TEF generated for C3&4 using the MBA (0.033) is very similar to the average TEF (0.036) of C3 (0.013) and C4 (0.058) reported using the MBA by Prof Oshima (EFSA, 2009).

Conclusion

The overall aim of this project has been achieved: to generate acute **oral** toxicity data from a purified epimeric mixture of C3&4 to allow a more valid TEF (0.028) to be generated.

In the process of generating the toxicological information for C3&4 all of the project objectives have been achieved; starting material obtained, a sufficient quantity of purified C3&4 isolated, and the oral toxicity of the material determined.

Although the TEF generated for C3&4 is very similar to that determined by the MBA, the authors are of the firm opinion that values for all regulated saxitoxin analogues be generated through oral administration and be implemented for monitoring purposes. This is because TEFs that are based on oral potency will afford a more appropriate assessment of potential risk to shellfish consumers.

This work complements research activities that have focused on generating acute oral toxicity data for all of the major regulated saxitoxin analogues. This list includes STX; NEO; dcSTX; GTX1&4; GTX2&3; GTX5; GTX6; dcGTX2&3, dcNEO; C1&2 and now C3&4. Data for some of these analogues has already been presented. A manuscript detailing the remainder, including C3&4, is in the final stages of preparation and will be submitted to Toxicon.

Implications

Determining the contribution of C3&4 to shellfish toxicity is dependent on the method of analysis, and in the case of analytical methods, the TEF applied.

- Having C3&4 reference material allows instrument calibration, enabling accurate quantitation of this toxin in samples. This is important, as in the absence of a C3&4 CRM various approaches have been adopted in analytical laboratories around the world. These include; calibrating C3&4 off another PST analogue and applying a relative response factor, using an in-house standard for direct measurement, performing an acid hydrolysis to convert C3&4 to GTX1&4, or not including C3&4 at all in assessments of shellfish toxicity.

- Adoption of the oral TEF for C3&4 would result in a change from 0.036 (ave TEF for C3 and C4 reported by Oshima) to 0.028 (oral TEF generated in this study). This represents a small but real decrease of approximately 20%. Applying this new factor would decrease the contribution of C3&4 to the calculated toxicity of the sample, should it be present in the shellfish tested.

- Adoption of orally derived TEFs for the saxitoxin group, including C3&4, when employing analytical test methods would provide greater protection of human health. However, adoption of the value for C3&4 alone, would only result in small changes to the overall approximation of sample toxicity due to the small change in toxicity determined by oral administration compared to the status quo.

Recommendations

- At the stakeholder level, broadcast information about findings of project and raise awareness of the reasons for overestimations of sample toxicity when C3&4 is present in shellfish samples and the PreCOX screen method is used. See ‘Extension and Adoption’ plan.

- At the regulator level, provide reports and manuscript generated as part of this project and raise awareness of the reasons for overestimations of sample toxicity when C3&4 is present in shellfish samples and the PreCOX screen method is used. Also, encourage adoption of TEFs generated via oral administration for use in the routine regulatory environment.

- At the analytical laboratory level, there is a need for C3&4 certified reference material to allow accurate quantitation of the congener in PST-contaminated shellfish samples. This material is not currently available commercially (see ‘Project materials developed’ section).

Extension and Adoption

- Comprehensive oral toxicity evaluation of C3&4 is now complete and the data generated will be submitted to a peer-reviewed scientific journal (Toxicon). This will be publicly available.
- Australian and New Zealand delegations have been working at Codex to revise the methods of analysis for marine biotoxins. The Codex standard for live and raw bivalve molluscs (Codex Stan 292-2008) now allows for determination of marine biotoxins by chemical methods that can distinguish and quantify the toxin analogues. The standard then states: "Total toxicity is estimated as the sum of the molar concentrations of detected analogues multiplied by the relevant specific toxicity equivalency factors (TEFs). Internationally scientifically validated TEFs must be used. The science behind TEFs is developing. Current internationally validated TEF's can be found on the FAO website. Information on TEFs could be incorporated in this standard at a future date." During the development of these words, the Codex Committee of Fish and Fisheries Products (CCFFP) acknowledged that oral TEFs were more relevant than the currently available mouse bioassay determined TEFs. FAO offered the use of their website in order that oral TEFs become available for regulatory use as soon as they have passed the peer review process. The work from this project will thus be submitted to the FAO for uploading to their website as appropriate TEFs for international use when they are published in a peer reviewed journal. Although CCFFP is unlikely to meet in the near future, the Australian and New Zealand delegations to the Codex Committee of Measurement Analysis and Sampling (CCMAS) were also involved in moving the new standard through Codex. CCMAS may consider altering Codex Stan 292-2008 in the future to list the oral toxicities, but adoption of the new TEFs will not be held back for this process, due to the ability to list the orally derived TEFs on the FAO website.

  Australian delegate leader to CCFFP: Lynda Hayden (DAWR)

  Technical representative during discussion on methods for the determination of marine biotoxins: Alison Turnbull (SARDI)

  Australian delegate leader to CCMAS and technical representative during discussion on methods for the determination of marine biotoxins: Richard Coughlan (NMI)

  See **Table 9** below for other forms of communication to promote extension and adoption of the information generated as part of this project:

Table 9. Communication plan to promote extension and adoption of project (2013/054) findings.

Communication type	Description	Audience	Person responsible	Time frame
Teleconference	Teleconference with regulators and Codex CCMAS delegate leader to describe work, outcomes and international uptake path, and role of CCMAS in this process.	DAWR export branch, Tas Department Health and Human Services, Tas Dep. Primary Industries Parks, Water and Environment Shellfish QA Program, Codex CCMAS delegate leader	Ali Turnbull to facilitate; Tim Harwood to summarise project and outcomes	March 2016
Project summary/Full report	Executive summary and outcomes to be provided to Oysters Tasmania (& Phil Lamb) for distribution industry wide via email. Full report to be available to those interested.	Tasmania shellfish industry	Tim Harwood	March 2016
Full report on SafeFish Website	Full FRDC report	Seafood stakeholders	Ali Turnbull	April 2016
Fishing today article	One page with picture. Why was the project conducted? Why did Cawthron do the work and not local researchers? What were the outcomes? How does this feed into the whole oral toxicity picture? What does this mean for industry? What will be the pathway to international use?	Tasmanian fishing industry, fishing managers and regulators	Tim Harwood/ Ali Turnbull	March /April 2016
Presentation	Powerpoint presentation to ASQAAC during their annual conference/meeting in September to describe work, outcomes and international uptake path	SQAP managers Industry representatives FSANZ DAWR Shellfish industry stakeholders	Tim Harwood	September 2016
Presentation	Powerpoint presentation to Shellfish futures during their annual conference/meeting in September to describe work, outcomes and international uptake path. Note: this will be offered to industry. Given recent developments with POMS, they may not choose to take this up.	Tasmanian shellfish industry stakeholders	Tim Harwood	October 2016

Project materials developed

- C3&4 material used for toxicological evaluation (see certificate of analysis in Appendix A).
- Manuscript describing toxicity of C3&4, and for other regulated STX analogues, is in the final stages of preparation (see Appendix B). This is to be submitted to Toxicon.
- A small amount of C3&4 calibration material has been generated and is currently being used for routine analysis at Cawthron for accurate calibration of instrument response and quantitation of C3&4 in samples. In the future, Cawthron aims to isolate more C3&4 from a micro-algal source to be able to generate a CRM to allow other analytical laboratories to calibrate their instrument response.

Appendices

- List of researchers and project staff

Researchers:

Dr Tim Harwood (Cawthron Institute, New Zealand)

Andy Selwood (Cawthron Institute, New Zealand)

Craig Waugh (Cawthron Institute, New Zealand)

Dr Paul McNabb (Cawthron Institute, New Zealand)

Dr Rex Munday (AgResearch, New Zealand)

Co-investigators:

Ali Turnbull (Sub-programme leader Food Safety & Innovation – SARDI)

Neil Stump (Tasmanian Seafood Industry Council)

Dr Mark Veitch (Acting Director of Public Health | Department of Health and Human Services)

- Intellectual Property

All information associated with the toxicological evaluation of purified C3&4 material has been disclosed and is to be made public through publication in an internationally peer-reviewed scientific journal.

The separation techniques employed to isolate and purify C3&4 material from micro-algal cells used proprietary isolation methods developed at the Cawthron Institute over the past 20 years for a suite of saxitoxin analogues. The processes include activated carbon chromatography, gel filtration chromatography and ion-exchange chromatography. For this reason the intellectual property associated with the isolation work remains in the domain of the Cawthron Institute.

References

Anon, 2005. Paralytic shellfish poison. Biological method. Final action. AOAC Official Method 959.08. AOAC International, 79-80.

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.

EFSA, 2009. Marine biotoxins in shellfish – saxitoxin group. Opinion of the panel on contaminants in the food chain. The EFSA Journal 1019, 76.

Hall, S., Darling, S.D., Boyer, G.L., Reichardt, P.B., Liu, H.W., 1984. Dinoflagellate neurotoxins related to saxitoxin: Structures of toxins C3 and C4, and confirmation of the structure of neosaxitoxin. Tetrahedron Lett. 25, 3537-3538.

Lawrence, J.F., Niedzwiadek, B., Menard, C., 2005. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: Collaborative study. J. AOAC. Int. 88, 1714-1732.

Munday, R., Thomas, K., Gibbs, R., Murphy, C., Quilliam, M.A., 2013. Acute toxicities of saxitoxin, neosaxitoxin, decarbamoyl saxitoxin and gonyautoxins 1&4 and 2&3 to mice by various routes of administration. Toxicon 76, 77-83.

OECD, 2008. Guidelines for the Testing of Chemicals. Guideline 425. Acute Oral Toxicity - Up-and-Down procedure.

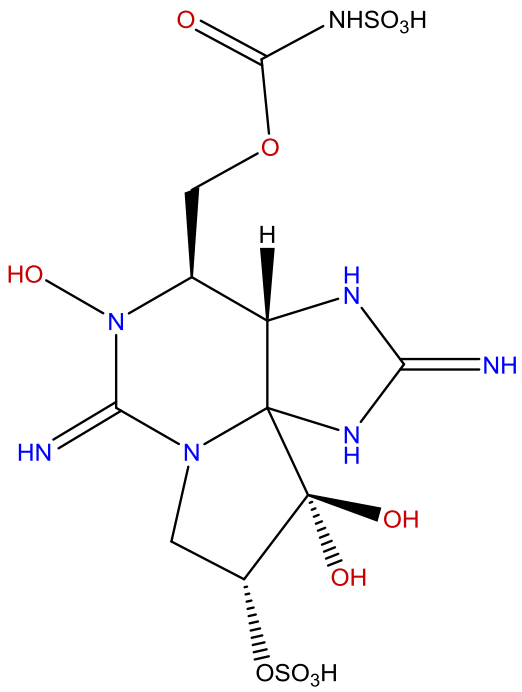
Sommer, H., Meyer, K.F., 1937. Paralytic Shellfish Poisoning. Archives of Pathology 24, 560-598.

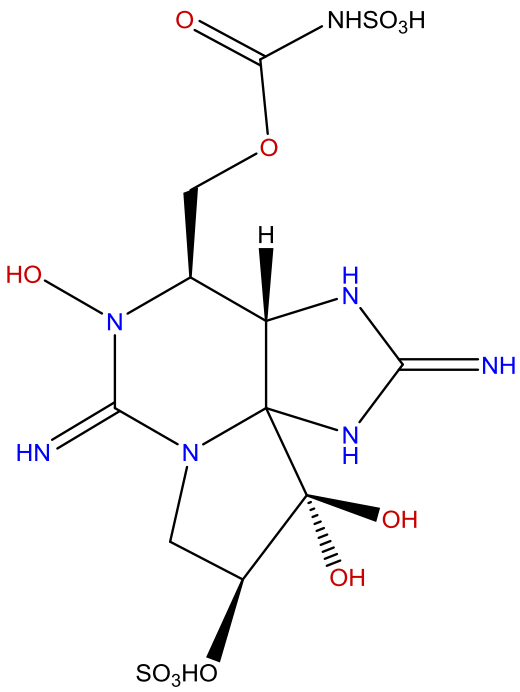
van de Riet, J., Gibbs, R.S., Muggah, P.M., Rourke, W.A., MacNeil, J.D., Quilliam, M.A., 2011. Liquid Chromatography Post-Column Oxidation (PCOX) Method for the Determination of Paralytic Shellfish Toxins in Mussels, Clams, Oysters, and Scallops: Collaborative Study. J. AOAC. Int. 94, 1154-1176.

Wiese, M., D'Agostino, P.M., Mihali, T.K., Moffitt, M.C., Neilan, B.A., 2010. Neurotoxic Alkaloids: Saxitoxin and Its Analogs. Marine Drugs 8, 2185-2211.

Appendix A

Certificate of Analysis for C3&4





N-sulfocarbamoyl-gonyautoxin-1 (C3)

Molecular formula: $C_{10}H_{17}N_7O_{12}S_2$

Molecular weight: 491.40

Monoisotopic molecular weight: 491.0377

N-sulfocarbamoyl-gonyautoxin- 4 (C4)

Molecular formula: $C_{10}H_{17}N_7O_{12}S_2$

Molecular weight: 491.40

Monoisotopic molecular weight: 491.0377

Lot number: CNC00045

Purity: 94% by LC-MS monitoring other PST congeners.

Source: *Alexandrium catenella*, Cawthron Institute Nelson, New Zealand

Storage: Store frozen at <-20 °C.

Description: An ampoule containing 0.5 mL of C3&4 in 20 µM acetic acid pH 4.8.

Concentration: C3 29.5 µM and C4 12 µM (C3 14.5 µg/mL and C4 5.85 µg/mL counter ion free).

Assessment of purity and quantity:

The purified C3&4 were analysed by HILIC LC-MS [1] and HPLC-FLD with post column oxidation [2]. The purity was determined from a negative ion scan, assuming all compounds measured shared an equal molar response. The concentration of C3&4 were determined by hydrolysing to gonyautoxins 1&4, with 0.1 M HCl, then calibrating with a certified reference material from the National Research Council Canada [3].

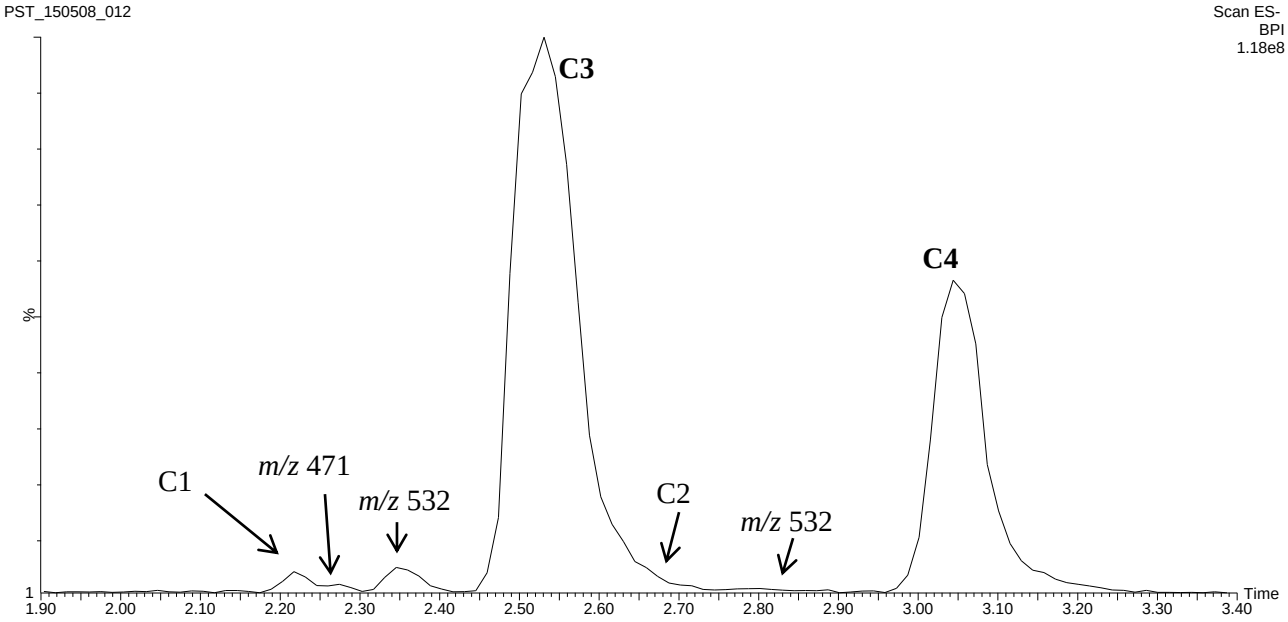


Figure 1. LC-MS negative ion chromatogram of CNC00045

Table 1. Percentage contribution of PST congeners detected by HILIC LC-MS integrating $[M-H]^-$ ions.

Compound Name	Percentage contribution
C3&4 $[M-H]^-$ *m/z* 490	94%
$[M-H]^-$ *m/z 532*	3%
C1&2 $[M-H]^-$ *m/z* 474	2%
$[M-H]^-$ *m/z 471*	1%

Reviewed by: Approved by:

Date:09 November 2015 Date:09 November 2015

References:

1. Boundy, M.J., et al., 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. Journal of Chromatography A, 2015. 1387: p. 1-12.

2. Oshima, Y. (1995). "Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. ." Journal of AOAC International 78(2): 528-532.

3. K. Thomas, E. Bond, P. LeBlanc, K. Reeves, I. Burton, J. A. Walter and M. A. Quilliam. "NRC CRM-GTX1&4-c, a certified calibration solution reference material for gonyautoxins-1 and -4", CRMP Technical Report CRM-GTX1&4-c-20080709, National Research Council Canada, Halifax, September 2008.

Appendix B

Draft manuscript titled:

Acute toxicities of the saxitoxin congeners gonyautoxin 5, gonyautoxin 6, decarbamoyl gonyautoxin 2&3, decarbamoyl neosaxitoxin, C-1&2 and C-3&4 to mice by various routes of administration.

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Abstract

Paralytic shellfish poisoning results from consumption of seafood naturally contaminated by saxitoxin and its congeners, the paralytic shellfish toxins (PSTs). The levels of such toxins are regulated, and maximum permitted levels in seafood have been established in many countries. Until recently, a mouse bioassay was the reference method for estimating the levels of PSTs in seafood, but this has now been superseded by instrumental methods of analysis. Such analyses provide data on the levels of many PSTs in seafood, but for risk assessment, knowledge of the relative toxicities of the congeners is required. These are expressed as "Toxicity Equivalence Factors" (TEFs). At present, TEFs are largely based on relative specific activities following intraperitoneal injection in a mouse bioassay rather than on acute toxicity determinations. A more relevant parameter for comparison would be median lethal

doses via oral administration. In the present study, the median lethal doses of gonyautoxin 5, gonyautoxin 6, decarbamoyl neosaxitoxin and of equilibrium mixtures of decarbamoyl gonyautoxins 2&3, C-1&2 and C-3&4 by oral administration to mice have been determined and compared with toxicities via intraperitoneal injection. The results indicate that the TEFs of several of these substances require revision.

Keywords: Paralytic shellfish toxins; Gonyautoxins; Decarbamoyl neosaxitoxin; Decarbamoyl gonyautoxins, C-1&2; C-3&4; Acute toxicity; Toxicity equivalence factors.

1. Introduction

Paralytic shellfish poisoning (PSP) is a serious and sometimes fatal outcome of consumption of seafood contaminated with saxitoxin and its congeners, which are produced by marine dinoflagellates of the genera *Alexandrium*, *Gymnodinium* and *Pyrodinium* and by several genera of freshwater cyanobacteria (Anderson et al., 2012; Pearson et al., 2010). The geographic distribution of PSP-inducing organisms is increasing, and on a global scale, around 2000 cases of PSP are reported each year, with a mortality rate of 15% (Hallegraeff, 1993).

For many years, evaluation of the safety of seafood for human consumption has been based on a mouse bioassay (MBA), which involves intraperitoneal injection of an extract of the seafood in mice, with death as the endpoint. This assay has been approved as a reference method for paralytic shellfish toxins by the Association of Official Analytical Chemists (AOAC, 2005). Such an assay is, however, ethically unacceptable and its validity is questionable since it involves intraperitoneal injection rather than the oral route through which humans are exposed to the PSP toxins. The use of the MBA is now forbidden in several countries, and alternative means of assessing the risk of seafood contaminated with saxitoxin and its congeners have been developed.

As of 2010, more than 50 analogues of saxitoxin had been identified (Wiese et al., 2010). Instrumental methods for estimation of saxitoxin and many of its congeners in seafood are now available. Such methods permit the assessment of the concentration of the toxins in a seafood sample and this, together with knowledge of the relative toxicity of the various compounds, permits the overall toxicity of the sample to be determined, enabling assessment of the potential risk to human health.

The relative toxicities of saxitoxin congeners are expressed as "Toxicity Equivalence Factors" (TEFs), which define the toxicities of these substances as a ratio of that of saxitoxin itself. Again, an MBA has been used for the estimation of TEFs for saxitoxin congeners. An assay for saxitoxin itself was developed by Sommer and Meyer in the 1930's (Sommer and Meyer, 1937), based on the relationship between the dose of pure saxitoxin administered to mice by intraperitoneal injection and the time to death of the animals. The amount of saxitoxin in the sample injected, expressed as "Mouse Units", was determined from the table of death-times established by these authors. Although validated only for saxitoxin itself, this MBA has more recently been applied to saxitoxin congeners, and TEFs for such congeners have been estimated from this data (EFSA, 2009).

The validity of this approach is questionable. Again, the assay depends upon intraperitoneal injection. Furthermore, the MBA is a bioassay, not a toxicological parameter, and it has been shown that TEFs derived from this method do not correlate with median lethal doses determined by approved toxicological methods. The use of the MBA also assumes that the dose death-time relationship for saxitoxin congeners are the same as that for saxitoxin itself. This again has been shown to be untrue (Munday et al., 2013). The inadequacy of the present TEFs for risk assessment was implied in the Scientific Opinion of the European Food Safety Authority Panel on Contaminants in the Food Chain, which indicated the need for establishing robust TEFs based on the relative oral toxicities of the saxitoxin congeners (EFSA, 2009).

Such data are now available for neosaxitoxin, decarbamoyl saxitoxin, gonyautoxins 1&4 and gonyautoxins 2&3 (Munday et al., 2013). As a continuation of these studies, we now report the acute toxicities of gonyautoxin 5 (GTX-5), gonyautoxin 6 (GTX-6), decarbamoyl gonyautoxin 2&3 (dcGTX-2&3), decarbamoyl neosaxitoxin (dcNeoSTX), *N*-sulfocarbamoyl gonyautoxin 2&3 (C-1&2) and *N*-sulfocarbamoyl gonyautoxin 1&4 (C-3&4) by two methods

of oral administration and a comparison of these data with the acute toxicities of these substances by intraperitoneal injection.

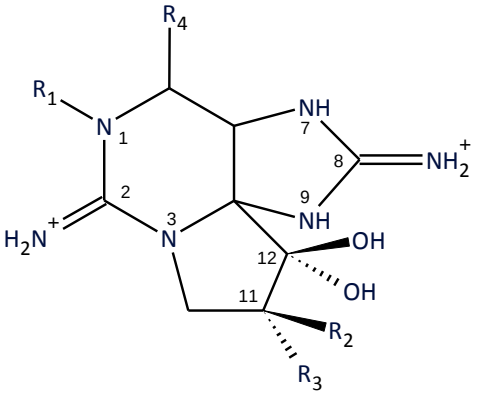
2. Materials and methods

2.1 Purification and analysis of toxins

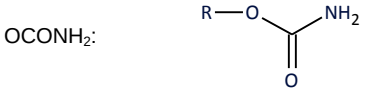
Structures of the PSTs are shown in Figure 1. The toxins used in this study were purified from *Alexandrium catenella* cells collected from a bloom event that occurred in Opua Bay, Marlborough Sounds, New Zealand in 2013. The toxins were extracted and purified using preparative column chromatography and chemically converted to other analogues (if required) using techniques previously described (Koehn et al, 1981; Laycock et al, 1994). Briefly, for toxin isolation bulk cultures of *A. catenella* were extracted with hot dilute acetic acid. Cell debris was removed by centrifugation and filtration. The toxins were recovered using activated carbon column chromatography. Further isolation used gel filtration and ion-exchange chromatography.

The purified toxins were dissolved in 10 mM acetic acid to give concentrated stock solutions. Dilutions of these stock solutions were accurately prepared volumetrically with purity and concentration determined using liquid chromatography with fluorescence detection (Van de Riet et al 2011) and liquid chromatography with mass spectrometric detection (Boundy et al 2015). National Research Council of Canada (NRC) certified reference materials (CRMs) were used as calibrants for all of the toxins generated except for C-3&4 and GTX6, for which no CRMs were available. Instead, C-3&4 was quantified by measuring the concentration of GTX-1&4 formed by acid hydrolysis (Costa et al, 2014, Oshima 1995) using the conversion of C-1&2 to GTX-2&3 as a control. The concentration assigned from this approach was in good agreement with direct measurement using non-certified C-3&4 reference materials from

NRC and the Japanese National Research Institute of Fisheries Science. GTX-6 was quantified directly using a non-certified reference material from NRC and confirmed by quantifying neoSTX generated by acid hydrolysis. C-1&2, C-3&4, and dcGTX-2&3, exist as pairs of epimers. These mixtures were equilibrated prior to toxicological analysis to give a ratio of approximately 3:1 (Table 1). This represents the same ratio that is found in shellfish contaminated with these toxins.



Group	Analogue	R1	R2	R3	R4
C toxins	C1	H	H	OSO_3^-	$OCONHSO_3^-$
	C2	H	OSO_3^-	H	$OCONHSO_3^-$
	C3	OH	H	OSO_3^-	$OCONHSO_3^-$
	C4	OH	OSO_3^-	H	$OCONHSO_3^-$
GTXs	dcGTX2	H	H	OSO_3^-	OH
	dcGTX2	H	OSO_3^-	H	OH
	dcGTX1	OH	H	OSO_3^-	OH
	dcGTX4	OH	OSO_3^-	H	OH
	GTX2	H	H	OSO_3^-	$OCONH_2$
	GTX3	H	OSO_3^-	H	$OCONH_2$
	GTX1	OH	H	OSO_3^-	$OCONH_2$
	GTX4	OH	OSO_3^-	H	$OCONH_2$
	GTX5 (B1)	H	H	H	$OCONHSO_3^-$
	GTX6 (B2)	OH	H	H	$OCONHSO_3^-$
STXs	doSTX	H	H	H	H
	dcSTX	H	H	H	OH
	dcNEO	OH	H	H	OH
	STX	H	H	H	$OCONH_2$
	NEO	OH	H	H	$OCONH_2$



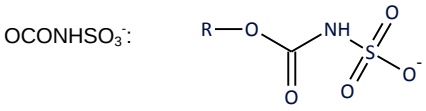


Fig 1. Structure of the major paralytic shellfish toxins.

Table 1.

Percentage molar concentration of the test materials

Toxin	Percentage composition														
	GTX5	GTX6	dcGTX-2	dcGTX-3	GTX2	GTX3	GTX1	GTX4	NeoSTX	dcSTX	dcNeoSTX	C-1	C-2	C-3	C-4
GTX5	98.42	0.87	0.12	0.22	0.15	0.03	0.11	0.02		0.03					
GTX6	0.15	99.60	0.04	0.01			0.05	0.11							
dcGTX-2&3			74.40	25.60											
dcNeoSTX									0.85	2.11	97.04				
C-1&2												72.50	27.50		
C-3&4												2.70	1.20	68.80	27.30

2.2. Animals

Female Swiss albino mice, bred at Ruakura, were employed in all experiments. The initial body weights of the mice were between 18 and 22 g. They were housed in solid-bottomed cages containing bedding of softwood shavings. The animals were allowed unrestricted

access to food (Rat and Mouse Cubes, Speciality Feeds Ltd, Glen Forrest, Western Australia) and tap water throughout the experimental period. All experiments were approved by the Institutional Animal Ethics Committee.

2.3. Determination of median lethal doses

Acute toxicities were determined according to the principles of OECD Guideline 425 (OECD 2008). Mice were weighed immediately before dosing, and the test substances were administered on a µmole/kg body weight basis. Aliquots of the test materials were diluted in 3 mM HCl. For intraperitoneal injection, the volume administered was 1 mL, while for gavage the volume was 200 µL. For determination of toxicity by voluntary consumption, mice were trained to eat small amounts of cream cheese, as described previously (Munday et al., 2013). For dosing, toxins were mixed with ~ 150 mg of cheese and immediately fed to the mice, who readily ate the food within 45 seconds. In order to avoid diurnal variations in response, dosing by all routes of administration was conducted between 8.00 and 9.30 a.m. The mice were monitored intensively during the day of dosing. Those dying during the course of the experiments were necropsied, while survivors were weighed and examined each day for 14 days, after which time they were killed and necropsied.

2.4. Determination of the No Observable Adverse Effect Levels (NOAELs)

Mice were dosed by gavage or by feeding with the test materials at doses below the LD_{50}. A logarithmic dose-progression was employed, using the protocol of OECD Guideline 425, but with "toxic effect" rather than death as the parameter. Exploratory behaviour was assessed by transferring the mice to a new cage and observing their movements. Abdominal breathing and lethargy were assessed visually.

2.5. Determination of the specific activities of C-1&2, C-3&4 and dcNeoSTX by the MBA

Aliquots of the test materials, diluted to 1 ml with 3 mM HCl, were injected intraperitoneally in mice according to the protocol of AOAC Official Test Method 959.08 (AOAC, 2005). Median death times were calculated, and MU/ml determined from Table 959.08A in the AOAC method. Specific activities were calculated as MU/µmole.

3. Results

3.1. Acute toxicity by intraperitoneal injection

The median lethal doses of the test substances by intraperitoneal injection are shown in Table 2. At lethal doses of the test compounds, the mice became lethargic soon after dosing, with rapid abdominal breathing. They subsequently became immobile. Their respiration became irregular and the rate of respiration declined. Respiration rates continued to decrease until breathing ceased completely. Exophthalmia and cyanosis were observed shortly before death, which occurred within 20 minutes of dosing. At sublethal doses, mice became lethargic, with abdominal breathing, and at doses close to the LD_{50}, a decrease in respiration rate was also observed. The animals recovered over a period of 2 – 4 hours, and their appearance and behaviour remained normal throughout the subsequent 14-day observation period. No abnormalities were observed at necropsy.

Table 2.

Acute toxicities of the test substances by intraperitoneal injection.

Compound	LD_{50} (µmole/kg)*
GTX-5	0.125 (0.065 – 0.155)
GTX-6	0.227 (0.173 – 0.277)
dcGTX-2&3	0.040 (0.032 – 0.050)
dcNeoSTX	0.478 (0.439 – 0.493)
C-1&2	0.400 (0.327 – 0.663)
C-3&4	0.480 (0.472 – 0.500)

*Figures in brackets indicate 95% confidence limits

3.2 Acute toxicities by oral administration

The median lethal doses and the NOAELs of the test compounds by gavage are shown in Table 3. Those by feeding are given in Table 4.

The symptoms of intoxication via the oral route were the same as those recorded after intraperitoneal injection, although the time to onset of the changes was greater, with signs of intoxication appearing at up to 45 minutes after dosing. Death times were also extended, particularly with dcNeoSTX, with which deaths were seen at up to 7 hours after dosing. Time to recovery after sublethal doses of the toxins was also extended, and tremors were observed in some animals at 6 – 9 hours after dosing, particularly in mice dosed orally with GTX-5, GTX-6 and dcNeoSTX.

Table 3.

Acute toxicities and NOAELs of the test substances by gavage

Compound	LD_{50} (µmole/kg)*	NOAEL (µmole/kg)*
GTX-5	18.9 (14.1– 21.7)	5.12 (4.80 – 6.00)
GTX-6	31.1 (29.5 – 36.5)	7.90 (7.42 – 9.31)
dcGTX-2&3	7.13 (6.00 – 7.60)	2.53 (2.38 – 3.00)
dcNeoSTX	5.50 (4.13 – 6.34)	2.13 (1.96 – 2.20)
C-1&2	35.0 (30.6 – 46.7)	15.0 (10.5 – 19.9)
C-3&4	42.7 (40.0 – 50.0)	25.5 (23.8 – 30.0)

*Figures in brackets indicate 95% confidence limits

Table 4.

Acute toxicities and NOAELs by feeding

Compound	LD_{50} (µmole/kg)*	NOAEL (µmole/kg)*
GTX-5	50.0 (37.5 – 72.9)	17.1(16.0– 20.1
GTX-6	>188	ND
dcGTX-2&3	29.6 (25.0 – 32.0)	10.0 (7.01 – 13.4)
dcNeoSTX	14.3 (10.8 – 15.9)	4.36 (4.00 – 4.49)
C1&2	74.0 (69.0 – 87.0)	17.4 (8.93 – 21.6)
C3&4	ND	ND

*Figures in brackets indicate 95% confidence limits
ND, Not determined

3.3. Specific activities of C-1&2, C-3&4 and dcNeoSTX by the MBA

The specific activities of C-1&2, C-3&4 and dcNeoSTX were 367, 69.5 and 43.0 MU/µmole respectively.

Discussion

As expected, the acute toxicities of the saxitoxin congeners by gavage were lower than those by intraperitoneal injection, most likely due to slower absorption via the oral route. Materials

injected intraperitoneally are generally rapidly and extensively absorbed, leading to high tissue levels and toxicity. Slower absorption via oral administration may allow more time for detoxification and/or excretion of the test material before toxic levels are reached. It should be noted, however, that there were wide variations in the ratios between the toxicities by the two routes of administration. This difference was most pronounced with dcNeoSTX, which showed one of the lowest toxicities by injection, but the highest by gavage. It is likely that differences in absorption rate account for such variations, since passage across the intestinal wall will vary according to the physical properties of the substance, particularly its lipid solubility.

It has been argued that administration by feeding, rather than by gavage, is the most relevant route for toxicity determinations in rodents, since the semi-solid content of the stomach of these animals does not permit mixing of the material given by gavage, which may flow around the stomach contents and rapidly enter the duodenum. When given by feeding, however, the test material becomes mixed with the stomach contents of rodents in the same way that substances are distributed throughout the liquid contents of the human stomach, leading to relatively slow release into the absorptive areas of the gastrointestinal tract (Munday, 2014). This is consistent with the observation that the absolute values of the acute toxicities of the saxitoxin derivatives were lower by feeding than by gavage. The ratio between the toxicity by feeding and that by gavage ranged from 2.1 to 2.6 for C-1&2, GTX-5 and dcNeoSTX, which is consistent with results with other saxitoxin congeners (Munday et al., 2013). The ratios for dcGTX-2&3 and GTX-6 were higher, however (4.1 and > 6, respectively). The reason for this disparity is not presently known. Possibilities include the conversion of these compounds into less toxic substances during the relatively long residence time in the stomach of the animals or inhibition of stomach contraction or of opening of the pyloric sphincter, leading to slower release into the duodenum.

For accurate risk assessment, it is essential that relevant and accurate TEFs for saxitoxin and its congeners are available. At present, the relative risk to human health of saxitoxin derivatives is largely based on TEFs calculated from the specific activities of these substances determined in the MBA. As shown previously (Munday et al., 2013), the relative acute toxicities of a number of saxitoxin congeners by intraperitoneal injection do not correlate with their relative specific activities in the MBA. This is consistent with the observation that the death time-dose curves for the saxitoxin derivatives are not the same as that for saxitoxin itself (Munday et al., 2013).

In the present study, the acute toxicities of GTX-5, GTX-6, dcGTX-2&3, dcNeoSTX, C-1&2 and C-3&4 were determined. MBA data are available for GTX-5, GTX-6 and dcGTX-2&3 (EFSA, 2009). No MBA data on epimeric mixtures of C-1&2 or C-3&4 are available. Also, the MBA figure given by EFSA for dcNeoSTX (EFSA, 2009) is regarded as incorrect. The figure given is that from Sullivan et al. (1985), but these authors did not determine the specific activity of dcNeoSTX, but assumed that it was the same as that of decarbamoyl saxitoxin. In order to facilitate comparison, we determined the specific activities of the C-toxin equilibrium mixtures and that of dcNeoSTX. It should be noted that the equilibrium mixtures of the epimers of dcGTX-2&3, C-1&2 and C-3&4 were evaluated in these studies, rather than the individual epimers, since the latter substances are never found in isolation in seafood, but invariably as equilibrium mixtures.

A comparison of the TEFs derived from the MBA, acute toxicity by intraperitoneal injection and by oral administration of the above toxins is shown in Table 5. Again, there was no correlation between the TEFs derived by the MBA and those from acute toxicity by intraperitoneal injection. The TEFs based on the MBA were similar to those based on oral toxicity for GTX-5 and C-3&4, but were higher for GTX-6, dcGTX-2&3 and C-1&2 but

lower for dcNeoSTX. The TEFs based on toxicity by feeding were ~ 40% lower than those proposed by EFSA for GTX-5 and dcNeoSTX, and more than 5 times lower for GTX-6.

The results of the present study suggest that TEFs for some of the above compounds should be revised. In this way, appropriate regulatory limits can be set that are not so high as to endanger human health and not so low that they cause unnecessary loss to the seafood industry through destruction of product or closure of harvesting.

Table 5.

Comparison of TEFs derived from the MBA, i.p. injection and oral administration.

Compound	TEF proposed by EFSA (EFSA 2009)	TEF based on MBA	TEF based on LD_{50} by i.p. injection	TEF based on LD_{50} by gavage	TEF based on LD_{50} by feeding
Saxitoxin	1.00	1.00	1.00	1.0	1.0
GTX-5	0.1	0.06 (Oshima, 1995)	0.22	0.063	0.064
GTX-6	0.1	0.08 (Koehn et al., 1982)	0.12	0.038	< 0.017
dcGTX-2&3	-	0.19 (Vale et al., 2008)	0.70	0.17	0.11
dcNeoSTX	0.4	0.02 (This study)	0.058	0.22	0.22
C-1&2	-	0.18 (This study)	0.070	0.034	0.043
C-3&4	-	0.03 (This study)	0.058	0.028	ND

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Conflict of interest

The authors declare that they have no conflicts of interest.

References

AOAC Official Method 959.08. Paralytic Shellfish Poison. Biological Method, in: Horwitz, W., Latimer, G.W. (Eds.), Official Methods of Analysis of AOAC International. 18th Edition, 2005. AOAC International, Gaithersburg, USA, pp. 79-82.

Anderson, D.M., Alpermann, T..J., Cembella, A.D., Collos, Y., Masseret, E., Montresor, M. 2012. The globally distributed genus *Alexandrium*: multifaceted roles in marine ecosystems and impacts on human health. Harmful Algae 14, 10-35.

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. Journal of Chromatography A **1387**, 1-12.

Costa, P.R., T. Moita, T., Rodrigues, S.M. 2014. Estimating the contribution of N-sulfocarbamoyl paralytic shellfish toxin analogs GTX6 and C3+4 to the toxicity of mussels (*Mytilus galloprovincialis*) over a bloom of *Gymnodinium catenatum*. Harmful Algae 31, 35-40.

EFSA, 2009. EFSA Panel on Contaminants in the Food Chain. Marine biotoxins in shellfish - saxitoxin group. EFSA J. 1019, 1-76.

Hallegraeff, G., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32, 79-99.

Koehn, F.E., Ghazarossian, V.E., Schantz, E.J. Schnoes, H.K., Strong, F.M. 1981. Derivatives of saxitoxin. Bioorganic Chemistry 10, 412-428.

Koehn, F. E., Hall, S., Wichmann, C. F., Schnoes, H. K., Reichardt, P. B., 1982. Dinoflagellate neurotoxins related to saxitoxin: Structure and latent activity of toxins B1 and B2. Tetrahedron Letters 23, 2247-2248.

Laycock, M.V., Thibault, P., Ayer S.W., Walter, J.A. 1994. Isolation and purification procedures for the preparation of paralytic shellfish poisoning toxin standards. Natural Toxins 2**,** 175-183.

Munday, R., 2014. Toxicolology of seafood toxins: a critial review, in: Botana, L. (Ed.), Seafood and Freshwater Toxins. Pharmacology, Physiology and Detection, Third Edition. Taylor & Francis, Boca Raton, pp. 197-290.

Munday, R., Thomas, K., Gibbs, R., Murphy, C., Quilliam, M.A., 2013. Acute toxicities of saxitoxin, neosaxitoxin, decarbamoyl saxitoxin and gonyautoxins 1&4 and 2&3 to mice by various routes of administration. Toxicon 76, 77-83.

Oshima, Y., 1995. Post-column derivitization HPLC methods for paralytic shellfish poisons, in: Hallegraeff, G., Anderson, D., Cembella, A. (Eds.), Manual on Harmful Marine Microalgae, IOC Manual and Guides, No. 33, pp. 81-94.

Pearson, L., Mihali, T., Moffitt, M., Kellmann, R., Neilan, B., 2010. On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. Marine Drugs 8, 1650-1680.

Sommer, H., Meyer, K.F., 1937. Paralytic shell-fish poisoning. Archives of Pathology 24, 560-598.

Vale, C., Alfonso, A., Vieytes, M., Romaris, X., Arévalo, F., Botana, A., Botana, L., 2008. In vitro and in vivo evaluation of paralytic shellfish poisoning toxin potency and the influence of the pH of extraction. Analytical Chemistry 80, 1770-1776.

van de Riet, J.R., Gibbs, S., Muggah, P.M., Rourke, W.A. MacNeil, J.D, Quilliam, M. A. (2011). Liquid Chromatography 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, 1154-1176.

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