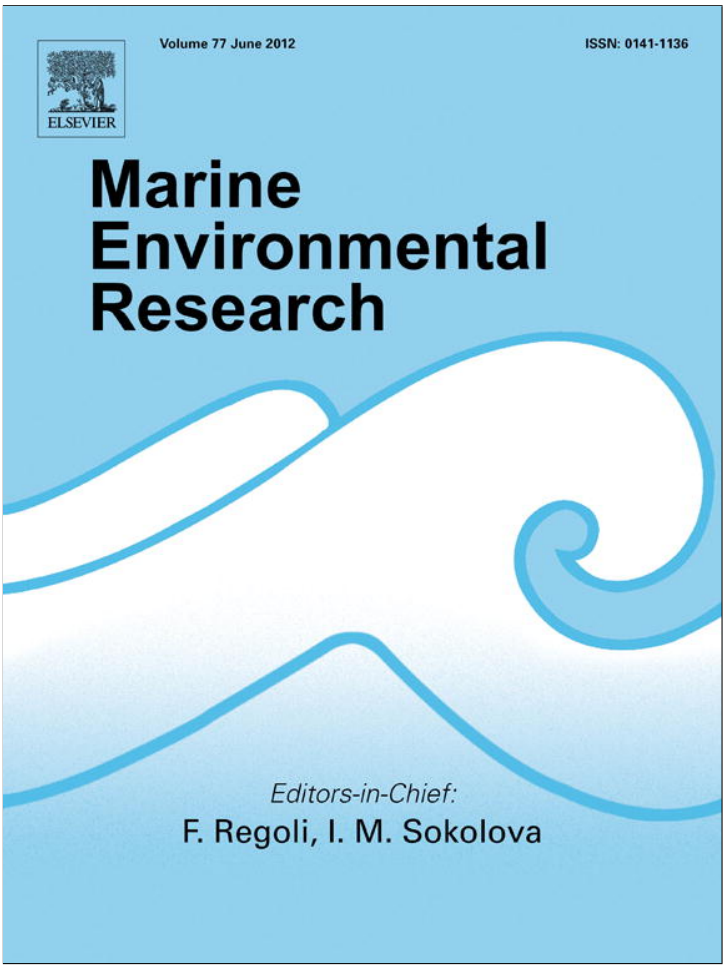
Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

Marine Environmental Research 77 (2012) 23–29

Contents lists available at SciVerse ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Uptake and release of paralytic shellfish toxins by the clam *Ruditapes decussatus* exposed to *Gymnodinium catenatum* and subsequent depuration

Maria João Botelho [a,*], Carlos Vale [a], Rita Velez Grilo [a], João Gomes Ferreira [b]

[a] IPIMAR, Av. Brasília, 1449-006 Lisbon, Portugal
[b] CMA, Department of Environmental Sciences and Engineering, Faculty of Sciences and Technology, New University of Lisbon, Qta Torre, 2829-516 Monte de Caparica, Portugal

ARTICLE INFO

Article history:
Received 20 October 2011
Received in revised form
11 January 2012
Accepted 11 January 2012

Keywords:
Paralytic shellfish toxins
Ruditapes decussatus
Particulate organic matter
Uptake
Depuration
Mass balance

ABSTRACT

A laboratory experiment was performed with the clam *Ruditapes decussatus*, fed with the toxic dinoflagellate *Gymnodinium catenatum* and the non-toxic algae *Isochrysis galbana* (14 days) and subsequently only with *I. galbana* (15 days). Individual paralytic shellfish toxins were determined by LC-FLD in *G. catenatum* cells, whole clam tissues, and particulate organic matter (POM) produced by clams. The toxins dcSTX and dcGTX2 + 3 in the algae were less abundant than C1 + 2 and B1, but were predominant in clams during both the exposure and depuration phases. The toxin dcNEO was only detected in clams during a short period, indicating conversion from other compounds. The toxin composition of the POM indicated the export of dcSTX as faeces or pseudo-faeces along the entire experiment (2.5–14 nmol mg^{-1}), B1 was present in a short period of the exposure and C1 + 2 and dcGTX2 + 3 absent. A mass balance calculation indicated that approximately 95% of C1 + 2 and 85% of B1 supplied to the clams were converted into other toxins or lost in solution. Conversely, the net gain of 512, 61 and 31 nmol for dcSTX, dcGTX2 + 3 and dcNEO, respectively, suggests the conversion from other assimilated compounds by clams during exposure and depuration phases.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Paralytic shellfish toxins (PSTs) are a group of neurotoxic alkaloids produced in the marine environment by dinoflagellates like *Alexandrium* spp., *Pyrodinium bahamense* and *Gymnodinium catenatum* (Llewellyn, 2006). The documented PSTs are grouped into three structural families in decreasing order of toxicity (Oshima, 1995a; Anon, 2009): carbamate (saxitoxin-STX, neosaxitoxin-NEO and gonyautoxins-GTX1 to GTX4), decarbamoyl (dcGTX1 to dcGTX4, dcSTX and dcNEO), and *N*-sulfocarbamoyl (B1, B2, C1 to C4). Most of the *Alexandrium* species have a carbamate-dominated toxin profile, while decarbamoyl and *N*-sulfocarbamoyl toxins are dominant components in *G. catenatum* (Cembella et al., 1987; Chou et al., 2004; Ordás et al., 2004; Band-Schmidt et al., 2005; Costa et al., 2010). It is well documented that PSTs are efficiently accumulated by filter-feeders during blooms of toxic phytoplankton species, being causative agents of paralytic shellfish poisoning in humans (Sommer and Meyers, 1937; Gessner and Middaugh, 1980).

Various studies have proved that shellfish exposed to dinoflagellates exhibit different PSTs profiles from the toxin producers (Bricelj et al., 1990; Oshima et al., 1990; Cembella et al., 1993; Samsur et al., 2006). Metabolic interconversion of assimilated PSTs achieved by enzymatic and chemical reactions in shellfish tissues may contribute to those differences (Shimizu and Yoshioka, 1981; Kotaki et al., 1985; Oshima, 1995b; Bricelj and Shumway, 1998). Incubation in vitro of toxic dinoflagellates or purified toxins extracts allowed elucidating the role of enzymatic activities (Sullivan et al., 1983; Fast et al., 2006; Artigas et al., 2007). Different uptake and depuration kinetics of individual PSTs may also contribute to the registered modifications on toxin profiles between the toxic algae and the exposed shellfish (Blanco et al., 2003; Yu et al., 2007; Botelho et al., 2010a).

Despite the low concentration of individual toxins in the dissolved fraction their quantification provides additional information on the elimination of individual PSTs (Bricelj and Shumway, 1998; Sekiguchi et al., 2001). Toxins in particulate organic matter rejected by shellfish are at higher concentrations, but may be difficult to interpret if encompassing particles from different pathways, namely faeces, pseudo-faeces and wasted food (Samsur et al., 2006; Estrada et al., 2007; Samsur et al., 2007).

This work reports the levels of the toxins C1 + 2, B1, dcSTX, dcGTX2 + 3 and dcNEO in whole tissues of the clam *Ruditapes decussatus* and in particulate organic matter rejected by the clams during a 29-day feeding experiment, including exposure to

* Corresponding author. Tel.: +351 21 3027172; fax: +351 21 3015948.
E-mail address: mjoao@ipimar.pt (M.J. Botelho).

0141-1136/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marenvres.2012.01.002

Author's personal copy

G. catenatum and subsequent depuration. Toxin composition of toxic algae, clams and particulate fraction in conjunction with mass balance calculations for individual toxins, allowed identifying the toxins exported as faeces or pseudo-faeces, as well as the compounds biotransformed during exposure and depuration phases.

2. Material and methods

2.1. Algal culture

The culture of the dinoflagellate *G. catenatum* (strain C37-07, IPIMAR collection) was maintained in natural seawater enriched with GSe medium (salinity 28, Blackburn et al., 2001) at 18 °C under a 16 h light: 8 h dark photocycle with a light intensity of 15 μmol photons m^{-2} s^{-1}. The strain was then mass cultured in 10-L culture flasks under the same conditions to supply to the clam feeding experiment. Cells of *G. catenatum* were harvested in the late exponential growth phase. The non-toxic microalgae *Isochrysis galbana* was grown in Wallerstein and Miquel medium (Bandarra et al., 2003) at 18 °C in 75-L plastic bags under constant illumination at salinity 25. Cells of the toxic microalgae were counted in Palmer-Maloney chambers under an Zeiss IM 35 inverted microscope and of *I. galbana* in an automatic particle counter (Coulter EPICS XL).

2.2. Clams

A total of 450 clams (*R. decussatus*) were obtained from growth banks at Ria Formosa, a coastal lagoon located in southern Portugal with an annual production of 5000 T. Clams were collected in November 2008, after several years of undetected *G. catenatum* blooms in the lagoon or adjacent coastal zone. Animals were acclimatized during 15 days to the laboratory conditions in a 50-L aerated polyethylene tank with filtered seawater and fed daily with the non-toxic microalga *I. galbana* culture (2×10^9 cells). Whole tissue wet weight and shell length of the clams ranged within 1.3–1.8 g and 2.8–3.9 cm, respectively.

2.3. Feeding experiment

Clams were divided into 42 groups of 10 individuals each and placed into 5-L plastic tanks filled with filtered natural seawater stored in a reservoir. The seawater in the tanks was continuously aerated and renewed daily. Water temperature remained at 12 ± 1 °C. The feeding experiment consisted of two phases schematically illustrated in Fig. 1. During phase I (14 days) each 10-clam group was fed twice a day with 1×10^4 cells of *G. catenatum* culture and 5×10^7 cells of *I. galbana* culture. During phase II (15 days) clams were fed twice a day only with 5×10^7 cells of *I. galbana* culture. No control animals were considered in the experiment, since *I. galbana* does not produce toxins and clam responses to toxin exposure was not studied. Throughout the 29 days of the experiment no clam deaths occurred and changes in the whole tissue weight were negligible.

2.4. Samples

Toxic algae. Aliquots of 400-mL *G. catenatum* culture were harvested at days 3, 9 and 14 of the phase I for toxin analysis. Algal mass was obtained by filtering the culture under light vacuum pressure (100 mmHg) through GF/C glass filters (1.2 μm), and freezing in 0.1 M acetic acid at −80 °C until analysis.

Clams. Specimens from three pools of 10 clams each were sacrificed in days 0, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 and 29, dissected and composite samples ($n = 10$) of whole soft tissues were prepared and stored at −80 °C until analysis.

Particulate organic matter. Particulate organic matter (POM) produced by clams was collected from the experimental tanks daily after day 3. Seawater of three tanks was transferred to an inverted conic recipient and particles were left settling for 12 h. Material was then collected by filtration through 0.45 μm polycarbonate membranes, and kept frozen in 0.1 M acetic acid at −80 °C until analysis.

2.5. Reagents

All chemicals and solvents used were LC or analytical grade. Sodium hydroxide and hydrogen peroxide were from Merck (Germany). Ultra-pure water was obtained by a *Milli-Q* system Millipore (France). Acetonitrile, acetic acid, methanol and ammonium formate were purchased from Sigma–Aldrich (Germany).

2.6. Toxin extraction and oxidation

Toxic algae. Toxins of *G. catenatum* cells retained on filters were extracted according to the method described in Artigas et al. (2007), with the following modifications. The extraction was done by freeze/thaw cycle followed by ultrasonication in an ice bath for 30 s at 60% amplitude and 20 W (*Vibra Cell*, Sonics & Materials Inc.). Cell debris after ultrasonification was examined under an inverted microscope revealing full disruption of the surveyed samples. The crude extract was centrifuged at 2500 g for 10 min, and then cleanup using a Solid Phase Extraction (SPE) C18 cartridge (*Sep-Pak Light*, Waters, USA). The 130-mg C18 cartridge was conditioned with 1.5 mL methanol and 1.5 mL ultra-pure water. Subsequently an aliquot of 250 μl of supernatant was loaded, then

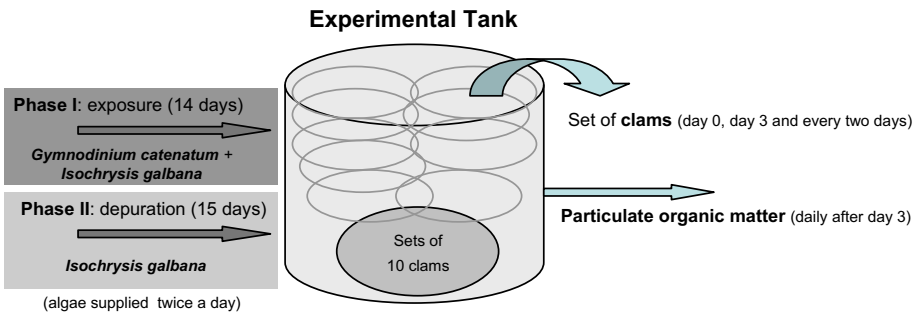


Fig. 1. Schematic representation of the laboratory feeding experiment.

Author's personal copy

washed with 500 μl of ultra-pure water. The pH of the extract was adjusted to 6.5 with 0.2 mol L^{-1} NaOH, and diluted to exactly 1 mL.

POM. Toxins in the collected POM were extracted using the same procedure as for dinoflagellate cells, except for the cleanup step, which was skipped due to the low toxin content. After centrifugation the extract was filtered through disposable 0.2 μm regenerated cellulose syringe filters.

Clams. Approximately 5 g of clam homogenate were double-extracted with 1% acetic acid solution (first extraction with heating), following AOAC 2005.06 (Anon, 2005). The extract passed through a SPE C18 cartridge (500 mg/3 ml, *Supelclean*, Supelco, USA).

Aliquots of *G. catenatum*, clams and particles extracts were used for peroxide and periodate oxidation of PSTs prior to liquid chromatography with fluorescence detection (LC-FLD) analyses. The procedure used in the oxidation of PSTs was based on the AOAC method (Anon, 2005) with a procedural modification due to dominance of *N*-sulfocarbamoyl and decarbamoyl compounds in the *G. catenatum* toxic profile (Botelho et al., 2010b). Similar procedures of peroxide and periodate oxidations were followed, substituting the oxidant reagent by water in order to detect naturally fluorescent compounds.

2.7. LC-FLD analysis

The LC system consisted of a Hewlett–Packard/Agilent (Germany) Model 1050 quaternary pump, Model 1100 in-line degasser, autosampler, column oven, and Model 1200 fluorescence detector. The Hewlett–Packard *Chemstation* software performed data acquisition and peak integration. The PST oxidation products were separated using a reversed-phase *Supelcosil* LC-18, 150 × 4.6 mm, 5 μm column (Supelco, USA) equipped with a guard column *Supelguard Supelcosil* C18, 20 × 4.0 mm id, 5 μm (Supelco, USA). The column was kept in an oven at 30 °C. The mobile phase gradient used 2 mobile phases: A (0.1 M ammonium formate, pH = 6) and B (0.1 M ammonium formate in 5% acetonitrile, pH = 6). The elution gradient consisted of 0–5% B in the first 5 min, 5–70% B in the next 4 min and back to 0% B in the next 5 min. Flow rate was 1 mL/min and the injection volumes were 50 μL and 100 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.

2.8. Quality control

The certified reference materials, CRM-STX-e, CRM-NEO-b, CRM-GTX2&3-b, CRM-GTX1&4-b, CRM-dcSTX, CRM-dcGTX2&3, CRM-GTX5-b (B1), CRM-C1&2 and CRM-dcNEO-b were obtained from the Institute for Marine Biosciences, National Research Council Canada (IMB, NRCC, Halifax, NS, Canada). For PSTs quantification in shellfish tissues, matrix matched calibration curves with cleaned-up clam tissue extract were used as described in Botelho et al. (2010a). Calibration curves prepared in solvent were used for toxin quantification in *G. catenatum* and particles extracts. Evaluation of linear ranges for PSTs and instrumental limits of detection (LOD), are described in Botelho et al. (2010b). Instrumental LODs were 3.9 nmol L^{-1} (C1 + 2); 4.0 nmol L^{-1} (B1); 4.0 nmol L^{-1} (STX); 4.9 nmol L^{-1} (dcSTX); 8.2 nmol L^{-1} (dcGTX2 + 3); 8.5 nmol L^{-1} (GTX2 + 3); 25 nmol L^{-1} (dcNEO); 30 nmol L^{-1} (GTX1+4) and 31 nmol L^{-1} (NEO). Recoveries of the analytical procedure were assessed through clam tissues spiked in triplicate, with the addition of a toxin mixture at two concentration levels. Intervals of the mean recoveries for the quantified PSTs in clams were: 71–74% (C1 + 2), 97–98% (B1), 77–114% (dcSTX), 85–107% (dcGTX2 + 3) and 55–56% (dcNEO). Repeatability values in terms of relative standard deviation were from 1 to 18%. Moreover, recoveries were assessed in POM spiked with B1 and dcSTX in triplicate at two concentration levels. Only the toxins quantified in POM were considered. The following intervals were obtained: 101–107% (B1) and 76–97% (dcSTX). Repeatability values were from 12 to 18%.

2.9. Mass balance calculation

The mass balance for individual toxins in clams was calculated for a basis of 100 clams by the following equation:

$$T_a + T_g = T_c + T_p + T_l + T_r \quad (1)$$

where:

T_a: mass of individual toxin in the toxic algae supplied to the clams
T_g: mass of individual toxin in clams gained by conversion of other assimilated compounds
T_c: mass of individual toxin accumulated in whole clam tissues
T_p: mass of individual toxin in particulate organic matter of the experimental tanks
T_l: mass of individual toxin in clams lost by conversion
T_r: mass of individual toxin released by clams to solution

The mass balance was calculated assuming that clams have ingested the whole algal mass supplied, and consequently the particulate organic matter collected in the experiments tanks consisted of material produced by clams. This assumption is consistent with the lack of *G. catenatum* intact cells in seawater from the tanks that was examined microscopically on a daily basis. In equation (1) T_g, T_l and T_r are unknown terms, their sum being substituted by T_{ng} that may be considered as a net gain quantity. By resolving the equation (1) T_{ng} was calculated by:

$$T_{ng} = T_c + T_p - T_a \quad (2)$$

2.10. Statistical analysis

Data statistical analysis were performed using the STATISTICA 6.0 Statistical Software System. The fitting of time-course variation of toxin concentrations to the experimental points was assessed by SigmaPlot 8.0 (Systat Software Inc.).

3. Results

3.1. Toxin profile of G. catenatum

Fig. 2 shows the median, the percentile 25% and 75%, minimum and maximum of concentrations of the toxins C1 + 2, B1, dcSTX and dcGTX2 + 3 quantified in the culture of *G. catenatum*. Samples were taken in replicates ($n = 4$) every 5 days during the 14 days of the exposure phase of the experiment. The *N*-sulfocarbamoyl analogues B1 and C1 + 2 were the major toxins produced by this culture, concentrations exceeding one order of magnitude the values found for the decarbamoyl analogues dcSTX and dcGTX2 + 3. Variation of the concentrations for the three sampling periods was comparable to values of the four replicates at each sampling period for C1 + 2 (33 and 31%), B1 (41 and 31%) and dcSTX (31 and 31%). A larger discrepancy was observed for dcGTX2 + 3 (42 and 19%). Modifications on cell size, chain length, and toxin leakage may contribute to the observed variability (Granéli and Flynn, 2006; Band-Schmidt et al., 2010).

Author's personal copy

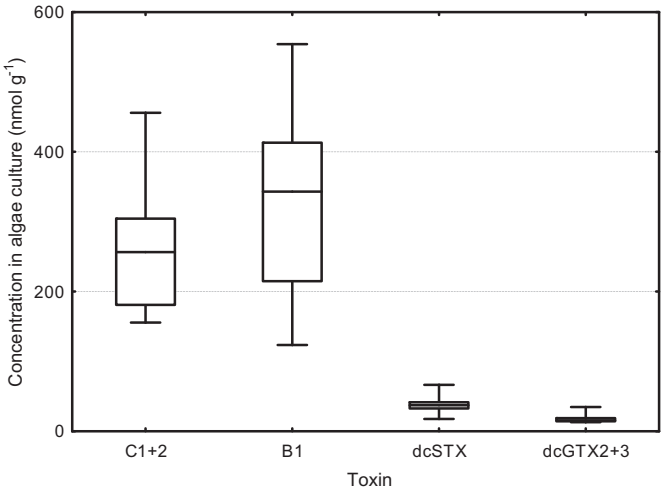


Fig. 2. Median, percentile 25% and 75%, minimum and maximum of C1 + 2, B1, dcSTX, dcGTX2 + 3 concentrations (nmol g^{-1}) in the culture of *Gymnodinium catenatum* at days 2, 7 and 12 of the clam exposure experiment; four replicates at each sampling date.

3.2. Toxin concentrations in R. decussatus

Mean concentrations (±one standard deviation) of the toxins C1 + 2, B1, dcSTX, and dcGTX2 + 3 in whole soft tissues of *R. decussatus* collected at days 0, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 and 29 are presented in Fig. 3. In general, toxin concentrations in clams increased during the feeding with *G. catenatum*, although the accumulation pattern differed among the compounds. The first concentration augment was registered for dcSTX in day 3, enhancements of B1 and dcGTX2 + 3 were found in day 5, while a sharp increase of C1 + 2 was only observed 11 days after exposure to the toxic algae. The maximum levels of C1 + 2 and dcGTX2 + 3 were registered by the end of the exposure period, while of B1 and dcSTX were found three days after, during the subsequent depuration period. The maximum concentrations pointed to a higher accumulation of decarbamoyl toxins (dcSTX and dcGTX2 + 3) than *N*-sulfocarbamoyl (B1 and C1 + 2). In addition to the above mentioned toxins, dcNEO was also found in clams (not shown). However, values were only above detection limit in days 11, 13 and 15 not exceeding 0.30 nmol g^{-1}. Results showed an opposite PSTs composition between clams and *G. catenatum* (Figs. 2 and 3).

Clams fed by a non-toxic diet exhibited a substantial decrease of C1 + 2 concentrations (18% of the peak value) eight days after depuration. Conversely, values of B1 and dcSTX remained at 68% and 50%, respectively, of the maximum concentrations after 14 days. An intermediate situation was found for dcGTX2 + 3 that exhibited a reduction of 37% at the end of the experiment. Furthermore a higher dispersion of dcGTX2 + 3 concentrations was registered between replicates in days 9, 17, 19 and 21.

3.3. Best fitting curves

The best fitting curves of the experimental points were searched for each quantified toxin. Table 1 shows the two equation types for exposure and depuration conditions, power equations ($y = ax^b$) and exponential decay equations ($y = ae^{-bx}$), respectively. Correlation coefficients and levels of significance are also given.

The calculated *b* values of the power equations were 2.45 ± 1.07 (C1 + 2), 1.55 ± 0.202 (B1), 1.18 ± 0.122 (dcSTX) and 1.33 ± 0.298 (dcGTX2 + 3). The fitting curve for C1 + 2 presented a level of significance above 0.05 (Table 1). The equations governing the time-dependent toxin concentrations under depuration conditions mirror a first-order kinetic, where *a* is the toxin concentration in the clam at the initial depuration conditions and *b* is the depuration rate. The levels of significance for C1 + 2, dcSTX and dcGTX2 + 3 curves were below 0.05, corroborating the validity of the first-order decay approach. The calculated depuration rates (*b*) were

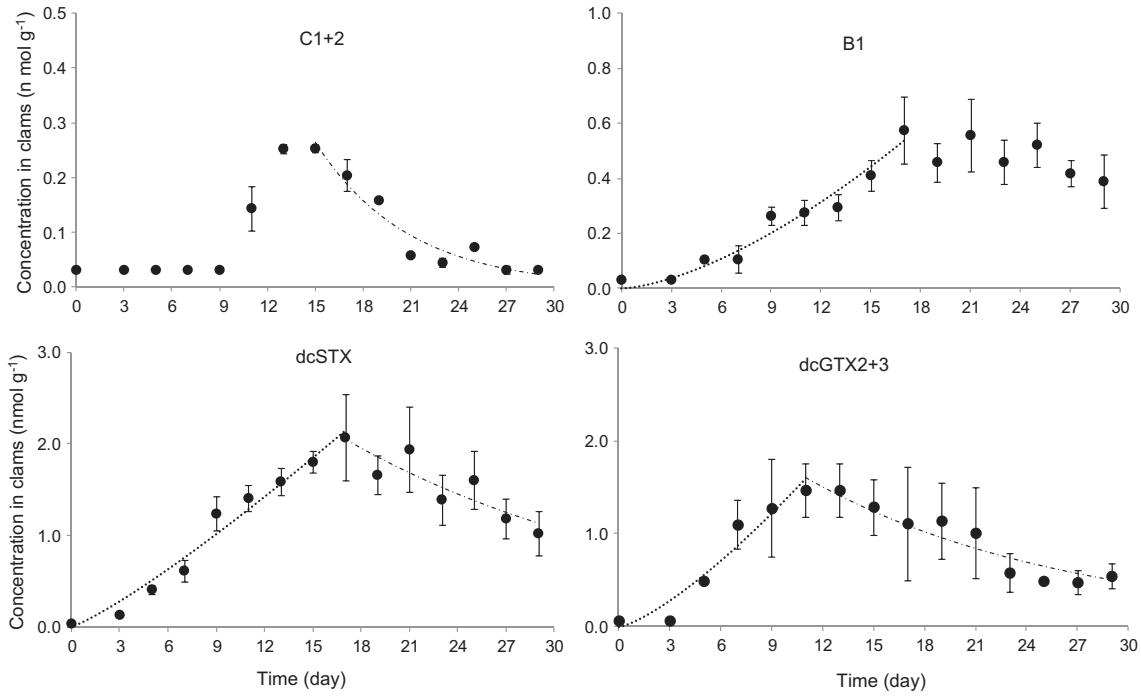


Fig. 3. Concentrations of the toxins C1 + 2, B1, dcSTX and dcGTX2 + 3 (nmol g^{-1}) in the clam *Ruditates decussatus* exposed 14 days to *Gymnodinium catenatum* and 15 days under depuration conditions; mean concentrations ($n = 3$; ±SD) and best fitting curves for exposure and depuration periods.

Author's personal copy

Table 1
Best fitting curves for C1 + 2, B1, dcSTX and dcGTX2 + 3 in *Ruditapes decussatus* during phase I (exposure) and phase II (depuration); calculated parameters (standard error); correlation coefficients (r) and levels of significance (p).

Phase I (exposure)			
Equation type: $y = ax^b$			
Toxin	a	b	r
C1 + 2[a]	0.0004 (0.001)	2.45 (1.07)	0.812**
B1	0.007 (0.004)	1.55 (0.202)	0.976*
dcSTX	0.076 (0.024)	1.18 (0.122)	0.986*
dcGTX2 + 3	0.065 (0.043)	1.33 (0.298)	0.967*
Phase II (depuration)			
Equation type: $y = a\,e^{-bx}$			
Toxin	a	b	r
C1 + 2	0.26 (0.021)	0.17 (0.024)	0.965*
B1	0.56 (0.038)	0.025 (0.010)	0.733**
dcSTX	2.06 (0.146)	0.049 (0.012)	0.886*
dcGTX2 + 3	1.61 (0.097)	0.065 (0.008)	0.948*

*$p < 0.05$ and **$p > 0.05$.
[a] Valid to $x > 9$.

0.17 ± 0.024 day^{-1} for C1 + 2, 0.049 ± 0.012 day^{-1} for dcSTX and 0.065 ± 0.0085 day^{-1} for dcGTX2 + 3. The fitting curve for B1 presented a level of significance above 0.05 (Table 1), indicating a poorer first-order decay approach. Fig. 3 shows computed and measured toxin concentrations in clams during the two phases of the experiment (Fig. 3).

3.4. Toxin composition of particulate organic matter

The mass of particulate organic matter (POM) produced daily by 100 clams, from 10 experimental tanks, during phase I increased from 0.50 to 2.5 mg (Fig. 4). The amount was more irregular under depuration conditions, varying between 0.83 and 5.5 mg. Concentrations of the toxins C1 + 2, dcGTX2 + 3 and dcNEO in POM were always below the detection limit. Conversely, the toxin dcSTX was quantified, concentrations increasing from 2.5 to 5.9 nmol mg^{-1} as clams were fed with the toxic algae, and ranging between 3.8 and 14 nmol mg^{-1}, under a non-toxic diet (Fig. 4). Furthermore, three periods of dcSTX enrichment were observed in the produced POM. An intermediate situation was found for B1, concentration being undetectable, except during 4 days of the phase I of the experiment that reached a maximum of 2.5 nmol mg^{-1}.

3.5. Mass balance calculation

Table 2 gives the amounts of toxins supplied to clams, accumulated in whole clam tissues and in POM produced by clams during the exposure phase (I), depuration phase (II) and the entire experiment. The quantities of C1 + 2, B1, dcSTX,dcGTX2 + 3 and dcNEO, expressed in nmol, were computed on a basis of 100 clams, corresponding to 10 experimental tanks. In the mass balance calculation, the value of detection limit for each compound was accounted when concentrations were undetected.

Table 2 presents also the net gain values (T_{ng}) of each toxin calculated according to the equation (2). Negative values of T_{ng}, like for C1 + 2 and B1 in the phase I, mean that amounts supplied to the clams were not accounted in the whole clam tissues or POM. Approximately 95 and 85% of the mass of C1 + 2 (T_a = 392 nmol) and B1 (T_a = 470 nmol), respectively, added to the experiment tanks were converted into other toxins by clams or lost in solution (T_{ng} = −372 nmol and T_{ng} = −401 nmol, respectively). Conversely, positive values of dcSTX, dcGTX2 + 3 and dcNEO, imply a net gain most likely resulted from the conversion of other assimilated

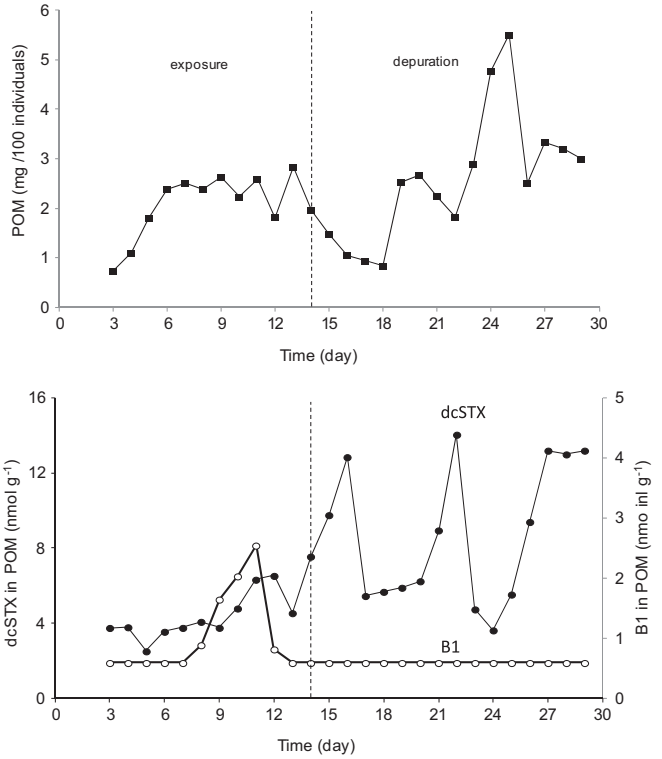


Fig. 4. Variation of the amount (mg) of particulate organic matter (POM) produced by 100 individuals of *Ruditapes decussatus* exposure to *Gymnodinium catenatum* and under depuration conditions; concentrations (nmol g^{-1}) of the toxins B1 and dcSTX in the POM.

compounds by clams. The net gain (T_{ng}) of dcSTX (250 nmol) and dcGTX2 + 3 (108 nmol) were approximately 5 times the amounts added to the tanks (50 and 21 nmol, respectively). The net gain of dcNEO (22 nmol) has no correspondence in the algae supplied. Under depuration (phase II), T_{ng} for C1 + 2, B1 and dcGTX2 + 3 were one order of magnitude lower (−15, 38 and −47 nmol, respectively) than during the phase I. Only 9 nmol of dcNEO were accounted. Contrarily, comparable T_{ng} were obtained for dcSTX in phases I (250 nmol), and phase II (262 nmol). The calculation of the T_{ng} values for the entire experiment, points to a substantial loss of

Table 2
Mass (nmol) of toxins accumulated in *Ruditapes decussatus* (T_c), supplied to the experiment (T_a) and present in particulate organic matter (T_p) after phase I, phase II, and phases I + II. Values of T_{ng} were computed according to equation (2). Amounts were calculated for a basis of 100 clams.

	Toxin	T_c	T_a	T_p	T_{ng}
		nmol	nmol	nmol	nmol
Phase I	C1 + 2	20	392	–	−372
	B1	41	470	28	−401
	dcSTX	180	50	120	250
	dcGTX2 + 3	129	21	–	108
	dcNEO	22	–	–	22
Phase II	C1 + 2	−15	–	–	−15
	B1	17	–	21	38
	dcSTX	−26	–	288	262
	dcGTX2 + 3	−47	–	–	−47
	dcNEO	9	–	–	9
Phase I + II	C1 + 2	5	392	–	−387
	B1	58	470	49	−363
	dcSTX	154	50	408	512
	dcGTX2 + 3	82	21	–	61
	dcNEO	31	–	–	31

Author's personal copy

C1 + 2 and B1, amounting −750 nmol and a gain of 604 nmol of the sum of dcSTX, dcGTX2 + 3 and dcNEO.

4. Discussion

4.1. Conversion of toxins assimilated by clams into decarbamoyl toxins

The concentrations of the toxins C1 + 2, B1, dcSTX, dcGTX2 + 3 and dcNEO as well as their relative proportion differed considerably in whole tissues of the clam *R. decussatus* and *G. catenatum* culture. The dcSTX and dcGTX2 + 3 were predominant in clams, although being detected at minor concentrations in the algae. Conversely, C1 + 2 and B1 were more abundant in the algae and minor in clams. In particular, C1 + 2, which is dominant in the supplied toxic algae, was detected later than the other toxins in the clams during the exposure period (Fig. 3). This delay on the accumulation of C1 + 2 pointed to different behaviour during the uptake, namely specific metabolic transformations. The undetected concentrations of dcNEO in the algae in conjunction with the presence in clams in days 11, 13 and 15 indicate the conversion of other assimilated toxins into dcNEO.

The modifications observed on PSTs profile between clams and the supplied algae cells were less drastic than those reported in other experiments with the clam *Tapes japonica* fed by *Alexandrium catenella* (Samsur et al., 2006) and *G. catenatum* (Samsur et al., 2007). In these feeding experiments, decarbamoyl derivatives were found in clams (*T. japonica*) being undetectable in *A. catenella* or *G. catenatum* cells that were used as food. The appearance of those compounds in clams was attributed to enzymatic hydrolysis of *N*-sulfocarbamoyl compounds supplied only once at the beginning of the experiment (Sullivan et al., 1983; Oshima, 1995b). The same explanation may be invoked to the results obtained in the current work. However, hydrolysis may have been more effective since *G. catenatum* cells were supplied daily during the exposure phase. Ingestion by clams during 14 days may have resulted in an ongoing biotransformation process, explaining the progressive difference of toxin profiles between *R. decussatus* and the ingested *G. catenatum* cells. The predominance of decarbamoyl toxins during the depuration phase of the experiment, indicates that biotransformation of other assimilated toxins into dcSTX and dcGTX2 + 3 pursued beyond the ingestion of the toxic algae. It is recurrently observed in national monitoring programmes that toxicity in shellfish remains beyond the presence of high density of toxic algae cells. The results of the current work show that dcSTX may be responsible for the prolonged toxicity in clams exposed to a *G. catenatum* bloom. Two factors contribute to this extension of toxicity. Firstly, the slower depuration rate of dcSTX in shellfish (Botelho et al., 2010a), and the high toxicity equivalent factor in comparison to the other toxins produced by *G. catenatum* (Oshima, 1995a; Anon, 2009). For example, the toxicity equivalent factor of dcSTX is 10 times higher than of B1 and C1 + 2.

The mass balance calculated for the entire experiment (Table 2) evidenced the net gain of decarbamoyl compounds and the conversion of C1 + 2 and B1 in comparable quantities (750 and 604 nmol, respectively). It appears that dcSTX and dcGTX2 + 3 resulted mainly from the biotransformation of C1 + 2 and B1, although other toxins assimilated by clams should not be negligible. This hypothesis is in line with several works pointing to the transformation of saxitoxin derivatives in shellfish contaminated by *G. catenatum* into dcSTX (Oshima et al., 1990; Lin et al., 2004; Vale, 2008). The slower depuration rates of dcSTX and dcGTX2 + 3 in comparison to C1 + 2 observed in the current experiment indicate a tendency to higher retention time of those compounds in clams, either resulted from assimilation or conversion from other toxins.

4.2. Elimination of dcSTX through clam faeces/pseudo-faeces

Toxin composition of particulate organic matter in rearing tanks is of difficult interpretation because often it reflects the mixture of wasted food, faeces and pseudo-faeces produced by shellfish (Samsur et al., 2006; Estrada et al., 2007; Samsur et al., 2007). The contribution of wasted food to the POM collected in the current experiment is most likely negligible. Firstly, because the dominant toxic component of the *G. catenatum* culture added to the experimental tanks (C1 + 2) were below the detection limit in the POM. Moreover, daily microscopic examination of seawater from the tanks, showed no *G. catenatum* intact cells. This indicates that clams have ingested the algal mass supplied daily, and particulate organic matter collected during the period that clams were exposed to the toxic algae (phase I) is, thus, admittedly constituted by a mixture of clam faeces and pseudo-faeces.

A relevant aspect of this work is that dcSTX was the only toxin quantified in the POM produced by clams during the exposure and depuration phases of the 28-day experiment. The toxin B1 was only detected during a short period of the exposure phase. The toxin profile of the particles produced by clams is different from that in the whole clam tissues. Estrada et al. (2007) proposed that PSTs in faeces are an indicator of the toxin content in the digestive system. The validity of this relationship cannot be tested in the current work because clam tissues were analyzed as a whole, and toxin concentration in the clam gut content may have been diluted with the low values in other tissues, as shown for other shellfish (Cembella et al., 1994; Shumway et al., 1994).

The toxin composition of faeces or pseudo-faeces produced during the exposure phase should reflect the ingested toxic algae composition and metabolic transformations of the assimilated toxins. However, only dcSTX, and punctually B1, were found in the POM. The absence of C1 + 2 and dcGTX2 + 3 suggests their biotransformation as ingested by clams. A similar explanation could be invoked regarding B1, since it was only quantified in the 8th day of exposure to the toxic algae. Although It should not be excluded the possibility of toxins being released in solution due to the hydrophilic nature of PSTs (Shimizu, 2000), the contribution of the soluble fraction to the mass balance should be low as suggested by Sekiguchi et al. (2001).

Under depuration, dcSTX concentrations in the POM were irregular with pronounced maximum (Fig. 4). These concentration peaks cannot reflect the composition of ingested food since the diet was only composed by non-toxic algae. Presumably, metabolic processes have a key role on the composition of the eliminated particles. Furthermore, dcSTX was the major excreted toxin by *R. decussatus* after fed by *G. catenatum*, which indicates its provenance from reactions associated with the metabolic transformation. Results of the two phases of the experiments pointed to the conversion of assimilated toxins, namely B1 and C1 + 2, into dcSTX and dcGTX2 + 3. This hypothesis is in accordance with previous works with the clam *Mactra chinensis* (Lin et al., 2004) and *Perdonidia venulosa* (Cho et al., 2008). The gain of decarbomoyl toxins evidenced in the mass balance approach is in line with conversion of toxins by clams. The toxin composition of the POM showed the export of dcSTX as faeces or pseudo-faeces. The elimination of C1 + 2 and dcGTX2 + 3 as rejected particles were negligible, being likely transformed into other compounds or loss in solution.

5. Conclusion

Toxin composition of particulate organic matter rejected by the clam *R. decussatus* under depuration conditions after being exposed

Author's personal copy

to *G. catenatum* indicates the release of dcSTX, pointing to interconversion of other assimilated toxins during the exposure.

Acknowledgments

This work had been performed under the projects Quasus (Environmental quality and sustainability of biologic resources in Ria Formosa) and Forward (Framework for Ria Formosa water quality, aquaculture, and resource development) financed by Sociedade Polis Litoral Ria Formosa. We are grateful to M.T. Moita for the *G. catenatum* strain from IPIMAR culture collection, and to B. Frazão for maintaining the toxic algal culture.

References

Anon, 2005. AOAC Official Method 2005.06. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using pre-chromatographic oxidation and liquid chromatography with fluorescence detection. AOAC International, Gaithersburg, MD, USA.

Anon, 2009. Scientific opinion of the panel of contaminants in the food chain on a request from the European Commission on marine biotoxins in shellfish – Saxitoxin Group. EFSA J. 1019, 1–76.

Artigas, M.L., Vale, P., Gomes, S.S., Botelho, M.J., Rodrigues, S.M., Amorim, A., 2007. Profiles of paralytic shellfish poisoning toxins in shellfish from Portugal explained by carbamoylase activity. J. Chromatogr. A 1160, 99–105.

Bandarra, N.M., Pereira, P.A., Batista, I., Vilela, M.H., 2003. Fatty acids, sterols and α-tocopherol in *Isochrysis galbana*. J. Food Lipids 10, 25–34.

Band-Schmidt, C.J., Bustillos-Guzmán, J., Gárate-Lizárraga, I., Lechuga-Devéze, C.H., Reinhardt, K., Luckas, B., 2005. Paralytic shellfish toxin profile in strains of the dinoflagellate *Gymnodinium catenatum* Graham and the scallop *Argopecten ventricosus* G.B. Sowerby II from Bahía Concepción, Gulf of California, Mexico. Harmful Algae 4, 21–31.

Band-Schmidt, C.J., Bustillos-Guzmán, J.J., López-Cortés, D.J., Gárate-Lizárraga, I., Núnez-Vázquez, E.J., Hernández-Sandoval, F.E., 2010. Ecological and physiological studies of *Gymmodinium catenatum* in the Mexican Pacific: a review. Mar. Drugs 8, 1935–1961.

Blackburn, S.I., Bolch, C.J.S., Haskard, K.A., Hallegraeff, G.M., 2001. Reproductive compatibility among four global populations of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). Phycologia 40 (1), 78–87.

Blanco, J., Reyero, M.I., Franco, J., 2003. Kinetics of accumulation and transformation of paralytic shellfish toxins in the blue mussel *Mytilus galloprovincialis*. Toxicon 42, 777–784.

Botelho, M.J., Vale, C., Mota, A.M., Simões Gonçalves, M.L.S., 2010a. Depuration kinetics of paralytic shellfish toxins in *Mytilus galloprovincialis* exposed to *Gymnodinium catenatum*: laboratory and field experiments. J. Environ. Monit. 12, 2269–2275.

Botelho, M.J., Vale, C., Mota, A.M., Rodrigues, S.M., Costa, P.R., Simões Gonçalves, M.L.S., 2010b. Matrix effect on paralytic shellfish toxins quantification and toxicity estimation in mussels exposed to *Gymnodinium catenatum*. Food Addit. Contam. A 27, 1724–1732.

Bricelj, V.M., Lee, J.H., Cembella, A.D., Anderson, D.M., 1990. Uptake kinetics of paralytic shellfish toxins from the dinoflagellate *Alexandrium fundyense* in the mussel *Mytilus edulis*. Mar. Ecol. Prog. Ser. 63, 177–188.

Bricelj, V.M., Shumway, S.E., 1998. Paralytic shellfish toxins in bivalve molluscs: occurrence, transfer kinetics, and biotransformation. Rev. Fish. Sci. 6 (4), 315–383.

Cembella, A.D., Sullivan, J.J., Boyer, G.L., Taylor, F.J.R., Anderson, R.J., 1987. Variation in paralytic shellfish toxin composition within the *Protogonyaulax tamarensis*/*catenella* species complex; red tide dinoflagellates. Biochem. Syst. Ecol. 15 (2), 171–186.

Cembella, A.D., Shumway, S.E., Lewis, N.I., 1993. Anatomical distribution and spatiotemporal variation in paralytic shellfish toxin composition in two bivalve species from the Gulf of Maine. J. Shellfish Res. 12, 389–403.

Cembella, A.D., Shumway, S.E., Larocque, R., 1994. Sequestering and putative biotransformation of paralytic shellfish toxins by the sea scallop *Placopecten magellanicus*: seasonal and spatial scales in natural populations. J. Exp. Mar. Biol. Ecol. 180, 1–22.

Cho, Y., Ogawa, N., Takahashi, M., Lin, H.-P., 2008. Purification and characterization of paralytic shellfish toxin-transforming enzyme, sulfocarbamoylase I, from the Japanese bivalve *Peronidia venulosa*. Biochim. Biophys. Acta 1784 (9), 1277–1285.

Chou, H.N., Chen, Y.M., Chen, C.Y., 2004. Variety of PSP toxins in four culture strains in *Alexandrium minutum* collected from southern Taiwan. Toxicon 43 (3), 337–340.

Costa, P.R., Botelho, M.J., Lefebvre, K.A., 2010. Characterization of paralytic shellfish toxins in seawater and sardines (*Sardina pilchardus*) during blooms of *Gymnodinium catenatum*. Hydrobiologia 655 (1), 89–97.

Estrada, N.A., Lagos, N., Garcia, C., Maeda-Martínez, A.N., Ascencio, F., 2007. Effects of the toxic dinoflagellate *Gymnodinium catenatum* on uptake and fate of paralytic shellfish poisons in the Pacific giant lions-paw scallop *Nodipecten subnodosus*. Mar. Biol. 151, 1205–1214.

Fast, M.D., Cembella, A.D., Ross, N.W., 2006. In vitro transformation of paralytic shellfish toxins in the clams *Mya arenaria* and *Protothaca staminea*. Harmful Algae 5 (1), 79–90.

Gessner, B.D., Middaugh, J., 1980. Paralytic shellfish poisoning in Alaska: a 20-year retrospective analysis. Am. J. Epidemiol. 141, 766–770.

Granéli, E., Flynn, K., 2006. Chemical and physical factors influencing toxin content. In: Granéli, E., Turner, J. (Eds.), Ecology of Harmful Algae. Springer, Heidelberg, Germany, pp. 229–241.

Kotaki, Y., Oshima, Y., Yasumoto, T., 1985. Bacterial transformation of paralytic shellfish toxins. In: Anderson, D.M., White, A.W., Baden, D.G. (Eds.), Toxic Dinoflagellates. Elsevier Science Publishers, New York, pp. 287–292.

Lin, H.-P., Cho, Y., Yashiro, H., Yamada, T., Oshima, Y., 2004. Purification and characterization of paralytic shellfish toxin transforming enzyme from *Mactra chinensis*. Toxicon 44, 657–668.

Llewellyn, L.E., 2006. Saxitoxin, a toxic marine natural product that targets a multitude of receptors. Nat. Prod. Rep. 23, 200–222.

Ordás, M.C., Fraga, S., Franco, J.M., Ordás, A., Figueras, A., 2004. Toxin and molecular analysis of *Gymnodinium catenatum* (Dinophyceae) strains from Galicia (NW Spain) and Andalucia (S Spain). J. Plankton Res. 26 (3), 341–349.

Oshima, Y., Sugino, K., Itakura, H., Hiraota, M., Yasumoto, T., 1990. Comparative studies on paralytic shellfish toxin profile of dinoflagellates and bivalves. In: Granéli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, Amsterdam, pp. 479–485.

Oshima, Y., 1995a. Postcolumn derivatization liquid-chromatography method for paralytic shellfish toxins. J. AOAC Int. 78, 528–532.

Oshima, Y., 1995b. Chemical and enzymatic transformation of paralytic shellfish toxins in marine organisms. In: Lassus, P., Arzul, G., Erard, E., Gentien, P., Marcaillou, C. (Eds.), Harmful Marine Algae Blooms. Intercept Ltd., pp. 475–480.

Samsur, M., Yamaguchi, Y., Sagara, T., Takatani, T., Arakawa, O., Noguchi, T., 2006. Accumulation and depuration profiles of PSP toxins in the short-necked clam *Tapes japonica* fed with the toxic dinoflagellate *Alexandrium catenella*. Toxicon 48, 323–330.

Samsur, M., Takatani, T., Yamaguchi, Y., Sagara, T., Noguchi, T., Arakawa, O., 2007. Accumulation and elimination profiles of paralytic shellfish poison in the short-necked calm *Tapes japonica* fed with the toxic dinoflagellate *Gymnodinium catenatum*. J. Food Hyg. Soc. Japan 48, 13–18.

Sekiguchi, K., Sato, S., Ogata, T., Kaga, S., Kodama, M., 2001. Accumulation and depuration kinetics of paralytic shellfish toxins in the scallop *Patinopecten yessoensis* fed *Alexandrium tamarense*. Mar. Ecol. Prog. Ser. 220, 213–218.

Shimizu, Y., Yoshioka, M., 1981. Transformation of paralytic shellfish toxins as demonstrated in scallop homogenates. Science 212, 547–549.

Shimizu, Y., 2000. Paralytic shellfish poisoning. Chemistry and mechanism of action. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins. Marcel Dekker, New York, pp. 151–172.

Shumway, S.E., Sherman, S.A., Cembella, A.D., Selvin, R., 1994. Accumulation of paralytic shellfish toxins by surfclams, *Spisula solidissima* (Dilwyn, 1897) in the Gulf of Maine: seasonal changes, distribution between tissues, and notes on feeding habits. Nat. Toxins 2, 236–251.

Sommer, H., Meyers, K.F., 1937. Paralytic shellfish poisoning. Arch. Pathol. 24, 560–598.

Sullivan, J.J., Iwaoka, W.T., Liston, J., 1983. Enzymatic transformation of PSP toxins in the littleneck clam (*Protothaca staminea*). Biochem. Biophys. Res. Commun. 114, 465–472.

Vale, P., 2008. Fate of benzoate paralytic shellfish poisoning toxins from *Gymnodinium catenatum* in shellfish and fish detected by pre-column oxidation and liquid chromatography with fluorescence detection. J. Chromatogr. A 1190, 191–197.

Yu, K.N., Kwong, R.W.M., Wang, W.-X., Lam, P.K.S., 2007. Biokinetics of paralytic shellfish toxins in the green-lipped mussel, *Perna viridis*. Mar. Pollut. Bull. 54, 1031–1071.