ANALYSIS OF PARALYTIC SHELLFISH TOXINS IN NATURAL SAMPLES: LIQUID CHROMATOGRAPHY COUPLED TO FLUORESCENT DETECTION VERSUS LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY

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DECLARATION OF AUTHORSHIP OF WORK

ANALYSIS OF PARALYTIC SHELLFISH TOXINS IN NATURAL SAMPLES: LIQUID CHROMATOGRAPHY COUPLED TO FLUORESCENT DETECTION VERSUS LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY

I declare that I am the author of this work, which is original and published. The sources consulted have been duly cited in the text and included in the list of references.

MA. LLORINA R. MESTIZO

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DEDICATION

This had been one of the fondest and satisfying journeys I had to take. The longing I have with my family will be replaced by the memories I have shared with all the people I had an opportunity to meet and became friends with.

To my wonderful husband Bobby and our children, Ash and Dos… *uuwi na si Mama* (Mama is coming home soon).

Ang paglalakbay na ito ay nagtatapos upang makapagsimula ng panibagong kapitulo…
This journey closes for a new chapter to begin…

SUMÁRIO

As toxinas PSP (Paralytic Shellfish Poison), conhecidas pelos seus efeitos paralisantes, são produzidas por várias espécies de dinoflagelados marinhos e subsequentemente bioacumulam-se em bivalves filtradores. Estes bivalves tornam-se vetores para o consumo humano, podendo causar sintomas tóxicos como dormência, diarreia, náuseas e vómitos, e, em casos graves, levar à morte por paralisia respiratória. Estas toxinas são monitorizadas por diversos países para proteger a saúde pública. No entanto, algumas toxinas PSP não são monitorizadas nem regulamentadas, pois foram recentemente descobertas através de técnicas analíticas avançadas.

Neste estudo, comparamos o método AOAC de cromatografia líquida com deteção por fluorescência com o método desenvolvido no nosso laboratório, que utiliza cromatografia líquida acoplada à espectrometria de massa de alta resolução (LC-HRMS), seguida de extração de cromatogramas da massa exata (AMXIC) para quantificar as toxinas PSP regulamentadas e não regulamentadas. O procedimento AMXIC demonstrou ser seletivo, fornecendo perfis individuais claros tanto para toxinas regulamentadas quanto para não regulamentadas. Devido à indisponibilidade de CRMs (Certified Reference Materials) para esses análogos, foi realizada uma estimativa usando o procedimento AMXIC e a resposta ao análogo de toxina regulamentada mais semelhante. Analisaram-se extratos de amostras de mexilhão naturalmente contaminadas (*Mytilus galloprovincialis*) e do dinoflagelado marinho *Gymnodinium catenatum* utilizando os métodos mencionados. Além disso, os efeitos de matriz foram avaliados em extratos preparados com cartuchos de C18 e de carbono grafitizado, utilizando saxitoxina como composto de referência.

As toxinas PSP não regulamentadas com um grupo hidroxilo no C-11, conhecidas como toxinas M, foram detetadas na amostra de mexilhão contaminado, enquanto as toxinas GC foram observadas apenas na amostra de dinoflagelado. A identificação foi feita com base nas massas exatas e nos padrões de fragmentação. No entanto, nem todas as toxinas não regulamentadas presentes nas amostras foram identificadas devido à baixa intensidade dos sinais destes analitos após fragmentação. A falta de CRMs para essas toxinas não regulamentadas limita a sua identificação. Em conclusão, o método HPLC-FLD é eficaz para triagem, especialmente em análises de rotina. No entanto, o método LC-HRMS permite uma identificação mais precisa do perfil completo das toxinas PSP nas amostras, contribuindo para uma melhor avaliação da toxicidade total.

ABSTRACT

Paralytic shellfish toxins (PST) are produced by several marine dinoflagellate species that bioaccumulate in bivalves after feeding. These filter-feeder bivalves then serve as vector for human consumption and cause toxic symptoms such as numbness, diarrhea, nausea and vomiting leading to death due to respiratory paralysis. These toxins are monitored by different countries to protect national public health. However, only regulated PSTs are covered. On the other hand, non-regulated PSTs toxins, which are new analogues discovered in the recent years using advanced analytical techniques, may pose unknown threats.

We compared the AOAC method, that uses liquid chromatography coupled to fluorescent detection, with the in-house-developed liquid chromatography-high resolution mass spectrometry (LC-HRMS) method, which involves full scan acquisition followed by accurate mass extracted ion chromatograms (AMXIC) to quantify the regulated and the non-regulated PSTs. The AMXIC procedure is selective as it gives clean individual profiles for regulated and non-regulated toxins. As CRMs for these analogues are not available, an estimate was obtained using AMXIC procedure and the response to the most similar regulated toxin analogue. Extracts from naturally contaminated mussel sample *Mytilus galloprovincialis* and marine dinoflagellate *Gymnodinium catenatum* were analyzed using the mentioned methods. Matrix effects evaluated in extracts prepared using C18 and graphitized carbon cartridges using saxitoxin as the reference compound.

C-11 hydroxyl metabolites (so-called M-toxins) were detected in the contaminated mussel sample, while GC-toxins were only observed from the dinoflagellate sample. The identification was based on the exact expected *m/z* values and on the fragmentation patterns. However, not all identified non-regulated toxins were identified due to the low intensity of signals of searched analytes after fragmentation. The unavailability of CRM counterparts of non-regulated toxins limits to identify toxin analogue. Overall, the HPLC-FLD is very good as a screening method especially in routine analyze. The LC-HRMS method, on the other hand, is notable in establishing toxin profile of samples that contributes to better evaluation of overall toxicity.

Keywords: paralytic shellfish toxins, saxitoxins, liquid chromatography-high resolution mass spectrometry, accurate mass extracted ion chromatograms, liquid chromatography coupled to fluorescent detection.

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List of formulae, symbols

amu	atomic mass units
BODIPY	fluorinated boron-dipyrromethene
°C	Celsius or Centigrade
C-18	octadecyl
Ca^{2+}	calcium ion
ESI^+ and ESI^-	electrospray ionization in the positive or negative mode
fmol	femtomole
g	gram
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HIO_4	periodic acid
H	hydrogen
i.d.	internal diameter
K^+	potassium ion
keV	energy in kilo electron-volt
kg	kilogram
L	liter
LD_{50}	lethal dose 50
m	meter
mg	milligram
mins	minutes
mL	milliliter
mL/min	milliliter per minute
mm	millimeter
mM	millimolar
m/z	mass-to-charge ratio
N-sulfocarbamoyl	nitrogen attached to the sulfocarbonyl
Na^+	sodium ion
NaOH	sodium hydroxide
Na_2HPO_4	sodium periodate
nm	nanometer
nM	nanomolar
OH	hydroxyl
OSO_3H	sulfonate
ppb	parts per billion
pg	picogram
R1-R4	Different functional groups that can be used to categorize the saxitoxin analogues
rpm	revolutions per minute
S/N	signal-to-noise ratio
SPE-COOH	cationic exchange SPE
µg	microgram
µg/kg	microgram per kilogram
µL	microliter
µM	micromolar
µS/cm	microsiemens per centimeter

List of acronyms and abbreviations

AF	ammonium formate
AgNPs	silver nanoparticles
AMXIC	accurate-mass extracted ion chromatogram
AOAC	Association of Analytical Chemists
AZA	azaspiracid
CBA-n2a	Neuroblastoma cell-based assay
CID	collision-induced dissociation
CRM	certified reference material
CTX	ciguatoxins
DA	domoic acid
dc	decarbamoyl
ELISA	enzyme-linked immunosorbent assay
EURLMB	EU Reference Laboratory for Monitoring of Marine Biotoxins
FA	formic acid
fRBA	fluorescence receptor binding assays
GC-toxins	Saxitoxin analogues with benzoate group present in R1 of the structure
GTX	gonyautoxins
HABs	harmful algal blooms
HAc	acetic acid
HCD	higher energy C trap dissociation
HESI	heated electrospray ionization source
HPLC	high performance liquid chromatography
HILIC-MS	hydrophilic interaction liquid chromatography mass spectrometry tandem mean MS/MS, fragmentation
HPLC-FLD	high performance liquid chromatography with fluorescence detection
HPLC-MS	high performance liquid chromatography coupled to mass spectrometry
HPLC-HRMS	high performance liquid chromatography-high resolution mass spectrometry
IP	intraperitoneally
IPMA	Instituto Português do Mar e da Atmosfera
LFI	lateral flow immunchromatography
LOD	limit of detection
LOQ	limit of quantification
M-toxins	C-11 hydroxyl
MBA	mouse bioassay
MeCN	acetonitrile
MeOH	methanol
MTT	((3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)
MTX	maitotoxins
NEO	neosaxitoxin
OA	okadaic acid
PbTx	brevetoxin
PCOX	post-column oxidation
PET	photoinduced electron transfer
PP	polypropylene
PSP	paralytic shellfish poisoning
PSTs	paralytic shellfish toxins

PITX	palytoxins
PTX	pectenotoxin
QC	quality control
QCM	quartz crystal microbalance
RBA	receptor binding assay
RIA	radioimmunoassay
rRBA	radioligand receptor binding assay
SD	standard deviation
SERS	surface-enhanced Raman spectroscopy
SPE	solid phase extraction
STX	saxitoxin
TEF	toxicity equivalency factor
TOC	total organic carbon
TTX	tetrodotoxin
VSGC	voltage-gated sodium channels
YTX	yessotoxin

INTRODUCTION

Harmful algal blooms

Harmful algal blooms (HABs) are extreme populations of microalgae occurring in a body of water at a specific time. These blooms are often caused by excessive nutrient loading, or eutrophication, which can result from urban and rural wastewater sources, agricultural runoff, industrial discharges, among others. The excess foam and scum produced by HABs reduces light penetration and leads to hypoxia and the degradation of ecosystems. Furthermore, the closure of areas affected by HABs is often necessary due to the production of toxins, which pose significant health risks to humans and marine life. These toxins are characterized by the symptoms they bring upon human consumption and can be divided into Paralytic Shellfish Poisoning (PSP), Neurotoxic Shellfish Poisoning (NSP), Diarrhetic Shellfish Poisoning (DSP), Amnesic Shellfish Poisoning (ASP), and Ciguatera fish poisoning. Saxitoxin family or STX family is one of the most researched PSP toxins of the PSP type, which is produced by the dinoflagellates *Gymnodinium catenatum*[1–3], *Alexandrium catenella, A. tamarense*[4–7], *and Pyrodinium bahamense* var. *compressum*[8–10]. PSP symptoms vary from a slight tingling sensation, numbness, diarrhea, nausea and vomiting which can lead to death due to respiratory paralysis[10,11]. It has been reported an LD_{50} of 8-10 µg/kg[12]. STX family is a group of toxins including tetrahydropurines or guanidinium derivatives that accumulate into filter-feeder bivalves. These bivalves, such as oysters, mussels, clams, and scallops[13–15] consume particulate matter through its ability to siphon water[16], in this case seawater. Clams are the largest marine bivalves in production while mussels are considered as the bioindicator of this toxin[10,17] because of higher uptake rate.

Toxins within the STX family are thermally stable under acidic conditions[18]. Their backbone structure categorizes the toxin family in subgroups based on R1 (Figure 1, Table 1), namely in carbamoyl, decarbamoyl, N-sulfocarbamoyl, and benzoate groups, where STX is the parent structure being the first toxin analogue discovered[15,19]. The N-sulfocarbamoyl (also known as C-11 hydroxyl) and benzoate moieties were discovered in recent years and identified as M- and GC- toxins, respectively. R2 identifies the presence of N-hydroxylated toxins[20]. R3 and R4, on the other hand, can be a hydroxyl or a sulfonyl moiety (Table *1*). A derivative form B (Figure 1) was reportedly formed after enzymatic and/or chemical reactions within the bivalves[21]. Knowing the different subgroups of STX family is of utmost importance as different structures show different toxicities, uptake, depuration and metabolism by the shellfish.

Overall, moving forward with public health safety regulations, at national or international levels[22,23], for instance, the Codex Alimentarius Commission has set the maximum level at 800 µg/kg as adopted by Canada, US, and the EU[14,24]. The Codex guide provides the risk level alerts for harvest of bivalves for consumption wherein criteria is based on animal testing data[25,26].

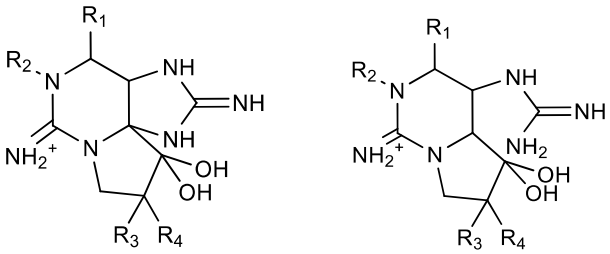
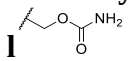
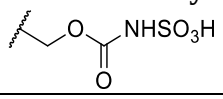
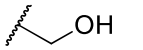
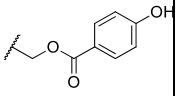


Figure 1. Parent structure of saxitoxin (STX) family, A, and its derivative form, B[27] where R1 can be carbamoyl, decarbamoyl, N-sulfocarbamoyl, or benzoate moieties (see Table 1 below).

Table 1. Chemical structures of paralytic shellfish toxin (PST) analogues, reported by Lage etal.[28]

R_1*				**R_2**	**R_3**	**R_4**	**Ring**
carbamoyl 	**N-sulfocarbamoyl** 	**Decarbamoyl** 	**Benzoate** 				
STX	GTX5	dcSTX	GC3	H	H	H	A
GTX2	C1	dcGTX2	GC2	H	H	OSO_3H	A
GTX3	C2	dcGTX3	GC1	H	OSO_3H	H	A
NEO	GTX6	dcNEO	GC6	OH	H	H	A
GTX1	C3	dcGTX1	GC5	OH	H	OSO_3H	A
GTX4	C4	dcGTX4	GC4	OH	OSO_3H	H	A
M2	M1	dcM2	--	H	H	OH	A
M4	M3	dcM4	--	H	OH	OH	A
M8	M7	dcM8	--	OH	H	OH	A
M10	M9	dcM10	--	OH	OH	OH	A
M6	M5	dcM6	--	H	OH	OH	B
M12	M11	dcM12	--	OH	OH	OH	B

**STX: saxitoxin, GTX: gonyautoxins, NEO: neosaxitoxin, dc: decarbamoyl, M: C-11 hydroxyl analogues or M-toxins, GC: GC-toxins*

Discovery of new analogues

STXs are produced by dinoflagellates but some congeners may also be formed through enzymatic and chemical reactions within the bivalve tissues[15]. For instance, M-toxins are considered to be metabolic products with lower toxicity.

Ding and his coworkers reported[29] that 11-hydroxy-C2 toxin (M1) and 11,11-dihydroxy-C2 (M3) toxin result from C2 via o-desulfation, while 11-hydroxy-C4 toxin (M7) and 11,11-dihydroxy-C4 (M9) are formed after reaction of C4. In addition, the metabolites M2, M4, and M6 appear to be products of GTX2 and GTX3, and the metabolites M8 and M10 are likely to derive from GTX1 and GTX4. It was also reported[11] that toxin analogues M1 to M5 were found in mussels but they are not present during the intense bloom of the *Alexandrium tamarense*. M1 and M3 productions are reported as a combination of enzymatic and chemical transformations while M5 is formed after chemical conversion because it is formed during sample storage[30].

GC-toxins or toxin analogues with benzoate group have strong affinity to sodium channels, but less than that of STX. These are relatively hydrophobic in nature due to the benzoate structure that distinguished them from the other toxin analogues. It is reported that its formed only by *G. catenatum*[31] and limited methods had been so far to quantify such toxins[17]. These newly-discovered analogues, the GC- and M-toxins will be referred herein as non-regulated analogues. Methods for the analysis of these newly-discovered toxin analogues have not been validated. Their determination is important to account for their toxicity contribution, preventing this way the underestimation of this parameter[24].

STX family is extracted using low concentration of acid, HCl or acetic acid, at pH 2-4, preferably pH 3 – where most conversions into different toxin analogues take place. This pH is to standardized reproducibility in using the method on regulatory basis, from one country to another[32].

Quantification: bioassays

The mouse bioassay (MBA) is the reference biological method for toxicity evaluation of the saxitoxin family[19] until the high-performance liquid chromatography with fluorescence detection (HPLC-FLD) was validated as an AOAC official method[20,33]. For example, the Philippines still uses MBA as official method[34], while EU uses HPLC-FLD as official method

for saxitoxin detection[35]**.** MBA is a direct method wherein toxin targets voltage-gated sodium channels (VGSC) in live animal. In this method a sample extract or toxin standard is injected intraperitoneally (IP) into a laboratory mouse and paralytic symptoms are observed for a given time frame and dosage. The procedure is repeated thrice at the correct concentration of the sample where symptoms must be observed within 5-7 minutes after injection[32]. MBA reports toxicity through IP potency[14]. However, this method has been questioned due to unethical practices. Therefore, in the last five decades, other methods and applications have been developed to quantify STX[9–12,14,15,23,36–41] to avoid the use of living animals (EU Council Directive 86/609/EEC). Additionally, MBA has shortcomings as not considered an appropriate tool for control purposes because of high variability in results, insufficient detection capability, high limit of detection (LOD), and limited specificity[13,19,23].

Neuroblastoma cell-based assay CBA-n2a[42,43] is a widely-used assay for marine biotoxins that is tandem with analytical methods for quantitation. It is a step-by-step guided method starting from MTT assay ((3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) with ouabain and veratridine treatments, that can last for 72 hours. This method can either detect the 2 activators CTXs and PbTxs and 2 inhibitors STXs and dcSTXs. Coefficient of variations can range from 3-29%[42]. Also, the addition of ouabain is warranted to initiate the inhibition of STX and TTXs, together with the function of veratridine as an activator. A drawback of this method is the high technical skillset required, together with visual approximation of cell propagation. This is a crucial step as results are dependent on the population providing the intensity of color change due to the ongoing reaction. It also requires laboratories adapted for cell rearing thus extending its research on other forms of marine biotoxins is necessary. As for other analytical methods, the neuroblastoma cell-based assay requires high initial investment in laboratory resources[44].

Receptor binding assay (RBA) utilizes the receptor-ligand interactions to capture the target molecule for quantitation. RBA is classified based on the type of receptor and ligand are used, and dictate the quantifying method such as radioligand receptor binding or rRBA[10,36,45,46], and fluorescence receptor binding assays or fRBA[47,48]. Radioligand receptor binding assay[49,50] uses a radiolabeled ligand to compete with its unlabeled counterparts (i.e. calibration standard solutions, certified reference materials, sample extracts) for the VGSC receptor site of the rat membrane. Porcine membrane is another commercially available receptor used that is part of the RBA kit[50]. The receptor-ligand interaction is based on MBA. This method was validated

for saxitoxin[51] and is also applied to quantify CTX and PbTx[52–54]. Ruberu and coworkers[36] made a single-laboratory validation of this method by following the Association of Analytical Chemists (AOAC) method proposed[50]. The fRBA approach, on the other hand, was also developed for another HABs, namely those that release ciguatoxins. However, in this case, a fluorescent-labelled BODIPY is the ligand used[47,48] to compete with the same standard and samples. Both rRBA and fRBA offer total toxicity equivalence wherein only one certified reference material (CRM) of toxin is needed, presenting only one toxicity equivalence factor or TEF is needed, as recommended on official regulations[32]. Consequently, it is favorable for seafood safety monitoring encompassing a greater scope that may lead to a wider yet intrinsic safety regulations[23]. It is also a cost-effective plan by using only one toxin analogue as CRM. Most methods developed for STXs are extended to other marine biotoxins such as CTX, TTX, PbTx, OA, DA, etc[55].

Enzyme-linked immunosorbent assay or ELISA is another method also applied to quantify STX. It is an antibody-based assay known for its high sensitivity and high throughput. However, is does not provide a toxin profile[32] similar to RBAs. The Jellett rapid test kit[44] employs a lateral flow immunchromatography (LFI), a type of ELISA but an alternative format, commonly used on immediate medical and veterinary diagnostics such as home pregnancy test kits. Its format makes it the best on initial on-site analysis but is still recommended for suspected samples to undergo quantitative confirmatory testing. A positive result yields a colored strip with a toxicity at half the regulatory limit. Theoretically, antibodies are sensitive, however, there is a lack of good cross reactivity to all members of the toxin group. Hence variability is high. Similar to ELISA, radioimmunoassay or RIA, and surface plasmon resonance or SPR showed promising toxicity results except on issues related with matrix effects. Development of commercial quantification kits like Jellett rapid test kits[44,56] and CiguaCheck for CTX were developed for these types of toxins to improve seafood safety monitoring programs with on-site toxin detection kits. Unfortunately, these products were discontinued. Another research[57] utilize a microplate format for automated determinations employing ELISA but it was found to be unsatisfactory during the AOAC international collaborative study[32].

Quantification: biosensors

Biosensors are analytical devices incorporating biomolecules transmitted as analytical signals through measurable format utilized in broad topics in environment, health and medicine,

agriculture, etc[58–60]. For example, Bergantin and Sevilla III[37] reported the use of quartz crystal microbalance (QCM) to identify STX binding to a host of immobilized receptor in the quartz crystal. STX concentration is interpreted as the change in frequency of the quartz crystal due to change in weight from the binding of STX. The analyte binds to the quartz crystal, then natural frequency of the quartz decreases because it becomes heavier to move. This change is indirectly proportional to the content of the analyte that bound in the QCM. Another type is a potentiometric sensor[61] on graphene nanosheets with incorporated lipid films polymerized with a natural STX receptor from rabbit serum purified IgG. It is reported to have a variable response time of 5-20 minutes, shows LOD of 1 nM, and was evaluated both in water and shellfish samples. Nanoparticles such as colloidal hydrosol of silver (AgNPs)[62] were also studied where the guanidinium moiety of STX binds with the colloidal surface at low concentrations. The detection utilizes surface-enhanced Raman spectroscopy (SERS) and is reported to have LOD and limit of quantification (LOQ) of 3 nM and 20 nM, respectively. Consequently, Huai and colleagues[63] utilized the same with laser optical tweezers and obtained LOD of 2 nM and LOQ of 16 nM. Both methods address the rapid detection which is crucial in implementing food safety. Jigyasa and colleagues made an in-depth review on the development of nanomaterial-based biosensor for marine biotoxins to address food safety concerns[64]. In a related topic, a photoinduced electron transfer (PET) chemosensor made from an aza-crown ether and a coumaryl-linked fluorophore had been studied to capture saxitoxin in the presence of sodium and potassium ions in water[65].

Quantification: chemical methods

Most research discussed herewith made use of the oxidation products of saxitoxin family and its fluorescence properties. In recent years, liquid chromatography tandem with mass spectrometry has become a promising method as well.

Chromatographic methods can quantify individual analogues of the marine toxin in the same sample. This is advantageous for regulation as they allow to identify the type of toxin that is more likely to be found in specific areas. It will then provide a more comprehensive seafood regulation program, for example, the pre-column derivatization HPLC-FLD and LCMS-MS methods are implemented under the EU legislation[32] for hydrophilic and lipophilic marine biotoxins, respectively. Also incorporating into regulations are high-throughput and automated methods as results are immediately needed. Thus far, no method can be used to detect all algal toxins simultaneously. Different chromatographic conditions have to be used to maximize

facility, unifying the process of food safety overall. First reports of HPLC method[66] utilizes reverse-phased C-18 with fluorescence detection. Generally, regulated PST analogues are identified and validated with this method. Separation of non-regulated toxin analogues was improved using a HILIC column and an HPLC-MS[67,68].

As mentioned, samples are derivatized before analysis on HPLC-FLD. STX analogues are not fluorescent[69]. However, after oxidation they generate a conjugated core structure[70] based on the saxitoxin oxidation product Figure 2a that undergoes fluorescence. Another oxidation product Figure 2b is non fluorescent may be due to the presence of the carbonyl that deactivates the excitation process. Moreover, this core structure is common to oxidation products of the different toxin analogues and therefore can potentially be detected by fluorescence. The presence of hydroxyl as R2 (see Figure 1) lack sufficient sensitivity to alkaline peroxide oxidation[71], and therefore oxidized using periodate instead. In the case of NEO, for example, it hinders the aromatization of the core structure. For this reason, the pre-sample treatment of the sample or standard prior quantitation is mandatory.

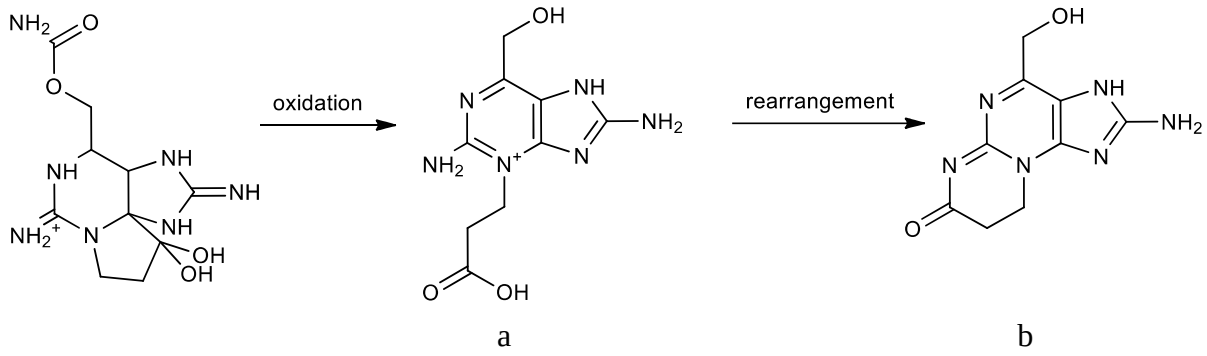


Figure 2. Conjugated core structure of oxidized saxitoxin analogues. Both products show the same conjugated purine nucleus, where (a) is the fluorescent product, and (b) is the non-fluorescent product via rearrangement of a. Corresponding moieties of R1-R4 can be viewed from Table 1

A reverse-phased HPLC-FLD was used in 2 methods of officially accepted methods, pre- and post-column oxidation[14,18,20,69]. Pre- treatment of samples by oxidation is crucial prior to chromatographic analysis of the analyte. The oxidation products are measured, identifying distinctly each analogue present in the sample. Not all analogues will be fully separated due to stereochemistry between them. The AOAC 2005.06 or the Lawrence method[72] quantifies the epimeric pairs together, GTX1,4; GTX2,3; C1,2; C3,4. This method effectively separates

carbamoyl, N-sulfocarbamoyl, and decarbamoyl[32], toxins. However, it is not effective for M-toxins and GC-toxins.

The research of van de Riet and colleagues[14] focused on a collaborative study on post-column oxidation (PCOX) for PST determination in mussels, oysters, and clams. It operates in a similar C18-column specifications in a step-gradient phosphate buffer system. Samples are then oxidized with periodic acid before detected at 330 nm excitation and 395 nm emission wavelengths.

Similar to its pre-column oxidation counterpart, where epimeric pairs are analyzed together, the PCOX of GTX2 and GTX3 achieve better resolution at lower and higher temperature, respectively, to eliminate coelution. It is also pointed out that analysis of scallop can be challenging as early eluting peak can be misidentified to be GTX4. Correspondingly, matrix-matched spiked samples are also made available for this study, in comparison to other methods PCOX from Oshima[69] and the Lawrence method.

HPLC-MS method had been incorporated to efficiently identify the STX analogues, including the newfound analogues discovered in the recent years. This method had been widely used to individually identify toxin analogues through MS/MS acquisition method specifically dynamic MRM transitions[73,74] for the different toxin analogues. This dynamic MRM transition method was used for both quantitative and qualitative purposes. This method maximizes analyte-specific sensitivity with the optimum collision energies and retention times for specific analogues, then thus, identifying individual toxin analogues within the same compound family. It satisfies the selectivity, detection, and quantitation for this type of complex analyte[75]. A pre-column HPLC-MS set-up was used to elucidate structures of the oxidized products and gas-phased dissociation of STX and neoSTX[70,76]. In addition, it may answer the scarcity of CRMs to quantify the different analogues. For instance, toxin analogs C-11 toxins were recently identified as metabolites of saxitoxin, yet these analogues do not have recommended TEF values to be accounted for total toxicity. Qu and colleagues offer semi-quantitation using relative molar ratio response[77] e.g. molar ratio response from GTX-2 is used to quantify similar analogues under the same subgroup of toxin family. It was also reported[75] that it is advantageous to use mass analyzers over HPLC-FLD set-up to reduce matrix effects . The HILIC-MS, on the other hand, has been effectively used in the detection of PSTs, both regulated and non-regulated analogues[28,74,78,79].

Due to the structural similarities of the toxin analogues, LC-HRMS is employed due to its high selectivity. It allows for the detection of analytes at the highest precision[80], up to 0.00001 atomic mass units (amu) by comprehensively profiling the samples through nontargeted analysis identifying this way unknown analytes[81]. In this study[81], 35 toxin analogues were identified in a single sample. This technique determines analytes using the accurate molecular weights and the analysis of specific chemical structures of chemical species, without the need for prior chromatographic separation[82]. In mass spectrometry, non-volatile salts suppress or even completely quench the ionization signal. Detergents and surfactants interfere with the ionization process. To improve sensitivity and chromatographic efficiency by removing these contaminants and co-eluting compounds, purification and clean-up of samples using SPE are recommended. Sensitivity is typically reported from the signal-to-noise ratio of the analytical instrumentation that can distinguish the slightest change in low concentration levels of analyte.

Analytical challenges in quantifying saxitoxin analogues

Analytical methods are becoming more and more complex in both, sample preparation and instrumentation, making them unusually expensive and requiring well-trained high technical level operators. Primary challenge on analytical methods for saxitoxin, specifically on FLD, is that these compounds lack the fluorophore for it to be directly detected. Hence, these compounds are subjected to oxidation, whether pre- or post-column, using peroxide or periodate, so that each congener can be quantified. Peroxide can only oxidize non-N-hydroxylated saxitoxin analogues, while periodate solution is used for the N-hydroxylated toxin analogues. The pre-column oxidation method was validated, and a collaborative study is reported[14,20,41]. The use of certified reference materials for this method is highly important. And with the discovery of new saxitoxin analogues, not all toxin analogues are available for these analyses. Because of this, the use of HPLC-FLD is expensive in both investment on equipment and highly skilled technicians. This method has also been deemed laborious due to the oxidation process that is analogue-specific, and if oxidation is not in-line with the HPLC system used.

In mass spectrometry, on the other hand, isotope-labeled compounds to be used as internal standards have also been suggested, which is the so-called isotope dilution method. However, the use of these standards makes the analysis even more expensive. Furthermore, not all target molecules have the correspondent labeled standard commercially available. As the core system

of equipment is similar to HPLC-FLD and LC-HRMS, initial investment for the latter is also very expensive.

Another possibility is the use of a naturally contaminated matrix as a reference analyte. Different bivalve matrices with known concentration of STX and toxin analogues had been studied and storage stability is tested over a period of time[83]. The preparation of such matrices can be used as reference materials to be used on the mentioned analytical techniques[84]. However, this will require the matrix to have high toxicity levels and high amount of the sample for reference material production to get through the stability study process. An additional difficulty of mass spectrometric methods is the identification and selectivity towards small molecules in complex organic samples due to presence of matrix affecting spatial arrangement of elemental composition and its isomeric forms[82]. Further cleanup procedures are needed in many cases prior to analysis to enhance the signal of targeted compounds. However, these procedures may cause sample loss due to the extra processing and clean-up steps before analysis. A solution is the use of matrix-matched calibration obtained using PST-free mussel extract or other matrices[14,85] spiked with known concentrations of regulated PSTs[86]. Fortified samples or spiking negative matrix are also studied to understand matrix effects[23].

Sample clean-up is necessary to reduce and eventually eliminate the matrix effects. This SPE method is a reverse-phase type (SPE-C18), in tandem with a reversed-phase column of HPLC-FLD. In recent years, the use of cationic exchange SPE or SPE-COOH is performed after the SPE-C18[72]. As STX is a cation in physiological pH, it will interact with the anionic resin first, as well as the toxin analogues, being then separated by playing with the eluent phase to change the ionic strength. Thus, coelution of the different toxin analogues in HPLC is prevented. The LODs and LOQs are always dependent on matrix content. Table 2 summarizes the LODs and LOQs for saxitoxin as analyzed using the different methods discussed.

Table 2. Summary of limit of detection (LOD) and limit of quantification (LOQ) of identified methods in detecting STX as the parent toxin derivative.

Source	Method	Reported values	
		LOD	LOQ
Bratakou, etal.[61]	Biological receptor on potentiometric sensor	1 nM	

FAO[87]	MBA	~ 40 µg STX/100g of shellfish ±15-20% variability	
Huai, etal.[63]	SERS with laser optical tweezers	2 nM	16 nM
Jellet, etal.[56]	ELISA	~ 40 mg STX equiv./100 g shellfish	
Lage, etal.[28]	LCMS orbitrap	0.01 µg • kg^{-1}	
Lawrence and Niedzwiadek[72]	HPLC pre-column oxidation, FLD	For every injection measurement at 3:1 S/N: 12 pg from peroxide oxidation 79 pg from periodate oxidation In the presence of sample: 0.02 ug/g	
Oshima etal.[66]	PCOX HPLC-FLD	< 0.01 fmol • $cell^{-1}$	
Pearman, etal.[62]	SERS with AgNPs	3 nM	20 nM
Ruberu etal.[36]	rRBA	105 µg STX equiv. • kg^{-1} shellfish	198 µg STX equiv. • kg^{-1} shellfish
Soliño etal.[86]	HPLC-FLD Lawrence method for pufferfish	3.4 µg • kg^{-1}	28.9 µg • kg^{-1}
Turner and Tölgyesi[73]	HILIC/MS/MS	0.02 nM	0.08 nM
Van Dolah, etal.[51]	rRBA	64 µg STX equiv. • kg^{-1} shellfish	130 µg STX equiv. • kg^{-1} shellfish

Objectives of this study

This study compares HPLC-FLD and LC-HRMS for the detection and quantification of regulated and non-regulated saxitoxin analogues. The acid extraction of the different toxin analogues is the same method used on the two analytical methods used. The sample clean-up procedures using SPE are with C18 and graphitized carbon cartridges for HPLC-FLD and LC-HRMS, respectively, which was also compared in this study. The comparison study provided an understanding of the matrix effects on different methods.

The HPLC-FLD method followed in this study the AOAC pre-column oxidation method[20,33] with slight modifications. To cater to the analytical system available for this study, comparison of emission and excitation spectra of the regulated toxin analogues is conducted prior to main study. Although CRMs of the regulated STX analogues are quantified in this method, this study did not target to validate the method as a routine protocol conducted in the laboratory.

The comparative method, LC-HRMS method applied, on the other hand, had been previously developed[28] for saxitoxin and related analogues in this laboratory. The use of accurate-mass extracted ion chromatography (AMXIC) and fragmentation analyses can distinguish the different toxin analogues. Applying these may in turn address the identification of non-regulated toxin analogues even in the absence of CRM solutions for comparison in which HPLC-FLD method is not validated to identify. Naturally-contaminated samples (mussel *Mytilus galloprovincialis*, and dinoflagellate *Gymnodinium catenatum*) and certified reference materials are also used to understand this comparison further.

MATERIALS AND METHODS

Chemicals

High-quality water referred hereafter to as water (H_2O), acetonitrile, ≥99.0% ammonium formate, and 99% formic acid (CARLO ERBA Reagents S.A.S., France), methanol (Honeywell, France), 25% ammonia solution (Scharlau, Spain) are all LCMS-grade. Glacial acetic acid and 30% H_2O_2 (CARLO ERBA Reagents S.A.S., France) are analytical-grade reagents. Ultrapure water used for HPLC-FLD analysis was collected from Milli-Q EQ 7000 Type 1 water purifier system (Merck Millipore, USA) at conductivity of 0.055 µS/cm at 25 °C, and 33 ppb total organic carbon (TOC).

Saxitoxin standards

Saxitoxin analogues are CRMs obtained from Laboratorio CIFGA, S.A. (Spain). These standards were extracted from dinoflagellate *Alexandrium tamarense* and were supplied in 3 mM HCl aqueous solution. The used saxitoxin standards and the corresponding stock solutions are listed in Table 3.

Table 3. Saxitoxin analogues standards available as CRMs

Toxin Analogue	Short name	Stock concentration (µmol/kg)
Saxitoxin	STX	55.1 ± 3.5
N-sulfocarbamoyl gonyautoxins-2	C1	84.4 ± 5.0
N-sulfocarbamoyl gonyautoxins-3	C2	24.2 ± 1.8
N-sulfocarbamoyl gonyautoxins-1	C3	25.6 ± 1.8
N-sulfocarbamoyl gonyautoxins-4	C4	6.81 ± 0.64
decarbamoylgonyautoxins-2	dcGTX2	99.5 ± 5.5
decarbamoylgonyautoxins-3	dcGTX3	22.7 ± 2.7
decarbamoylsaxitoxin	dcSTX	59.1 ± 5.1
gonyautoxins-2	GTX2	56.3 ± 3.8
gonyautoxins-3	GTX3	20.6 ± 1.4
gonyautoxins-5	GTX5	47.6 ± 3.1
gonyautoxins-6	GTX6	25.3 ± 1.3
gonyautoxins-1	GTX1	66.4 ± 3.9
gonyautoxins-4	GTX4	17.7 ± 1.5
neosaxitoxin	NEO	52.7 ±3.0
decarbamoylneosaxitoxin	dcNEO	26.2 ± 1.4

± *U* (k=2, 95%)

Natural Samples

The naturally contaminated mussel *M. galloprovincialis* was collected through the Instituto Português do Mar e da Atmosfera (IPMA) monitoring program in December 2016, after a

Gymnodinium catenatum bloom in Aveiro, Portugal. The non-contaminated (PSTs-free) mussels *Mytilus galloprovinciallis* that serves as the negative matrix sample were provided by Fenisterrra S. A. (Sagres, Portugal) in November 2023.

The marine dinoflagellate was cultured in a laboratory with ALISU code IO13-27-02, from Cascais collected on 28.09.2018. The culture is originally a cyst, a clonal culture reisolated from mother culture with media L1, salinity 33 ppm, maintained at 19ºC ± 1, and light cycle 12L:12D.

Materials

SPE cartridges used were from the Supelclean™ line (Sigma-Aldrich, Germany), the ENVI-Carb™ (250mg/3 mL) and LC-18 (500 mg/3mL) SPE cartridges, referred hereafter as the graphitized carbon and C18 cartridges, respectively. Oasis® MCX Plus cationic exchange extraction cartridge (SPE-COOH, 225 mg short cartridge; Waters, USA) was used for fractionation of samples before the periodate oxidation. Sample containers are all made up of polypropylene PP tubes (ALWSCI Corporation, China) to ensure the least interaction possible of toxins to the surface of the container.

Working solutions

- 1:100 (v/v) acetic acid (HAc)

  1-mL glacial acetic acid was added into a small amount of H_2O then diluted to 100-mL total volume.
- 20:80:0.25 (v/v/v) acetonitrile/ water/ acetic acid (MeCN/H_2O/HAc)

  250 µL of glacial acetic acid and 20.0 mL MeCN are mixed, then diluted with 80.0 mL of H_2O.
- 1000:1 (v/v) H_2O/ ammonia (NH_3) 25%

  100 µL of 25% NH_3 was diluted with H_2O to 100-mL total volume.
- 80% aqueous methanol (MeOH)

  80 mL of LCMS-grade MeOH was diluted with H_2O to 100-mL total solution.
- 100 mM ammonium formate (AF)

  315.28 mg of AF was dissolved in H_2O to 50-mL total solution.
- 10% peroxide (H_2O_2) oxidant solution

  3 ml of 30% H_2O_2 was combined with 6 mL H_2O.

- 0.1 M periodic acid (HIO_4) oxidant solution

  The following reagents were combined to yield the HIO_4 oxidant solution: 5 ml 0.03 M HIO_4, 5 mL 0.3 AF, 5 mL 0.3 M disodium phosphate (Na_2HPO_4), maintained at pH 8.2 with 1 M NaOH.

- LCMS/MS mobile phase:

  Solution A: aqueous 0.1% formic acid (FA), 10 mM AF

  500 µL of FA and 50 mL of 100 mM AF are mixed, then diluted with H_2O to provide a 500-mL total solution.

  Solution B: 0.1% FA, 2% 100mM AF in MeCN

  500 µL of FA and 10 mL of 100 mM AF are mixed, then diluted with MeCN to provide a 500-mL total solution.

- HPLC-FLD mobile phase:

  Solution C: 0.1M AF, 6 ml 0.1M HAc in H_2O

  6.31g AF, 6 mL HAc were dissolved in H_2O to provide a 1000-mL total solution.

  Solution D: 0.1M AF, 6 ml 0.1M HAc in MeCN

  6.31 mg AF, 6 mL HAc, and 50 mL MeCN were diluted to 1000-mL total solution with H_2O.

METHODS

Preparation of standard solutions

Certified reference materials of each individual toxin were used to prepare concentrations between 0.05-2 µM and mixtures of standards to be used as working standard solutions (Table *4*). The mixtures were prepared by following the recommended protocol of the EU Reference Laboratory for Monitoring of Marine Biotoxins (EURLMB)[35], modified by considering the Lawrence method[20], and Lage et al. method[28], for HPLC-FLD and LC-HRMS analysis, respectively. A matrix-matched calibration solution was also prepared for the latter analysis. The matrix matching was achieved using the negative mussel extract, which was pre-analyzed to ensure absence of the marine toxins before used for this study, providing a matrix with a ratio of 1g per mL. Table *4* summarizes the concentrations prepared for each toxin analogue to be used in each method, and Table *5* the different standard solutions prepared for HPLC-FLD analysis. For LCMS-MS analysis, on the other hand, calibration solutions were diluted in 1:3 H_2O: MeCN (v:v).

Table 4. Concentration of the studied toxins in the used working solutions.

Toxin analogue	Working standard solution (μM)	
	HPLC-FLD	HPLC-HRMS
STX	4.00	2.00
C1	4.01	2.01
C2	1.15	0.576
dcGTX2	3.99	2.00
dcGTX3	0.91	0.455
dcSTX	3.97	1.98
GTX2	3.99	2.00
GTX3	1.46	0.730
GTX5	4.00	2.00
GTX6	4.01	2.01
GTX1	4.09	2.04
GTX4	1.09	0.545
NEO	3.93	1.97
dcNEO	4.01	2.00
C3	4.01	---
C4	1.07	---

Table 5. Working solutions used for HPLC-FLD analysis.[35]

Label	Description
MIX-I	A mixture of non-N-hydroxylated compounds, where the stock solution contains STX, dcSTX, dcGTX2&3, C1&2, GTX2&3 and GTX5
MIX-II	A mixture of stock solutions containing GTX1&4 and NEO
MIX-III	A stock solution containing dcNEO
MIX IV	A stock solution containing dcSTX. On the other hand, MIX I can also be used
MIX-V	A stock solution containing GTX6
MIX-VI*	A stock solution containing C3&4

**prepared for this work*

An additional mixture, MIX VI, was prepared and studied due to the availability of C3,4 CRM. The mixtures of standards referred in Table 5 were categorized based on the hydroxylation at the N- position (see Figure 1, Table 1) . A 2-μM concentration of each individual saxitoxin analogue was also prepared and processed to determine the optimal fluorescence detection parameters.

Toxin extraction from mussels *Mytilus galloprovincialis*

Both, non-contaminated and natural contaminated mussel samples were processed. The whole tissue of non-contaminated mussels was homogenized to achieve approximately 100-150 g of

total weight, referred hereafter as the matrix sample. This sample was extracted using a modified AOAC Official Method 2005.06[20]. Briefly, five (5) mL of 1% HAc was added to five (5) grams of homogenized mussel to provide a 1:1 weight-per-volume ratio. Mixtures were vortexed for 3 minutes at 2000 rpm, prior to heating in a boiling water bath for 5 minutes. The samples were then cooled down in an ice water bath and vortexed for additional two (2) minutes. To carefully decant the supernatant solution into clean polypropylene (PP) tubes, each mixture was centrifuged at 3500 rpm for 10 minutes at 15 °C. Another 3-mL of HAc was added and procedure was repeated without the water bath. The total solution is diluted to a 10-mL total volume of extract collected. A 1400-µL extract of each sample was separated into PP tubes containing 7-µl of 25% ammonia prior to SPE clean-up procedures[73]. The remaining of the collected extracts was kept at 4 °C until further analysis. The naturally contaminated mussel was also submitted to the same extraction procedure.

Toxin extraction from marine dinoflagellate *Gymnodinium catenatum*

The extraction of the dinoflagellate was performed by following the protocol described above for mussel samples but an additional homogenization step using Ultra-Turrax (T25 easy clean digital, IKA 107-Werke GmbH & Co. KG, Germany)[28] was performed prior to heating.

Solid-Phase Extraction: Graphitized carbon, C-18, and cation exchange SPE-COOH cartridges

The extracts were processed employing two (2) SPE methods (

Table *6*) by using graphitized carbon and C18 cartridges. The graphitized carbon and C18 processed extracts were analyzed by LC-HRMS and HPLC-FLD, respectively. The graphitized carbon cartridges were conditioned[19,79] with 3 mL of MeCN/H_2O/HAc (20:80:0.25 v/v/v) and 5 mL H_2O/ 25% NH_3 (1000:1 v/v), both then eluting to waste. Then, 500 µL of the extract was loaded onto the conditioned cartridge. The retained fraction was then washed with 700 µL of H_2O to cleanup non-retained molecules, before collecting the eluate with 2 mL of MeCN/H_2O/HAc (20:80:0.25 v/v/v). On the other hand, the C18 cartridge was conditioned with 3 ml MeOH, followed by 3 ml of H_2O, before loading the 500 µl of extract. The retained fraction was washed with two consecutive 2 ml of H_2O, both collected and marked as C18 eluate 1. In a second step 2 ml of 80% aqueous MeOH, was used to remove the retained molecules and this eluate was labeled as C18 eluate 2. The latter eluate was collected to identify presence of the less hydrophilic saxitoxin analogues[67] namely non-regulated analogues. The

cartridges were not allowed to dry during the intermediate steps to ensure retention of the targeted analytes. All eluates were then stored into PP tubes for LC-HRMS analyses and oxidation experiments before HPLC-FLD analysis. Each clean-up method yielded approximately 2-mL eluates, except for C18 eluate 2, which gave ~1 mL. The clean-up methods were conducted from one (1) extract only; hence, reproducibility in the extraction procedure is not accounted in this study.

Table 6 SPE clean-up procedures used

	C18 cartridge	graphitized carbon cartridge	Decision
Prepare	3 mL MeOH 3 ml H_2O	3 mL 20/80/1 MeCN/H_2O/HAc 5 ml 1000/1 H_2O/NH_3	
Load	500 µL sample	500 µL sample	
Wash	---	700 µL H_2O	To discard; contains salts
Elute	2 x 1000 µL H_2O	2 mL 20/80/1 MeCN/H_2O/HAc	To collect Labeled as C18 eluate 1
	1000 uL 80% aq. MeOH	---	To collect; Labeled as C18 eluate 2

75 µl of each eluate was diluted with 225-µL acetonitrile before analysis to prepare the calibration curve[19]. The goal was to evaluate the contribution of matrix before the clean-up step. Additionally, the fractionation C-18 Eluate 1 was further cleaned-up using SPE-COOH cartridges before HPLC-FLD analysis. Summing up, three (3) fractions were collected to fractionate the different saxitoxin analogues and improve individual detection[20].

Pre-column oxidation: Peroxide and Periodate oxidation

Oxidation of standards and samples prior to fluorescence analysis were conducted manually and individually, to better distinguish the resulting signals. Samples collected after C18 and SPE-COOH clean-ups were submitted to peroxide and periodate oxidation, respectively.

The H_2O_2 oxidation was achieved by first mixing 25ul 10% H_2O_2 and 250 µL 1M NaOH in a 1.5 PP vial. Then, 100 µL of the standard solution or sample was added, vortexed in the vial

and allowed to react for 2 minutes. After this time 20 µL of glacial acetic acid was added to stop the oxidation process.

On the other hand, the HIO_4 oxidation was conducted by combining 100 µl of standard or sample solution with 100 µl matrix sample (working as matrix modifier) in a 1.5 PP vial. This solution was oxidized by adding 500 µl 0.1M HIO_4 oxidant solution, 5 µl glacial acetic acid and the resulting solution vortexed for 1 minute. The reaction solution was kept at room temperature for additional 10 minutes before analysis.

All standard mixtures (Table *5*) were subjected to periodate oxidation. MIX I standard solution was subjected to peroxide oxidation only, which allowed the determination of dcSTX and dcNEO instead of MIX IV which contains dcSTX only.

Instrumentation: HPLC Fluorescence Detection

Samples were analyzed by reversed-phase liquid chromatography with fluorescence detection using a modified Lawrence based method[20]. Chromatography was conducted using a Dionex™ UltiMate 3000 UHPLC System with fluorescence detector. A C18 column 4.6 x 150mm, 3 µm (Avantor® Alltima HP; VWR, Germany) was used to separate the oxidation products of saxitoxin analogues with the aid of a mobile phase prepared with solution C, containing 0.1M AF with 6 mL 0.1M HAc per L, and solution D, composed of 0.1 M AF, 6 mL 0.1M HAc, and 50 mL MeCN diluted to 1-L solution. A corresponding guard cartridge (≥ 3 µm, ODS, SecurityGuard™ Guard Column System, Phenomenex, USA) was connected at the inflow of the LC column. The gradient program starts at 0% D, then increases to 5% in 5 min, and then to 70% in 4 min, and this composition maintained for 2 min. A 5-minute equilibration time came after returning to 0% D in one minute, concluding the 17-minute run time. Standards and samples collected from C-18 and SPE-COOH clean-ups underwent pre-column oxidation prior to injection. The injection volume was 20 µL. Initial analyses were conducted with 2 µM of the CRMs to checking for the appropriate excitation and emission wavelengths. Oxidation using peroxide and periodate were used for categorizing CRMs as non-N-hydroxylated and N-hydroxylated toxin analogues, respectively[66]. The emission spectra were collected after excitation at 320 nm and emission between 340 nm and 650 nm to obtain the full emission spectra. The excitation spectra were obtained by monitoring the emission at 400 nm and registering the excitation between 250 and 380 nm. STX was used as exemplar compound.

After analysis of fluorescence data obtained for the oxidation products of each individual toxin, 337 nm was selected as the excitation wavelength while the emission was registered between 357 and 650 nm. However, the quantitation was performed using the FLD profile obtained with emission registered at 392 nm. Toxin analogues that are determined together as epimeric pairs are dcGTX2 and dcGTX3, GTX2 and GTX3, GTX1 and GTX 4, C1 and C2, and C3 and C4. Data were analyzed using the Chromeleon 7.2.10 software (ThermoFisher Scientific Inc., USA). Also, three (3) standard point-solutions between 0.03 to 0.05 µM saxitoxin were prepared to estimate limit of detection by evaluating the signal-to-noise ratio S/N≥3, while maintaining variability at <30%[88] between results. Built-in analysis tool pack in Prism 10 is used for simple linear regression using the calibration arrange was between 0.05 and 2.0 µM.

Instrumentation: High Resolution Mass Spectrometry (HRMS)

Samples were analyzed by HPLC-high resolution mass spectrometry (HRMS) using AMXIC-based quantitation[28]. Only samples collected from graphitized carbon clean-up were analyzed in this method. Chromatographic separation was carried out using an UltiMate 3000 UHPLC system coupled to a Orbitrap Elite mass spectrometer (ThermoFisher Scientific Inc., USA) equipped with a heated electrospray ionization source (HESI II). The PST analogues were separated using an ACQUITY Premier BEH Amide (2.1 x 100 mm, 1.7 µm; Waters, USA) at 35 °C. Mobile phase composition is as follows: (A) aqueous 0.1% FA and 10 mM AF and (B) 0.1% FA and 2% 10 mM AF solution in MeCN. The gradient condition starts at 5% B with increasing linearly the hydrophilicity until reaching 95% B at the 11-minute run time. This composition is maintained one minute, and then reverts to 5% of A in 1 minute. The mobile phase is maintained with this composition for 2 minutes prior to the next injection. Total run time is 15 minutes at a flow rate of 0.30 mL/min. Injection volume was 5 µL. An HILIC guard column (≥ 3 µm, HILIC, SecurityGuard™ Guard Column System, Phenomenex, USA) was used. Samples were maintained at 4°C (autosampler).

Data were acquired under positive and negative polarity ionization (ESI) using the following parameters: capillary temperature, 325 °C; heater temperature, 300 °C; sheath gas, 40 arbitrary units; auxiliary gas, 10 arbitrary units; spray voltage, 3.8 kV; and S-Lenses RF level, 69.06%. The acquisition was performed under full scan high resolution (HRMS) between 100 and 800 m/z using the XCalibur™ (Thermo Fisher Scientific Inc., USA). To identify, toxin analogues are analyzed through comparable *m/z* values (Table 7) and retention times of CRMs.

Fragmentation analyses were performed on exact masses of each of the analogues that were present in the samples. The collision energies for fragmentation of each *m/z* values are determined by infusing the CRM solution into the MS system (Table *8*) for the contaminated mussel sample. For unavailable toxin analogues as CRMs, the collision-induced dissociation (CID) and higher energy C trap dissociation (HCD) parameters were taken from the literature[28,73]. The system was run under product ion scan (MS^2 spectra) using a detection range between 100-500 m/z. These analyses allowed an additional evaluation (besides the exact m/z value) of the GC- and M-toxins through their reported fragments.

Table 7. Calculated exact masses of regulated and non-regulated paralytic shellfish toxins on negative and positive ions (adapted from Lage et.al.[28])

	PST Analogue	**Positive ion**	**Calculated exact mass**	**Negative ion**	**Calculated exact mass**
carbamoyl	STX	$[M + H]^+$	300.141	$[M + HCOO]^-$	344.132
	GTX2	$[M + H – SO_3]^+$	316.136	$[M – H]^-$	394.078
	GTX3	$[M + H]^+$	396.093		
	NEO	$[M + H]^+$	316.136	$[M + HCOO]^-$	360.127
	GTX1	$[M + H – SO_3]^+$	332.131	$[M – H]^-$	410.073
	GTX4	$[M + H]^+$	412.088		
	M2	$[M + H]^+$	316.136	$[M + HCOO]^-$	360.127
	M4/M8	$[M + H]^+$	332.131	$[M + HCOO]^-$	376.122
	M10	$[M + H]^+$	348.126	$[M + HCOO]^-$	392.117
	M6	$[M + H – H_2O]^+$	316.136	$[M + HCOO - H_2O]^-$	360.127
	M12	$[M + H]$	350.141	$[M – H]^-$	348.126
n-sulfocarbamoyl	GTX5	$[M + H]^+$	380.098	$[M – H]^-$	378.083
	C1/C2	$[M + H]^+$	476.050	$[M – H]^-$	474.034
		$[M + H – SO_3]^+$	396.093		
	GTX6	$[M + H]^+$	396.093	$[M – H]^-$	394.078
	C3/C4	$[M + H]^+$	492.044	$[M – H]^-$	490.029
		$[M + H – SO_3]^+$	412.088		
	M1	$[M + H]^+$	396.093	$[M – H]^-$	394.078
	M3/M7	$[M + H]^+$	412.088	$[M – H]^-$	410.073
	M9	$[M + H]^+$	428.083	$[M – H]^-$	426.340
	M5	$[M + H – H_2O]^+$	396.093	$[M – H – H_2O]^-$	394.078
	M11	$[M + H]^+$	430.098	$[M – H]^-$	428.083
decarbamoyl	dcSTX	$[M + H]^+$	257.135	$[M + HCOO]^-$	301.126
	dcGTX2	$[M + H – SO_3]^+$	273.130	$[M – H]^-$	351.072
	dcGTX3	$[M + H]^+$	353.087		
	dcNEO	$[M + H]^+$	273.130	$[M + HCOO]^-$	317.121
	dcGTX1	$[M + H – SO_3]^+$	289.125	$[M – H]^-$	367.067
	dcGTX4	$[M + H]^+$	369.082		
	dcM2	$[M + H]^+$	273.130	$[M + HCOO]^-$	317.121
	dcM4/M8	$[M + H]^+$	289.125	$[M + HCOO]^-$	333.116

	dcM10	$[M + H]^+$	305.120	$[M + HCOO]^-$	349.111
	dcM6	$[M + H - H_2O]^+$	273.130	$[M + HCOO - H_2O]^-$	317.121
	dcM12	$[M + H]^+$	307.136	$[M + HCOO - H_2O]^-$	279.141
benozate	GC1/GC2	$[M + H]^+$	473.108		
	GC3	$[M + H]^+$	377.156		
	GC4/GC5	$[M + H]^+$	489.103		
	GC6	$[M + H]^+$	393.151		

Table 8. Experimental CID and HCD values of select CRMs determined from infusion

	ESI polarity*	**m/z used****	**CID, a.u.**	**Dominant m/z fragments**	**HCD, a.u.**	**Dominant m/z fragments**
STX†	pos	300	25	282(54.64), 282.08(100), 282.17(96.16)	46	106.06 (100), 201.08 (43.13), 221.02 (46.83)
NEO	pos	316	28	298.08(100), 298.17(93.57, 316.25(63.68)	45	225.11(29.77), 298.13(47.67), 316.14(100)
GTX6	pos	396	25	316.14(100), 378.13(5.66), 396.3(10.02)	35	237.11(4.78), 298.13(15.88), 316.14(100)
	neg	394	20	376.11(100), 394.24(10.64),351.09(9.46)	---	
GTX5	pos	380	20	300.14(100), 257.14(5.72), 380.21(2.99)	20	300.14(100), 380.10(12.6), 300.10(2.74)
	neg	378	19	360.08(100), 360.17 (71.6), 360.00(73.19)	65	121.95(100), 121.96(46.36), 378.07(45.36)
GTX2,3	neg	394	18	351.08(100), 351.17 (80.01), 351.00 (64.37)	55	351.06(100), 333.05(46.79), 394.06(41.06)
GTX1,4	pos	412	25	394.11(100),412.28(50.31), 332.10(49.58)	55	124.05 (100), 128.05 (95.89), 314.12 (93.51)
	neg	412	25	367.14 (100), 392.10 (7.87), 349.12 (3.71)	55	367.05(100), 349.04(36.85), 367.10(36.12)
dcNEO	pos	273	25	255.12 (100), 241.15 (52.69), 273.18 (26.69)	55	255.17 (100), 225.11 (53.51), 273.13 (41.32)
dcSTX	pos	257	28	239.08 (100), 239.17 (83.22), 239.00 (67.44)	55	126.07 (100), 257.19 (78.01), 222.10 (75.68)

dcGTX2,3	neg	351	18	333.05 (100), 351.06 (26.76), 333.09 (11.49)	85	333.05 (100), 164.05 (44.77), 253.11 (39.22)
C1,2	neg	474	18	456.08 (100), 456 (83.23), 351.08 (75.99)	55	394.08 (100), 351.07 (44.94), 474.04 (38.56)
			20	456.03 (100), 351.08 (77.15), 431.03 (35.22)	---	---

[*] *pos: positive; neg: negative*
[**] *whole numbers only*
[†] *HCD acquired from product ion fragmentation of contaminated mussel sample confirmed with the presence of STX*

Data analysis

Complementing the built-in softwares Chromeleon 7 and Xcalibur™ 4.1.0 that came with the instrumentations throughout this study, related data treatment and statistical analyses were performed using the Data Analysis of Microsoft Excel (Office 365, Microsoft, USA), and Linear Regression analysis from GraphPad Prism version 10.2.2 (GraphPad Software, USA). Key aspects in method verification such as calibration, precision, use of certified reference materials (CRM), and method suitability were also determined using these programs. Key equations are as follows:

$$y = mx + b$$

Equation 1. Equation of the line (GraphPad)

y	Analytical signal at a given concentration
m	slope of the least linear squares
x	toxin concentration
b	y-intercept

$$s_c = \frac{s_r}{m}\sqrt{\frac{1}{M} + \frac{1}{N} + \frac{(\bar{y}_c + \bar{y})^2}{m^2 S_{xx}}}$$

Equation 2. Uncertainty of the toxicity in sample established from the suitable calibration curve

s_c	standard deviation of the result based on the calibration curve
s_r	standard deviation about regression
M	number of replicate analyses
N	number of x and y pair points
$\bar{y}c$	the result
$\bar{y}$	mean value of analytical signal y
S_{xx}	sample corrected sum of squares
m	Slope, reported as m from Equation 1

$$C_x\left(\mu g\ STX \cdot 2HCl\frac{eq}{kg}\right) = \left[\frac{A_x - A_0}{m}\ x\ TEF\right](\mu M)\text{x MW}\ \left(\frac{\text{g}}{\text{mol}}\right)\text{x}\ \frac{V_E(\text{mL})}{m_H(g)}\ x\ D_f$$

Equation 3. Relation used for calculation of toxicity in µgSTX·2HCl equivalence per kilogram of mussel meat from HPLC-FLD analysis. (Lawrence method)

C_x	concentration, reported as µgSTX·2HCl eq/kg
A_x	Analytical signal produced for the sample extract
A_0	y-intercept, reported as b from Equation 1Equation 1. Equation of the line (GraphPad)
m	Slope, reported as m from Equation 1
TEF	Toxin equivalence factor
MW	Molecular weight of saxitoxin dihydrochloride, 372.2 g/mol
V_e	Volume of extract
m_H	Weight of homogenized extract, in g
D_f	Dilution factor

Matrix effects were evaluated by comparing the slope of the calibration curves of the CRMs dissolved the negative mussel matrix with the CRMs dissolve in 1%(v:v) acetic acid. The 1:1 ratio of toxin to matrix provides how much matrix affects the analytical signal (loss or gain) through the concentration range of toxin, for HPLC-FLD, or whether there is ion suppression or enhancement for LC-HRMS. If no matrix effect is present ratio is 1.0. Under suppression the value is < 1.0 while enhancement leads to ratios > 1.0.

Calculations and treatments were based on the protocols of Lawrence and EURLMB for HPLC-FLD[20,35], and the protocol of Lage etal[28] for the LC-HRMS. LODs were determined using 2 methods where (1) the standard deviation (SD) from repeated analyses (n = 3) of the lowest analyte concentration[89], and (2) identifying the lowest standard concentration where the analytical signal[90] achieved at least S/N≥3.

RESULTS AND DISCUSSION

In this study, we determined the saxitoxin analogues, both regulated and non-regulated, in naturally contaminated *Mytillus galloprovincialis* and *Gymnodinium catenatum* using two analytical methods: (i) high-performance liquid chromatography high-resolution mass spectrometry (LC-HRMS) method developed in-house published by Lage et al.[29]and (ii) the AOAC Official Method 2005.06 with pre-chromatographic oxidation and liquid chromatography with fluorescence detection, with minor modifications[20,33].

Application of AOAC Lawrence method for saxitoxin and its analogues

This study was conducted followed the AOAC pre-column oxidation method[20,33] with slight modifications applicable for the analytical system available for this study. The fluorescence spectra of the different regulated analogues are shown on Figure 3, Figure 4.

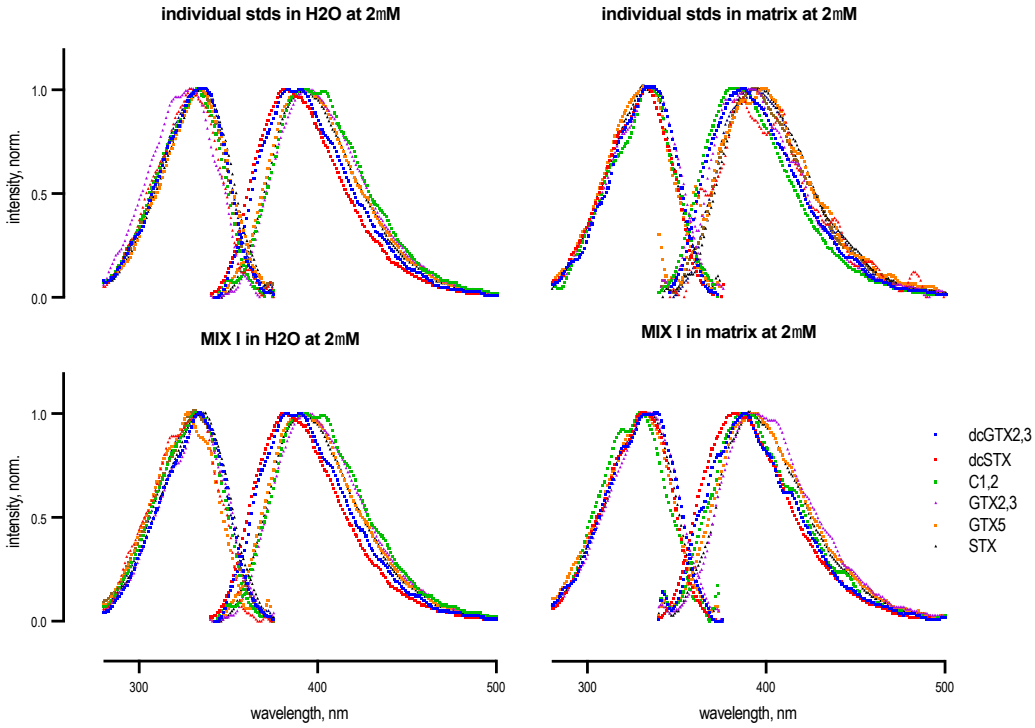


Figure 3. Excitation (between 250-380nm) and emission (between 350-500nm; 501-640 nm not shown) normalized (to unit) spectra of the oxidation products of the certified reference materials of saxitoxin analogues of MIX I. Products were obtained after peroxide oxidation in the absence and presence of matrix. Concentration at ~2 µM, using the individual toxin concentrations shown in Table 4

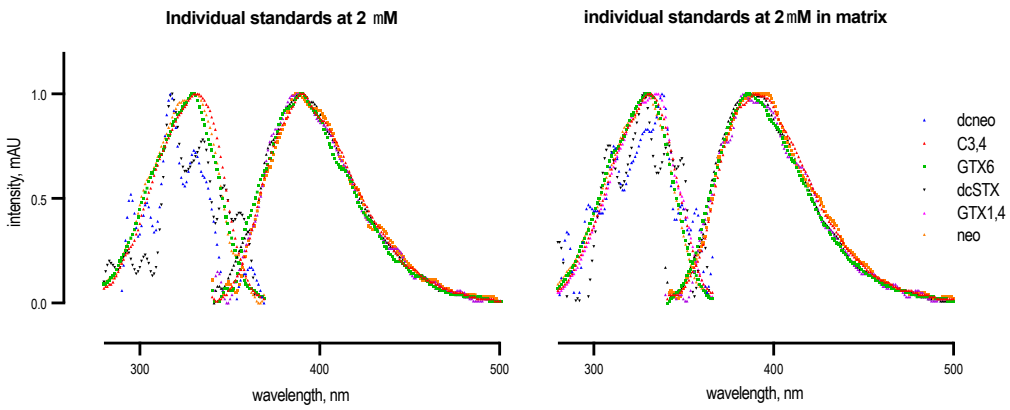


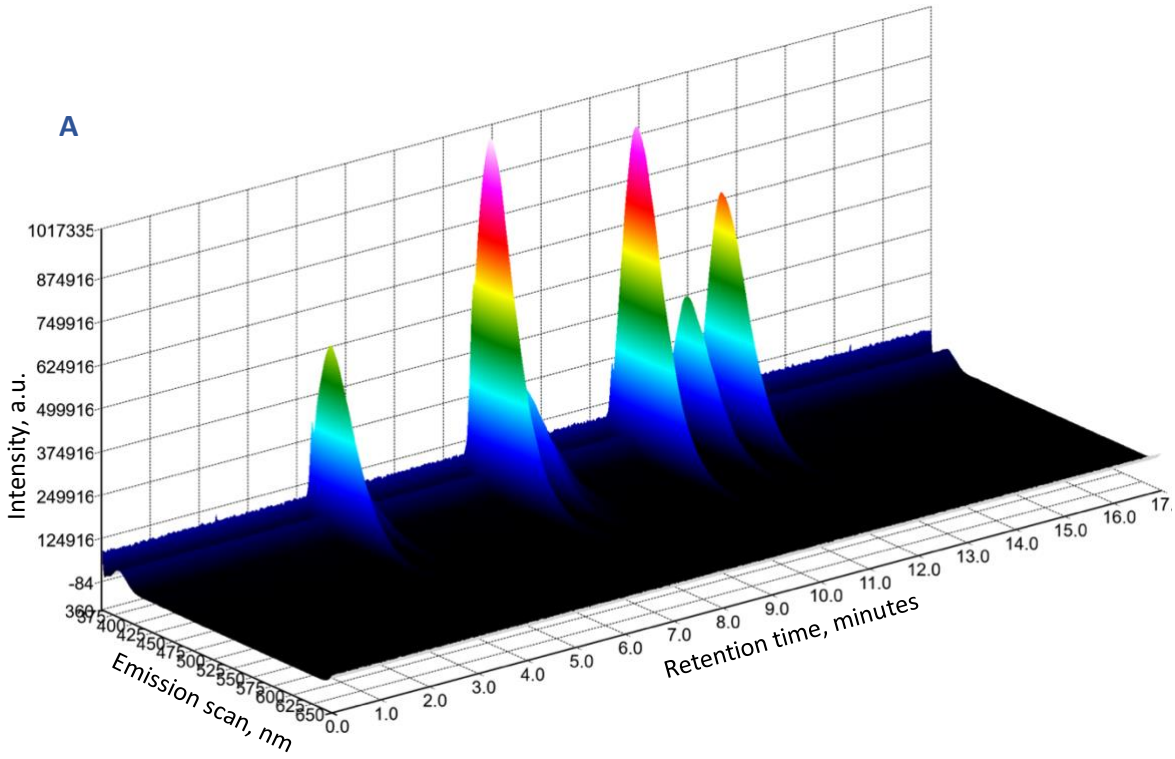
Figure 4. Excitation (between 250-380nm) and emission (between 350-500nm; 501-640 nm not shown) normalized (to unit) spectra of certified reference materials of saxitoxin analogues at ~2 µM after periodate oxidation in the absence (left) and presence (right) of matrix. See Table 4 for individual toxin concentrations.

As can be seen from Figure 3, all oxidation products of non-N-hydroxylated toxin analogues (MIX I) showed similar emission and excitation spectra. This was expected as all compounds possess the same fluorescent core structure being the differences only due the different R groups (R2-R4, refer to Table 1) that may affect the complete formation of the conjugated group[70,71] but do not strongly affect the emitting moiety.

The periodate oxidation products of dcSTX and dcNEO produce spectra slightly different from the N-hydroxylated saxitoxin analogues (Figure 4). dcSTX is highly sensitive to H_2O_2 oxidation and is quantified after this process. dcNEO is quantified after periodate oxidation, during which dcSTX, which if present, co-elutes at the same time. Although both toxins share the same elution time due to similar oxidation products, they can be differentiated by their specific oxidation processes. Both toxins produce two peaks, and when both are present, the chromatographic peaks represent the total from both dcSTX and dcNEO oxidation products. The dcSTX concentration is evaluated by the amount detected through periodate oxidation, leaving the dcNEO peak to be quantified directly.

We didn't explore in detail the origin and nature of the different oxidation products for this study. Overall, the maximum wavelengths for excitation and emission were selected by overlapping spectra of the different oxidation products and choosing the ones that potentially lead to the highest sensitivity for all compounds. For excitation the wavelength selected was

337 nm while the emission was followed at 392 nm. However, the recommended values, 340 nm for excitation and 395 nm for emission, fall within the wavelength range selected as the spectral bandwidth of the used fluorescence detector is ± 10 nm. This application of fluorescence spectroscopy is highly selective as emission spectra are unique to specific molecular species only, and in this case, it is the conjugated oxidation product of saxitoxin analogues that is fluorescent, as shown for saxitoxin in Figure 2. Also, narrow wavelengths were used for both excitation and emission, wherein a single excitation and its complementary emission wavelength determined in this study achieve the best analytical signal for the analyte. HPLC conditions are summarized on Table *9*. Complementing the discussion herewith is Figure 5A showing the chromatographic profile of MIX I-VI (see Table *5*) based on excitation and emission spectra shown from Figure 4.



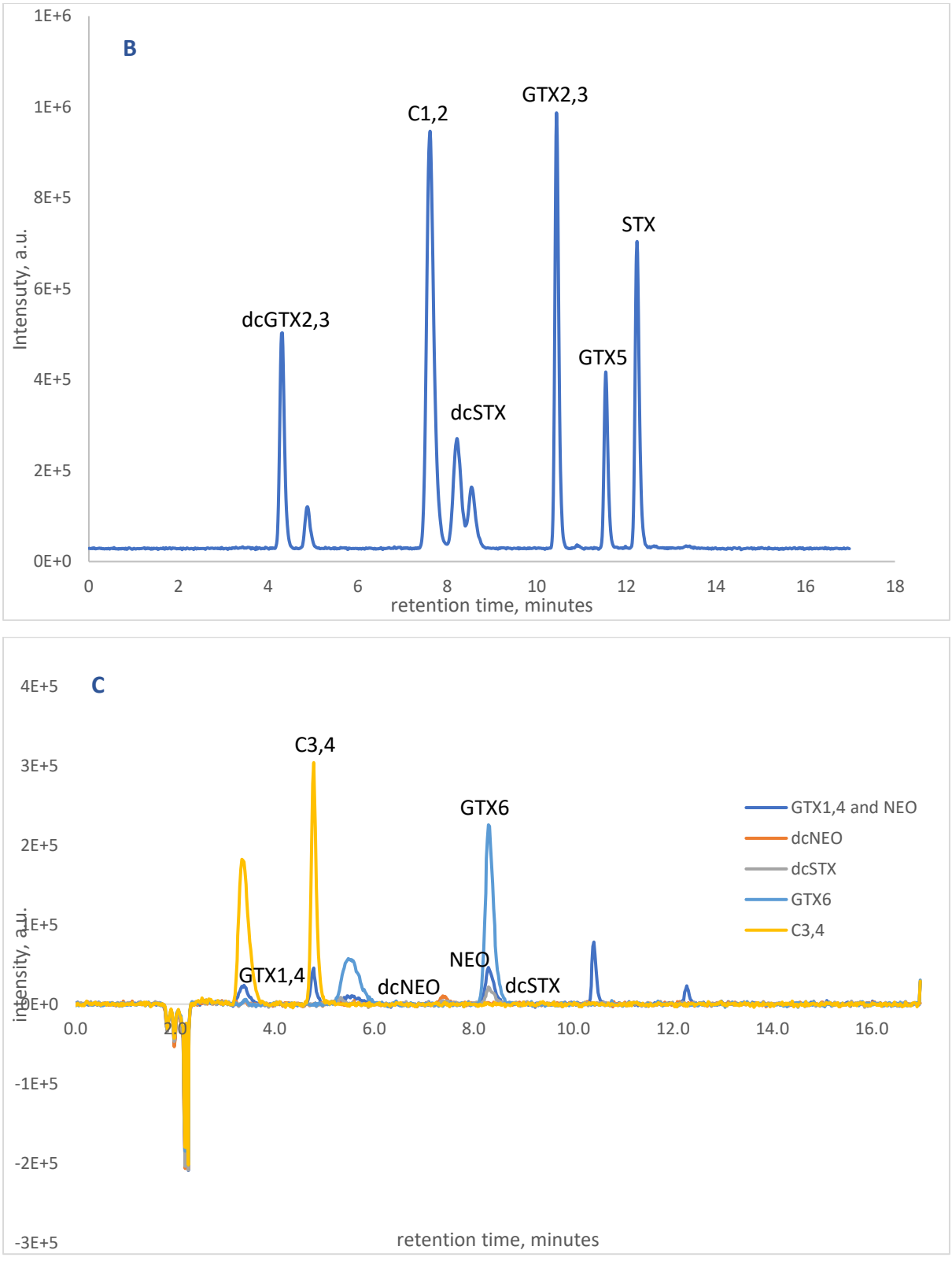


Figure 5. 3D view of emission spectra of MIX I standards over time (A) and its corresponding chromatographic separation of its oxidation products (B) are shown. The chromatograms of MIX II-VI labeled here as the corresponding saxitoxin analogues after periodate oxidation (C) also show complete separation of oxidation products. Individual concentration of toxins are at ~2 µM concentration. Excitation wavelength was at 337 nm and the at 392 nm for data presented in B and C.

Table 9. Instrumentation parameters (HPLC-FLD) used in this study developed from the reference method AOAC Official Method 2005.06

HPLC parameter	EURLMB protocol modified based on AOAC Official Method 2005.06	This study
column	C18 column 4.6 x 150mm, 5 µm	C18 column 4.6 x 150mm, 3 µm
mobile phase C*	0.1M AF with 6mL 0.1M HAc in 1-L solution	0.1M AF with 6mL 0.1M HAc in 1-L solution
mobile phase D*	0.1M AF, 6mL 0.1M HAc, and 50 ml MeCN diluted to 1-L solution	0.1M AF, 6mL 0.1M HAc, and 50 ml MeCN diluted to 1-L solution
flow rate at	1mL/min	1mL/min
sample injection	5 uL for peroxide oxidation 20uL for periodate oxidation	20 uL for peroxide and periodate oxidation
Excitation wavelength	340 nm	337 nm
Emission wavelength	395 nm	392 nm
Gradient method (in reference to the mobile phase D)	0 min 0% 5 mins 5% 5-9 mins 5-70% 9-11 mins 70%-0% 11-15 mins 0% as equilibration period for the next injection	0 min 0% 5 mins 5% 5-9 mins 5-70% 9-11 mins 70% 11-13 mins 70%-0% 13-17 mins 0% as equilibration period for the next injection

**The use of C and D as labels for mobile phase is discretion of the author in reference to the equipment used. This did not alter the overall method as hereto referred.*

Analyses was also conducted in the presence and absence of matrix. The oxidation products of most toxin analogues show weaker fluorescence signals when measured in mussel extract. The role of the matrix components on the fluorescence intensities was conducted using a toxin concentration of 1 µM and would give a better understanding on the matrix effects on non-N-hydroxylated toxin analogues (Figure 6). It is evident that the mussel matrix decreases fluorescence intensity, which also agrees to previous report that presence of matrix quenches the signal but still provides sufficient intensity for detection[91]. However, on N-hydroxylated toxin analogues, the use a matrix modifier is recommended[33] but no evidence of enhancing peak intensities is reported[92]. A negative matrix of *M. galloprovincialis* was analyzed as a blank and the observed signals were subtracted to the chromatogram of the sample, a critical step in quantifying N-hydroxylated toxin analogues. This justifies the similar matrix, being it of the same species. For the marine dinoflagellate, H_2O was used as blank. It is clearly noted that the intensities for the peaks for the oxidation products from periodate oxidation are not as intense

as those from the peroxide oxidation. With the absence of the recommended matrix modifier, N-hydroxylated toxin analogues were detected only at higher concentrations after periodate oxidation, which is evident from the LOD values (Figure 7) and from the minimum value of the dynamic range shown in Table 10. The procedure might not detect lower concentrations; however, it provides an overestimation of toxicity when noted as positive, which is more plausible for the purpose of the method used in seafood safety monitoring[32]. Overall, spectral patterns of all toxin analogues are similar for individual standards and when in their mixture, in the presence and absence of mussel matrix.

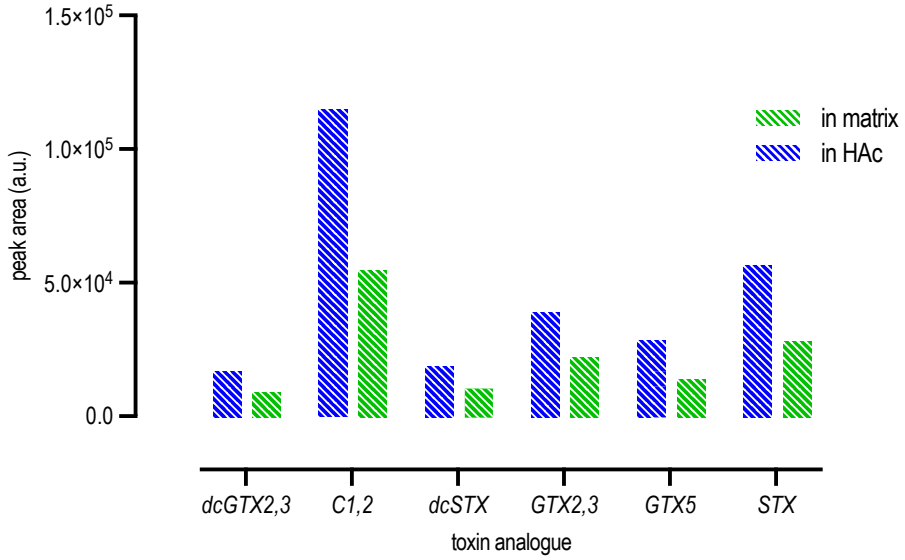


Figure 6. Effect of mussel matrix in HPLC-fluorescence analysis at ~1uM concentration of non-N-hydroxylated saxitoxin analogues. Signal loss is between 47-53%, with C1,2 showing the highest values.

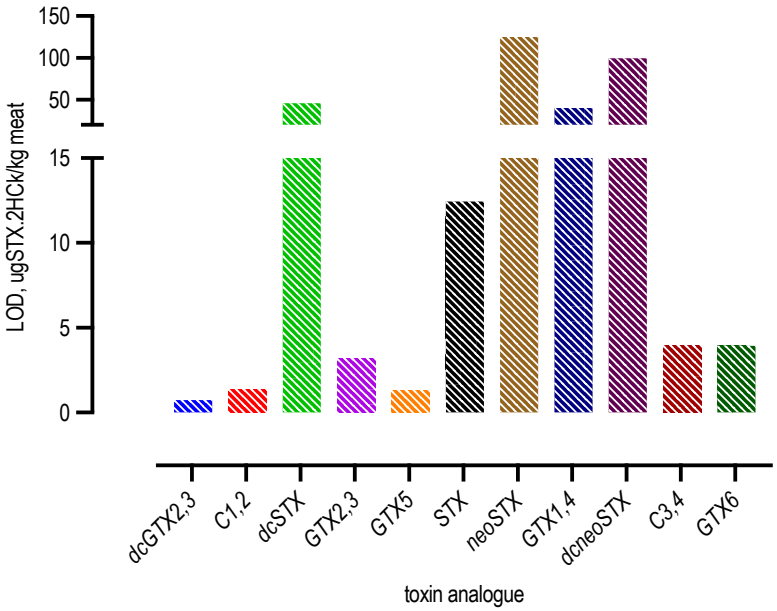


Figure 7. Limit of detection (LOD) of saxitoxin analogues determined using HPLC-FLD based on Equation 2. Also refer to Table 10 for the corresponding values

Prior to quantitation, the C18 eluate of naturally contaminated samples of *M. galloprovincialis* and dinoflagellate *G. catenatum* were used for peroxide oxidation (see Figure 9) and fractionation using SPE-COOH, to completely separate those toxin analogues that co-elute with each other, before periodate oxidation (see Figure 9). The hydrolysis step is skipped due to the availability of C3,4 CRM. Each chromatographic peak was identified based on the retention times referring from Table *2*, the non N-hydroxylated and N-hydroxylated toxin analogues for peroxide and periodate oxidation, consecutively. Several of the non N-hydroxylated toxin analogues were present in both samples, which are easily identified by single identifiable peaks corresponding to each toxin analogue. With this, the quantification is straightforward process.

Three fractions were collected from SPE-COOH which separates into (1) C3,4; (2) GTX1,4 and GTX6; and (3) dcNEO and NEO. At most, 3 oxidation products can be collected from N-hydroxylated toxin analogues. Not all peaks are necessary for quantification but are essential to identify which individual peak is the most important[17]. The low analytical signals of the regulated toxin analogues prevented us to quantify all toxins. From the fractions collected, only C3,4 were found in *G. catenatum*. All identified saxitoxin analogues in the samples are shown in Figure 10 reporting in the corresponding concentration in µM, and complementing the data in Table *11*. By applying the adapted AOAC Lawrence method[20,33], under the followed conditions R^2 results are near or lower than 0.92 for most N-hydroxylated toxin analogues. The

data at hand still follows a linear model with values ≥ 0.82 where variations can be addressed due to low number of replicates. The concentration of these toxins is not reported and their LODs are therefore estimates. The LOD can be estimated following recommended calculations[90] where Lawrence et.al.[33] followed the LOD calculations using the 3:1 S/N and reported toxin content as "not quantitative" in pg values. Comparing the LOD values gathered for STX (n = 3), as the parent toxin analogue, following the 3:1 S/N per injection is 0.576 ng (not quantitative), at least 50-fold over. The analytical technique in this work, specifically the sample preparation reaction conditions need to be further improved. Also needed is to increase number of replicates. Summary of data is presented in *Table 10*.

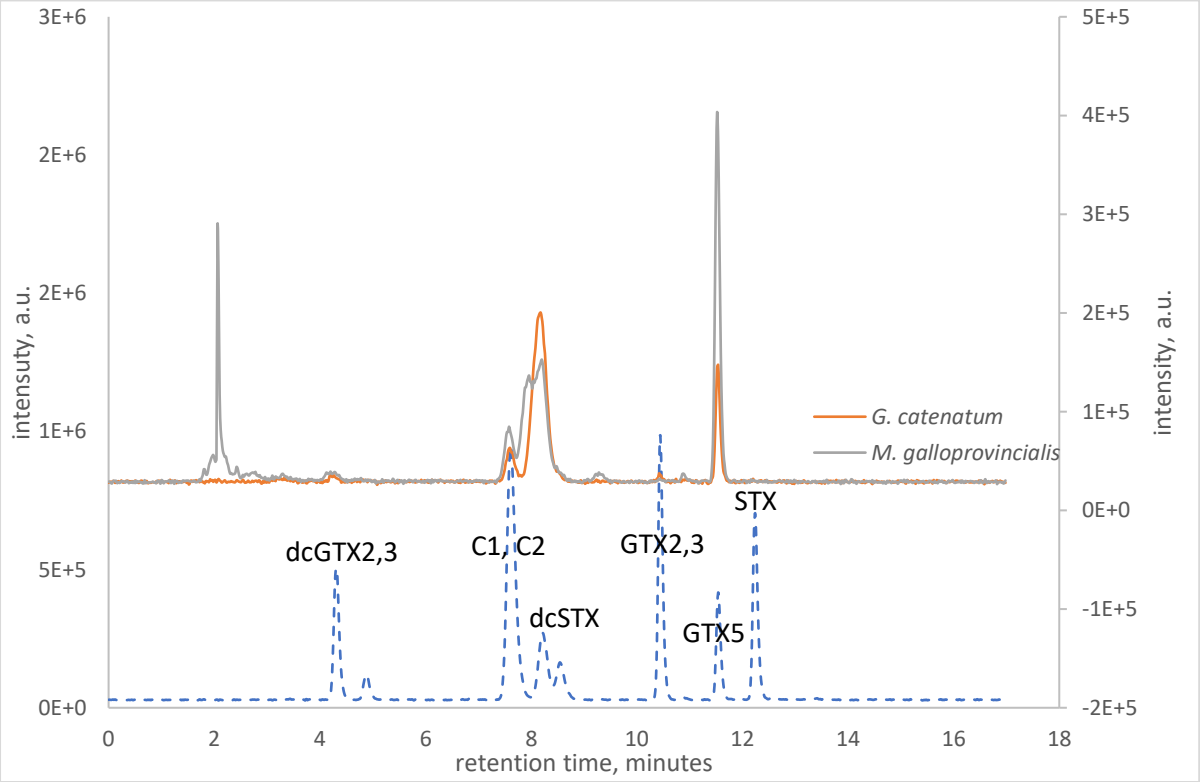


Figure 8. Chromatographic profile (from HPLC-FLD) of samples M. galloprovinialis and G. catenatum undergone peroxided oxidation in comparison to non N-hydroxylated certified reference saxitoxin. Each non N-hydroxylated toxin analogue predominantly produced one fluorescent compound and are separated efficiently using the AOAC Lawrence method. With single peaks distinctly describing each individual analogue, quantitation is rather direct by interpolation from calibration curve.

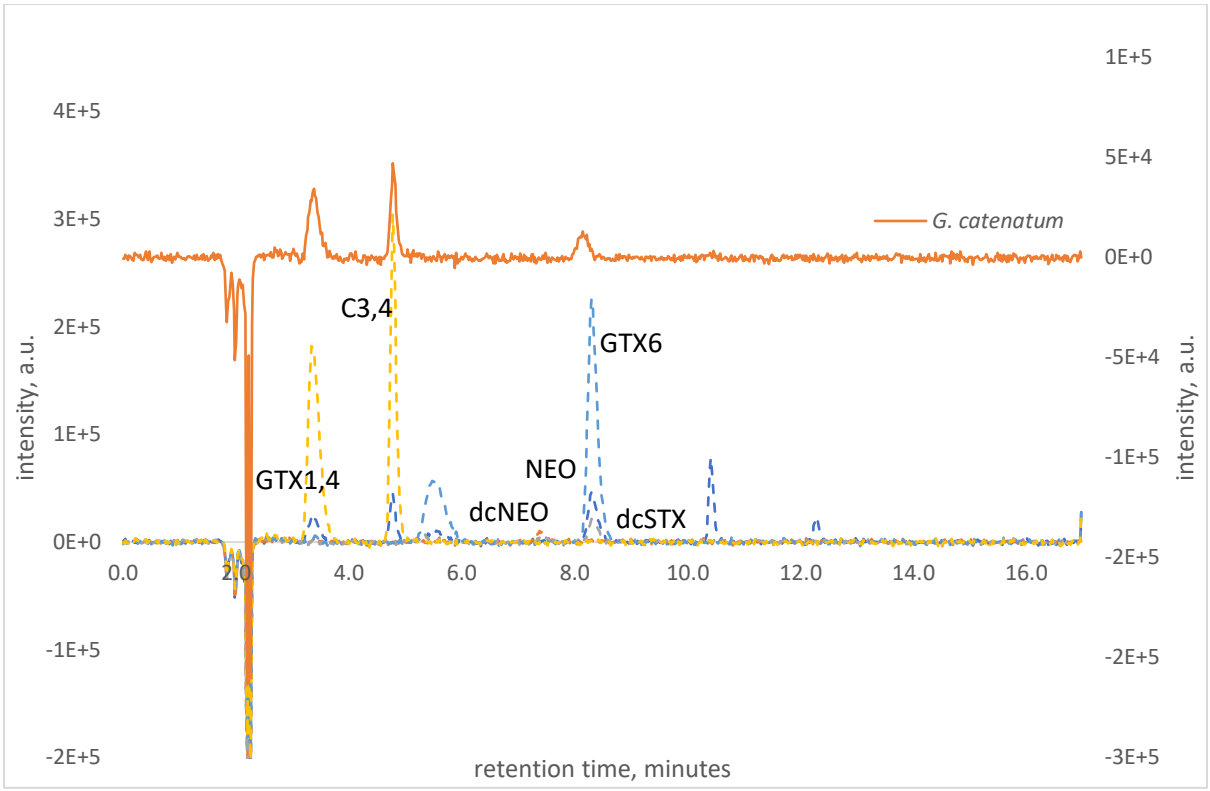


Figure 9. Chromatographic profile (from HPLC-FLD) of samples M. galloprovinialis and G. catenatum with comparison to N-hydroxylated certified reference saxitoxin analogues from periodate oxidations. Periodate oxidation of saxitoxin produces 2-3 distinct peaks, indicating possible fluorescent compounds. Only one peak for every toxin analogue is used for quantitation. The other peaks are used to qualify the toxin analogue present in sample. Only fraction 1 from SPE-COOH provided peaks comparable to CRMs, with both oxidation products are evaluated with excitation wavelength at 337 nm, then emission scanning (357-640 nm) quantified at 392 nm. Fractions 2 and 3 (not shown) from SPE-COOH of the samples were evaluated as <LOD from saxitoxin analogues.

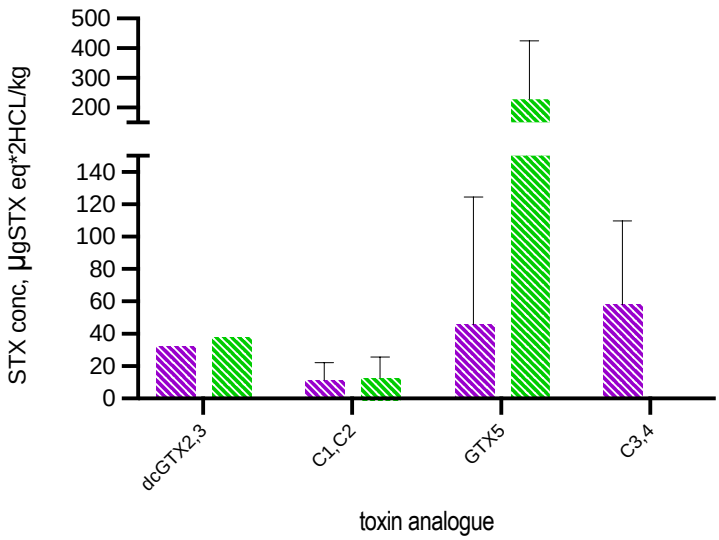


Figure 10. Concentration of the individual toxin analogues analyzed using the modified HPLC-FLD method for M. galloprovincialis and G. catenatum. Saxitoxin analogues not shown are < LOD (n=2).

Table 10. Quantitative description of the adapted AOAC Lawrence method for saxitoxin analogues

	Oxidation process	**Toxin analogue**	**Retention time*, minutes**	**range, µM**	**R^2**	**n**	**LOD**	**LOQ†**
							(µM)	
non N-hydroxylated toxins	Peroxide, H_2O_2	dcGTX2,3	4.28	0.0798 - 1.60	0.962	2	0.02	0.07
		C1,2	7.42	0.0522 - 2.00	0.975	3	0.02	0.07
		dcSTX	8.36	0.331 - 0.397	0.985	1	0.08	0.2
		GTX2,3	10.5	0.313- 1.60	0.976	3	0.06	0.2
		GTX5	11.7	0.0800 - 2.00	0.982	3	0.01	0.04
		STX	12.38	0.0520 - 2.00	0.986	3	0.02	0.05
N-hydroxylated toxins	Periodate, HIO_4	GTX6	4.74	0.106 - 1.34	0.921	1	0.1	0.3
		GTX1,4	8.28	0.108 - 0.681	0.919	2	0.1	0.3
		neo‡	7.4	0.103 – 1.31	0.847	1	0.1	0.3
		dcneo	4.78	0.501 - 0.859	0.838	2	0.1	0.3
		C3,4	8.32	0.106 - 1.34	0.973	1	0.1	0.3

*matching peaks for quantitation[17,20,35]

†available only for toxin analogues calculated by following EURACHEM guide[89]

Table 11. Toxicity content of the natural samples in STX equivalence analyzed by HPLC-FLD.

Toxin analogue	**contaminated mussel, reported in µgSTX·2HCl/ kg meat sample**	**Dinoflagellate, reported in µgSTX·2HCl/ kg cell sample**
dcGTX2,3	18.88 ± 2.0	16.08 ± 2.1
C1,2	4.80 ± 2.8	4.23 ± 2.8
GTX5	85.47 ± 2.0	34.11 ± 2.0
C3,4	<LOD	7.22 ± 0.006
Total toxicity	**109.16 ± 6.8 µgSTX·2HCl/ kg meat sample**	**61.64 ± 6.9 µgSTX·2HCl/ kg cell sample**

The negative mussel matrix was analyzed using the same procedure before used as the matrix modifier for this study. It was ensured that no toxin or pre-oxidized components are found on non-oxidized and oxidized forms. It also showed that its absence provided an acceptable result in other study[93]. The presence of a negative matrix helps in high and uniform toxin recovery as reported by Lawrence and his colleagues[33]. Their use of oyster extract was because of a cleaner chromatographic profile during their study in comparison to clams and mussels. This criterion of a clean chromatographic profile, free from any fluorescent components both natural and STX-related components, was also applied to the negative mussel matrix in our study. By choosing the same mussel, same species matrix, it is best fit applying fundamental concept of subtracting the blank sample injection to HPLC chromatogram to ensure a cleaner chromatogram with peaks based only on the contribution of the analyte in question. The use of a negative mussel extract, with the same species of the contaminated mussel, as matrix modifier justifies the similar mussel matrix for this study.

Application of a High-Resolution Mass Spectrometry Method: Identification and quantitation of toxin analogues using certified reference materials

There are different saxitoxin analogues that can be quantified using HPLC-FLD. However, for each new discovered analogue, a method should be applied and validated. For the method to be applied and validated, the corresponding CRM of the new toxin must be commercially available. In recent works, HPLC-MS/MS has been proposed as an alternative method[73,74] for these toxins due to high selectivity and capability to quantify each individual compound. An additional method is HPLC-HRMS using accurate-mass extracted ion chromatography (AMXIC)-based quantitation[28] that has been used to complement the HPLC-FLD method. Full scan profiles Figure 11 were acquired for the negative mussel matrix, contaminated mussel and marine dinoflagellate extracts to evaluate the presence of toxin analogues. The method involves

the acquisition under full scan followed by the extraction of the AMXIC using a ± 5ppm window[94]. Quantification was then performed by preparing calibration curves using the areas of obtained peaks. This AMXIC procedure, unlike the MS/MS procedure, has the advantage of keeping information from the sample so that it can be analyzed later on for other toxin analogues. The application of AMXIC to regulated toxins allowed us to obtain the retention times (Table 12, Figure 12) of all CRM. The retention times should coincide in both the ESI^+ and ESI^- analyses of each saxitoxin analogue. By applying this method, the presence of any specific toxin can be searched in the sample profiles. It also shows which ionization is deemed appropriate for quantitation by checking the signals under both the positive and negative modes[19,73]. The same procedure was applied to all samples to detect which toxin analogues are present. By combining both the corresponding retention times and AMXIC, we were able to identify the regulated toxins in the samples. As expected[28], AMXIC procedure gives rise to a single peak for each toxin, therefore, the procedure is highly selective to identify and quantify the regulated toxins (Figure 12).

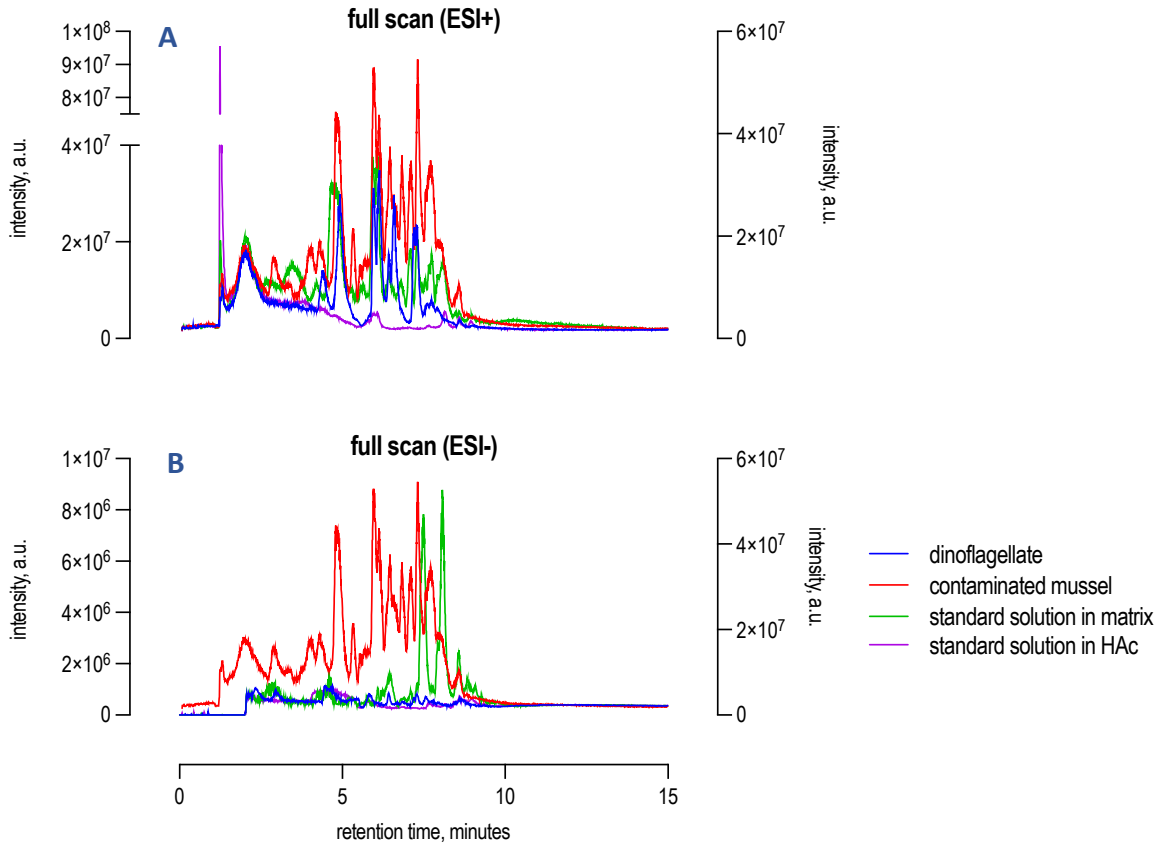


Figure 11 Full scan LC-HRMS in the (A) positive and (B) negative ESI modes of the standard mixture of CRM toxin analogues in acetic acid, and in negative mussel sample matrix, and

the extracts contaminated mussel M. galloprovincialis (intensity refer to y-axis on the right).and dinoflagellate G. catenatum.

Table 12. Retention times for regulated toxins (detected under ESI^+).

Toxin analogue	Retention times, minutes
GTX2	7.58
GTX1	7.64
dcGTX2,3	7.70
GTX3	7.77
C1	7.78
GTX4	7.85
C2	7.99
dcNEO	8.08
STX	8.09
dcSTX	8.12
neoSTX	8.13
GTX5	8.14
GTX6	8.29

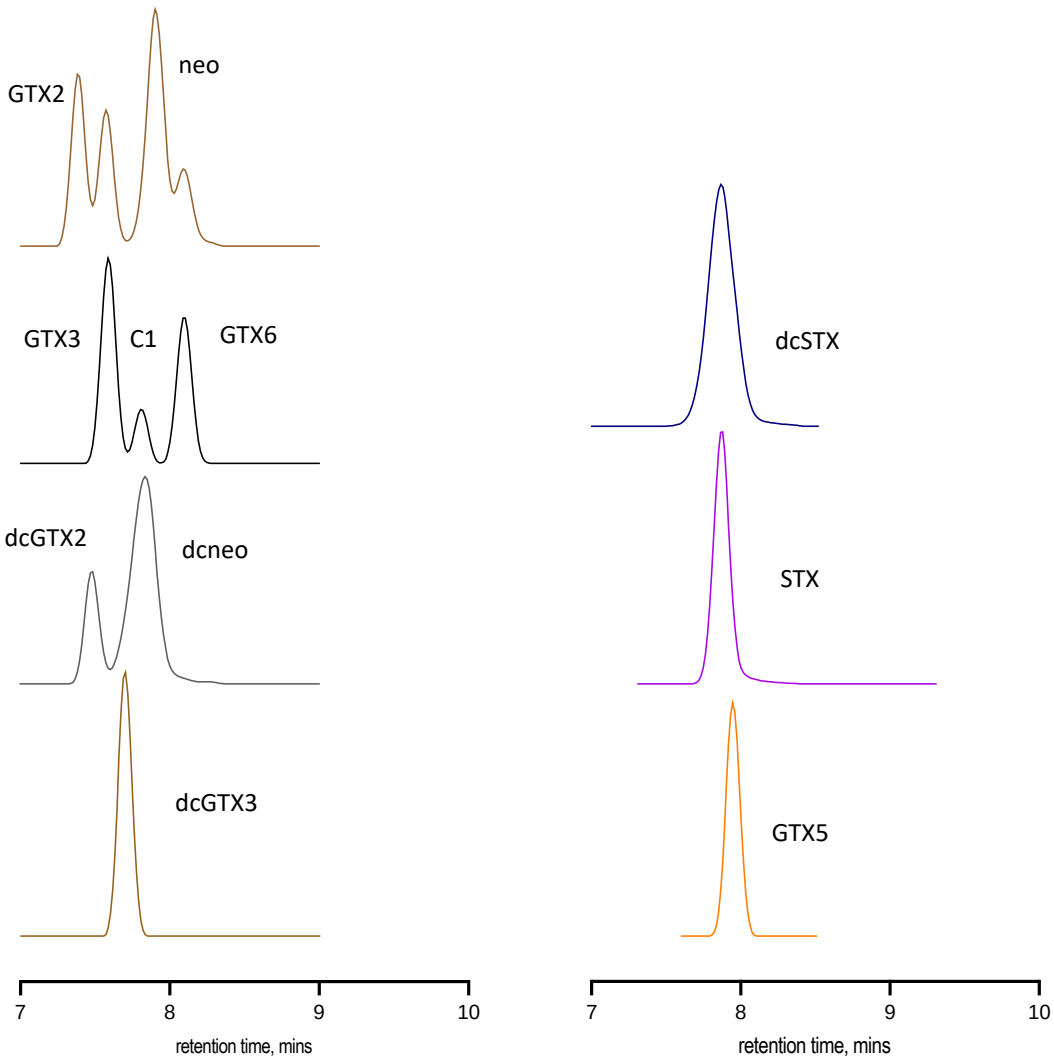


Figure 12. Accurate mass extracted ion chromatograms (AMXIC) of regulated saxitoxin analogues in a standard mixture obtained from full scan in ESI$^+$ mode. See Table 7 for corresponding m/z values (±5ppm).

Having been able to identify the presence of the regulated toxins, we proceeded to quantify the corresponding toxins in the samples. We evaluated the clean-up procedure prior to quantitation. Samples were processed using both graphitized carbon and C18 SPEs cartridges. In routine analyses for HPLC-FLD[20], the use of C18 SPE is sufficient to screen contaminated samples. This concept was followed for LC-HRMS, but an additional washing step was also conducted (C18 eluate 2) using 80% MeOH. This second eluate is intended for the toxin analogues that did not elute after the first elution such as the GC toxins[74]. Our data show that regulated toxins are present in both C18 eluates meaning that toxins are not fully washed out from the cartridge if the second washing is not conducted. Graphitized carbon SPE, on the other hand, is expected to retain nonpolar to polar analytes[95]. Hence, graphitized carbon is advisable for the hydrophilic

toxins such as saxitoxin and its analogues[72]. This SPE type of cartridge, according to our results, was also able to retain the toxin analogues and clean-up the samples.

Figure 13 presents the calibration curves for saxitoxin, a representative compound of the STX family in both, the solvent and the mussel matrix. The R^2 were 0.9697 and 0.9619 in the matrix and the solvent, respectively. A matrix effect of 0.408 from the clean-up of C18 compared to the 0.884 in the graphitized carbon clean-up was obtained meaning that the latter cleanup procedure is more efficient in removing compounds that cause matrix effects. The results also explain the weak signals of saxitoxin at lower concentration in the presence of the mussel matrix.

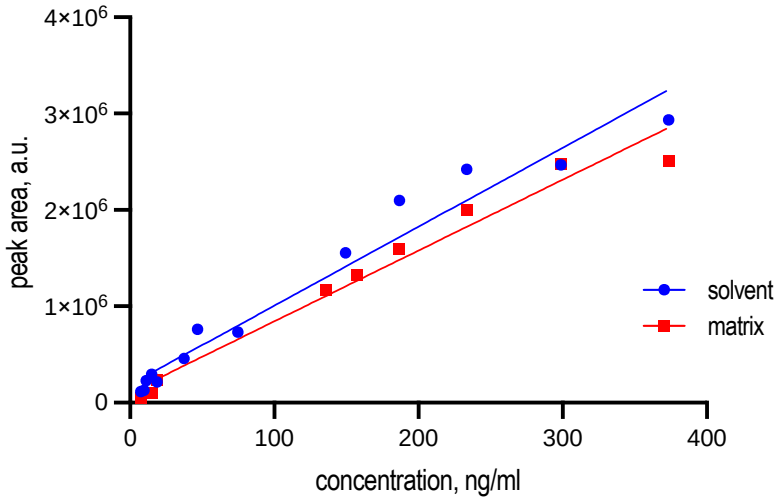


Figure 13. Comparison of standard curve of saxitoxin, as a reference compound, in solvent and in negative mussel matrix after graphitized carbon SPE, detected through LC-HRMS. STX shows weak signals at low concentrations due to ion suppression in the presence of matrix. Detection method is HPLC-HRMS.

Ions suppression of STX in the presence of a mussel matrix was also observed for other toxin analogues (Figure 13). On the other hand, neoSTX shows a value >1.0, indicating ion enhancement effect (Table 13). Under ESI^-, 4 toxin analogues, namely dcGTX3, GTX4, GTX3 and C2, were not detected in the C18 eluates[32]. Overall, the use of graphitized carbon was found to be a more suitable SPE clean-up for the analytes. Hence this procedure was evaluated on the matrix effects (Table *13*). The quantitation of STX, dcSTX, neoSTX, and dcneoSTX was made under ESI^+[28,73]. The toxin analogues analogues were evaluated in either the positive or negative ESI modes. C1,2 and dcGTX2,3 were analyzed in the ESI^- mode, while the remaining analogues GTX2,3, GTX5 and GTX6 are evaluated in the ESI^+. The complementary

ESI modes of positive and negative charges are used qualitatively to confirm that it is indeed the same toxin analogue at a given average retention time during analysis (Table *14*).

Table 13. Matrix effects obtained for the different regulated toxin analogues in the mussel sample after following the graphitized carbon SPE procedure.

Toxin analogue	Matrix effect
GTX2	0.734
GTX1	0.581
dcGTX2	0.692
GTX3	0.757
C1	0.758
GTX4	0.987
C2	0.708
dcneoSTX	0.737
STX	0.884
dcSTX	0.491
neoSTX	1.44
GTX5	0.821
GTX6	0.795

There are several approaches to obtaining the limit of detection and quantitation for this procedure. The values reported here are reported in the presence of the matrix as the samples analyzed using the LC-HRMS procedure are mussel samples.

The marine dinoflagellate was found with the toxin analogues GC-toxins, while the contaminated mussel was found with several M-toxin analogues, similarly identified from the same species in the preceding study[28]. With this study is the first for these natural samples, comparison cannot be conducted on the exact toxin analogues present and the corresponding toxicities. On the other hand, the data agree with previous publications wherein M-toxins are present in the bivalves and GC-toxins are present in the marine dinoflagellate, except for the presence of M4 in the marine dinoflagellate. M-toxins are considered "metabolites" of STX and the regulated toxin analogues in bivalves[77].This may serve the first report on this. Each toxin analogue was identified and quantified using the correspondent calibration curve. The concentrations of the different toxin analogues obtained from the contaminated mussel and dinoflagellate are presented in Figure 14. Total toxicities calculated from these values, taking into the account the toxicity equivalence factor (TEF)[32], were 58.85 and 161.92

µgSTXeq·2HCl/kg of sample for *M. galloprovincialis* and *G. catenatum*, respectively. Both values are below the regulatory limit of 800 µg/kg[32]. The implemented procedure allowed us to evaluate if the toxicity levels of the studied samples meet the regulatory limits.

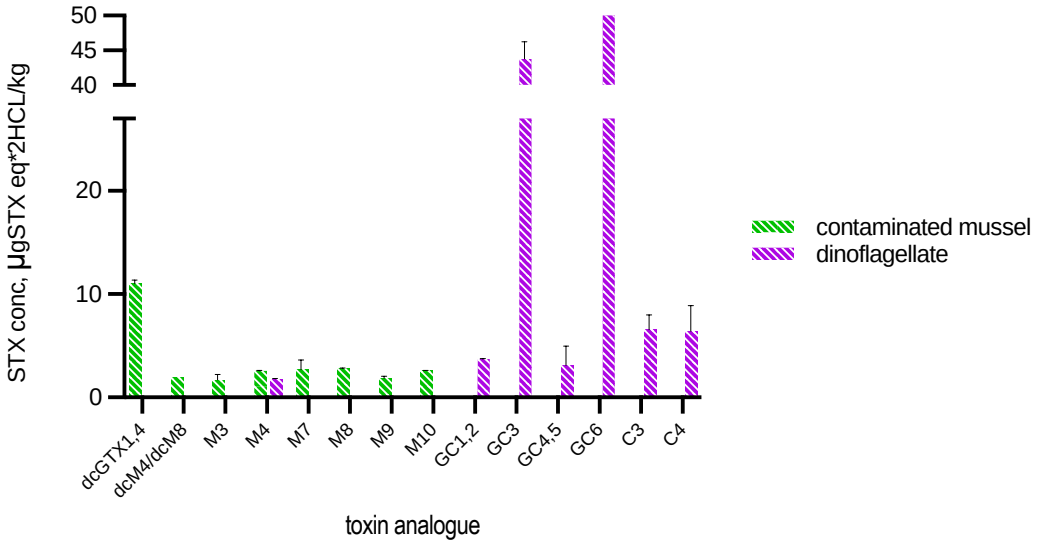


Figure 14. Toxicity levels (n=3) of the different saxitoxin analogues using HPLC-HMRS, reported as ugSTXeq·2HCl.

Table 14. Average retention times, R2 values and concentration ranges obtained for the regulated toxins in the presence and absence of matrix, as detected using LC-HRMS.

Toxin analogue	**Retention times, minutes**	**R^2**	**Concentration range, ng/mL**
GTX2	7.58	0.971	0.980-19.58
GTX1	7.64	0.938	1.51-23.02
dcGTX2	7.70	0.949	1.72-34.46
GTX3	7.77	0.975	0.131-2.622
dcGTX3	7.77	0.952	0.448-1.79
C1	7.78	0.998	1.48-29.75
GTX4	7.85	0.911	0.0818-1.64
C2	7.99	0.918	0.122-2.45
dcNEO	8.08	0.948	1.13-9.08
STX	8.09	0.984	6.88-137.7
dcSTX	8.12	0.930	1.03-20.67
neoSTX	8.13	0.961	0.918-18.37
GTX5	8.14	0.975	0.828-16.56
GTX6	8.29	0.976	0.401-8.020

Application of a High-Resolution Mass Spectrometry Method: Identification and quantification of non-regulated saxitoxin analogues

LC-HRMS can be used to identify the presence and quantify non-regulated STX analogues. First, we made a AMXIC using the expected m/z values of non-regulated STX analogues reported in the literature to evaluate their presence in our samples. Figure 15 shows the profiles obtained after extraction of the m/z 396.093 from full scan profiles of contaminated mussel and of the fortified negative mussel sample. The regulated GTX2, GTX3 and GTX6 also possess this exact mass. Additionally, C1 and C2 can undergo in-source fragmentation, leading to a daughter ion also possessing this m/z value. Finally, the non-regulated toxins M1 and M5 also show the same exact mass. As can be seen from Figure 15 the contaminated sample shows at least one additional peak observed above 8.5 min, that can be due to M1, M5 or both. We proceed by getting additional mass spectral information, namely the correspondent fragmentation data. The fragmentation of the signals near 8.5 minutes were obtained by running the samples under product ion scan by fragmentation of the ion at m/z 396.093 over the entire chromatographic separation (LC-HRMS^2) and detection of the product ions between 100 and 500 *m/z*.

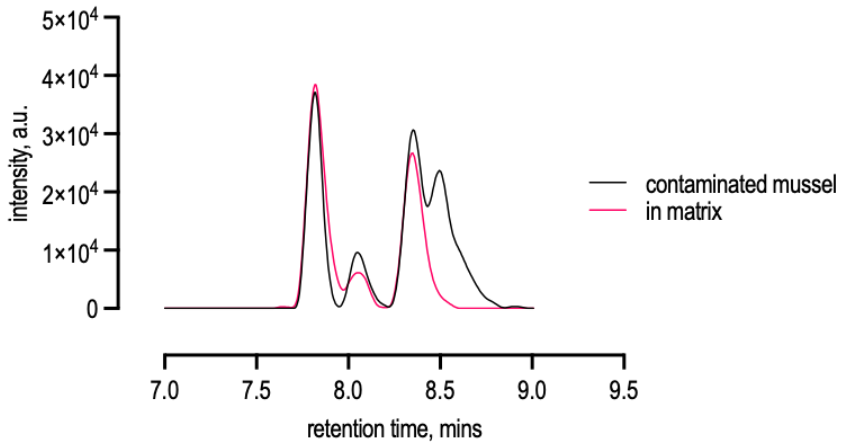


Figure 15. AMXIC obtained after extraction of m/z 396.0932, corresponding to GTX3, C1, GTX6, M1, and M5, from the contaminated mussel sample (black) and from the fortified mussel sample (pink). In this chromatogram you can identify both the regulated and non-regulated toxin analogues present in the sample by comparing it with the retention time of the CRM available. With peaks that are incomparable to the CRM, it may be identified as non-regulated toxins, and fragmentation analysis conducted after.

The profiles obtained under HCD above 8.5 min and by following this procedure didn't show clear fragmentation spectra. However, the correspondent profile obtained under CID showed

the presence of fragments at m/z 316 and 298, characteristic of M1 and M5 (see Figure 16). Additionally, a fragment at m/z 257 was also observed indicating the signal corresponds to M5[11]. It should be noted that the spectra obtained by us under CID are not expected to be similar to the reported in reference[11] as the fragmentation procedures are different. However, the main peaks as well and the signal observed at m/z 257 allowed us to identify M5 in the analyzed sample. Signal at m/z 378 corresponds to a neutral loss of water which reflects the presence of OH groups in M5 structure.

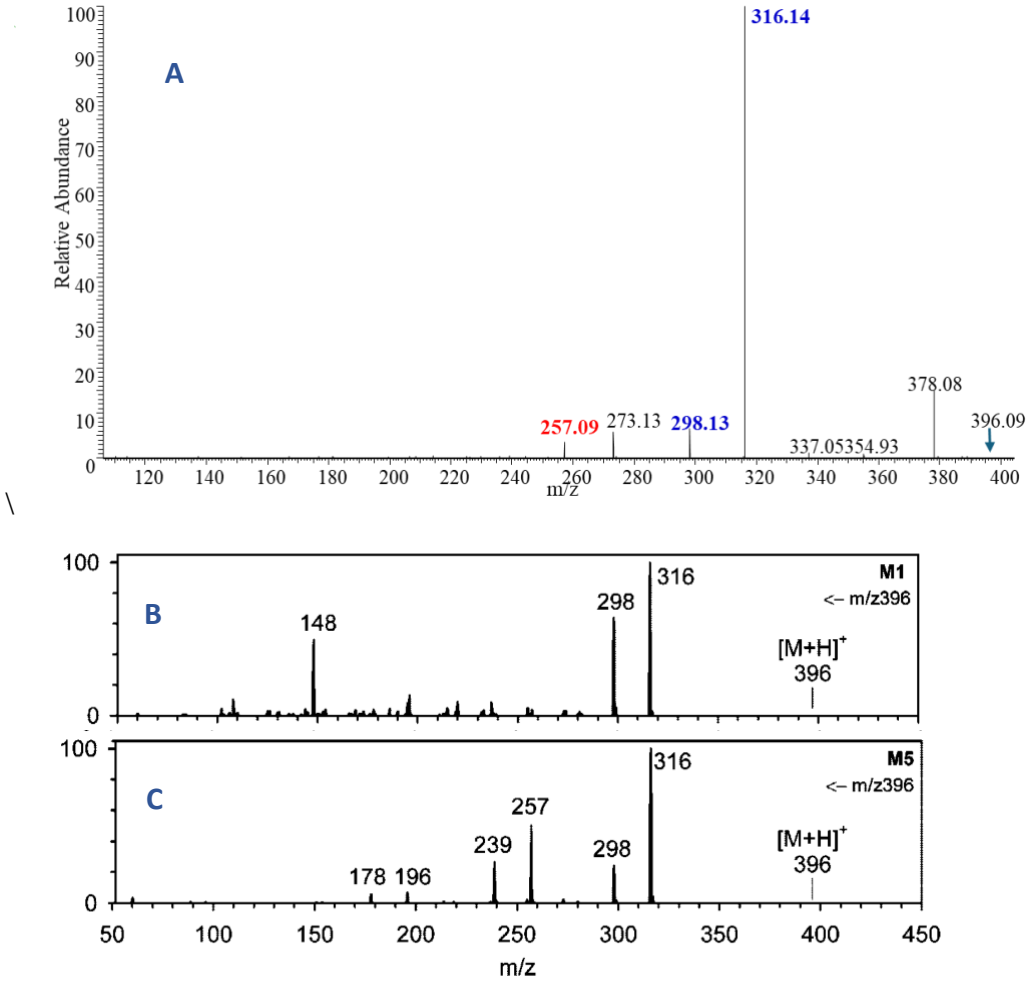


Figure 16. Spectrum A shows the fragmentation spectrum of M5 obtained under product ion scan by fragmentation of the ion at m/z 396.093 between 8.5 and 9.0 minutes. It is compared with the images labeled (B) and (C) as fragmentation spectrums of M1 and M5 as reported by Dell'Aversano, et.al.[11]

We follow this procedure to identify other non-regulated STX toxins in our samples. Basically, the exact mass of the selected compounds is searched in the full scan profiles and then the structure of the observed signal, if any, is verified by comparing the obtained experimental spectrum with data reported in the literature. After the identification the quantification was performed assuming each detected non-regulated toxin shows an instrument response similar

to the regulated compound showing the most similar structure. Fragmentation analyses of each non-regulated saxitoxin analogues can aid in confirmation, however, not all spectra provided clear unambiguously data for identification using the fragments detected (Annex C, Annex *D*) The obtained results are presented in Figure 17, Figure 18, and .

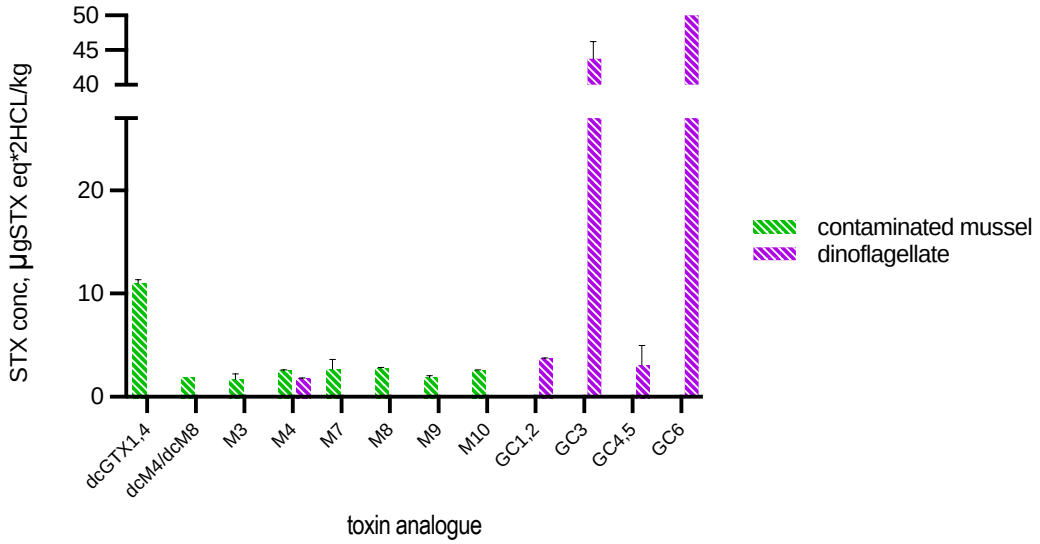


Figure 17. Toxicity levels (n=3) of the different toxin analogues, reported as ugSTXeq·2HCl.

Table 15 Identified non-regulated toxins from the contaminated mussel using LC-HRMS at different ionization polarity of (ESI).

m/z		**Saxitoxin analogues**	
(+)	**(-)**	*M. galloprovincialis*	*G. catenatum*
332.131		M4; M8	M4
	367.067	dcM4, dcM8	
	333.116	M3, M7	
	410.073	dcM4, dcM8	
	392.117	M3, M7	
472.109			GC1/GC2
377.157			GC3
489.103			GC4/GC5
393.152			GC6

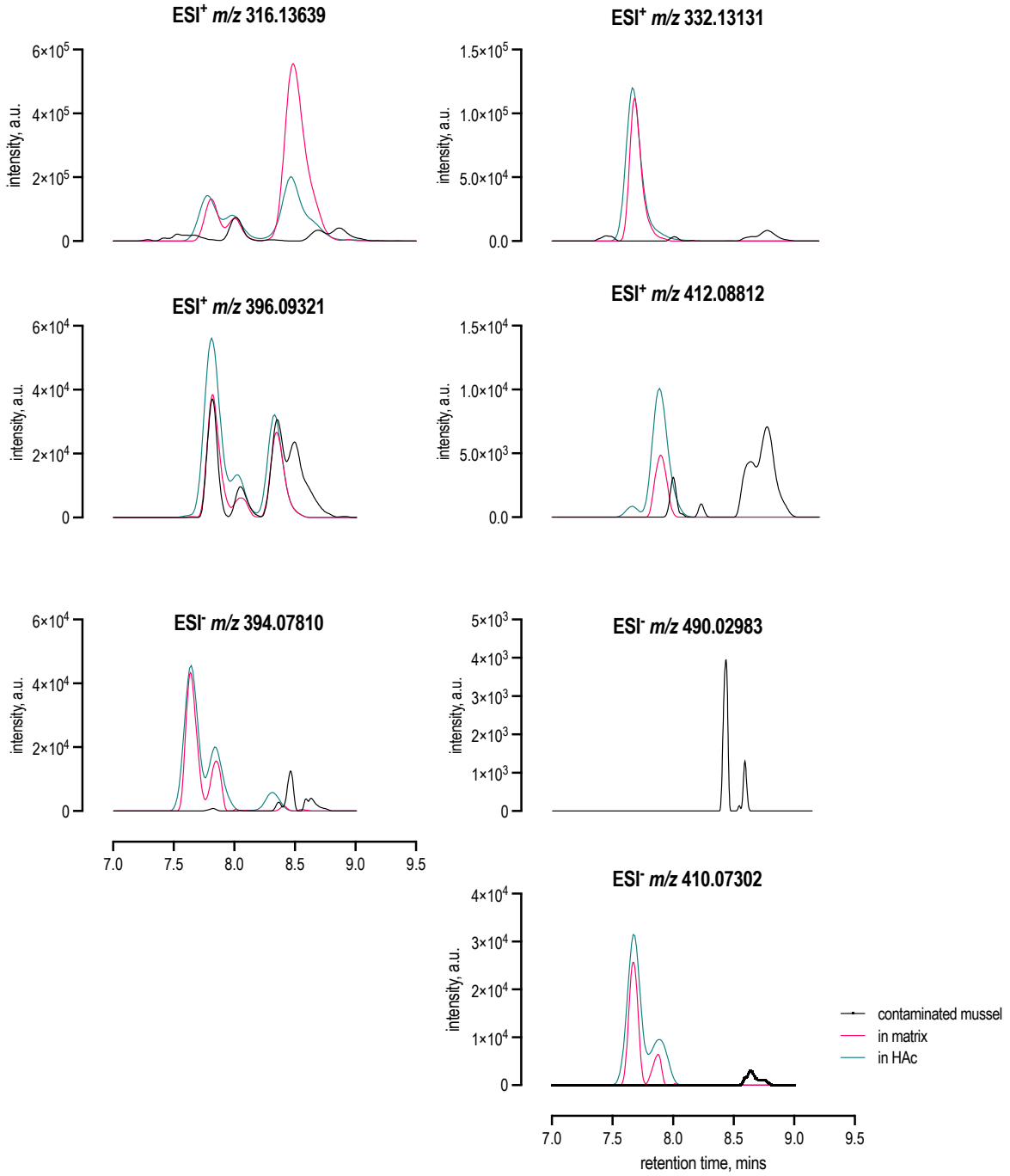


Figure 18. AMXIC of standard solution in the presence and absence of matrix and in the sample M. galloprovincialis for ESI+ m/z 316.13639, m/z 332.13131, m/z 396.0932, and m/z 412.08812; ESI- m/z 394.0781, m/z 490.02983 and m/z 410.07302. These values are used to identify non regulated toxin analogues M2, M6, M1, M5, M4, M8, M3, and M7.

Consequently, identifying possible GC-toxins from extract of *G. catenatum* dinoflagellate follows the same procedure. These toxin analogues can only be analyzed in ESI^+ and resulted to a general spectrum across all *m/z* values. Only the fragmentation spectrum of *m/z* 393 is

shown here (Figure 19) to simplify presentation, The predominant product ion resulted to *m/z* 256.300. The use of AMXIC isolated 1 to 2 peaks identifying the possible GC-toxins content. However, this result does not provide a more comprehensive mass spectra to unambiguously identify the toxin analogues[28]. Re-analysis is recommended, specifically using a lower collision energy, which was not conducted in this period of study due to very limited amount of sample extract. The research of Costa and colleagues[74] reported the MS/MS product ion scans of *G. catenatum* and successfully identifying GC-toxins.

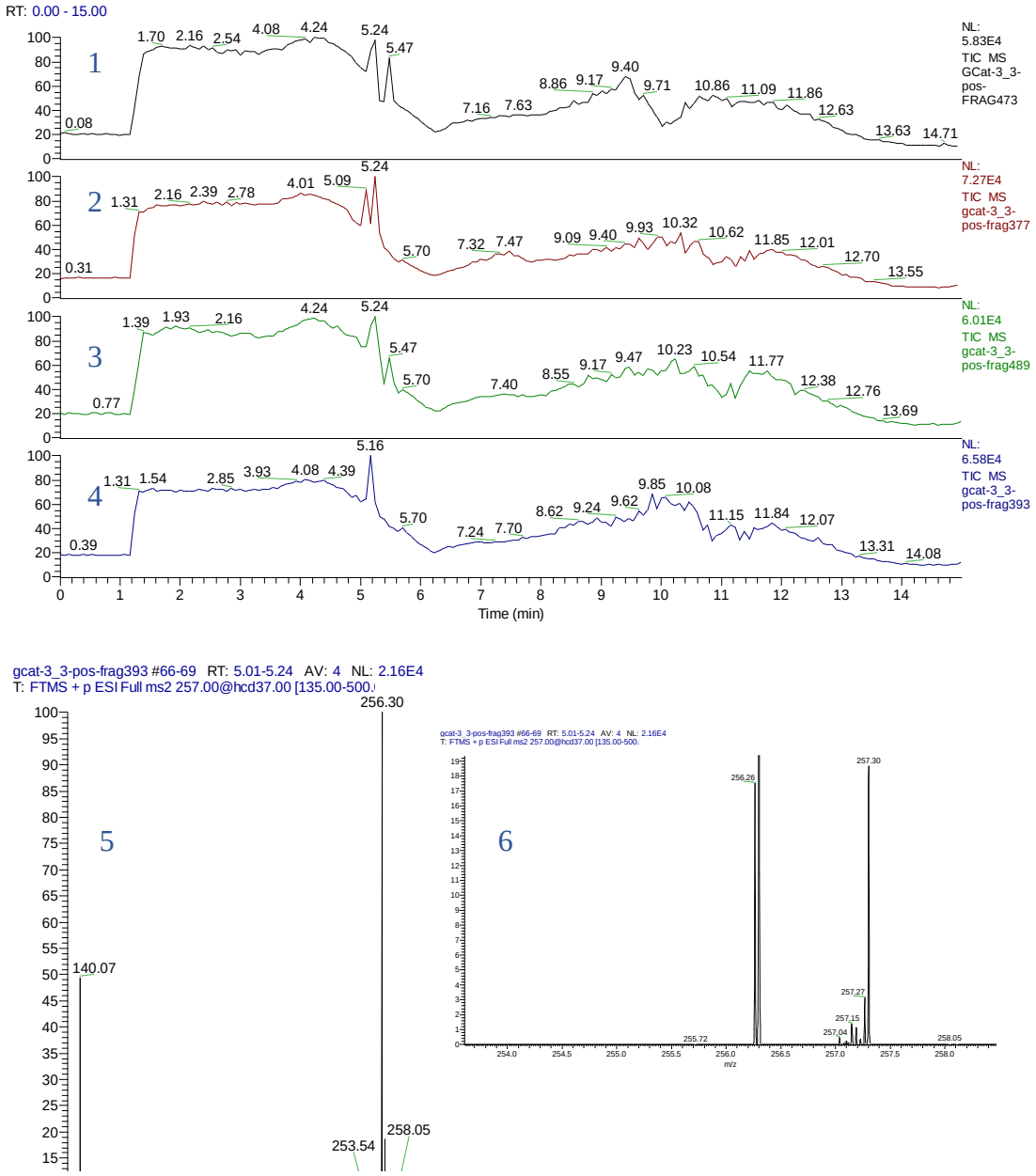


Figure 19. Fragmentation analysis of G. catenatum with peaks at 5.2 minutes at ESI$^+$ (1) m/z 473, (2) m/z 377 (3) m/z 489 and (4) m/z 393; full spectrum at m/z 393 (5) with (6) magnified section by the base peak of the spectrum. Dominant product ion of m/z 256.300 as shown in (5). Similar spectra were also obtained from all m/z values for GC-toxins

CONCLUSION

This work compares the performance of HPLC-FLD and LC-HRMS methods twoards the analysis of regulated and non-regulated STX toxins. Both methods were found capable to detect and quantify, these toxins in both CRMs and on naturally contaminated samples of mussel *M. galloprovinicalis* and marine dinoflagellate *G. catenatum*. Both methods suffer from matrix effects. Comparing the two methods, no significant difference (paired t-test) for every determined saxitoxin analogue in the natural samples: contaminated mussel *M. galloprovinicalis* $t = 1.298$, $df = 1$, $p = 0.418$, SD of differences = 130.4; marine dinoflagellate *G. catenatum* $t = 0.03293$, $df = 4$, $p = 0.9753$, SD of differences = 80.09 was observed.

FLD analysis is known for its high selectivity as only few compounds undergo fluorescence. While saxitoxin and its analogues are primarily non-fluorescent compounds, oxidation of samples as an additional step and the need of different fractionation steps for different toxin groups make the whole procedure more tedious and time-consuming. And with the similarities in the structure of the different toxin analogues, the fluorescent compounds yielded from oxidation overlap each other making the analysis more difficult. And with reporting toxicity levels as saxitoxin equivalence shows overestimation where the TEF values for the toxin analogues are lower than 1. Overestimation of the toxicity is beneficial for implementing a stricter seafood safety management, however, may prevent sale of bivalves and other seafood in the area question. The use of a matrix modifier, aside from the matrix of the sample analyzed, is also difficult for routine analysis. This method cannot identify the presence of non-regulated toxins. Detection method for such non-regulated toxins, case-in-point, for the natural samples used in this study, is LC-HRMS.

The LC-HRMS method proposed in this work is a conservative approach to quantify the different toxin analogue through AMXIC while preserving the sample information for further analysis. While both HPLC-FLD and LC-HRMS can quantify individually the regulated toxin analogues, LC-HRMS can also identify the presence of non-regulated toxin analogues. Fragmentation analysis can aid in confirming the detection of the saxitoxin analogue without commercially available CRMs, by comparing fragmentation spectra with the literature. The graphitized SPE is better to achieve lower matrix effects. However, due to lack of CRMs the identified non-regulated analogues will only be possible to be semi-quantitated in the natural samples, whether in the bivalve or in the dinoflagellate. Overall, the use of LC-HRMS in identifying non-regulated saxitoxin analogues expands its application to international

regulations and seafood safety program., the HPLC-FLD could be used for screening of toxicity levels, while HPLC-HRMS provides saxitoxin profile. Although both methods can provide toxin profile to determine which toxin analogues contributes to total toxicity of sample, HPLC-HRMS is a more comprehensive choice due to detecting both regulated and non-regulates saxitoxin analogues.

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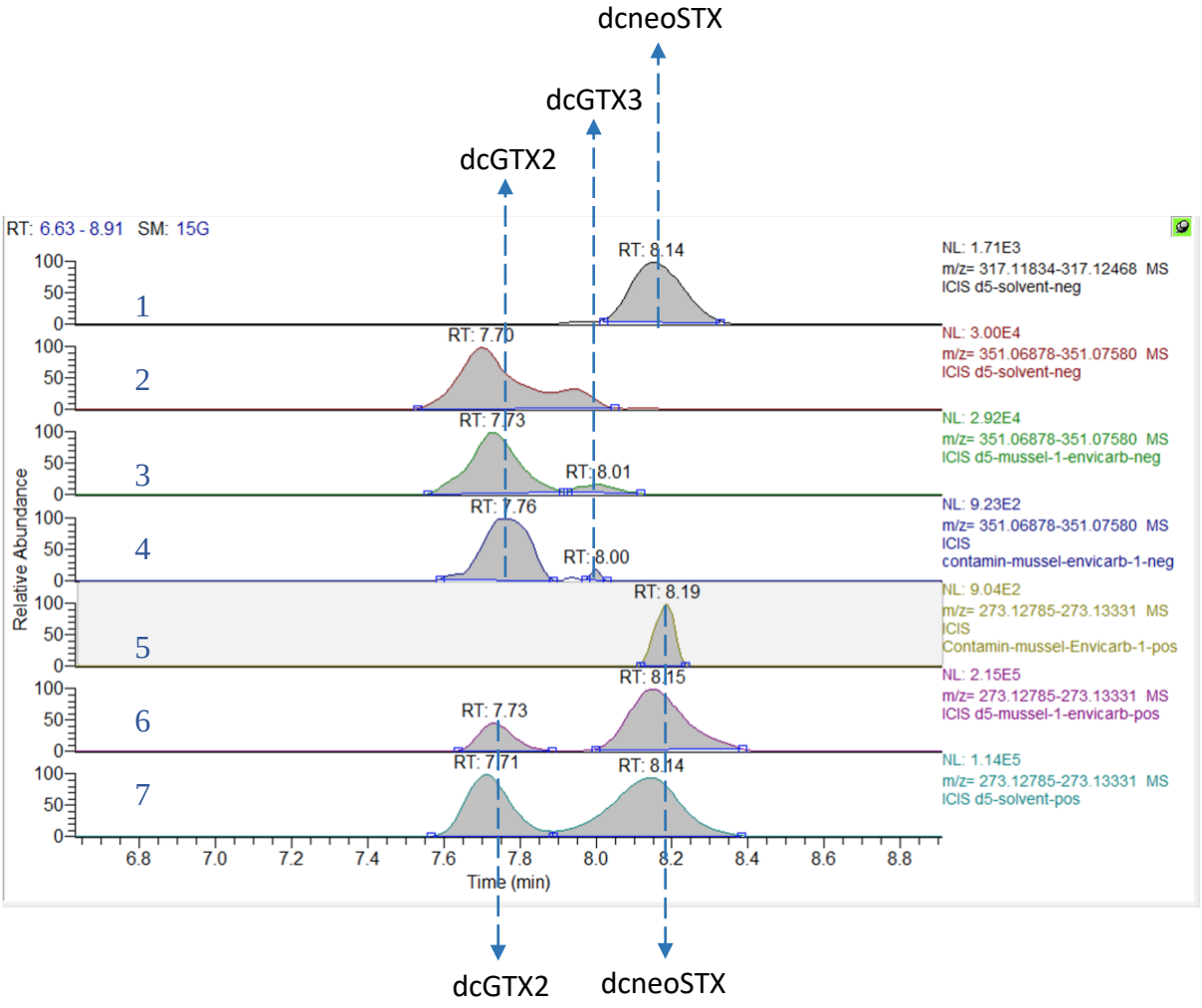
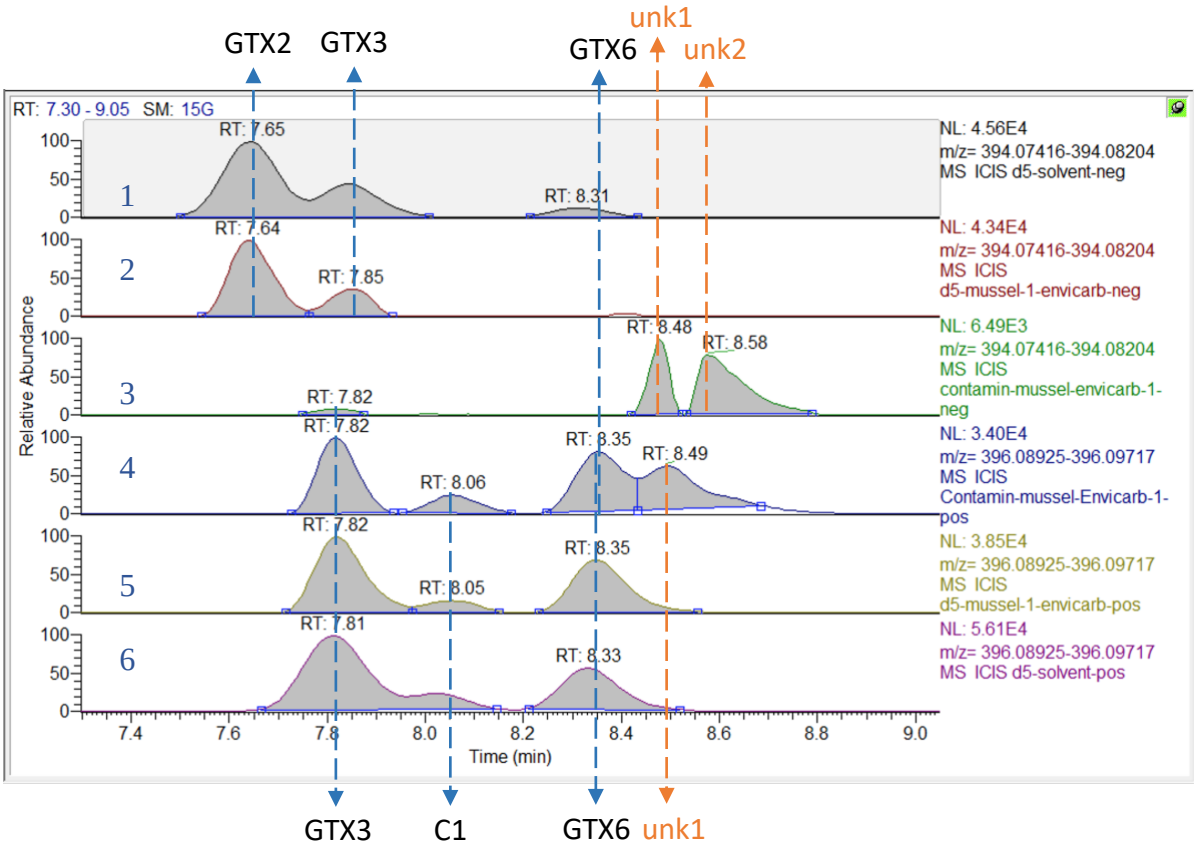
ANNEX

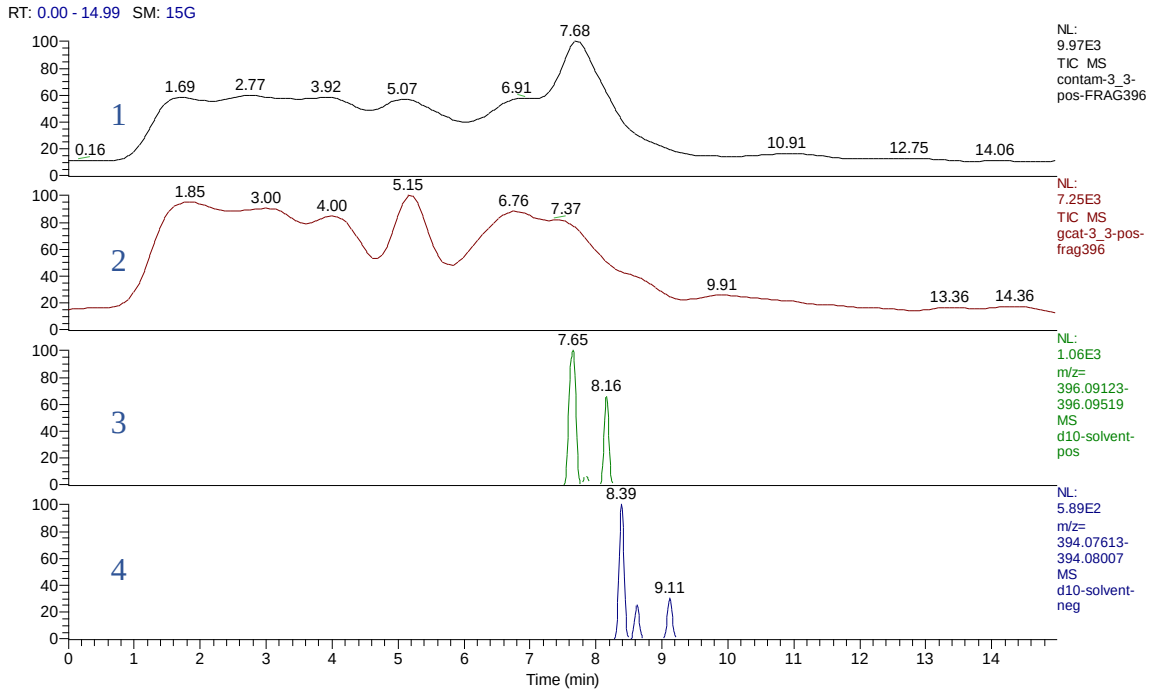
Annex A ESI^- m/z 351.07229 corresponds to saxitoxin analogues dcGTX2,3. ESI^- m/z 317.12151 and ESI^+ m/z 273.13058 corresponds to dcneoSTX; also shares the same values with dcM2 and dcM6, where it confirms its absence in the contaminated mussel sample. (1) Calibration standard in solvent, ESI^- m/z 317.12151 (2) Calibration standard in solvent, ESI^- m/z 351.07229 (3) Calibration standard in mussel matrix, graphitized carbon clean-up, ESI^- m/z 351.07229 (4) Contaminated mussel, graphitized carbon clean-up, ESI^- m/z 351.07229 (5) Contaminated mussel, graphitized carbon clean-up, ESI^+ m/z 273.13058 (6) Calibration standard in mussel matrix, graphitized carbon clean-up, ESI^+ m/z 273.13058 (7) Calibration standard in solvent, ESI^+ m/z 273.13058

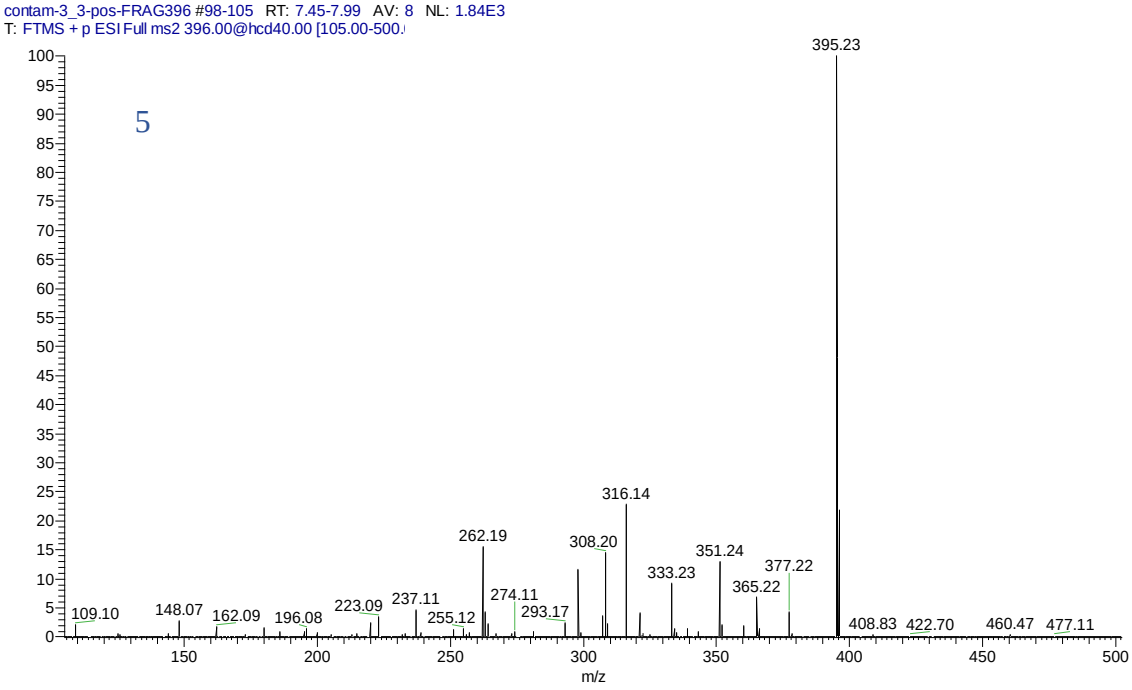


Annex B ESI$^-$ and ESI$^+$ m/z 394.07810 and 396.09321, respectively, corresponding to GTX3, GTX6, C2, M1, and M5. GTX2 is also accounted for but is quantified under ESI$^+$ m/z 316.13639. Unk1 and Unk2 can be attributed to the non-regulated analogues M1 and M5. (1) Calibration standard in solvent, ESI$^-$ m/z 394.07810; (2) Calibration standard in mussel matrix, graphitized carbon clean-up, ESI$^-$ m/z 394.07810; (3) Contaminated mussel, graphitized carbon clean-up, ESI$^-$ m/z 394.07810; (4) Contaminated mussel, graphitized carbon clean-up, ESI$^+$ m/z 396.09321; (5) Calibration standard in mussel matrix, graphitized carbon clean-up, ESI$^+$ m/z 396.09321; (6) Calibration standard in solvent, ESI$^+$ m/z 396.09321.

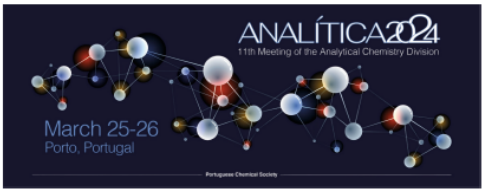


Annex C Fragmentation analysis of (1) contaminated mussel and (2) dinoflagellate compared to the CRM counterparts (3) at ESI^+ *m/z* 396 and (4) ESI^- 394. No clear identification as shown in spectrum (5); interference from unknown compounds which could potentially be M1 and/or M5.





Annex D Annotation study of contaminated mussel sample presented as poster at the Analítica 2024 in Porto, Portugal on 25-26 March 2024



DETECTION OF NON-REGULATED SAXITOXIN ANALOGUES IN MUSSELS BY LC-HRMS

UAlg FCT UNIVERSIDADE DO ALGARVE FACULDADE DE CIÊNCIAS E TECNOLOGIA CCMAR Centro de Ciências do Mar IPMA

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Saxitoxins (STX) are a family of marine biotoxins associated with paralytic shellfish poisoning. These compounds bioaccumulate in filter-feeder bivalves[1,2] through marine dinoflagellates. This family includes both regulated and non-regulated analogues. Regulated forms have been thoroughly researched and have commercially available CRMs. Most non-regulated toxin analogues, on the other hand, do not, and need to be annotated first and then quantified using a relative molar response of a structure related to regulated analogue with available CRM.
By using a natural sample, taken in December 2016 from contaminated mussel *Mytilus galloprovincialis* after a *Gymnodinium catenatum* bloom in Aveiro, Portugal, we demonstrate that LC-HRMS is a powerful technique to annotate non-regulated toxin analogues via chemical induced dissociation (CID) and higher-energy collisional dissociation (HCD). This mussel sample was cleaned up using a SPE-HILIC matrix, which gave rise to lower matrix effects than its C18 cartridge counterpart.

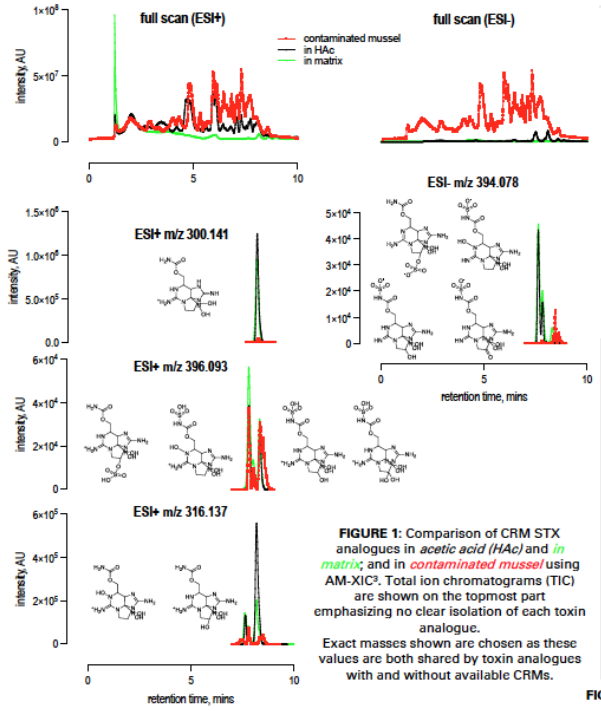

FIGURE 1: Comparison of CRM STX analogues in *acetic acid (HAc)* and *in matrix*; and in *contaminated mussel* using AM-XIC[3]. Total ion chromatograms (TIC) are shown on the topmost part emphasizing no clear isolation of each toxin analogue.
Exact masses shown are chosen as these values are both shared by toxin analogues with and without available CRMs.

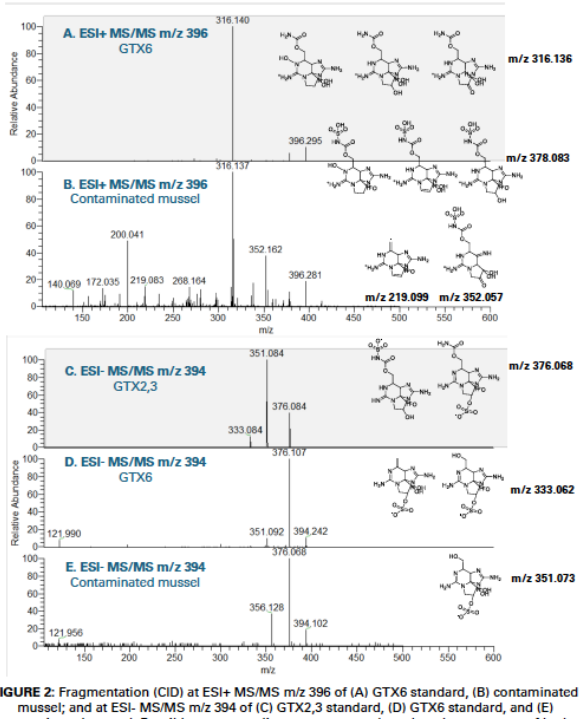

FIGURE 2: Fragmentation (CID) at ESI+ MS/MS m/z 396 of (A) GTX6 standard, (B) contaminated mussel; and at ESI- MS/MS m/z 394 of (C) GTX2,3 standard, (D) GTX6 standard, and (E) contaminated mussel. Possible corresponding structures are based on the structures of both regulated and non-regulated toxins.

TABLE 1: Identification of saxitoxin and analogues found in the contaminated mussel using different polarity of ionization (ESI)

m/z		Saxitoxin analogues	
(+)	(-)	With available CRM	Without CRM
300.141		STX	
257.136		dcSTX	
380.098		GTX5	
	317.121	dcGTX2; dcneo	
353.087		dcGTX3	
316.136		GTX2; neo	M2; M6
396.093		GTX3; C1,C2; GTX6	M1; M5
332.131		GTX1	M4;M8
412.088		GTX4	C3,C4; M3/M7
	351.072	dcGTX2,3	
	394.078	GTX2,3; GTX6	M1; M5
	474.035	C1,C2	
	410.073	GTX1,4	M3, M7
	392.117		M10
	333.116		dcM4, dcM8
	367.067		dcGTX1,4

TABLE 2: Annotation of possible saxitoxin analogues from the contaminated mussel

Precursor ion (m/z ± 0.50)	Possible analogues	LCMS data contaminated mussel (from Figure 1)	Interpretation
316	GTX2; neo M2; M6	<LOQ: GTX2 and neo	
396	GTX3; C1,C2; GTX6 M1; M5	Presence of C2 GTX6 has a different spectrum (Figure 2)	M-toxins present (Figure 2)
332	GTX1 M4; M8	<LOQ: GTX1,4	
412	GTX4 C3, C4; M3/M7	<LOQ: GTX4 Presence of C3, C4; same RT in the ESI-	2 analogues of M-toxins are present with shoulder peaks (Figure 1)
394	GTX2,3; GTX6 M1; M5	<LOQ: M-toxins	

<LOQ based on the calibration curve of each STX analogue.

Eight non-regulated toxins were annotated. All these fall under the M-toxins subgroup. Because these analogues have similar core structures, the detection method must be carefully chosen. The LC-HRMS method **is highly selective**, even for similar moieties, and can be used to annotate each analogue. It is also a valuable tool for reporting toxin profiles of contaminated samples that are analyzed for the first time, even in the absence of CRMs.

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