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## Matrix effect on paralytic shellfish toxins quantification and toxicity estimation in mussels exposed to *Gymnodinium catenatum*

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### ► To cite this version:

Maria João Botelho, Carlos Vale, Ana Maria Mota, Susana Margarida Rodrigues, Pedro Reis Costa, et al.. Matrix effect on paralytic shellfish toxins quantification and toxicity estimation in mussels exposed to *Gymnodinium catenatum*. *Food Additives and Contaminants*, 2010, 27 (12), pp.1724-1732. 10.1080/19440049.2010.525753 . hal-00643847

**HAL Id: hal-00643847**

**<https://hal.science/hal-00643847>**

Submitted on 23 Nov 2011

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**Matrix effect on paralytic shellfish toxins quantification and toxicity estimation in mussels exposed to *Gymnodinium catenatum***

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2010-200.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	06-Sep-2010
Complete List of Authors:	Botelho, Maria João; IPIMAR – Instituto Nacional de Recursos Biológicos Vale, Carlos; IPIMAR – Instituto Nacional de Recursos Biológicos Mota, Ana; Centro de Química Estrutural, Instituto Superior Técnico Rodrigues, Susana; IPIMAR – Instituto Nacional de Recursos Biológicos Costa, Pedro; IPIMAR – Instituto Nacional de Recursos Biológicos Gonçalves, Maria; Centro de Química Estrutural, Instituto Superior Técnico
Methods/Techniques:	Chromatography - HPLC
Additives/Contaminants:	Phycotoxins – PSP
Food Types:	Seafood

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Manuscripts

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3 **Matrix effect on paralytic shellfish toxins quantification and toxicity**  
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6 **estimation in mussels exposed to *Gymnodinium catenatum***  
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31  
32 **Abstract**  
33

34  
35 Paralytic shellfish toxins were quantified in whole tissues of the mussel *Mytilus*  
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37 *galloprovincialis* exposed to blooms of the dinoflagellate *Gymnodinium catenatum* in  
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39 Portuguese coastal waters. A validated HPLC method with fluorescence detection,  
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41 involving pre-chromatographic oxidation was used to quantify carbamoyl, N-  
42  
43 sulfocarbamoyl and decarbamoyl toxins. In order to test for any matrix effect in the  
44  
45 quantification of those toxins, concentrations obtained from solvent and matrix matched  
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47 calibration curves were compared. A suppression of the fluorescence signal was  
48  
49 observed in mussel extract or fraction in comparison to solvent for the compounds  
50  
51 dcGTX2+3, GTX2+3 and GTX1+4, while an enhancement was found for C1+2,  
52  
53 dcSTX, STX, B1, dcNEO and NEO. These results showed that a matrix effect varies  
54  
55 among compounds. The difference of concentrations between solvent and matrix  
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57 matched calibration curves for C1+2 (median=421 ng g<sup>-1</sup>) exceeded largely the values  
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3 for the other quantified compounds (0.09-58 ng g<sup>-1</sup>). Those differences were converted  
4 into toxicity differences, using Oshima toxicity equivalence factors. The compounds  
5 C1+2 and dcNEO were the major contributors to the differences of total toxicity in the  
6 mussel samples. The differences of total toxicity were calculated in ten mussel samples  
7 collected during a 10-week blooming period in Portuguese coastal lagoon. Values  
8 varied between 53 and 218 µg STX equivalents kg<sup>-1</sup>. The positive differences mean that  
9 the estimated toxicity using solvent calibration curves exceed the values taking into  
10 account the matrix. For the toxicity interval 200-800 µg STX equivalents kg<sup>-1</sup> an  
11 increase was found between 44 and 28%.

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27 **Keywords:** paralytic shellfish poisoning; phycotoxins; saxitoxin; LC-FLD

## 28 29 30 31 **Introduction**

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33 Paralytic shellfish toxins (PSTs) are responsible for one of the most severe forms of  
34 seafood poisoning (Gessner and Middaugh 1980; Sommer and Meyers 1937). At least  
35 20 toxins have been identified (Llewellyn 2006). These toxins are produced naturally by  
36 microalgae species of the dinoflagellate genus *Alexandrium*, *Pyrodinium* and  
37 *Gymnodinium* (Masó and Garcés 2006). The better documented were grouped in three  
38 classes according to their chemical structure: carbamoyl toxins (saxitoxin-STX,  
39 neosaxitoxin-NEO and gonyautoxins-GTX1 to GTX4), N-sulfocarbamoyl toxins (B1,  
40 B2, C1 to C4), and decarbamoyl toxins (dcGTX1 to dcGTX4, dcSTX and dcNEO)  
41 (Figure 1). The mechanism of these compounds consists of the blockage of the sodium  
42 channels of nerve membranes, resulting in stoppage of the propagation of neural  
43 impulses and the paralysis of neuromuscular systems (Lagos and Andrinolo 2000).  
44 Different toxicities result from specific binding affinity to voltage-gated sodium channel

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3 receptors (Genenah and Shimizu 1981). The toxicity of each compound has been  
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5 estimated by mouse bioassays and expressed in terms of mouse units. The carbamoyl  
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7 group contains the most toxic compounds, the N-sulfocarbamoyl group contains the  
8  
9 least toxic, and the decarbamoyl group have intermediate toxicities (Oshima 1995).  
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13 During the last decades, chemical methodologies for PSTs determination have been  
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15 under development and validation, attempting to replace the mouse bioassay as the  
16  
17 official method (Anon.1990). A liquid chromatography method with fluorescence  
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19 detection (LC-FLD) involving a pre-column oxidation using hydrogen peroxide and  
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21 periodate was adopted by the Association of Official Analytical Chemists as an official  
22  
23 method for determination of PSTs in shellfish (Anon. 2006). After validation through a  
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25 collaborative study for STX, NEO, GTX2+3, GTX1+4, dcSTX, B1 (GTX5), C1+2 and  
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27 C3+4 in shellfish (mussels, clams, oysters and scallops) the method was integrated in  
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29 European Directives to act as a legal alternative to the mouse bioassay (European Union  
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31 Commission 2006; Lawrence et al. 2005). Recently, a refinement and extension of the  
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33 AOAC method including the additional toxins dcNEO and dcGTX2+3 offers  
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35 possibilities to achieve reliable analytical alternatives (Turner et al. 2009).  
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41 The AOAC method, also called “the Lawrence method”, includes a C18 oyster  
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43 cleaned extract as a matrix modifier in the periodate reaction in order to enhance the  
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45 fluorescence intensity of N-hydroxylated toxins oxidation products (Lawrence and  
46  
47 Niedzwiadek , 2001). No added matrix modifier is foreseen for the peroxide oxidation  
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49 running under aqueous solution due to the adequate detection of the products of  
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51 oxidation. The method was primarily developed for shellfish samples contaminated by  
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53 *Alexandrium* spp blooms, having N-sulfocarbamoyl and decarbamoyl toxins, which are  
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55 quantified after peroxide oxidation and have a lower contribution to the PSTs profile  
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60 (Lawrence and Menard 1991). A different situation occurs with shellfish contaminated

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3 by *Gymnodinium catenatum* a common bloom in Portuguese and Galician waters. The  
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5 compounds dcGTX2+3, dcSTX (decarbamoyl) and C1+2, B1 (N-sulfocarbamoyl)  
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7 emerge as major components of the toxin profiles (Artigas et al. 2007; Franca et al.  
8  
9 1996; Oshima 1990; Vale et al. 2008) and, according to the AOAC method, their  
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11 quantification can be carried out without a matrix modifier.  
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15 This work tested whether the matrix effect is relevant in the quantification of N-  
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17 sulfocarbamoyl and decarbamoyl toxins, as well as other toxins, in mussels naturally  
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19 contaminated by *Gymnodinium catenatum* using solvent and matrix matched calibration  
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21 curves. The impact of this effect on the estimation of the total toxicity is examined.  
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## 27 **Materials and methods**

### 28 *Shellfish sample preparation*

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30 The shellfish samples used in this work were: (i) uncontaminated mussels (*Mytilus*  
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32 *galloprovincialis*) from Viana, west coast of Portugal, used as matrix on toxin  
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34 determination; (ii) mussels collected weekly during a 10-week period from Aveiro  
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36 lagoon, west coast of Portugal, exposed to repeated blooms of *Gymnodinium catenatum*,  
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38 to assessing the repercussion of matrix effect on the toxicity calculation; (iii)  
39  
40 uncontaminated oysters (*Crassostrea* spp) from Formosa lagoon, south of Portugal,  
41  
42 used as matrix modifier following the Lawrence method (Anon. 2006). Each bivalve  
43  
44 was cleaned externally with fresh water, opened and the whole soft tissues removed  
45  
46 from the shell, rinsed and drained. Composite mussel samples (n=30 individuals) of soft  
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48 tissues were blended, mechanically homogenised and stored at -18°C until required for  
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50 determinations. The same procedure was used for the preparation of the oyster  
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52 composite sample (n=15).  
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### *Extraction, cleanup and oxidation of samples*

The extraction, cleanup and oxidation procedures for PSTs determination were based on the AOAC method (Anon. 2006) involving the following steps: (i) acid duplicate extraction of the composite shellfish sample with acetic acid solution (first extraction with heating); (ii) cleanup using a solid phase extraction (SPE) 500 mg C18 cartridge (Supelclean<sup>®</sup>, Supelco, Bellefonte, PA, USA); (iii) an aliquot of C18-cleaned extract is added to a SPE ion exchange cartridge (SPE-COOH) (Bakerbond<sup>®</sup>, J.T. Baker, Phillipsburg, NJ, USA) being sequentially eluted to obtain three individual fractions (fraction 1 to 3); (iv) hydrogen peroxide (10% v/v) is added to 1M NaOH solution and vortex mixed, then a PST standard solution or sample extract after C18 is added, and the mixture thoroughly mixed and allowed to react for 2 min at room temperature; glacial acetic acid is added and vortex mixed; (v) a PST standard solution or a sample extract after C18 or SPE-COOH fractionation is added to a matrix modifier solution prepared with PST-free oysters, and then is added the periodate oxidant; the mixture is thoroughly mixed and allowed to react during 1 min., then glacial acetic acid is added and the mixture is allowed to stand for a further 10 min.

Aliquots of C18 extract are used for oxidation of PSTs with peroxide and periodate oxidants, while aliquots of SPE-COOH fractions are used for periodate oxidation, prior to LC analyses.

### *Liquid chromatography analysis*

The LC analysis was carried out by a Hewlett-Packard /Agilent system (Palo Alto, CA, USA) consisted of a 1050 Series quaternary pump, 1100 Series in-line degasser, autosampler, column oven, and 1200 Series fluorescence detector. The Agilent Chemstation<sup>®</sup> software (revision A.10) performed data acquisition and peak integration.

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3 Separation was performed using a reversed-phase column Supelcosil<sup>®</sup> C18, 15x4.6 mm  
4 id, 5 µm (Supelco, Bellefonte, PA, USA) equipped with a guard column Supelguard  
5  
6 Supelcosil<sup>®</sup> C18, 2x4.0 mm id, 5 µm (Supelco, Bellefonte, PA, USA). Column was  
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8 kept in a column oven at 30°C. Detection wavelengths were set at 340 nm for excitation  
9  
10 and 395 nm for emission. The mobile phase gradient used to elute the PST oxidation  
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12 products consisted of 2 mobile phases under the following conditions: 0-5 % B (0.1 M  
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14 ammonium formate in 5 % acetonitrile, pH = 6) in the first 5 min, 5-70% B for the next  
15  
16 4 min and 100% mobile phase A (0.1 M ammonium formate, pH = 6) was used for 5  
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18 min before next injection. The flow rate was 1 mL min<sup>-1</sup>. The injection volumes were 50  
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20 µL and 100 µL, for the oxidation products of peroxide and periodate reaction,  
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22 respectively.  
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29 Certified calibration solutions for PSTs were purchased from the Certified Reference  
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31 Materials Program of the Institute for Marine Biosciences, National Research Council,  
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33 Halifax, Canada (CRM-STX-e, CRM-NEO-b, CRM-GTX2&3-b, CRM-GTX1&4-b,  
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35 CRM-dcSTX, CRM-dcGTX2&3, CRM-GTX5-b (B1), CRM-C1&2 and CRM-dcNEO-  
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37 b). Working standard solutions were prepared by appropriate dilution of the  
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39 corresponding stock solution and stored at 4°C during 1 month. All PSTs were  
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41 identified and quantified by comparison peak areas of sample oxidation products and of  
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43 working standards. The compounds GTX2+3, GTX1+4, C1+2 and C3+4 were  
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45 quantified jointly due to the fact that the oxidation process creates the same oxidation  
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47 product.. Due to the lack of standards for C3+4 and B2 their quantification was not  
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49 achieved. However, identification was done taking into account their retention time in  
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51 the LC method (Ben-Gigirey et al. 2007; Lawrence and Niedzwiadek 2001). All  
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53 chemical and reagents were LC or analytical grade. Acetonitrile, acetic acid, methanol,  
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55 ammonium formate, ammonium acetate and disodium hydrogen phosphate were  
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3 purchased from Sigma-Aldrich (Steinheim, Germany). Periodic acid, sodium hydroxide,  
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5 hydrogen peroxide and sodium chloride were from Merck (Darmstadt, Germany).  
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8 Ultrapure water was obtained by Milli-Q 185 Plus system Millipore (Bedford, MA,  
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10 USA).  
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### 12 13 14 15 ***Procedural modification***

16  
17 The procedural modification used in this work is centered on the inversion of reactions  
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19 sequence of AOAC method (Anon. 2006), the peroxide oxidation being performed  
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21 before the periodate oxidation. Figure 2 shows a flow diagram highlighting the  
22  
23 procedural modification. The reason for this inversion comes from non-N-hydroxylated  
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25 toxins (B1, C1+2, dcSTX, dcGTX2+3) being the most abundant ones in shellfish  
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27 samples contaminated by *Gymnodinium catenatum* from Iberian waters. In the AOAC  
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29 method, these compounds are also quantified in the peroxide oxidation, due to a better  
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31 detection of the oxidation products, but after a periodate oxidation. The dcNEO toxin  
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33 was quantified after the periodate oxidation of the C18 extract, avoiding dilution effect  
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35 during the SPE-COOH fractionation.  
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41 To resolve the overlapped areas of dcSTX and dcNEO frequently observed in  
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43 shellfish samples contaminated by *Gymnodinium catenatum*, dcNEO was quantified by  
44  
45 subtracting the dcSTX contribution. This contribution of dcSTX was estimated from the  
46  
47 calibration curve oxidized with periodate, using the concentration obtained previously  
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49 under peroxide oxidation. A similar calculation was applied to quantify (i) GTX1+4 in  
50  
51 the extract from fraction 2 followed by periodate oxidation, subtracting the contribution  
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53 of dcGTX2+3, and (ii) NEO in the extract from fraction 3 followed by periodate  
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55 oxidation, subtracting the contribution of dcNEO and dcSTX.  
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### *Test to matrix effect*

To test the matrix effect in the quantification of toxins, two trials of working standard solutions for calibration curves were prepared by dilution of PSTs stock solutions in 0.1 mM acetic acid and in cleaned-up mussel tissue extract and subsequent fractions. Two standard toxin mixtures were prepared: Mix-I containing GTX1+4 and NEO; Mix-II including GTX2+3, dcGTX2+3, STX, dcSTX, B1 and C1+2. The dcNEO was used individually in working standard solutions. The matrix effect in the peroxide oxidation tested in this procedure is not foreseen in the Lawrence method. The oyster C18 cleaned extract is only used in the periodate reaction.

### *Limits of detection, linearity and recovery*

The instrumental limits of detection (LOD) were experimentally determined using a signal:noise ratio of 3:1. The LODs ranged from 12 ng g<sup>-1</sup> for B1 and STX to 99 ng g<sup>-1</sup> for GTX1+4. These values, expressed in µg STX equivalents kg<sup>-1</sup>, were comparable to those quoted in the AOAC method. The linear range of PSTs varied from 67-80 times the respective LOQ (calculated using signal:noise ratio of 10:1) to 100-250 times for GTX2+3, dcGTX2+3, STX, dcSTX, B1 and C1+2. Recoveries of the analytical procedure were assessed through mussel tissues spiked in triplicate, with the addition of two standard mixtures at two levels of contamination. For most of the non-N-hydroxylated compounds and for GTX1+4 and NEO recoveries varied between 77 and 116%. The lower recovery obtained for C1+2 (58%) may be attributed to their thermal instability during spiking procedure. This explanation is supported by results obtained in storage stability tests (Indrasena and Gill 2000).

## Results and discussion

### *Calibration curves in solvent and mussel matrix*

Equations for the calibration curves for the calculation of PST concentrations, including the compounds quantified after peroxide and periodate oxidations, showed linear fits with good correlation coefficients for the linearity intervals in solvent and C18-cleaned mussel extract or fractions (Table 1). The mussel extract or fraction exhibited a suppression of the fluorescence signal in comparison to solvent for dcGTX2+3, GTX2+3 and GTX1+4, while an enhancement was obtained for C1+2, STX, dcSTX, B1, dcNEO and NEO. These results corroborate previous findings, indicating that the chromatographic signal associated with matrix effect varied among the compounds (Turner et al 2009). The results of the current study are not fully comparable to ones reported by Turner et al. (2009) since some toxins were quantified in different steps of the analytical procedure.

Figure 3 illustrates three examples of calibration curves in solvent and mussel extract. The GTX2+3 in which the slope in solvent doubled the one in mussel extract, indicated a divergence between the two lines as concentration increases. Consequently, the matrix effect becomes more effective as mussels contain high levels of these compounds. The two lines for C1+2 presented similar slopes and higher Y-intercept value for mussel extract than for solvent. The parallelism of the two lines indicates that matrix effect is similar within the linearity concentration interval. Similar slopes and Y-intercept values were found in solvent and mussel extract for B1, indicating a negligible matrix effect.

### *PSTs in mussels exposed to toxic algae blooms*

Ten composite samples of mussels naturally exposed to blooms of *Gymnodinium catenatum* in Aveiro lagoon were analysed by the above mentioned procedure. Figure 4

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3 illustrates the LC-FLD chromatograms of a selected sample. The peaks obtained after  
4 peroxide or periodate oxidation in C18-cleaned extract are shown. Sharp peaks were  
5 seen at the response time of working standard solutions used for their quantification.  
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7 Naturally fluorescent co-extractives had no interference with toxin peaks. The peak  
8 areas of quantified compounds in the chromatograms were converted into  
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10 concentrations using the calibration curves calculated for solvent and C18-cleaned  
11 mussel extracts. Figure 5 shows the median, the percentile 25% and 75%, minimum  
12 and maximum of each toxin concentrations found in the mussel samples after  
13 calibration curves prepared with solvent and mussel extract. Except for dcGTX2+3 no  
14 significant differences ( $p < 0.05$ ) were found for all the quantified compounds. C1+2 and  
15 B1 were the most abundant toxins registered in the mussel samples, their concentrations  
16 being substantially higher than levels of dcGTX2+3, dcSTX, GTX2+3, STX and  
17 dcNEO. The observed abundance of N-sulfocarbamoyl (B1, C1+2) in mussel extracts is  
18 in agreement with the *Gymnodinium catenatum* profile described for Portuguese waters  
19 (Negri et al. 2007). Mussel metabolic processes may have enhanced the levels of  
20 decarbamoyl compounds reported for the algae (e.g., dcGTX2+3 and dcSTX) (Artigas  
21 et al. 2007; Oshima 1995). Values of dcNEO may also be related to either its presence  
22 in *Gymnodinium catenatum* (Costa et al. 2009), or resulted from metabolic products of  
23 assimilated toxins in mussels.  
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48 The broad range found for C1+2 and B1 concentrations, and in a less extent for other  
49 minor toxins, may have resulted from the repeated blooms of *Gymnodinium catenatum*  
50 exhibiting different toxin profiles. Indeed, a study of toxin profiles of this dinoflagellate  
51 in the Gulf of California pointed to differences among strains in different blooms  
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58 (Band-Schmidt et al. 2005).  
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### Impact of matrix effect on individual concentration and toxicity

The matrix effect was assessed by the differences of toxin concentrations ( $\Delta C_i$ , ng g<sup>-1</sup>) calculated for each compound  $i$  by the following expression:

$$\Delta C_i = C_{i\text{-solvent}} - C_{i\text{-matrix}} \quad (1)$$

where  $C_{i\text{-solvent}}$  and  $C_{i\text{-matrix}}$  are the concentrations of the compound  $i$  calculated by the calibration curves in solvent and mussel extract or fraction, respectively.

Using the Oshima toxicity equivalence factors ( $TEF_i$ ) and conversion factor for units ( $CF_i$ ,  $\mu\text{g STX equivalents ng}^{-1}$ ) (Quilliam 2007), the calculated  $\Delta C_i$  were converted into differences of individual toxicities ( $\Delta T_i$ ,  $\mu\text{g STX equivalents kg}^{-1}$ ) by the expression:

$$\Delta T_i = \Delta C_i \times TEF_i \times CF_i \quad (2)$$

In the case of isomeric pairs (GTX1+4, GTX2+3, C1+2 and dcGTX2+3), the highest toxicity factor was used for each pair, and the toxicity factor of dcSTX was applied for dcNEO.

Figure 6 gives the median, the percentile 25% and 75%, minimum and maximum of  $\Delta C_i$  and  $\Delta T_i$ , for each quantified compound in the mussel samples. The elevated  $\Delta C_i$  obtained for C1+2 (median=421 ng g<sup>-1</sup>) reflects the considerable difference of calibration equation parameters in solvent and matrix for the same chromatographic peak areas. The other dominant compound of the toxin profile is B1, although showing a small  $\Delta C_i$  (median=46 ng g<sup>-1</sup>). The similarity between the two calibration curves resulted in a negligible matrix effect for B1. All the other compounds presented also small  $\Delta C_i$  (medians between 0.09 and 58 ng g<sup>-1</sup>). By converting  $\Delta C_i$  into  $\Delta T_i$ , the differences between C1+2 and the other toxins are less pronounced. The  $\Delta T_{C1+2}$  and  $\Delta T_{dcNEO}$  were not significantly ( $p < 0.05$ ) different. The high  $TEF_{dcNEO}$  (0.51) in comparison to  $TEF_{C1+2}$  (0.10) compensates the differences between  $\Delta C_{C1+2}$   $\Delta C_{dcNEO}$ . In

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3 the contaminated mussels used in this study the two compounds became thus the major  
4 contributors to the differences on total toxicity.  
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### 10 ***Impact on total toxicity***

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12 For each sample, the difference on the total toxicity ( $\Delta T$ ,  $\mu\text{g STX equivalents kg}^{-1}$ ) was  
13 calculated by summing  $\Delta T_i$  for all quantified toxins. The  $\Delta T$  calculated for the ten  
14 mussel samples varied between +53 and +218  $\mu\text{g STX equivalents kg}^{-1}$ . The positive  
15 values indicate that the toxicity estimation using solvent calibration curves exceeds the  
16 values taking into account the matrix. The use of calibration curves in solvent leads thus  
17 to an overestimation of the total toxicity in mussels. Figure 7 presents the  $\Delta T$  values  
18 against the total toxicity estimated by the mussel calibration curves. The values  
19 increased with the toxicity of the mussel sample. Considering the toxicity interval  
20 between 200 and 800  $\mu\text{g STX equivalents kg}^{-1}$  (regulatory level for PSTs), the obtained  
21  $\Delta T$  varied between 88 to 223  $\mu\text{g STX equivalents kg}^{-1}$ , which corresponds to 44% and  
22 28% of the toxicity level, respectively. These results point to a considerable over-  
23 estimation of toxicity in mussels exposed to *Gymnodinium catenatum* that contain high  
24 levels of C1, C2 and B1. Further studies should be performed in order to assess whether  
25 matrix effect has an additional impact on toxicity calculation for a more diverse toxin  
26 profile.  
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### 50 **Acknowledgments**

51  
52 The Portuguese Foundation for Science and Technology supported this study through  
53 the research grant PTDC/MAR/78997/2006.  
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Table 1. Equations of the calibration curves in solvent and C18-cleaned mussel extract or fractions for PSTs; toxin concentration ranges and correlation coefficients.

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Toxin	Concentration range (ng g <sup>-1</sup> )	Matrix	Equation	r
dcGTX2+3	101-1023	Solvent	Y=0.25x-7.6	0.996
		Mussel extract	Y=0.13x+19	0.985
C1+2	274-2814	Solvent	Y=0.16x-23	0.995
		Mussel extract	Y=0.21x+17	0.988
dcSTX	40-406	Solvent	Y=1.1x-18	0.999
		Mussel extract	Y=1.1x+0.058	0.997
GTX2+3	120-1234	Solvent	Y=0.49x-14	0.997
		Mussel extract	Y=0.24x+14	0.993
B1	97-980	Solvent	Y=0.25x-9.1	0.999
		Mussel extract	Y=0.26x-1.2	0.999
STX	48-479	Solvent	Y=0.39x-9.7	0.997
		Mussel extract	Y=0.40x+0.68	0.997
dcNEO	146-1199	Solvent	Y=0.12x-0.57	0.994
		Mussel extract	Y=0.15x+4.6	0.996
GTX1+4	312-1870	Solvent	Y=0.18x+1.2	0.995
		Mussel fraction	Y=0.16x+15	0.997
NEO	272-1925	Solvent	Y=0.04x+2.6	0.988
		Mussel fraction	Y=0.05x-3.6	0.995

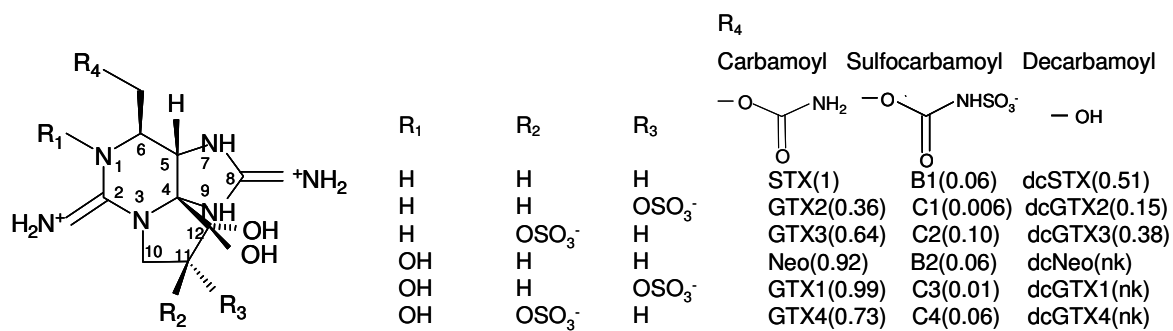


Figure 1.

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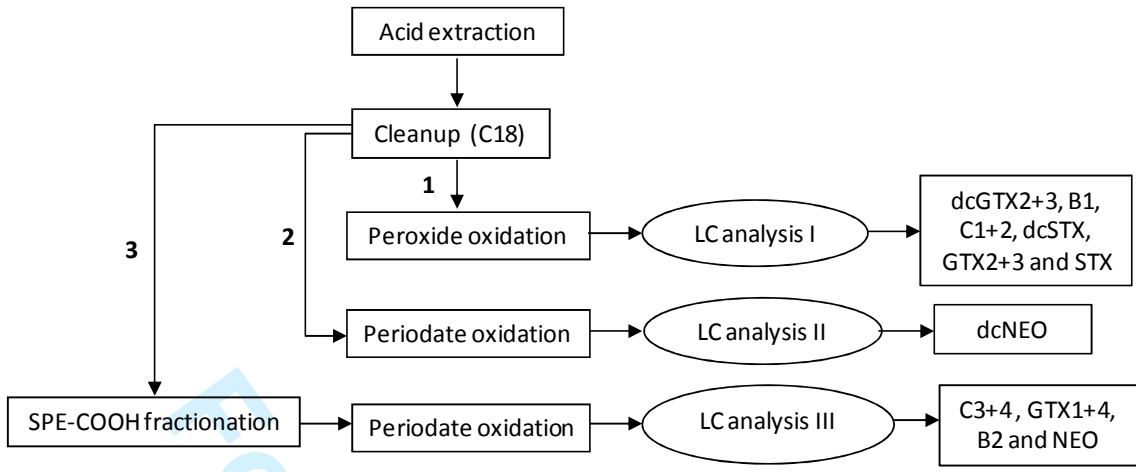


Figure 2.

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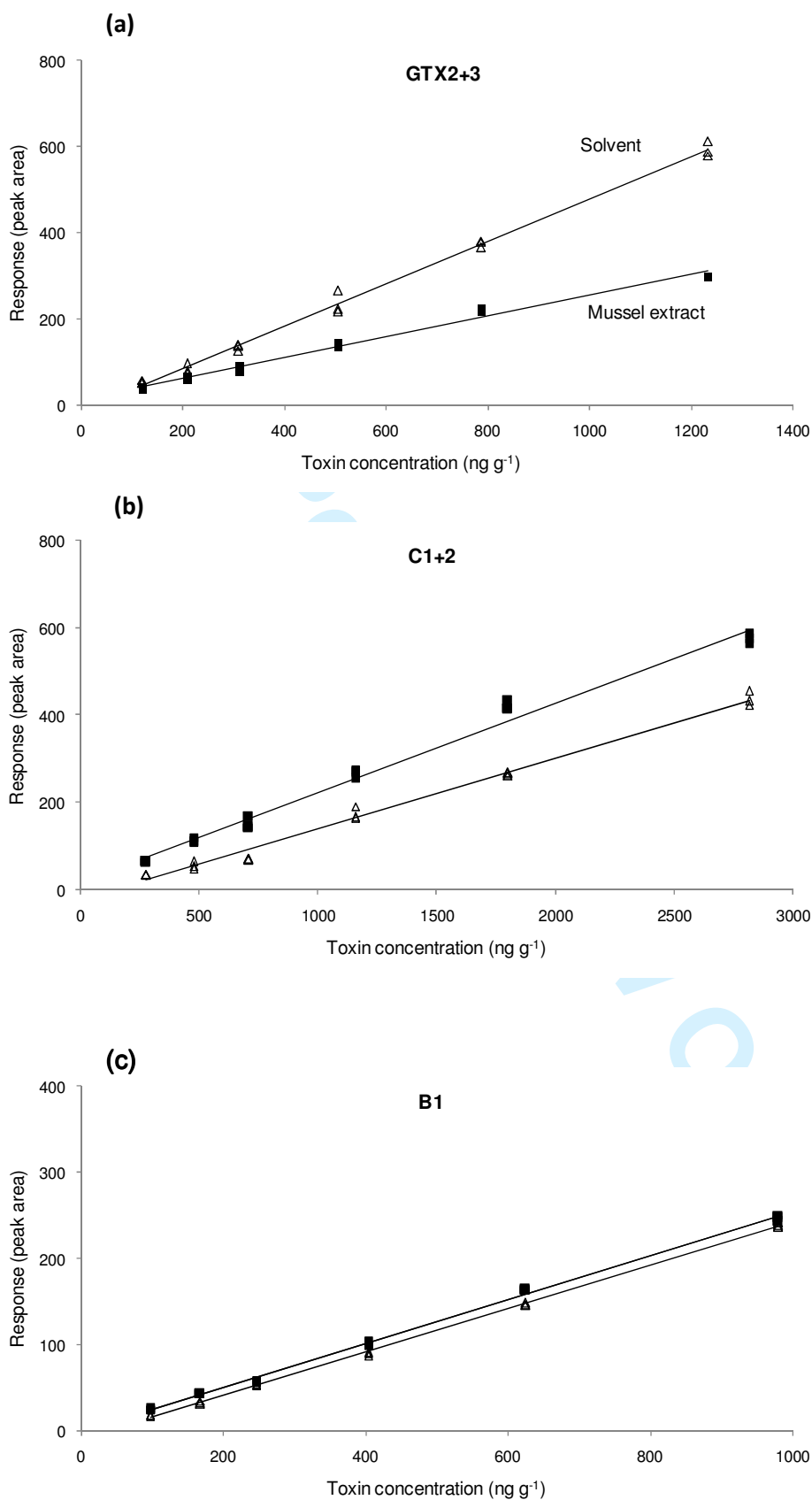


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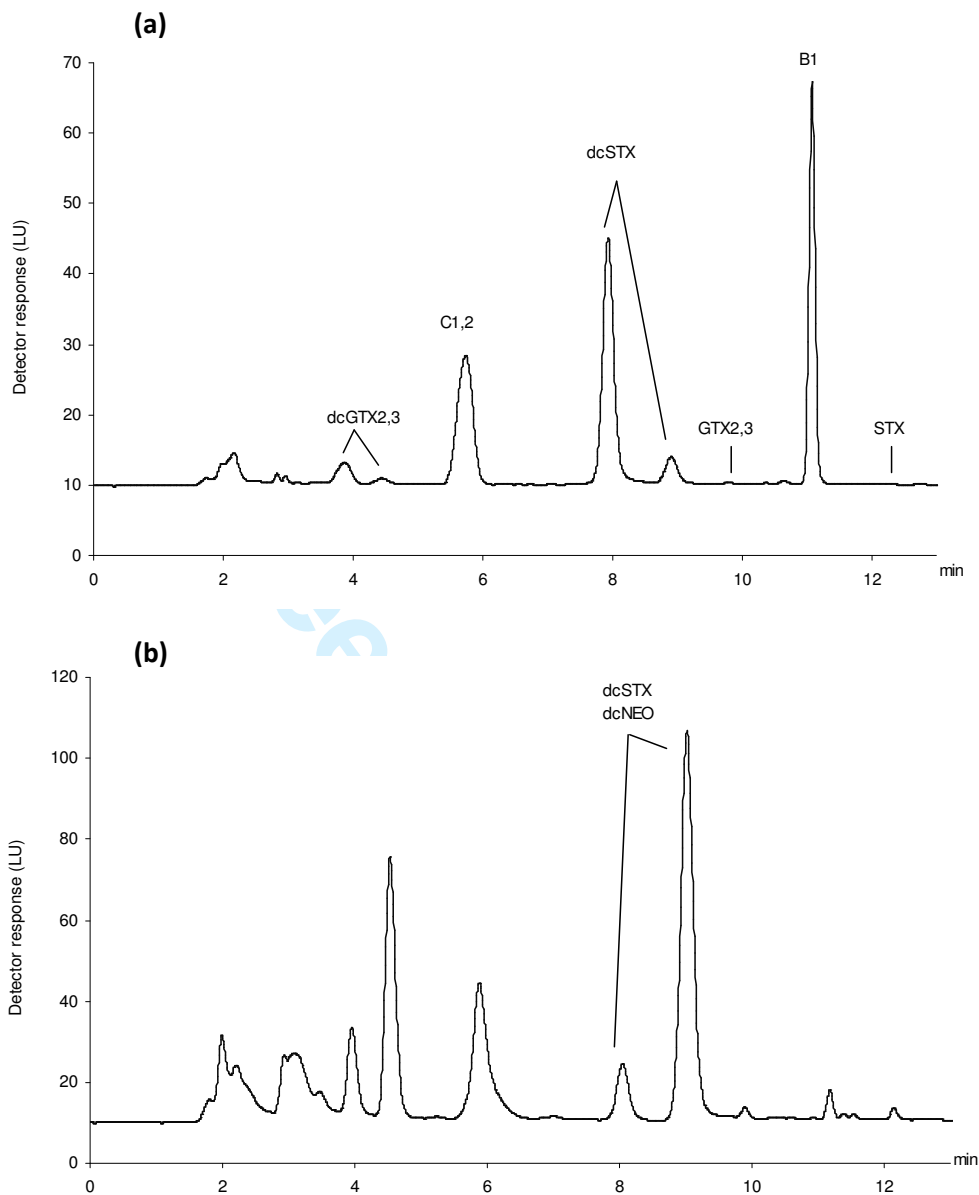
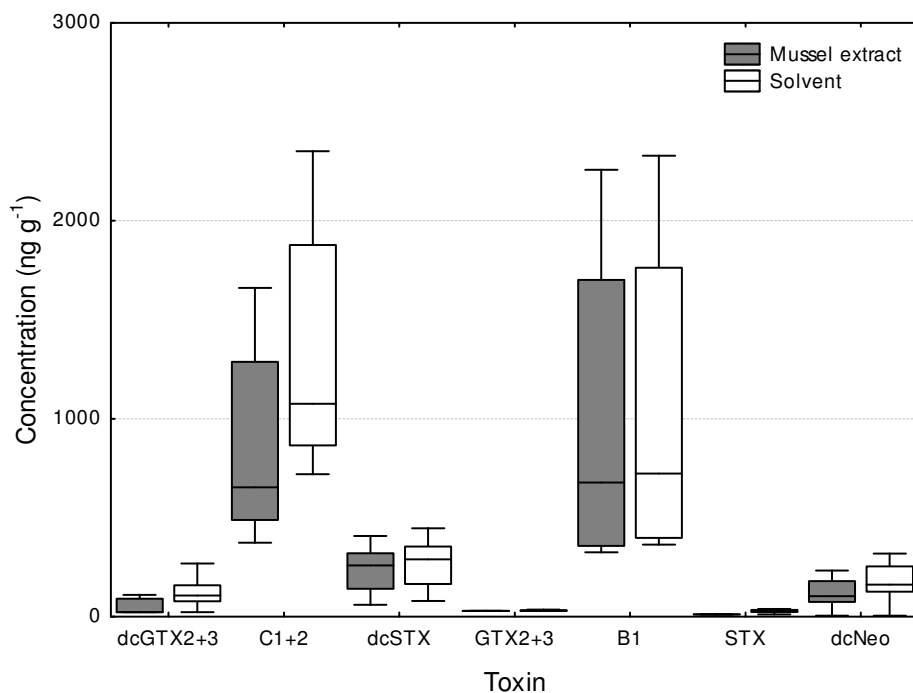


Figure 5.



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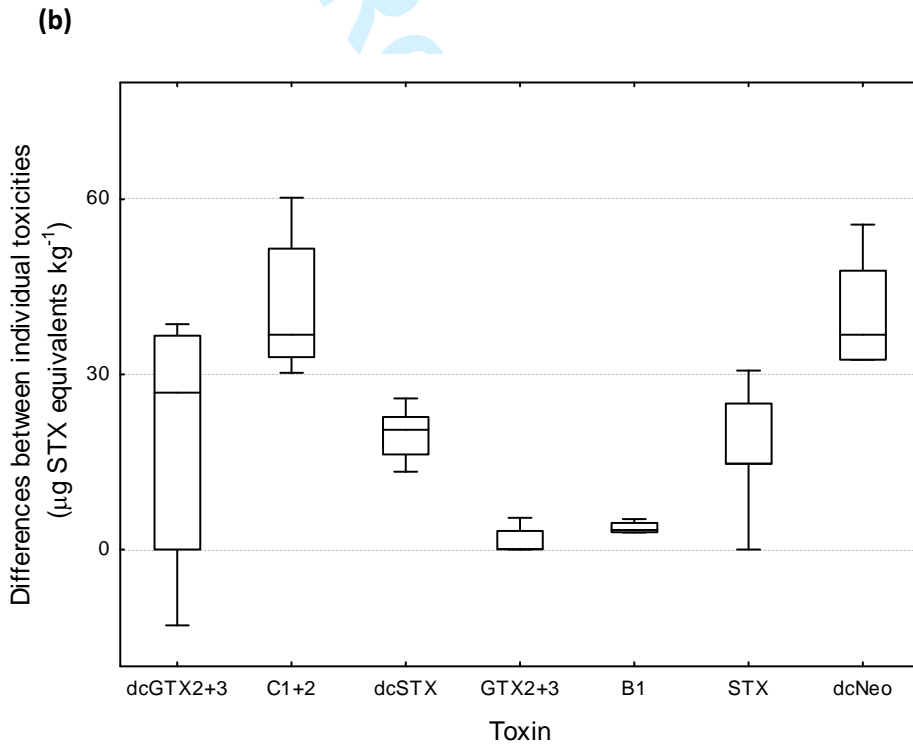
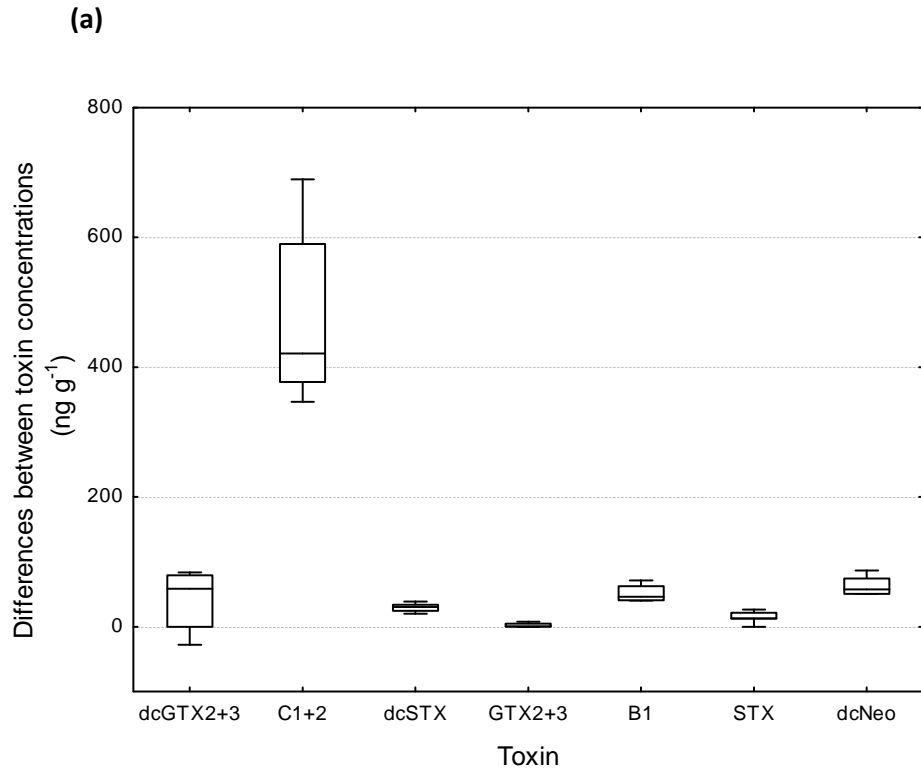
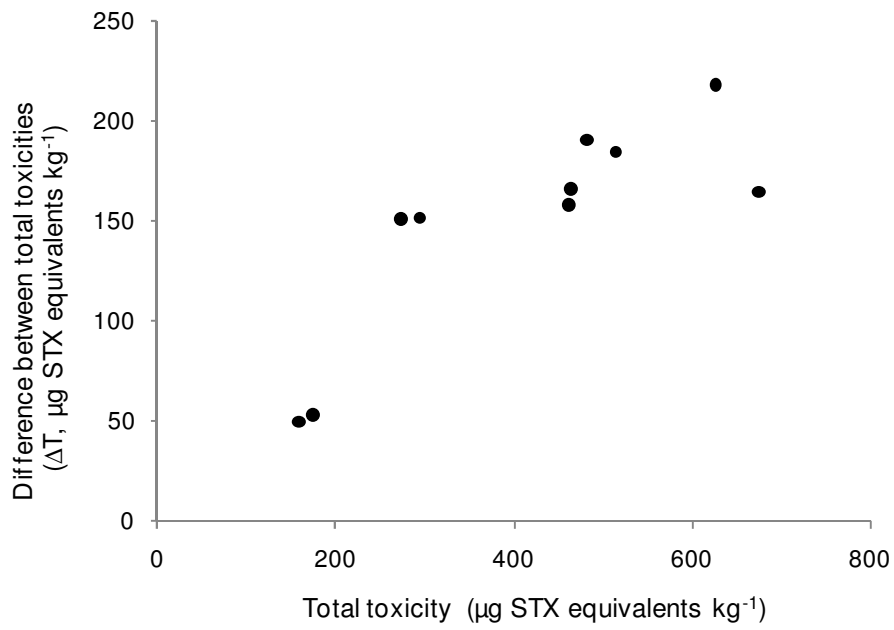


Figure 7.





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Figure captions

Figure 1. PSTs chemical structure and the Oshima toxicity equivalence factors (Quilliam, 2007) (nk = not known). STX = Saxitoxin, Neo = Neosaxitoxin, GTX = Gonyautoxins.

Figure 2. Flow diagram with the sequence of oxidation reactions (1 = peroxide oxidation of C18 extract; 2 = periodate oxidation of C18 extract; 3 = periodate oxidation of fractions 1 to 3).

Figure 3. Calibration plots of GTX2+3 (a), C1+2 (b) and B1 (c) concentrations against detector response for standards prepared in mussel extract after C18 cleanup (shaded boxes) and in solvent (open triangles), over the working calibration range

Figure 4. Chromatograms obtained for a composite sample of mussels exposed to blooms of *Gymnodinium catenatum* in Aveiro lagoon. (a) quantification of dcGTX2+3, C1+2, dcSTX, GTX2+3, B1 and STX was achieved after peroxide oxidation of the C18-cleaned extract; (b) dcNEO was quantified after periodate oxidation of the same extract.

Figure 5. Median, percentile 25% and 75%, minimum and maximum of dcGTX2+3, C1+2, dcSTX, GTX2+3, B1 and STX concentrations ( $\text{ng g}^{-1}$ ) in mussel extract and solvent; n=10.

Figure 6. Median, percentile 25% and 75%, minimum and maximum of the differences between toxin concentrations ( $\Delta C_i$ ,  $\text{ng g}^{-1}$ ) (a) and between individual toxicities ( $\Delta T_i$ ,  $\mu\text{g STX equivalents kg}^{-1}$ ) (b);  $\Delta C_i$  was calculated by the calibration curves in solvent and mussel extract;  $\Delta T_i$  was calculated by  $\Delta C_i$  and the corresponding toxicity factors and conversion unit factors; n=10.

Figure 7. Differences between total toxicities ( $\mu\text{g STX equivalents kg}^{-1}$ ), as sum of individual toxicities based on concentrations calculated by the calibration curves in solvent and mussel extract, against total toxicity estimated by the mussel calibration lines; n=10.