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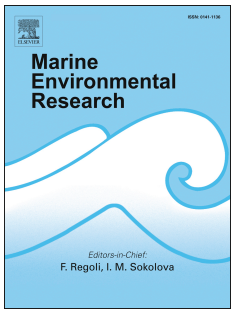

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Paralytic shellfish toxin profiles in mussel, cockle and razor shell under post-bloom natural conditions: evidence of higher biotransformation in razor shells and cockles

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Abstract

Concentrations of the paralytic shellfish toxins GTX6, C1+2, GTX5, C3+4, dcSTX, dcNEO and dcGTX2+3 were determined by LC-FLD in composite samples of whole soft tissues of mussels (*Mytilus galloprovincialis*), cockles (*Cerastoderma edule*) and razor shells (*Solen marginatus*) after exposure to a *Gymnodinium catenatum* bloom. Specimens were harvested weekly during three months under natural depuration conditions in the Mira branch of Aveiro lagoon, Portugal. Under the decline of *G. catenatum* cell densities, elimination or transformation of the uptake toxins associated with the ingestion of toxic cells differed among the surveyed species. Ratio between the toxins dcSTX plus dcGTX2+3 plus dcNEO and toxins GTX6 plus GTX5 plus C1+2 plus C3+4 was used to illustrate the biotransformation occurring in the bivalves. Enhancement of the ratios was

observed for razor shells and cockles seven weeks after the peak of the algal bloom. Most likely it reflects more intense biotransformation in razor shells and cockles than in mussels. Conversion into toxins of higher toxicity may prolong the bivalve toxicity. These results show the complexity of toxin elimination in bivalves under post-bloom conditions and emphasize the pertinence of monitoring programs of bivalve toxicity in order to protect human health.

Keywords: Paralytic shellfish toxins, biotransformation, *Mytilus galloprovincialis, Cerastoderma edule, Solen marginatus*

Introduction

Paralytic shellfish toxins (PST) are neurotoxic alkaloyds naturally produced by marine dinoflagellates belonging to the genera *Alexandrium, Gymnodinium* and *Pyrodinium* (Gedaria et al., 2007; Krock et al., 2007). This group of toxins comprises a vast range of saxitoxin derivatives. The most studied analogues are divided into three families, according to their side chains: carbamoil (saxitoxin-STX, neosaxitoxin-NEO and gonyautoxins-GTX1 to GTX4), N-sulfocarbamoil (GTX5, GTX6 and C1 to C4) and decarbamoil (dcGTX1 to dcGTX4, dcSTX and dcNEO) (Oshima, 1995a). Other families have been identified, such as deoxydecarbamoil (doSTX, doGTX2 and doGTX3), hydroxy- and sulfate-benzoate toxins (GC toxins), and hydroxylated saxitoxins (M toxins), whose origin and biosynthesis pathway is not yet fully understood (Qiu et al., 2018; Ding et al., 2017; Li et al., 2012; Vale, 2010; Wiese et al., 2010; Dell'Aversano et al., 2008; Negri et al., 2007, 2003). Bivalves as filter feeding organisms may accumulate and biotransform those compounds in their tissues during toxic algal blooms (Bricelj and Shumway, 1998). Oshima (1995b) proposed PSTs transformation pathways in shellfish tissues (Figure 1). As result of the balance among uptake of ingested toxins, biotransformation and elimination processes, consumption of contaminated bivalves may represent a serious risk for human health (Arnich and Thébault, 2018; García et al., 2005; Gessner et al., 1997).

Profiles of PST (analogues and their proportion) often differ among phytoplankton species. Moreover, strains of the same species may differ depending on various factors, such as population geographic location, life stage, nutrient availability and other environmental conditions (Silva et al., 2015; Negri et al., 2007, 2001; Granéli and Flynn, 2006; Ordás et al., 2004, Oshima et al., 1993). In the Portuguese coastal waters, the phytoplankton

species frequently associated with PST contamination in bivalves has been *Gymnodinium catenatum* (Botelho et al., 2019; Vale et al., 2008). Its toxin profile is typically dominated by N-sulfocarbamoil derivatives (C1 to C4 and GTX5, GTX6) and by GC toxins (GC1 to GC3, GC5, GC6) (Costa et al., 2015; Silva et al., 2015; Vale, 2008; Negri et al., 2007, 2003).

Due to the diverse biotransformation and metabolic pathways, toxin composition of contaminated bivalves in general differs from the corresponding PST producing phytoplankton (Ding et al., 2017; Marsden et al., 2016; Vale, 2010; Samsur et al., 2006; Silvert et al., 1998; Oshima et al., 1990). In bivalves, PST profiles are affected by the balance between the uptake of toxic phytoplankton cells, toxin transformation and elimination. Biotransformation of toxins in bivalves may occur as a result of epimerization, oxidation, reduction, desulfation and hydrolysis reactions, enzymatic action, and also as a consequence of transformation by bacteria present in their digestive tract (Qiu et al., 2018; Ding et al., 2017; Turner et al., 2013; Donovan et al., 2009; Artigas et al., 2007; Fast et al., 2006; Smith et al., 2001; Murakami et al., 1999; Oshima, 1995b; Kotaki et al., 1985; Shimizu and Yoshioka, 1981). Biotransformation processes play an important role on the toxin excretion, leading to possible differences in toxicity and depuration kinetics among bivalve species (Bricelj and Shumway, 1998).

The objective of the present study is to compare the elimination of PSTs (GTX6, C1+2, GTX5, C3+4, dcSTX, dcNEO and dcGTX2+3) in mussels (*Mytilus galloprovincialis*), cockles (*Cerastoderma edule*) and razor shells (*Solen marginatus*) after a bloom of *Gymnodinium catenatum* that occurred in the Mira branch of the Aveiro lagoon (Portugal).

2. Material and Methods

2.1. Reagents and chemicals

All reagents and chemicals used were liquid chromatography or analytical grade. Sodium hydroxide, sodium chloride, and hydrogen peroxide were purchased from Merck (Germany), acetonitrile and methanol from Riedel-de Haën (Germany) and ammonium formate, disodium hydrogen phosphate, periodic acid and acetic acid from Sigma-Aldrich (Germany). Ultra-pure water was obtained from a Purite Select Neptune system (Suez Water, United Kingdom). Tissue gDNA Isolation Kit, TE Buffer, magnesium chloride, dNTP solutions, Supreme Taq DNA polymerase, DNA ladders, GreenSafe staining and sterile water were purchased from Nzytech

(Portugal), NucleoSpin Tissue kit from Macherey-Nagel GmbH and Co. KG (Germany). TAE Solution and agarose were obtained from Merck (Germany) and ME15/Me16 and COI primers from Stabvida (Portugal).

2.2. Tissue samples

Mussels, cockles and razor shells were collected weekly from 9th January to 3rd April 2017, under post-bloom natural depuration, at a rocky substrate (mussel) and natural beds (cockles and razor shells) in the Mira branch of the Aveiro lagoon, located in the northwestern coast of Portugal (Figure 2). Shell lengths (mean ± standard deviation) of the collected specimens were: 6.5±1.1 cm (mussels), 3.1±0.3 cm (cockles), and 9.4± 1.1 cm (razor shells).

Twelve individuals of each species were randomly selected for molecular taxonomic analyses to assure the identification of the collected specimens. Taxonomic identification of the three species followed the procedure described in Wood et al. (2003) for mussels and Velez et al. (2016) for cockles and razor shells.

Ten specimens of mussels and razor shells, and 40 specimens of cockles were used for toxin determinations. These specimens were sacrificed and dissected to obtain composite samples of whole soft tissues. Aliquots of the homogenate tissues (5 g) were taken and stored at - 20 ^{0}C until toxin extraction. Triplicates of composite samples were prepared.

2.3. Toxin extraction, clean-up and oxidation

The extraction, clean-up and oxidation procedures for PSTs determination were based on the AOAC method (Lawrence et al., 2005) with a procedural modification (Botelho et al., 2010), involving the following steps: (i) an aliquot of the whole soft tissues homogenate were double-extracted with 1% acetic acid solution (first extraction with heating); (ii) the tissue extract passed through a solid phase extraction (SPE) C18 cartridge (500 mg/3 mL, Supelclean, Supelco, USA) whose pH was adjusted to 6.5; (iii) an aliquot of C18-cleaned extract was placed in an SPE-COOH cartridge (500 mg/3 mL, Bakerbond, J.T. Baker, USA) and sequentially eluted to obtain three individual fractions (1 to 3). Fractionation step was performed with increasing concentrations of aqueous NaCl (0.05 mol L^{-1} and 0.3 mol L^{-1}) for elution of the toxins.

For quantification of non N-hydroxylated toxins (GTX5, C1+2, dcSTX, dcGTX2+3, STX and GTX2+3), a hydrogen peroxide solution (10% v/v) was added to a 1 mol L^{-1} sodium hydroxide solution and vortex mixed, then the C18-cleaned extract or the PST standard solution was added, and the mixture was thoroughly mixed and allowed to react for 2 min at room temperature; then glacial acetic acid was added and vortex mixed before LC-FLD analysis. In parallel, in order to quantify N-hydroxylated toxins a C18-cleaned extract (for dcNEO), a SPE-COOH fraction (for NEO, GTX1+4, C3+4 and GTX6) or a PST standard solution (for calibration) was added to a matrix modifier solution prepared with PST-free oysters, and to that it was added the periodate oxidant (0.3 mol L^{-1} ammonium formate, 0.3 mol L^{-1} disodium phosphate, 0.03 mol L^{-1} periodic acid, with adjusted pH to 8.2); the solution was thoroughly mixed and allowed to react for 1 min, then glacial acetic acid was added and the mixture was allowed to stand for further 10 min before LC-FLD analysis.

2.4. Estimation of C3+4 toxin

Despite the unavailability of commercial certified reference material for the toxin C3+4, its concentration was determined by the conversion of C3+4 into GTX1+4, through hydrolysis. The analytical procedure was based on the European Union Reference Laboratory for Marine Biotoxins Report for the determination of PSTs in shellfish after hydrolysis (AESAN, 2007). Hydrolysis was carried out by adding 225 µL of 1 mol L^{-1} HCl solution to a 500 µL aliquot of fraction 1 (from SPE-COOH fractionation). Hydrolysis occurred in a water bath at 90 ^{0}C during 20 min. Samples cooled down at room temperature and the reaction was then neutralized by adding small volumes of 1 mol L^{-1} sodium hydroxide solution. The solution was mixed after each addition until a volume of 225 µL was attained. Following neutralization, extracts were submitted to periodate oxidation. Concentration of C3+4 toxin was thus indirectly quantified by conversion to GTX1+4, assuming that the molarity of the hydrolyzed C3+4 toxin was equal to the molarity of GTX1+4 toxin present in fraction 1 after hydrolysis.

2.5. Liquid chromatography and PST quantification

The LC system consisted of an Agilent Model 1290 Infinity II quaternary pump and in-line degasser Model 1290 Infinity autosampler and Model 1260 fluorescence detector and column oven. The OpenLAB CDS software performed data acquisition and peak integration. The PST oxidation products were separated using a reversed-

phase column Supelcosil LC-18, 150x4.6 mm i.d., 5 µm particle size (Supelco), equipped with a guard column Supelguard Supelcosil C18, 20x4.0 mm i.d., 5 µm particle size (Supelco). The column was kept in an oven at 30 ^{o}C. Two mobile phases were used for separation of PSTs: solution A (0.1 mol L^{-1} ammonium formate, pH=6) and solution B (0.1 mol L^{-1} ammonium formate in 5% acetonitrile, pH=6). The mobile phase gradient used in chromatography consisted of 0-10% B in the first 4 min, 10-90% B in the next 5 min, back to 10% B in the next 2 min and 0% B in the last 2 min. Flow rate was 1 mL min^{-1} and the injection volumes were 40 µL and 80 µL, for the oxidation products of peroxide and periodate reaction, respectively. The excitation and emission wavelengths for fluorimetric detection were set at 340 nm and 395 nm, respectively.

The quality control of the results was assured through the use of certified reference materials in solution (C1+2-b, GTX2+3-d, GTX1+4-d, GTX5-c, GTX6, STX-f, NEO-d, dcSTX-b, dcNEO-d and dcGTX2+3-c) purchased from the National Research Council Canada. Instrumental detection limits for individual toxins in C18-cleaned extracts were: 4 nmol L^{-1} for GTX2+3, GTX5, STX and dcSTX, 8 nmol L^{-1} for GTX6, 20 nmol L^{-1} for dcGTX2+3 and C1+2, 30 nmol L^{-1} for NEO, and 40 nmol L^{-1} for GTX1+4 and dcNEO. PST-free clam tissues were spiked with the toxin mixtures aforementioned to assess the recovery of the added quantities of C1+2, GTX5, dcGTX2+3, dcSTX, dcNEO, GTX2+3, GTX1+4, STX and NEO. Spiking of clam tissues were prepared at concentrations that give total toxicity close to half the regulatory limit (EC, 2004): 82 ng g^{-1} of dcSTX to 567 ng g^{-1} of C1+2. Recovery values obtained from triplicates were: 74±0.7% (C1+2), 98±6 % (GTX5), 107±3% (dcGTX2+3), 114±1 % (dcSTX), 56±11% (dcNEO), 104±2 % (GTX2+3), 59±12% (GTX1+4), 93±8 % (STX) and 97±16% (NEO). Most likely, biotransformation through enzymatic activities, which trigger rapid losses of selected toxins added to the tissues, explain the lower percentages obtained (Artigas et al., 2007; Fast et al., 2006). This constraint could be avoided if certified reference material in bivalve matrix would be commercially available.

Figure 3 presents chromatograms illustrating the toxin separation of two standard mixtures of dcGTX2+3, C1+2, dcSTX, GTX2+3, GTX5 and STX after peroxide oxidation (a), of GTX6 and GTX1+4 after periodic acid oxidation (b), for a selected cockle sample with quantification of GTX6 after C18-clean up, SPE-COOH fractionation and periodic acid oxidation (c), and for a selected mussel sample with quantification of dcGTX2+3, C1+2, dcSTX and GTX5 after peroxide oxidation of the C18-cleaned extract (d). Individual toxin concentrations are presented as µg g^{-1} of bivalve whole soft tissues. Bivalve toxicity values were estimated in terms of µg STX di-HCl equivalents per kg, multiplying the toxin concentration by the toxicity equivalence

factor (TEF) of each individual compound (EFSA, 2009). The regulatory limit (RL) for PSTs is 800 µg STX di-HCl equivalents per kg (EC, 2004).

2.7. Data analysis

The ratio between decarbamoil and N-sulfocarbamoil toxins (R) was calculated using individual toxin concentrations expressed in a molar basis for each sampling date and each bivalve species according to the following equation:

$$R = [(dcSTX)+(dcGTX2+3)+(dcNEO)]:[(GTX6)+(GTX5)+(C1+2)+(C3+4)] \quad \text{(Eq. 1)}$$

Statistical analyses were performed using R software (Version 1.1.463). Analysis of variance and post-hoc tests were performed with *car* and *PMCMR* packages, respectively. Data were tested for normality and homogeneity of variance with Shapiro-Wilk and Levene tests, respectively. Since these assumptions could not be met, the non-parametric Kruskal-Wallis test was used to assess differences in temporal variation of toxin concentrations and decarbamoil to N-sulfocarbamoil ratios over the study period. Whenever Kruskal-Wallis test was significant, the post-hoc Conover test was performed for multiple comparisons between groups. The probability lower than 0.05 was considered as statistically significant.

3. Results

3.1. Toxic phytoplankton cells

Weekly data of the cell density of phytoplankton species producers of PSTs in Aveiro lagoon from 5th December 2016 to 13th February 2017 were obtained from the monitoring programme of toxic phytoplankton from the Portuguese Institute for the Sea and the Atmosphere (IPMA, database available at https://www.ipma.pt/pt/bivalves/fito/index.jsp). Most likely, *Gymnodinium catenatum* was the PST-producer species as it has been reported in previous works in Portugal (Botelho et al., 2019). The highest density (4540 cells $L^{-1,}$) was recorded on 12th December and then decreased pronouncedly in the following two weeks (480 and 160 cells L^{-1}, respectively). A second enhancement was found on 2nd January (2320 cells L^{-1}) followed by a decrease until 13th February (60 cells L^{-1}). After this period and until 3rd April, cell densities remained below the

detection limit (20 cells L^{-1}). It was assumed that the abundance of toxic *G. catenatum* cells registered in Aveiro lagoon from 5th December 2016 to 13rd April 2017 contributed to the accumulation of PSTs in the bivalves harvested during the study period.

3.2. Identification of the bivalve species

Analysis of nuclear DNA markers showed that all the mussel samples exhibited the typical genotype of *Mytilus galloprovincialis*. Likelihood analysis of COI sequences demonstrated that cockles clustered together with *Cerastoderma edule* sequences deposited at EMBL GenBank database and that razor shells aligned unambiguously with those of *Solen marginatus* (supplementary Figure S1). Detailed information is presented as supplementary material.

3.3. Toxicity of bivalves exposed to an algal bloom

On 9th January the bivalve species *M. galloprovincialis, C. edule* and *S. marginatus* from Mira branch (Aveiro lagoon) were exposed to two enhancements of toxic phytoplankton cells. Accumulation of toxin compounds produced by *G. catenatum* resulted in the following toxicities of *M. galloprovinciallis, C. edule* and *S. marginatus*: 4013±355, 4721±757 and 1177±185 µg STX di-HCl equivalents kg^{-1}, respectively. These values exceeded largely the toxicity regulatory limit (RL) for PSTs (800 µg STX di-HCl equivalents kg^{-1}). Consequently, harvesting in that production area was interdicted by the National Authority (IPMA).

3.4. Toxin profiles of mussels, cockles and razor shells exposed to an algal bloom

Figure 4 shows the toxin profiles, expressed as molar fractions, of the quantified toxins in the specimens collected on 9th January. GTX6, C1+2, GTX5, C3+4, dcSTX and dcGTX2+3 were the major contributors to the toxin profiles in composite samples of the three species. Toxin dcNEO was a minor contributor in mussels and cockles, and was undetected in razor shells. The compound GTX6 accounted for 35, 33 and 30% of total quantified toxins in mussels, cockles and razor shells, respectively. Furthermore, the results showed higher proportion of C1+2 in cockles (34%) than in mussels (24%) and in razor shells (16%), while the compound GTX5 reached 24% in razor shells, clearly above the 13% in mussels and 11% in cockles. Differences among species were also observed in the toxins dcSTX, dcGTX2+3 and dcNEO that were present in lower proportions.

3.5. Toxin concentrations in mussels, cockles and razor shells under post-bloom conditions

Concentration of all detected PSTs in bivalve whole soft tissues collected weekly in the the study area between 9th January and 3rd April 2017 are given as supplementary data (supplementary Table S1). GTX6, C1+2, GTX5, C3+4, dcSTX, dcNEO and dcGTX2+3 were detected in most of the samples of the three species. The toxins GTX2+3, STX, GTX1+4 and NEO were below the detection limit throughout the observation period. Figure 5 shows the time-course variation of mean toxin concentrations (±one standard deviation) in whole soft tissues of mussels, cockles and razor shells collected under post-bloom natural conditions. In general, toxin concentrations decreased in line with the decline of the abundance of toxic algae, although the elimination pattern differed among compounds and species. On 9th January, concentrations of C1+2 and GTX6 in cockles (13.3 and 10.7 μg g^{-1}, respectively) and in mussels (7.1 and 8.7 μg g^{-1}) exceeded 13 to 5 times the toxin values found in razor shells (1.1 and 1.7 μg g^{-1}). The same pattern was observed for the toxins GTX5, C3+4, dcSTX and dcNEO, although toxin concentrations in cockles and mussels were only 2 to 4 times higher than in razor shells. Comparison of dcNEO could not be done because values in razor shells were below the detection limit. On 9th January, mean concentrations of dcGTX2+3 were higher in cockles (1.3 μg g^{-1}) than in mussels (0.44 μg g^{-1}) and razor shells (0.41 μg g^{-1}) that presented similar values. Initial concentrations of C1+2, GTX6, GTX5 and dcSTX were significantly different ($p < 0.05$) among the bivalve species. Cockles exhibited the fastest decrease in dcGTX2+3 concentrations, with a decline of 94% of the initial value after 7 days. Irregularities were recorded for this compound in razor shells throughout the observation period, with concentration peaks on 30th January and 1st March. Cockles also showed the fastest reduction in GTX6, with concentrations decreasing 81% within the first 7 days. Contrastingly, a concentration peak for GTX6 was registered in razor shells after six weeks (on 20th February), differing statistically ($p < 0.05$) from all the values with exception of 9th, 16th and 30th January. Mussels presented a relatively consistent decrease in GTX6, attaining low concentrations on 6th February. On the second week of the survey (23rd January), an enhancement of C1+2 concentration was observed in razor shells, being 49% above the initial value. This value was statistically different ($p < 0.05$) from all C1+2 concentrations, except from the value of 16th January. Smaller peaks were also observed on 20th February and 3rd April. A similar pattern was obtained for GTX5 concentrations in razor shells, which increased 10% above the initial value on 23rd January, and showed several peaks between 20th March and 3rd April. Concentrations

of dcSTX showed irregularities with time in razor shell and mussel samples after three and seven weeks, respectively, while cockles showed a steady decrease until the end of the observation period.

3.6. Time-course variation of bivalve toxicity

Figure 6 shows the variation of total toxicities in the bivalve specimens collected from 9th January to 3rd April, 2017. During this period, mean toxicities decreased from 4013 to 165 (mussels), 4721 to 125 (cockles) and 1176 to 133 (razor shells) µg STX di-HCl equivalents kg^{-1}. In all species higher toxicities were registered one week after the enhancement of toxic phytoplankton cells (2nd January, 2320 cells L^{-1}). Toxicity values of mussels and cockles were higher than of razor shells. Enhancement of toxic cells on 30th January seems to have contributed to the increase of mussel toxicities in the following dates, although relative constant values were observed in cockles and razor shells. During four weeks, toxicity in the three species displayed values above the RL for PSTs. Approximately three months after the initial time of observations, toxicity levels observed in razor shells, mussels and cockles decreased to 11, 5 and 3% of the respective initial values.

3.7. Ratios between decarbamoil and N-sulfocarbamoil toxins

Since decarbamoil derivatives have a minor contribution to the toxin profile of *G. catenatum* cells registered in the Portuguese coast, the molar ratio R (Eq. 1) was used as footprint of PSTs biotransformation in bivalves. Figure 7 shows the mean R calculated for mussels, cockles and razor shells under post-bloom conditions, between 9th January and 3rd April. During the first three weeks, the ratios were higher for razor shells than for cockles and mussels. Afterwards, from 30th January to 13th February, ratios calculated for cockles increased to values close to the ratios for razor shells, while mussel ratios remained relatively constant. Ratios calculated for razor shells (1st March and 6th March) and for cockles (1st March) enhanced approximately 2 to 3 times the initial values, showing significant differences ($p<0.05$) with the initial values. This variation contrasts with the narrow ratio interval obtained for mussels. Ratios for razor shells on 1st March and 6th March differed significantly ($p<0.05$) from all values except of 9th January, 16th January and 13rd February. The highest ratio (0.46) calculated for cockles was statistically different ($p<0.05$) from all values except 6th and 20th March. Elevated ratios for razor shells decreased abruptly from 0.38 to 0.06 between 6th and 13th March. In the same period, cockles ratios decreased from 0.36 to 0.21, and mussels ratios fluctuated between 0.14 and 0.08.

4. Discussion

The present study, performed under the decline of *G. catenatum* cell densities, shows differences in the elimination of PSTs among the bivalves *C. edule, M. galloprovincialis* and *S. marginatus*. Despite the sharper decline of toxins in mussels and cockles after the algal bloom (December), concentrations in razor shells showed enhancements in February (GTX6 and C1+2), and in February-March (dcGTX2+3). A possible explanation may be the influence of additional toxin sources, such as remobilisation of cysts or dead toxic cells associated with sediment re-suspension (Artigas et al., 2008; Li et al., 2019). Differences on biotransformation or elimination processes are clearly illustrated by the variation of the ratio between the toxins dcSTX plus dcGTX2+3 plus dcNEO and toxins GTX6 plus GTX5 plus C1+2 plus C3+4. Presumably, the first group of toxins resulted mainly from metabolic processes in the bivalve after the ingestion of toxic phytoplankton cells (Bricelj and Shumway, 1998). This hypothesis is supported by previous studies that showed the predominance of N-sulfocarbamoil toxins (GTX6, GTX5, C1+2 and C3+4) in *G. catenatum* cells (Botelho et al., 2015; Silva et al., 2015; Vale, 2008; Negri et al., 2007, 2003). Botelho et al. (2015) quantified PSTs in cells of *Gymnodinium catenatum* collected during an extreme event of mussel toxicity in the Óbidos lagoon (Portugal). The toxin profile was dominated by N-sulfocarbamoil analogues, with the median of the molar proportions of C1+2 (67%) and GTX5 (23%) exceeding the values found for decarbamoil analogues dcGTX2+3 (5%) and dcSTX (4%) by one order of magnitude. Furthermore, an enhancement of ratios between decarbamoil and N-sulfocarbamoil toxins was observed for razor shells and cockles seven weeks after the peak of the algal bloom, which reinforces the relevance of the metabolism on the alteration of toxin profiles in the bivalve. This enhancement was more pronounced for razor shells than for cockles which may mirror the specificity of the elimination metabolism.

The metabolic conversions of GTX5 and C1+2 into dcSTX and dcGTX2+3, respectively, are in line with previous works where N-sulfocarbamoil PSTs were showed to be enzymatically converted into the respective decarbamoil derivatives (Turner et al., 2013; Artigas et al 2007; Fast et al., 2006; Oshima, 1995b; Sullivan et al., 1983). Lin et al. (2004) demonstrated the role of the enzyme carbamoylase I on the hydrolysis of GTX5 into dcSTX. A similar pathway was suggested for the hydrolysis of C toxins into dcGTX2+3 (Cho et al., 2008). Other biotransformation pathways involving benzoate (GC1, GC2 and GC3) and M toxins should be considered, due to a possible contribution to the toxin profiles observed in the present study. Although the quantification of

these metabolites was not achieved with the analytical methodology used in this study, its presence in bivalve tissues could help to better understand the variation of toxin profiles in bivalves in post-bloom conditions. Conversion of the hydroxy-benzoate toxins GC1, GC2 and GC3 into the decarbamoil analogues dcGTX2, dcGTX3 and dcSTX, respectively, may occur in the presence of a carbamoylase enzyme, through the hydrolysis of the benzoate group (Vale, 2008). In addition, the conversion of C1+2 and C3+4 into M1 and M7 toxins, respectively, by desulfation of the 11-hydroxysulfate group and the conversion of GTX5 into M1, by hydroxylation of the same group, should also be considered (Qiu et al., 2018; Ding et al., 2017; Li et al., 2012; Vale, 2010).

Factors related with the trophic ecology of the species on the uptake of toxic cells should also be taken into account. Infaunal species that live buried in the sediment, such as razor shells and cockles feed mostly on particles present on the seabed. Epifaunal species, such as mussels live in rocky subtract above the sediment and collect suspended particles from the water column. The possible availability of *G. catenatum* cysts in the sediment after the bloom peak (Artigas et al., 2008) may have contributed to differences in the uptake of toxic cells by razor shells and cockles (deposit feeding) compared with mussels that exhibit suspension feeding. The ingestion of cysts by razor shells and cockles with an eventual distinct PST profile from phytoplankton cells should not be discarded, contributing also to differences in the toxin profiles.

From 9th January to 3rd April 2017 PSTs accumulated by the three surveyed species resulted in toxicities, expressed in µg STX di-HCl equivalents kg^{-1}, that ranged from 4013 to 165 for *M. galloprovinciallis,* 4721 to 175 for *C. edule* and 1177 to 608 for *S. marginatus*. During the first three weeks the toxicity values of the three species were above the regulatory limit for PSTs. For cockles and razor shells toxicity values decreased to below the RL in the following week, contrasting to toxicities of mussels that remained above RL for another four weeks. However, during the surveyed period the compounds that contributed to the total toxicity differed among the bivalve species. Contribution of decarbamoil derivatives (dcSTX, dcGTX2+3 and dcNEO) for the toxicity of razor shells was higher than in mussels and cockles that, on average, remained within the narrow interval of 54-58% during both periods, above and below the RL. One possible explanation for these results is the high toxicity equivalent factor of decarbamoil toxins in comparison to the other toxins produced by *G. catenatum* (EFSA, 2009). For example, the toxicity equivalent factor of dcSTX is 10 times higher than of GTX5 and C1+2.

Various studies have proved that toxicity of bivalves harvested in coastal waters results mainly from the ingestion of cells produced by toxic phytoplankton blooms (Bricelj et al., 1996; Shumway and Cucci, 1987). In addition, biotransformation of compounds produced by the toxic cells into other molecules eventually of higher toxicity may contribute to the prolongation of bivalve toxicity (Bricelj and Shumway, 1998; Botelho et al., 2015). The present work shows the different biotransformation of PSTs of three bivalve species collected in the same area when exposed to a toxic bloom of *G. catenatum*. Razor shells and cockles were able to convert N-sulfocarbamoil derivatives into decarbamoil derivatives within a few weeks after the bloom peak. The consequent enhancement of toxicity inverted the progressive decline in toxic cells abundance. This observation under post-bloom conditions illustrates the contribution of biotransformation of PSTs to the toxicity of razor shell. Presumably, unexpected enhancement of toxicity in marine toxin monitoring programmes may be related with that contribution and emphasises the pertinence of national programmes to survey the quality of bivalves.

5. Conclusions

Concentrations of GTX6, C1+2, GTX5, C3+4, dcSTX, dcNEO and dcGTX2+3 in whole soft tissues of *M. galloprovincialis, C. edule* and *S. marginatus* exposed to a bloom of *Gymnodinium catenatum* in the same area of the Aveiro lagoon allowed to identify the intense biotransformation occurring in *S. marginatus* and *C. edule* during the subsequent post-bloom period that may prolong the toxicity value of this bivalve species for human consumption. These results contribute to a better understanding of the variation of bivalve toxicity after a toxic phytoplankton bloom and the pertinence of monitoring programs to protect human health.

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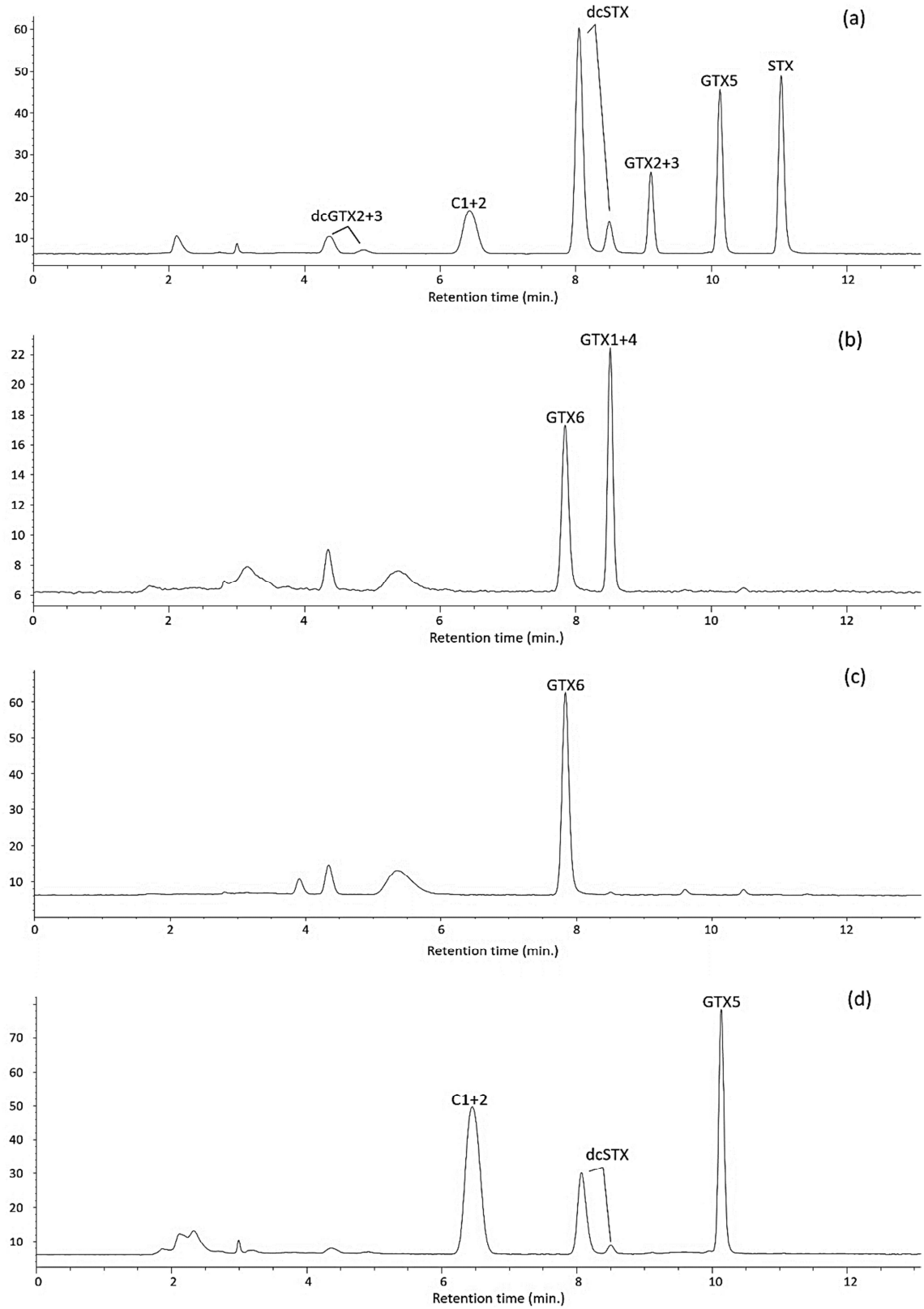

Figure 3. Chromatograms obtained for two standard mixtures of dcGTX2+3, C1+2, dcSTX, GTX2+3, GTX5 and STX after peroxide oxidation (a), of GTX6 and GTX1+4 after periodic acid oxidation (b), for a selected cockle sample with quantification of GTX6 after C18-clean up, SPE-COOH fractionation and periodic acid oxidation (c), for a selected mussel sample with quantification of C1+2, dcSTX and GTX5 after peroxide oxidation of the C18-cleaned extract (d).

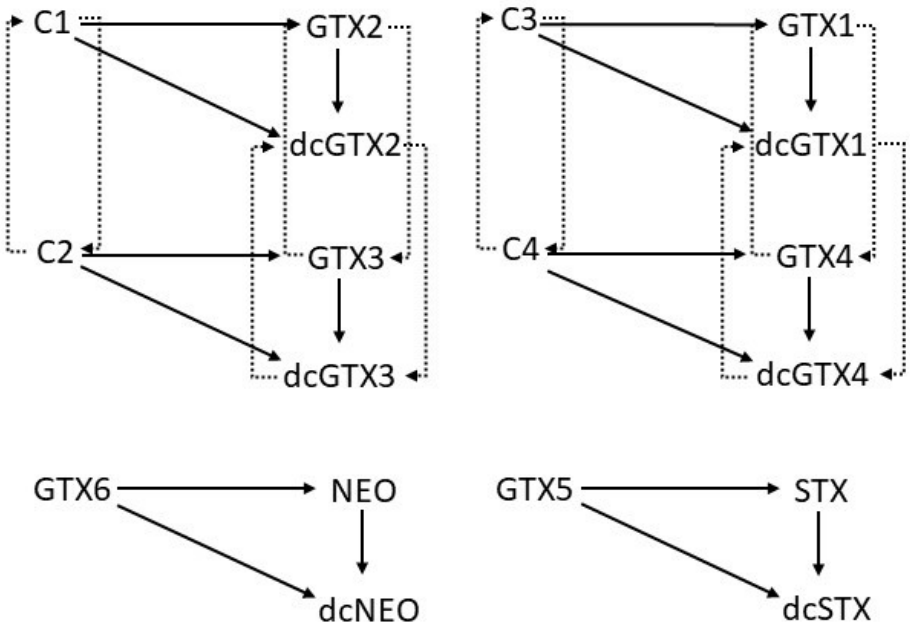

Figure 1. Paralytic shellfish toxin biotransformation pathways in shellfish tissues (Oshima, 1995b). Solid arrow: hydrolysis; dashed arrow: epimerisation. NEO - neosaxitoxin; STX - saxitoxin, GTX1 to GTX4 - gonyautoxins; GTX5, GTX6 and C1 to C4 - N-sulfocarbamoil toxins; dcGTX1 to dcGTX4 - decarbamoil toxins.

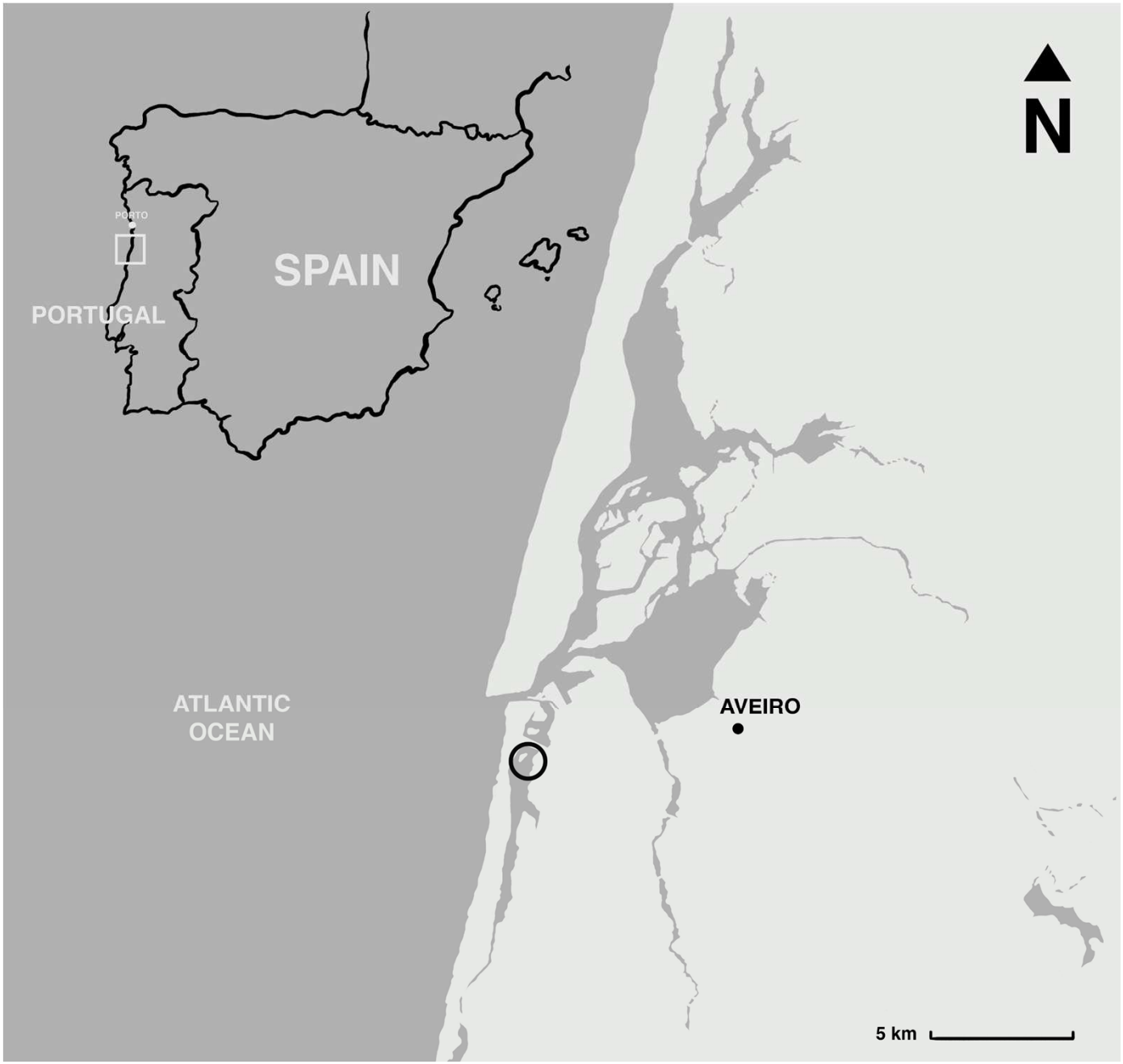

Figure 2. Harvesting area of mussels (*Mytilus galloprovincialis*), cockles (*Cerastoderma edule*) and razor shells (*Solen marginatus*) in the Mira branch, Aveiro lagoon, Portugal. Source: modified from Google Maps.

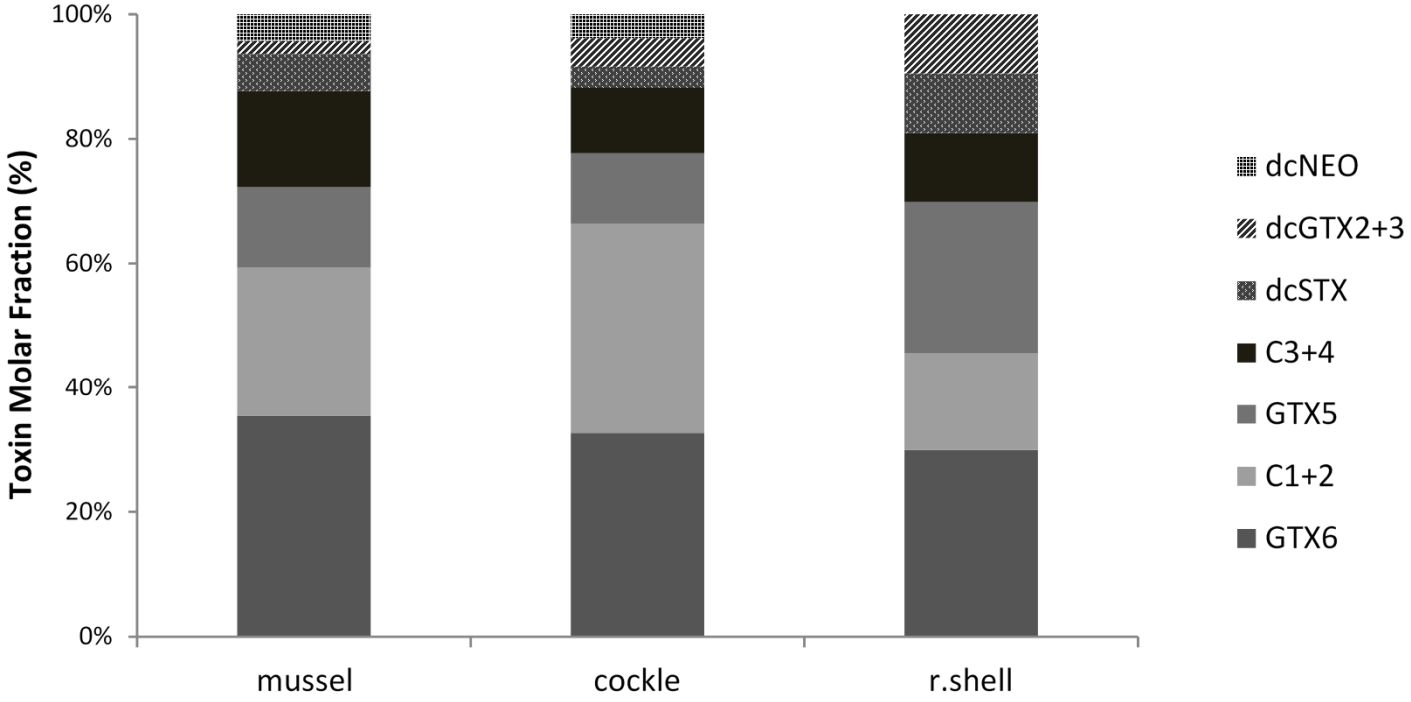

Figure 4. Toxin profiles of all quantified toxins (GTX6, C1+2, GTX5, C3+4, dcSTX, dcGTX2+3 and dcNEO) expressed as molar ratios (%) in whole soft tissues of mussels (*Mytilus galloprovincialis*), cockles (*Cerastoderma edule*) and razor shells (*Solen marginatus*), harvested in the Mira branch, Aveiro lagoon, on 9th January, 2017; mean values (n=3).

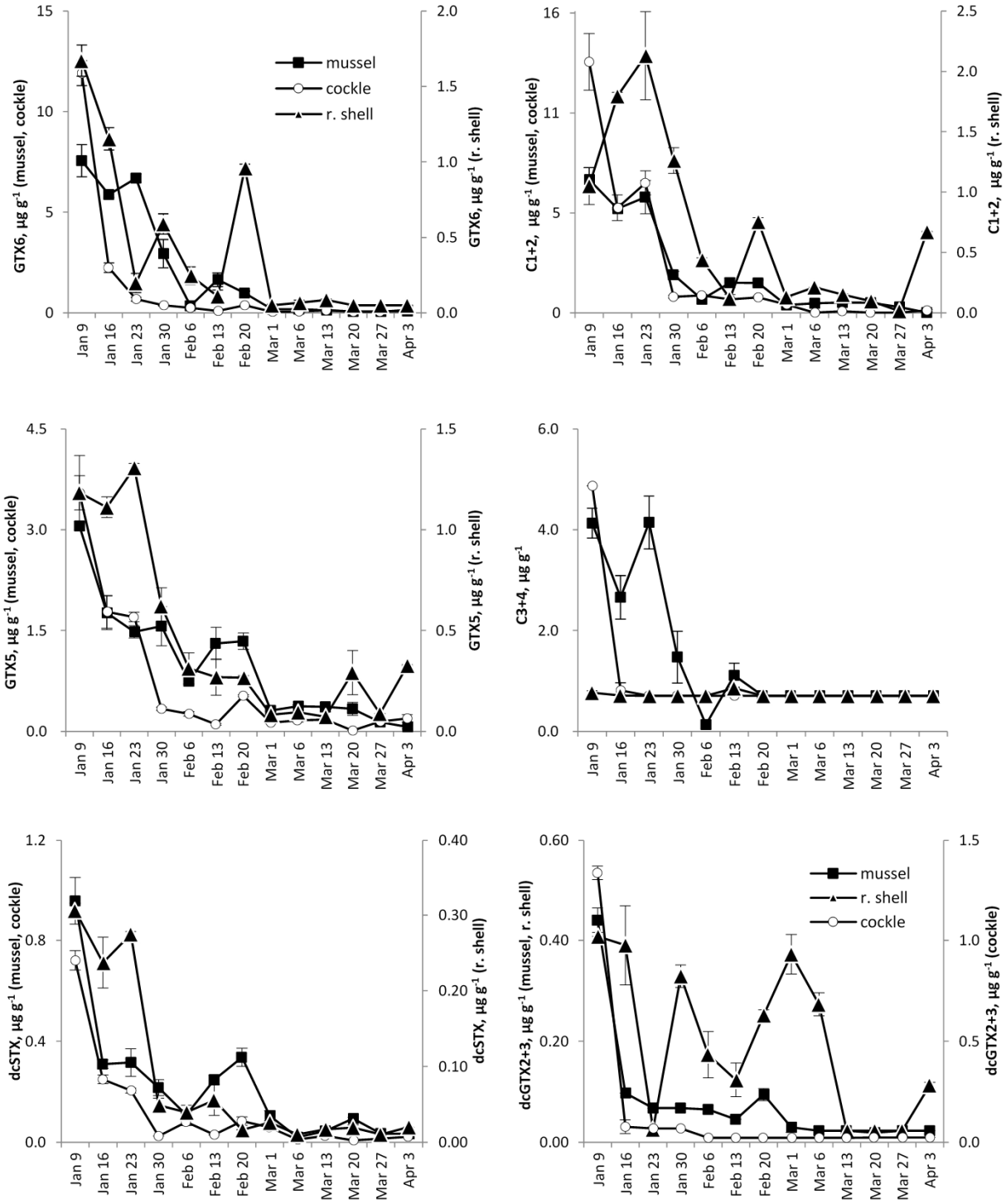

Figure 5. Individual paralytic shellfish toxin (GTX6, C1+2, GTX5, C3+4, dcSTX and dcGTX2+3) concentrations (µg g^{-1}, mean ± standard deviation, n=3) in soft tissues of mussel (*Mytilus galloprovincialis*), cockle (*Cerastoderma edule*) and razor shell (*Solen marginatus*), harvested weekly from 9th January to 3rd April, 2017, in the Mira branch, Aveiro lagoon, during post-bloom natural conditions.

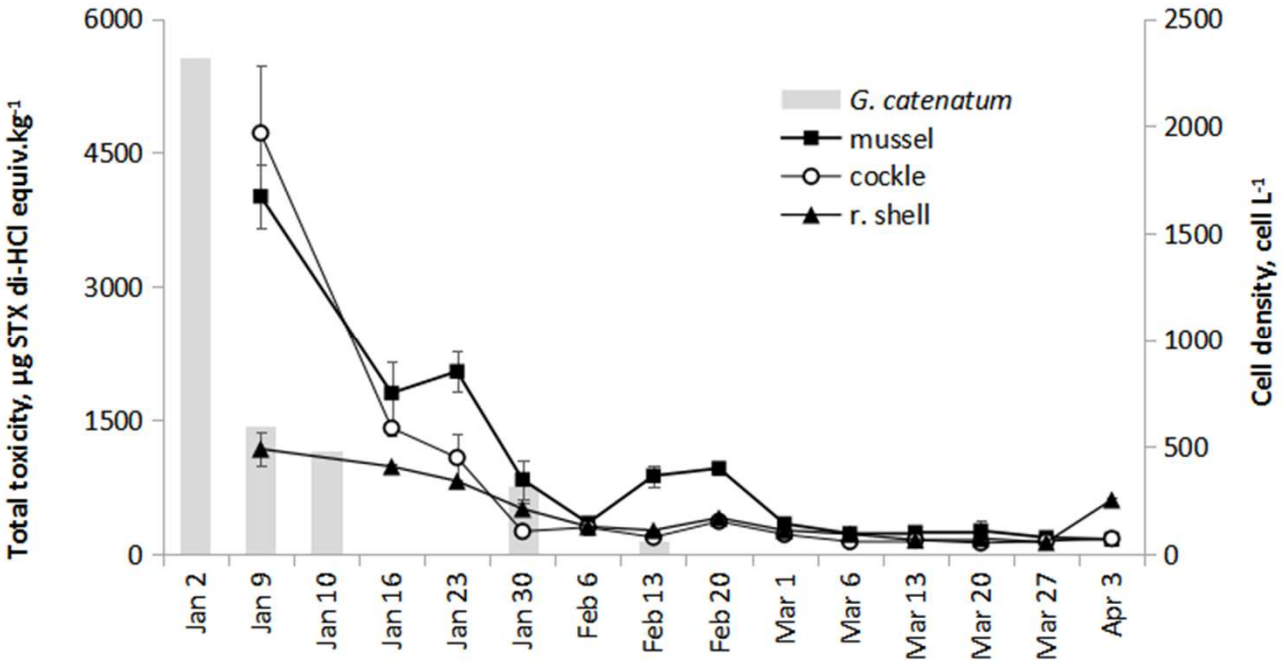

Figure 6. Total toxicity (µg STX di-HCl equiv. kg^{-1}, mean ± standard deviation, n=3) of whole soft tissues of mussel (*Mytilus galloprovincialis*), cockle (*Cerastoderma edule*) and razor shell (*Solen marginatus*) collected in the Mira Branch, Aveiro lagoon, Portugal (9th January to 3rd April, 2017). Grey bars represent *Gymnodinium catenatum* cell density (cell L^{-1}) at the same sampling site, from 2nd January to 13rd February 2017 (data were obtained from the monitoring programme of toxic phytoplankton from the IPMA database, available at https://www.ipma.pt/pt/bivalves/fito/index.jsp).

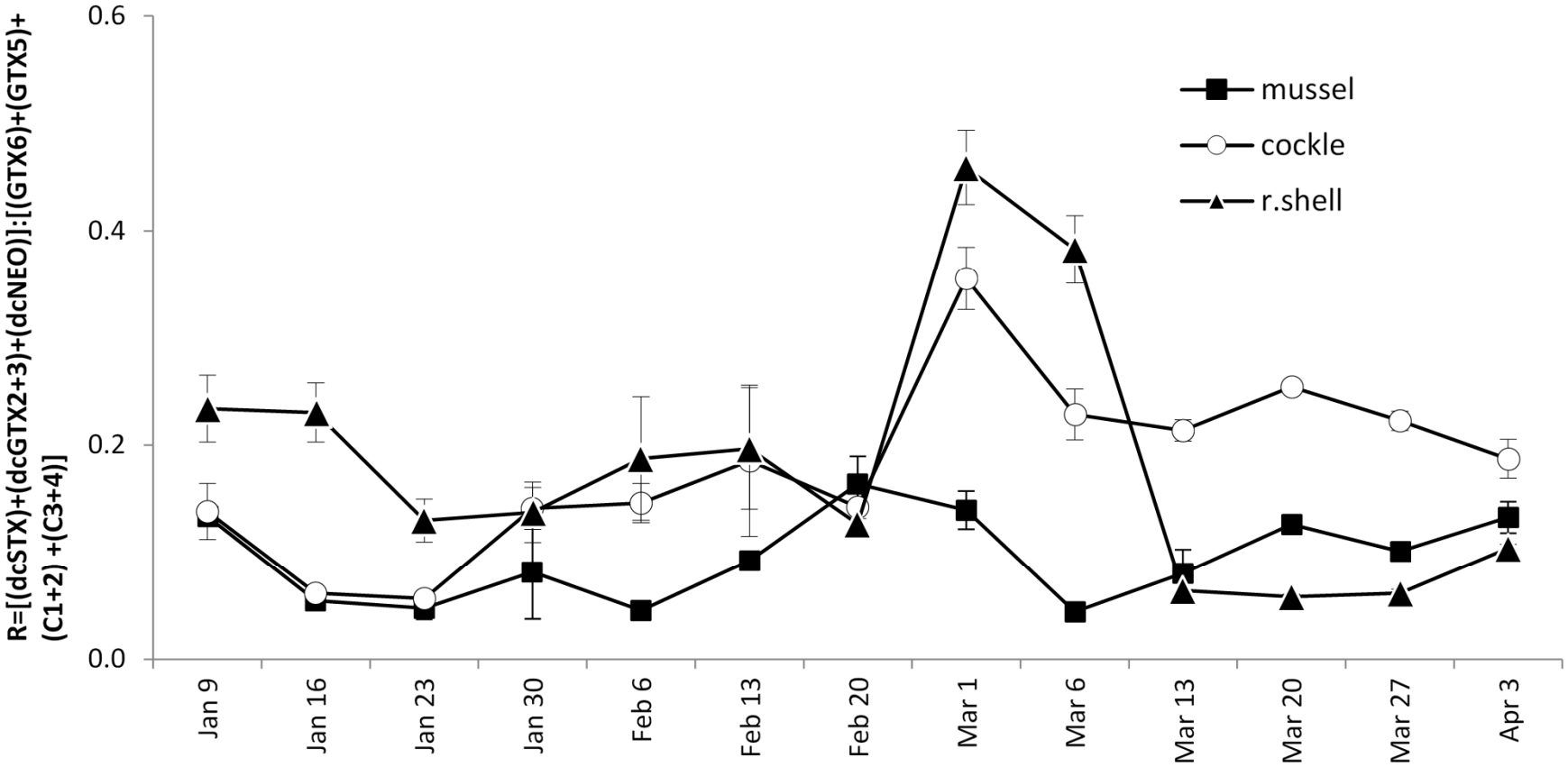

Figure 7. Molar ratios (R) of decarbamoil (dcSTX, dcGTX2+3, dcNEO) to N-sulfocarbamoil (GTX6, C1+2, GTX5, C3+4) toxins concentrations detected in soft tissues of mussel (*Mytilus galloprovincialis*), cockle (*Cerastoderma edule*) and razor shell (*Solen marginatus*), collected in the Mira branch, Aveiro lagoon, in post-bloom conditions (January to April). Ratios are presented as mean ± standard deviation (n=3).

Highlights:

- GTX6, C1+2, GTX5, dcSTX and dcGTX2+3 were the prevalent toxins in bivalves
- Toxin concentrations under post-bloom conditions declined with abundance of toxic cells
- Elimination pattern differed among toxins and bivalve species
- Ratios of decarbamoil to N-sulfocarbamoil toxins used as footprint of biotransformation
- Higher ratios found for cockles and razor shell