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Temperature effects on kinetics of paralytic shellfish toxin elimination in Atlantic surfclams, *Spisula solidissima*

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ABSTRACT

Surfclams, *Spisula solidissima*, pose a particular health risk for human consumption as they are characterized by accumulation of extremely high levels of toxins associated with paralytic shellfish poisoning (PSP), slow toxin elimination and an extremely high post-ingestive capacity for toxin bioconversion. Surfclam populations experience a wide range of temperatures along the NW Atlantic continental shelf, and are undergoing range contraction that has been attributed to global warming. In this study the influence of temperature (5, 12 and 21 °C) on detoxification kinetics of individual PSP toxins in two tissue compartments of juvenile surfclams (~35 mm shell length) was determined under controlled laboratory conditions, over prolonged (2.4 months) depuration. Clams were toxified with a representative regional Gulf of Maine isolate of the dinoflagellate *Alexandrium fundyense* of known toxin profile, allowing tracking of changes in toxin composition and calculated toxicity in surfclam tissues. The visceral mass detoxified at all temperatures, although toxin loss rate increased with increasing temperature. In contrast, total toxin content and calculated toxicities in other tissues remained constant or even increased during depuration, suggesting a physiological or biochemical toxin-retention mechanism in this tissue pool and temperature-independent detoxification. In vivo toxin compositional changes in surfclam tissues found in this study provide evidence of specific toxin conversion pathways, involving both reductive and decarbamoylation pathways. We conclude that such toxin biotransformations, especially in non-visceral tissues, may introduce a discrepancy in describing kinetics of total toxicity (in saxitoxin equivalents [STXeq]) of *S. solidissima* over prolonged detoxification. Nevertheless, use of total toxicity values generated by routine regulatory monitoring based upon mouse bioassays or calculated from chemical analytical determination of molar toxin concentrations is adequate for first-order modeling of toxin kinetics in this species. Furthermore, the differential detoxification response of viscera and other tissues in relation to temperature emphasizes the need for two-compartment modeling to describe the fate of PSP toxins in this species. Finally, key parameters were identified that may prove useful in hindcasting the timing of toxic blooms or new toxin input in deep offshore waters where routine monitoring of toxic phytoplankton is impractical.

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1. Introduction

Dinoflagellates of the *Alexandrium fundyense/tamarense* species complex, producers of paralytic shellfish poisoning (PSP) toxins in the NW Atlantic, are ingested by suspension-feeding bivalves, which can lead to accumulation of high toxin levels in soft tissues used for human consumption. This constitutes a public health hazard and causes major economic losses due to the need for shellfish harvest closures and extensive monitoring of shellfish toxicity. Different bivalve species pose a varying risk of PSP depending on their capacity for toxin uptake, conversion and elimination. Atlantic surfclams, *Spisula solidissima*, are relatively insensitive to the effects of PSP toxins (PSTs). This nerve insensitivity is evidenced by the fact that they show no burrowing incapacitation during exposure to high concentrations of these toxins (Bricelj et al., 1996), and therefore can accumulate extremely high toxin levels. Field populations from Georges Bank in the NW Atlantic attained a maximum of 6400 μg saxitoxin equivalents (STXeq) 100 g^{-1} whole tissues, whereas juveniles contaminated in the laboratory attained up to ~48000 μg STXeq 100 g^{-1} (Bricelj and Shumway, 1998). Surfclams eliminate PSTs relatively slowly compared to other bivalve species, such that whole tissue toxicities may require from 1 to 2.5 years to drop below the regulatory level

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of 80 μg STXeq 100 g^{-1}. In common with only a few Pacific clam species, *S. solidissima* are capable of extensive biotransformation of ingested individual PSP toxins (Cembella and Shumway, 1995a, 1995b; Bricelj et al., 1996; Laby, 1997), which could lead to an increase in net toxicity, given that toxin congeners differ greatly in specific potency (Fig. 1).

S. solidissima supports a valuable offshore commercial fishery in Atlantic North America from the Delmarva Peninsula to Georges Bank, with typical harvests from ~9 to 46 m depths (Weinberg et al., 2005). The recent shift in surfclam distributions to more northern and deeper waters in the Atlantic Bight has been associated with climate-driven global warming (Weinberg, 2005), and has led to increasing interest in the commercial exploitation of populations off the New England coast and on Georges Bank. The latter area has been closed to harvesting since 1990 due to PST-producing dinoflagellate blooms (presumably of *Alexandrium* spp.) that caused toxicity levels in surfclams that greatly exceeded the regulatory limit (Nassif and Timperi, 1993). These closures included surfclams collected from Georges Bank, where clams attained up to 4502 μg STXeq 100 g^{-1} whole tissues in mid-summer 1990.

Over its distributional range *S. solidissima* experiences wide temperature variation, with populations limited by an upper lethal limit of 26–28 °C for adults (Saila and Pratt, 1973). The current study therefore focused on determining the role of temperature in detoxification kinetics in a controlled experimental system, for potential extrapolation to explaining toxicity patterns of natural populations. Further objectives of this study were to determine the toxin transformations that occur in surfclam tissues under controlled laboratory conditions when exposed to a dinoflagellate of known PST profile. This information will assist in interpreting changes occurring in deep offshore waters where the toxin source, in terms of dinoflagellate species and life history stage (sinking vegetative cells, benthic sexual cysts, etc.) is often unknown. Additionally, we address the following questions: (a) can PST composition in surfclams be used to hindcast the timing and source of toxin input to offshore populations?; (b) do toxin composition changes need to be considered in modeling toxin kinetics in this species? Finally, improved understanding of the effects of temperature on toxin elimination, and of the chemical and metabolic transformations among individual toxin analogs has important implications for the development of effective shellfish detoxification methods.

2. Materials and methods

2.1. Algal culture

Toxic *A. fundyense* strain GTCA29 isolated from Cape Ann, Ipswich Bay, MA in the Gulf of Maine was cultured in a modified L1 medium with the addition of NH_4Cl at a final concentration of 5×10^{-5}M (Guillard and Hargraves, 1993). The non-toxic centric diatom *Thalassiosira weissflogii* (ACTIN, strain CCMP 1336) from the Provasoli-Guillard National Center for Marine Algae and Microbiota, Maine, was grown in a commercially prepared f/2 medium (Guillard and Ryther, 1962). Both species were cultured at 16 °C on a 14 h:10 h light:dark photocycle at a photon flux density of 146 μmol s^{-1} m^{-2}. Non-axenic cultures were batch-cultured in 20 L plastic carboys filled with cartridge-filtered (0.22 μm) autoclaved seawater medium provided with gentle sterile-aeration, in a temperature controlled environmental chamber. *A. fundyense* stock cell concentrations and those in experimental aquaria (see below) were determined microscopically, whereas those of *T. weissflogii* were determined with an electronic particle counter.

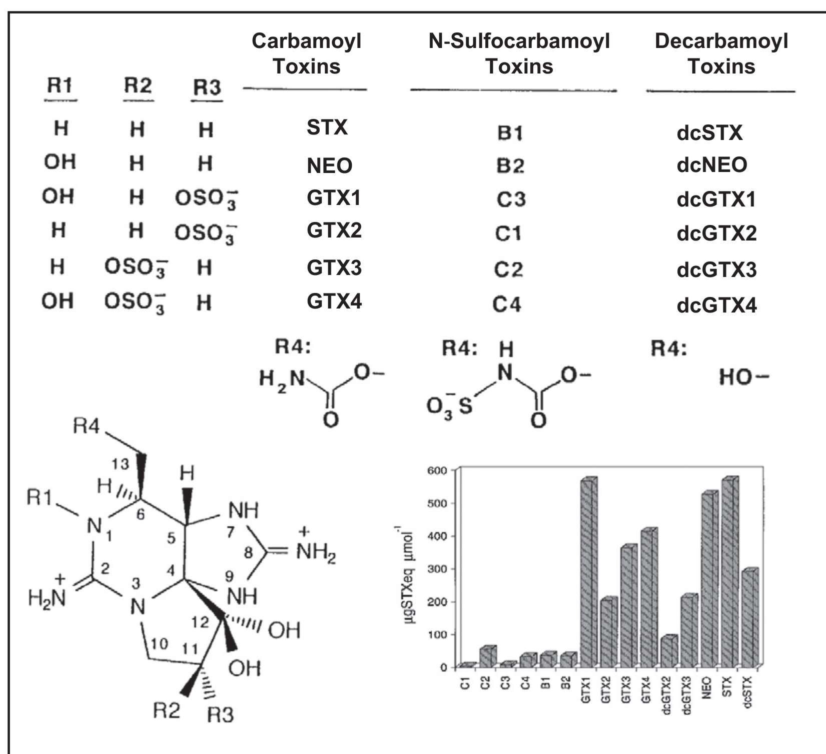

Fig. 1. Schematic of the major paralytic shellfish poisoning toxins (PSTs) produced by *Alexandrium* spp. and their grazers, including bivalves, and the relative potency of individual PSTs (in μgSTXeq μmol^{-1}) as determined by the mouse bioassay from purified toxin analogs. Additional saxitoxin analogs (M1–M4) found in mussels (*Mytilus* spp.) but not in the plankton (Dell'Aversano et al., 2008), are not included as they were not detected in this study and their potency remains unknown (see Wiese et al. (2010) for a current list of the 57 known PST analogs).

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2.2. Experimental design

Juvenile surfclams averaging 37.9 mm in mean shell length, SL (range = 35.3–41.2 mm) and ~2 g in mean wet weight (WW) of soft tissues were obtained from a hatchery (Mook Sea Farms, Damariscotta River, Maine). Clams were acclimated for ~3 weeks in flow-through raceways receiving ambient unfiltered seawater at 11–12 °C and 30 salinity. For toxification they were transferred to duplicate 70 L aquaria containing washed sand as substrate. The aquaria were held within a recirculating freshwater temperature bath at a mean temperature of 11.0 °C (range = 8.9–12.3 °C). Clams were exposed for 2 weeks to the high-toxicity *A. fundyense* isolate GTCA29 at a constant concentration of ~100 cells mL^{-1}. The supply of toxic cells was maintained via delivery of stock cultures from an aerated, Plexiglass feeding column by a peristaltic pump, for which the flow rate was adjusted to match the clams' consumption of dinoflagellate cells.

Following toxification, clams were depurated in the laboratory in sediment for 2.4 months (72 d) at 5, 11 and 21 °C. All toxified clams were first pooled and then distributed randomly among the 3 temperature treatments. Over the first 4 days of depuration one-third of the clams ($n = 120$) underwent a gradual raise in temperature from 11 to 21 °C, and another third a decrease from 11 to 5 °C, while the remaining clams remained at a constant ~12 °C. Depuration during the first 2 weeks was conducted in a closed system, drip-fed with *T. weissflogii*, to allow the release of live and partly digested dinoflagellate cells in feces. Clams were transferred to 3 flow-through raceways for the remaining depuration period, where they received unfiltered, ambient seawater at a constant temperature from a centrally-controlled seawater system, supplemented with *T. weissflogii* delivered from a feeding column by a peristaltic pump. The intent was to hold the clams at an approximate maintenance food ration to preclude confounding effects of growth on clam toxicities at any of the test temperatures. Actual mean temperatures obtained from continuous (hourly) temperature records during the depuration period in the 3 treatments were 20.9 °C (range = 19.5–21.2), 11.5 °C (9.9–12.5) and 5.2 °C (4.3–6.8).

Surfclams were subsampled 3 times during toxification (at 4, 9 and 14 days), and every 0.3–2 days during the first week of depuration, every 3–4 days during the subsequent month and every 5–9 days during the last 4 weeks of depuration. Tissues were dissected into the visceral mass (including the digestive gland, stomach complex and gonadal tissues), and other tissues, which included the adductor and pallial muscles, foot, mantle, siphons and remaining tissues. These two tissue compartments were tracked separately given that two-compartment models of toxin kinetics often more adequately describe toxin kinetics in bivalves (Blanco et al., 1997; Lassus et al., 2007). Replicate samples ($n = 3$ or 4) were obtained at each sampling date, each consisting of pooled viscera or other tissues from 3 clams. Drained WW of tissues (0.1 mg precision) was determined immediately following dissection. Tissues were immediately frozen in liquid nitrogen, stored at −80 °C and lyophilized prior to toxin extraction. Three control samples of clams prior to toxification were also run to confirm that they did not contain PSP toxins.

2.3. Toxin and data analysis

Cell toxin content and composition were determined for 8 samples of *A. fundyense* GTCA29 obtained in late exponential growth phase from 20 L-carboys used over the toxification period. Dinoflagellate samples were extracted in 0.03 M acetic acid (HOAc) and those of lyophilized clam tissues in 0.1 M HOAc following previously described methods (Bricelj et al., 1991; MacQuarrie, 2002; MacQuarrie and Bricelj, 2008). This method of extraction from lyophilized tissues has been shown to preserve the integrity of N-sulfocarbamoyl toxins in clam species, namely *Mercenaria mercenaria* (Bricelj et al., 1991) *Mya arenaria* (MacQuarrie, 2002), and *Ruditapes phillipinarum* (Bricelj et al., 2011), that show limited capacity for transformation of PSTs when fed *Alexandrium* strains containing predominantly C toxins.

Toxin analysis was performed by reverse-phase, ion-pair, high performance liquid chromatography coupled with post-column derivatization (oxidation) and fluorescence detection (LC-FD) according to the method of Oshima (1995a) with minor modifications. Naturally occurring paralytic shellfish toxins include 3 major structural groups, the N-sulfocarbamoyl, decarbamoyl and carbamoyl toxins of increasing potency (Fig. 1). With the Oshima (1995a) method, three sample injections are required to quantify separately each of the 3 toxin groups by LC-FD: (1) saxitoxin (STX), decarbamoyl saxitoxin (dcSTX) and neosaxitoxin (NEO); (2) N-sulfocarbamoyl C and B toxins; (3) gonyautoxins (GTXs) and their respective decarbamoyl derivatives (dcGTXs). Certified analytical toxin standards were obtained from the Certified Reference Materials Program (CRMP) of the Institute for Marine Biosciences (IMB), National Research Council (NRC), Halifax, Canada. As no certified standards were available for dcGTX2+3 and C1+2, these toxins were quantified from toxin calibration secondary standards provided by Maurice Laycock (IMB, NRC), which were calibrated at high concentration against the CRMP STX primary standard by capillary electrophoresis with UV detection. No standards were available at the time of this study for dcNEO and dcGTX1+4. Toxicities were calculated from toxin concentrations by conversion to STXeq units based on a conversion factor of 0.23 μg STXeq MU^{-1} (Cembella et al., 1993) using relative potency values in mouse units (MU) of individual toxins (MU μmol^{-1}) derived from the mouse bioassay specific toxicities provided by Oshima (1995a).

Given the scale of the undertaken experiment, which required a common, temperature controlled-raceway for long-term depuration, true replication was not possible within each temperature treatment. Therefore we did not attempt statistical comparison among temperature treatments, but rather analyzed the time series within each temperature experiment. Best fit functions were fitted to the total toxicities (in μg STXeq 100 g^{-1}) of viscera and other tissues, as well as to the toxin concentrations (in nmol g^{-1}) of individual toxin congeners in each of these tissues. The initial and final toxicity of viscera and other tissues, and those of whole tissues were also compared by one-way analysis of variance (ANOVA). For this purpose the first 6 samples (at 0.3 and 1.1 days of depuration) were compared with the last 8 samples (at 63 and 72 days of depuration), following testing to determine that they were not statistically different and could be pooled. The one exception was the 21 °C treatment, in which only the first 3 samples of viscera and whole tissues were used as the initial values, because the toxicity declined significantly between 0.3 and 1.1 days (ANOVA, $p \leq 0.05$). All statistical analysis was conducted using SYSTAT 10.0 (SPSS, Chicago, IL) software.

3. Results

3.1. Toxin uptake

The GTCA29 *A. fundyense* isolate fed to the surfclams exhibited a mean calculated toxicity of 13.8 pg STXeq $cell^{-1}$. The toxin profile on a molar basis (mean % ± standard deviation, SD, of each toxin), was composed predominantly of N-sulfocarbamoyl toxins C1+2 (51.5% ± 5.5) and NEO (27.8% ± 5.9), with ~12.5% (± 2.9) comprising GTX1+4 and 7.5% (± 2.3) GTX2+3. This strain contained only trace amounts of STX (0.34% ± 0.46), B1 (0.29% ± 0.07) and dcGTX2

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(0.12% ± 0.08). At the end of toxification (14 days) juvenile clams attained a mean toxicity of 3.16×10^4 μg STXeq $100\,g^{-1}$ whole tissues, 17.35×10^4 μg STXeq $100\,g^{-1}$ in viscera and 0.95×10^4 μg STXeq $100\,g^{-1}$ in other tissues. Thus juveniles accumulated toxin at a rate of ~2.2×10^3 μg STXeq $100\,g^{-1}$ whole tissues day^{-1}. Tissues of the three control surfclams contained no detectable concentrations of any PSTs, except for the appearance of a small chromatogram peak with a retention time coinciding with that of STX in viscera. This peak was likely a fluorescence artifact, but even if interpreted as STX, the concentration would have been three to four orders of magnitude lower than in clams exposed to the toxic GTCA29 isolate and thus was quantitatively unimportant.

3.2. Detoxification kinetics

The visceral mass detoxified significantly in all three temperature treatments (ANOVA comparing initial and final toxicities,

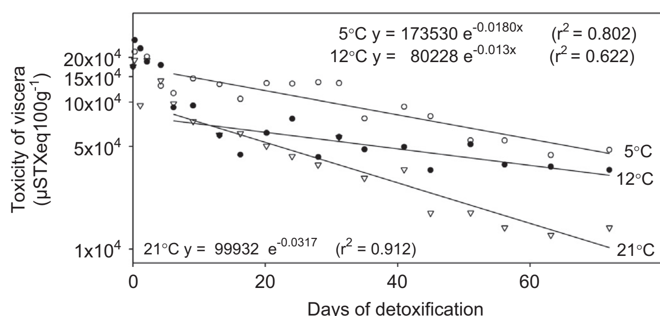

Fig. 2. Toxicity of viscera (in μg STXeq $100\,g^{-1}$ wet tissue weight) during detoxification of juvenile *Spisula solidissima* fed continuously a diet of the non-toxic diatom *Thalassiosira weissflogii* at three experimental temperatures. Fitted regressions of the form $Y = ae^{-bX}$, where Y = toxicity, X = time in days (data from the first 4 days of detoxification, the initial phase of more rapid detoxification, were excluded from curve fitting), r^2 = coefficient of determination. Each data point represents the mean of 3–4 replicate samples, each composed of pooled tissues from 3 clams.

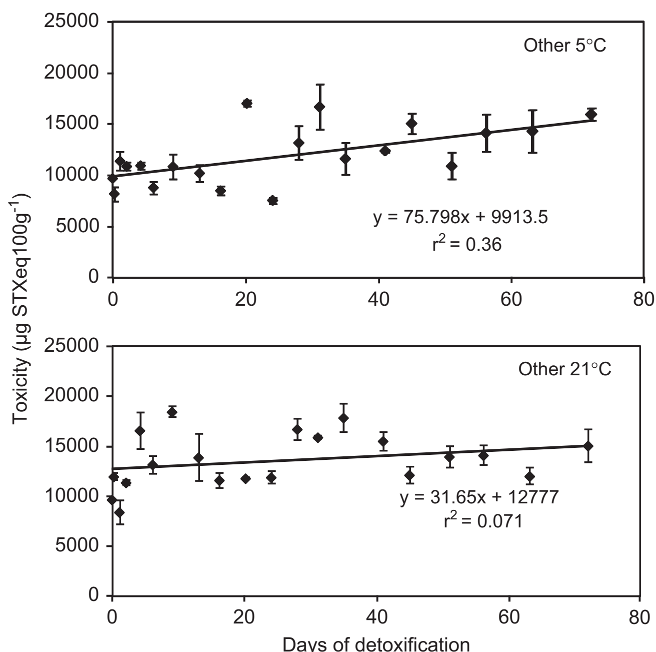

Fig. 3. Toxicity of other tissues (in μgSTXeq $100\,g^{-1}$ wet tissue weight, mean ± standard error, SE) during detoxification (up to 72 days of feeding on a non-toxic diet) of juvenile *Spisula solidissima* fed continuously a diet of *Thalassiosira weissflogii* at 5 °C and 21 °C, with fitted linear regressions and coefficient of determination (r^2). The regression equation for the intermediate temperature (12 °C, plot not shown) was: 64.135x+11126 (r^2 = 0.248).

$p < 0.001$), resulting in a toxin loss of 95.6%, 88.9% and 84.7% at 21 °C, 12 °C and 5 °C, respectively, by the end of detoxification. The detoxification rate of this tissue pool determined from fitted exponential equations was greater at 21 °C (3.1% loss d^{-1}) than at 12 °C and 5 °C (1.3% and 1.9% loss d^{-1}, respectively) (Fig. 2). In contrast, net toxicity of other tissues remained constant (e.g., at 21 °C) or increased during depuration (at 5 °C), as indicated by the positive slope in Fig. 3. This increase may be partly attributed to an increase in the molar concentration of the highest-potency toxin STX, as shown by the positive slopes of the detoxification curves in other tissues of this congener (Table 1). There was no significant difference between initial and final toxicities of other tissues at 12 °C and 21 °C (p = 0.47 and 0.10, respectively) but differences were significant at 5 °C (p = 0.04). Overall, individual toxins from other tissues were eliminated at much lower rates than viscera, as reflected by the magnitude of the negative slopes (b) of the fitted equations shown in Table 1.

The marked difference in detoxification between the two tissue pools results in a slowing down of the detoxification of whole tissues, which are monitored for regulatory purposes as a conservative measure, rather than only the edible tissues. Whole tissues showed a 34%, 60% and 54% reduction in toxicity by the end of the depuration period at 5 °C, 12 °C and 21 °C, respectively. These reductions were all significant ($p < 0.001$ for tests at 12 °C and 21 °C, and $p < 0.01$ for that at 5 °C), but final toxicities remained at least two orders of magnitude above the regulatory level. Detoxification of whole tissues at 12 °C is illustrated in Fig. 4 as surfclams were fully acclimated to this temperature prior to the experiment, and during toxification and depuration.

The time required for other tissues to exceed the toxin burden of viscera (due to the toxin exchange between these two tissue compartments and the more rapid detoxification of viscera) was positively related to temperature (Fig. 5). Thus, it took ~6.5, 11 and 53 d at 5, 12 and 21 °C respectively for other tissues to exceed the toxin burden of viscera. This reflects the finding in this study that detoxification of viscera (in μg STXeq $100\,g^{-1}$) is temperature-dependent whereas that of other tissues is not, or only weakly so (Figs. 2 and 3).

Clams showed no significant growth (ANOVA, $p < 0.01$) in shell length or tissue wet weight over the depuration period in any of the temperature treatments. Indeed, they showed a reduction in the wet weight of whole tissues in the 3 temperature treatments during depuration (20%, 25% and 36% at 5, 12 and 21 °C, respectively). This reduction was statistically significant only at the highest temperature (ANOVA, $p < 0.01$). On average, the viscera of surfclams made up only 15% of the total WW of soft tissues, although this tissue pool contributed the bulk of the toxin load during toxification (up to 85%) in the three temperature treatments (Fig. 5).

3.3. Changes in toxin composition in surfclam tissues

Our results indicate that N-sulfocarbamoyl toxins (C1,2) in the dinoflagellate diet were rapidly converted to decarbamoyl gonyautoxins (dcGTX2+3) in clam tissues (Figs. 6 and 7). The former were highly ephemeral following toxin ingestion: at the end of the toxification phase they contributed only on average 0.18% of total toxins in viscera and were non-detectable in other tissues. By the first sampling during detoxification (0.3 d), the dominant toxins in the dinoflagellate, the N-sulfocarbamoyl toxins C1,2, were only present in the viscera at 5 °C, where their molar contribution was reduced to 0.08%, and they were not detectable in other tissues at this first sampling. The N-sulfocarbamoyl toxins are thus not apparent in the clam toxin profiles shown in Figs. 6 and 7. These profiles showed gradual enrichment in the relative molar contribution of STX+dcSTX over detoxification time at all

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Table 1
Detoxification rates of individual PSTs (in nmol) in *Spisula solidissima* viscera (A) and other tissues (B), as determined by the slope of fitted exponential equations; r^2 = coefficient of determination; significance of the Pearson's correlation coefficient (r) (see text). ns = non-significant.

	5 °C			12 °C			21 °C		
	b	r^2	Significance	b	r^2	Significance	b	r^2	Significance
(A) Viscera									
STX	−0.012	0.65	***	−0.013	0.57	***	−0.026	0.86	***
dcSTX	0.013	0.61	***	0.008	0.26	*	−0.004	0.08	ns
NEO	−0.036	0.91	***	−0.050	0.86	***	−0.070	0.87	***
dcGTX2,3	−0.019	0.78	***	−0.023	0.66	***	−0.186	0.94	***
GTX2,3	−0.019	0.76	***	−0.030	0.72	***	−0.210	0.96	***
GTX1,4	−0.149	0.58	***	−0.273	0.76	***	−0.251	0.94	***
(B) Other tissues									
STX	0.016	0.81	***	0.019	0.65	***	0.020	0.55	***
dcSTX	0.029	0.92	***	0.023	0.44	**	0.028	0.54	***
NEO	0.004	0.11	ns	−0.004	0.12	ns	−0.021	0.74	***
dcGTX2,3	0.005	0.22	*	0.001	0.02	ns	−0.009	0.51	***
GTX2,3	0.004	0.18	ns	0.002	0.04	ns	−0.008	0.45	**
GTX1,4	−0.005	0.12	ns	−0.022	0.68	***	−0.053	0.84	***

* $0.01 < p \le 0.05$.
** $0.001 < p \le 0.01$.
*** $p \le 0.001$.

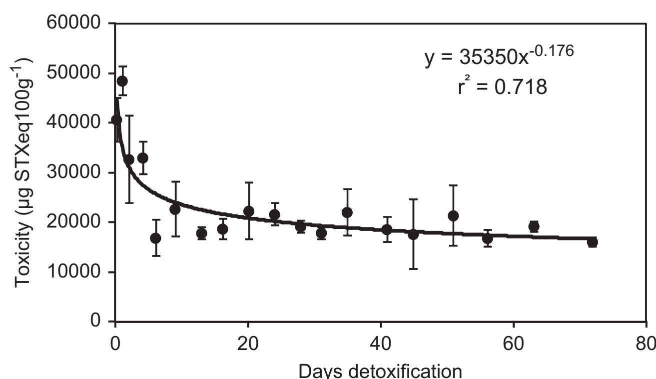

Fig. 4. Toxicity of whole tissues (mean ± standard error, SE, $n=3$ or 4) over the 72 days of depuration at 12 °C, with fitted power function (r^2 as in Fig. 2). Note the biphasic nature of detoxification with a rapid initial detoxification phase over the first week followed by a slower detoxification phase thereafter.

temperatures, and the contribution of these two toxins at any given time increased with temperature in both tissue pools. Thus in the viscera the mean molar contribution of STX+dcSTX at the end of depuration ($n=4$) increased from 27% (standard deviation, SD = 2.88) at 5 °C, to 35% (SD = 2.62) at 12 °C, to 56% (SD = 2.78) at 21 °C (Fig. 6). In other tissues STX+dcSTX similarly increased from 21% to 34% to 47% with increasing temperature (Fig. 7). Absolute STX and dcSTX molar concentrations increased significantly over time in other tissues at all temperatures, and this increase was positively related to temperature (Table 1). This indicates that temperature-dependent accumulation of these toxins via transformation, selective transfer from viscera and/or selective retention in other tissues exceeds their elimination rate.

Mean calculated toxicities and toxin concentration (in nmol) of the two tissue pools as a percent of initial are compared in Fig. 8 for the 21 °C treatment. The mean difference between the two values in other tissues during detoxification was 8.4% (maximum = 20.1%), and that in viscera was 5.9% (maximum = 13.6%). At 5 °C the mean difference in other tissues was only 6.2% (maximum = 10.4%). The % difference between toxicities and molar concentrations was found to increase linearly with detoxification time (shown in Fig. 8 inset). Thus the worst case scenario in modeling toxin kinetics in surfclams using toxicity values is expected to occur at higher temperatures in the non-visceral tissue compartment (maximum difference between toxicities and toxin concentrations = 20% at 2.4 months), and over prolonged detoxification.

Rapid epimerization of certain PSP toxins, from primarily β epimers (e.g. GTX3, GTX4) in the ingested dinoflagellate cells to their respective α isomers (e.g., GTX2, GTX1), is known to occur in bivalve tissues (reviewed by Bricelj and Shumway (1998)). This equilibration process, e.g., measured by the ratio GTX2/GTX3, is illustrated for surfclams that detoxified at 12 °C (Fig. 9, upper graph). In contrast to the ratio of β- to α-epimers, the ratio of STX/dcSTX, end products of metabolic enzymatic biotransformation, declined more rapidly over detoxification time in viscera than other tissues at all three experimental temperatures (illustrated in Fig. 9, at 5°C lower graph).

4. Discussion

4.1. Source of toxin

The toxin profile of *A. fundyense* isolate GTCA29 used in the current study was composed predominantly of C1+2 toxins (52%), followed by NEO (28%) and GTX1,2,3,4 (20%) (Fig. 7), whereas *A. fundyense* cells in Gulf of Maine coastal waters and on Georges Bank in 2007, 2008 and 2010 (summed for all depths and sampling stations) contained predominantly GTX toxins (up to 91% of the molar toxin concentration) (Deeds et al., this issue). The carbamoyl toxins STX and NEO contributed 8%, and C-toxins made up ≤1% of the total molar toxin composition in these dinoflagellate field populations. Yet N-sulfocarbamoyl toxins are a common and relatively large component in *Alexandrium* isolates from the Gulf of Maine (Anderson et al., 1994; D.M. Anderson, unpublished data) and toxin profiles of *Alexandrium* are known to be stable in culture and hence representative of their respective natural populations (reviewed by Cembella (1998)). The N-sulfocarbamoyl C-toxins also contributed to the molar toxin profile of *S. solidissima* viscera in three monthly samples (spring/summer) collected between March 1990 and July 1991 from an inshore site at Head Beach, coastal Maine (Cembella and Shumway, 1995a, 1995b). The rapid and almost total conversion of N-sulfocarbamoyl toxins to carbamoyl and decarbamoyl derivatives in our laboratory experiments with *Spisula* suggests that these field populations of *S. solidissima* (Cembella and Shumway, 1995a, 1995b) had experienced a recent

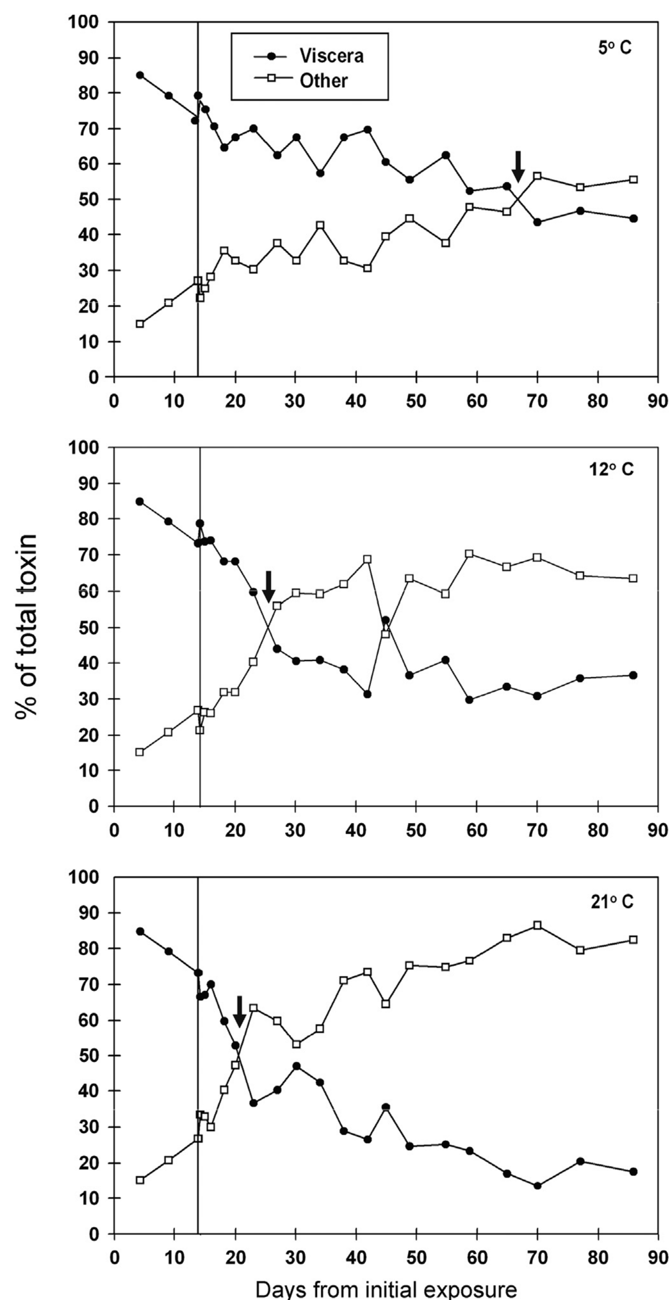

Fig. 5. Mean percent contribution of viscera and other tissues to the total toxin body burden (in μgSTXeq) of *Spisula solidissima* during toxin uptake (at a common temperature = 11 °C) and depuration at 5, 12 and 21 °C. Vertical lines mark the beginning of detoxification, and arrows mark the point of crossover after which the toxicity of other tissues exceeds that of the visceral mass.

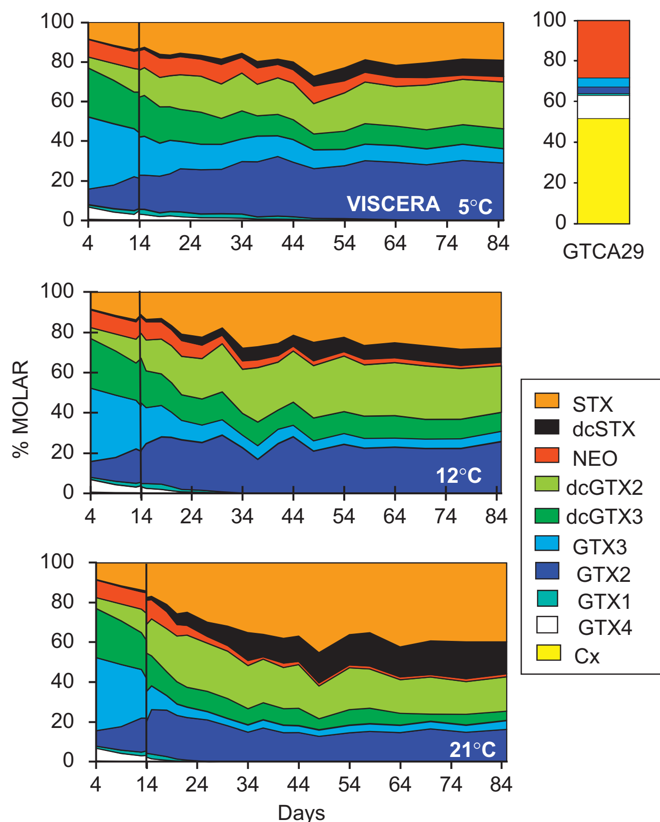

Fig. 6. Percent molar composition of individual PSTs in the ingested *Alexandrium fundyense* cells (strain GTCA29, upper right) and in the viscera of *Spisula solidissima* juveniles during toxin uptake (at a common temperature ~11 °C) and detoxification at 5, 12 and 21 °C (left). The vertical line marks the beginning of detoxification; day 4 indicates the first sampling of clams during toxification.

input of toxins from a dinoflagellate population rich in N-sulfocarbamoyl derivatives. Nevertheless, it remains to be determined whether *Alexandrium* strains containing N-sulfocarbamoyl toxins can be dominant contributors to toxic blooms in the region given that there is very limited past information on the toxin composition of natural blooms in the region.

4.2. Detoxification kinetics

The two lower experimental temperatures (5 and 12 °C) in this study are expected to be roughly representative of those experienced by surfclams in deep, offshore waters, where they live in a relatively stable, low temperature environment. Thus, near-bottom temperatures measured at least 10 m off-bottom on Georges Bank during the Gulf of Maine Toxicity (GOMTOX) cruises ranged from 5 to 13 °C in the spring (April/May), 5 to ~14.5 °C in June/July, and 5 to ~17 °C 5 during peak summer (July/August) (McGillicuddy et al., this issue (Fig. 3)).

At all three temperatures the viscera detoxified significantly over time, whereas other tissues showed a relatively constant toxicity (or even an increase in net toxicity) following prolonged (2.4 month depuration). The fact that this is observed both with respect to calculated toxicities and molar concentrations (Fig. 8) indicates that the effect cannot be explained only by conversion to more potent individual toxins and must be attributed to an as yet unidentified mechanism/s of toxin retention/concentration that may be largely independent of toxin composition in other tissues. This finding serves to explain the very prolonged detoxification (1 to 2 years) of natural *S. solidissima* populations (Bricelj and Shumway, 1998; Shumway et al., 1994). An additional consequence of the differential toxin retention between viscera and other tissues is that the difference in toxicity between these two tissue pools will decline initially with increasing detoxification time, as indicated until the crossover point marked by the arrow in Fig. 5, although this convergence will be delayed at low temperatures.

The differential detoxification rate of the two tissue pools was important to confirm under controlled conditions because the absence of a new input of toxins is difficult to confirm from field studies, especially in offshore waters. However, a time series of detoxification of adult surfclams collected from 2 stations in Georges Bank between May 2009 and December 2010 as part of the GOMTOX program supports our laboratory results. Although the surfclams tracked had a very low initial toxicity (~250 μg STXeq 100 g^{-1}), and no data were available between August 2009 and May 2010, non-edible tissues (viscera) were found to detoxify

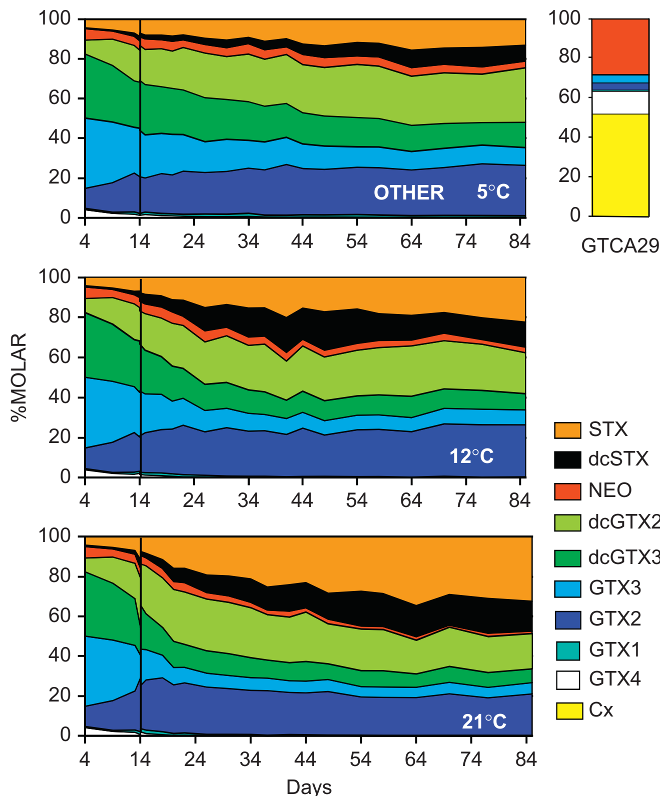

Fig. 7. Percent molar composition of individual PSTs in the ingested *Alexandrium fundyense* cells (strain GTCA29, right) and in other tissues of *Spisula solidissima* juveniles during toxin uptake (at a common temperature ~11 °C) and detoxification at 5, 12 and 21 °C (left). Vertical line as in Fig. 6; day 4 indicates the first sampling of clams during toxification.

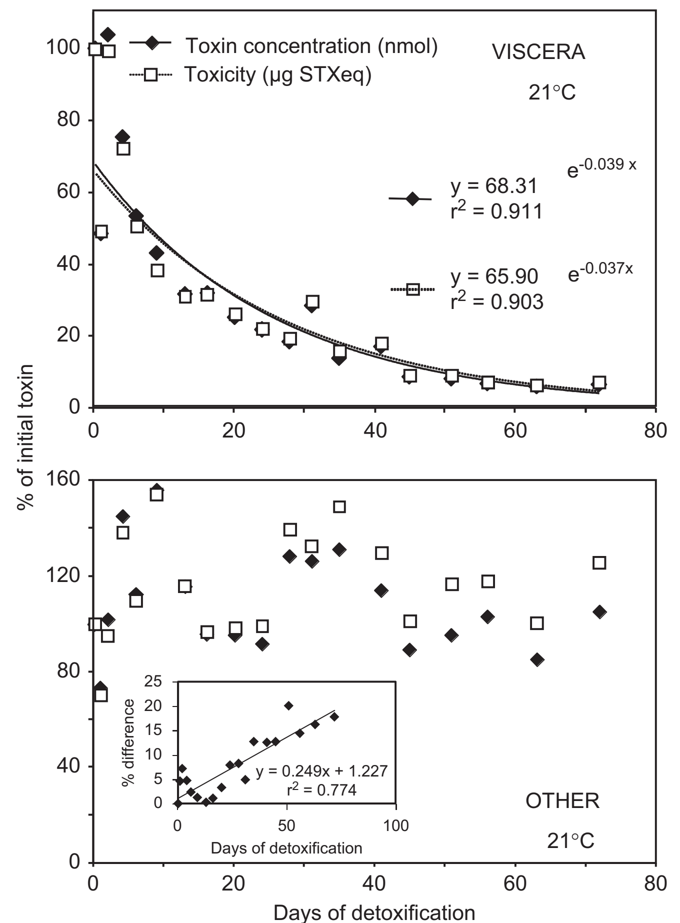

Fig. 8. Percent of initial toxicity (in µgSTXeq) or initial toxin concentration (in nmol) of the viscera (upper panel) and other tissues (lower panel) of *Spisula solidissima* during detoxification at 21 °C. For the viscera, fitted exponential decay equations are compared for toxicities and toxin concentrations. The % difference between toxin concentrations (in nmol) and toxicities (in µg STXeq) as a function of detoxification time in other tissues at 21 °C, was fitted to a linear regression shown in the lower panel inset; r^2 as in Fig. 2.

whereas the edible tissue pool (foot, mantle, gill, siphon and adductor muscles, equivalent to other tissues in the present study) did not [S. Lage et al., National Institute of Biological Resources (IPIMAR), Portugal, unpublished data].

Higher temperature resulted in an increase in the detoxification rate of the visceral mass, but had no detectable effect on that of other tissues. The % loss of toxicity from viscera was 1.9%, 1.3% and 3.1% per day at 5, 12 and 21 °C, respectively (Fig. 2), such that a ΔT of 16 °C over the range 5 to 21 °C yielded a temperature coefficient, Q_{10}, of 1.4, and the Q_{10} between 12 and 21 °C was equal to 1.8. a $Q_{10}=1$ indicates that a rate is temperature-independent, and acclimated physiological rates in bivalves typically attain a maximum Q_{10} of 2–3 (e.g., Peck, 1989; Grizzle et al., 2001). No temperature difference in detoxification rates was detected between 5 and 12 °C but this may be due to the high variability in visceral toxicities at 5 °C. Temperature-dependent differences in the detoxification rate of surfclam viscera cannot be attributed to toxin dilution caused by growth during the 2.4 month-depuration period, as clams were kept on an approximate maintenance diet and did not grow in any of the temperature treatments. Indeed, clams experienced some tissue weight loss, ranging from 20% to 36%, at the 3 experimental temperatures. Toxicities of other tissues, however, only increased significantly during depuration at 5 °C (Fig. 3), despite the fact that clams showed the lowest (and non-significant) weight loss in this temperature treatment. Thus changes in tissue mass do not explain the toxicity patterns observed in the present study.

While an increase in temperature is expected to increase the transfer rate of toxins from viscera to other tissues, the latter, which contribute ~83% to total tissue mass in juvenile surfclams, do not appear to detoxify irrespective of temperature. As a consequence, there was an overall slowing down of the detoxification of whole tissues and a reduction in the effect of temperature on the detoxification of whole tissues. No significant effect of temperature on detoxification rate of whole tissues was described for the butter clam, *Saxidomus giganteus*, over the range 7.5–16.5 °C and a 14 week-depuration period (Madenwald, 1985). Thus both *S. giganteus* and *S. solidissima* sequester PSP toxins—primarily in the siphons and in non-visceral tissues, respectively. In *S. giganteus*, as demonstrated with a STX radio-receptor assay, this was due to the presence of STX-binding soluble protein extracted from tissues, especially from the siphons (A. Robertson, FDA, Dauphin Island, AL, unpublished results). The fact that the STX-binding binding protein has highest affinity for this PST analog may explain why STX appears to be preferentially retained within the siphon. In any case, as for butter clams, temperature manipulation is therefore not recommended as an effective management tool to accelerate detoxification in *S. solidissima*.

Full acclimation of physiological rate functions to temperature in bivalves typically takes ~2 weeks of holding at a constant temperature, and may take longer for acclimation to low temperatures (Widdows and Bayne, 1971). Therefore the differential response to temperature found in this study is likely underestimated during the first few weeks of detoxification, as the

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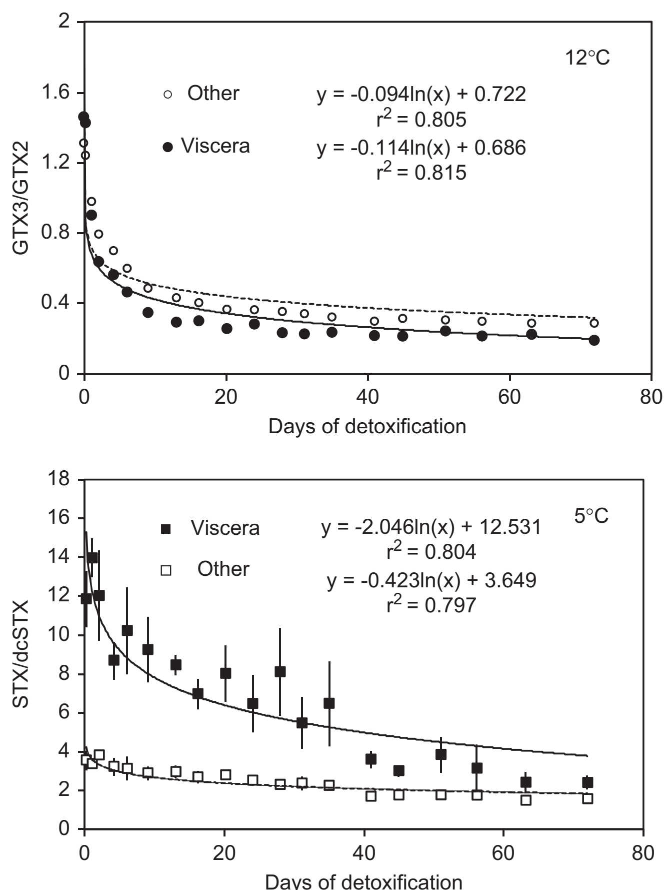

Fig. 9. The decline in the mean ratio of epimers GTX3/GTX2 (upper) and in that in the STX/dcSTX ratio (lower) over time in surfclam, *Spisula solidissima*, viscera and other tissues during depuration on a non-toxic diet at 12 °C and 5 °C, respectively. The equations represent the best (logarithmic) fit to the data (r^2=coefficient of determination). In the lower figure error bars that are not apparent are smaller than the data point.

relatively rapid change from the common 11 °C toxification temperature to 21 °C and 5 °C did not allow full acclimation to these experimental temperatures during early depuration.

4.3. Changes in toxin composition

Toxin bioconversions may lead to discrepancies between total toxin concentrations (in nmol) and toxicities measured by mouse bioassay (in μgSTXeq) by routine shellfish toxin monitoring programs or those calculated from toxin composition determined by chemical analytical methods such as liquid chromatography coupled with fluorescence (LC-FD) or mass spectrometric (LC-MS) detection. The large range in specific potency among the toxin analogs (over two orders of magnitude) makes this problem most acute when the toxin profile is complex, and particularly when the low potency N-sulfocarbamoyl toxins are initially dominant but subject to conversion in shellfish tissues. In the present study this bioconversion from N-sulfocarbamoyl to carbamoyl toxins is apparently so rapid over a wide temperature range that the former toxins are no longer significantly present during detoxification, thereby mitigating this discrepancy. In addition, the present study demonstrated that *S. solidissima* also has a high capacity for bioconversion (decarbamoylation) of PSTs, comparable to that of three other Pacific clams, *Protothaca staminea* (Fast et al., 2006), *Mactra chinensis* (Lin et al., 2004), and *Peronidia venulosa* (Cho et al., 2008), out of more than 20 bivalve species investigated to date. N-sulfocarbamoyl toxins C1+2 were rapidly converted to dcGTX2+3 in surfclam tissues – within ≤1 day (Fig. 6). A prior study showed that no STX was detected in surfclam tissues when they ingested *Alexandrium minutum* cells (strain ALIV) containing only GTXs [molar composition=97% GTX1+4 and 3% GTX2+3 (Bricelj and Cembella, 1995)]. Therefore STX in the present study is likely derived from reduction of NEO in surfclam tissues. In vitro studies have indicated that this reduction may be mediated via either the presence of natural reductants (e.g. glutathione) or enzymatically by oxidoreductase activity in shellfish tissue (e.g. Oshima, 1995b; Fast et al., 2006), but which of these mechanisms is dominant in *S. solidissima* has not been established in vivo. In addition, the potential role of bacterial gut flora cannot be ruled out, as certain bacteria isolated from bivalve shellfish are also known to be capable of reductive biotransformation of PSTs (Wiese, et al. 2010 and references therein). In whole-organism studies, conversion of toxins may occur in concert with selective depuration/retention of individual congeners, allowing only inference of specific conversion pathways. However, integration of results from studies in which a given bivalve species is fed strains with different, well characterized toxin profiles (see below) is particularly useful in defining specific pathways of toxin transformation.

The relative rate of enrichment in STX+dcSTX in surfclam tissues increased with increasing temperature. This is especially evident in viscera (Fig. 6), as the toxin composition of this tissue pool will be the first to reflect changes from those of the ingested toxin food source. Decarbamoylation of PSTs in *S. solidissima* is almost certainly enzyme-mediated as demonstrated in a few other clam species, but in vitro assays are needed to confirm this. Results of in vitro experiments with three Pacific clam species, the littleneck *P. staminea*, the Japanese clam *P. venulosa*, and *M. chinensis*, indicated that carbamoylase enzymes show higher conversion rates with β- (e.g., GTX3, C2) than α-epimers (Buzy et al., 1994; Artigas et al., 2007; Cho et al., 2008). In the two former species, N-sulfocarbamoyl toxins were also more rapidly converted to decarbamoyl than carbamoyl toxins (GTXs and STX), but the opposite was true in *M. chinensis*. In the Portuguese surfclam, *Spisula plana*, substrate-specific enzymatic decarbamoylation was high for all the toxin standards tested, varying by a factor of 2.8 among toxins (Artigas et al., 2007). Substrate specificity of carbamoylases can thus differ among bivalve species (Cho et al., 2008). It is noteworthy that in vivo decarbamoylation in *S. plana* was slower than that observed in vitro.

Decarbamoyl GTX2,3, resulting from decarbamoylation of GTX2,3 and/or corresponding N-sulfocarbamoyl C1,C2 toxins, made an important contribution to the molar toxin profile of juvenile *S. solidissima* in laboratory studies in which they were exposed to three strains of *Alexandrium* species: *A. tamarense* PR18b (Bricelj et al., 1996), *A. minutum* AL1V (Bricelj and Cembella, 1995), and *A. fundyense* GTCA29 (present study). Decarbamoyl GTX2,3 toxins were also present seasonally in both viscera and other (edible) tissues of adult surfclams collected from Georges Bank in 2009/2010 (S. Lage et al., IPIMAR, unpublished results). However, they were not found in adult surfclams from coastal Maine waters (Head Beach), despite the occasional presence of C-toxins and high molar contribution of GTX1,2,3,4 in their tissues (Cembella and Shumway, 1995a, 1995b). This discrepancy remains to be accounted for.

Results of the present study, combined with prior laboratory studies, provide strong evidence that *S. solidissima* is capable of reductive conversion of PSTs (e.g. NEO to STX, GTX1,4 to GTX2,3), and decarbamoylation of both carbamoyl (GTX2,3 to dcGTX2,3, and STX to dcSTX) and N-sulfocarbamoyl toxins (C1,2 to dcGTX2,3). Atlantic surfclams are thus comparable to *S. solida* (Artigas et al., 2007) and *M. chinensis* (Lin et al., 2004), that can convert both N-sulfocarbamoyl and carbamoyl toxins to their corresponding dc-derivatives, whereas in *P. venulosa* only the former undergo decarbamoylation (Oshima, 1995b). This

difference is attributed to different enzymes: sulfocarbamoylase I isolated from *P. venulosa* can only catalyze the conversion from N-sulfocarbamoyl- to dc-toxins (Cho et al., 2008), whereas carbamoylase I, isolated from *M. chinensis* can convert both N-sulfocarbamoyl and carbamoyl toxins to their corresponding dc-analogs (Lin et al., 2004). The optimum temperature for both enzymes is 25 °C, and enzymatic activity is highest in the digestive gland (73% in *M. chinensis* and 28% in *P. venulosa*).

Toxin bioconversions had limited effect on the net detoxification rate of viscera or in fully explaining the low or undetectable rate of detoxification in other tissues in the present study. This agrees with modeling of experimental results obtained with juvenile surfclams exposed to an *A. minutum* strain ALIV with a much simpler toxin profile consisting only of GTXs (Silvert et al., 1998). In our study, this is likely due to a compensation effect, i.e. some conversions led to an increase in toxicity (STX accumulation via biotransformation), whereas others led to a decrease in toxicity (e.g., decarbamoylation of STX to dcSTX, and epimerization from GTX3 to GTX2). Toxin bioconversion did have an effect on the net detoxification rate of other tissues (up to 20% in 2.4 months) which may need to be considered in modeling longer term toxin kinetics of *S. solidissima*.

This study identifies some of the parameters in surfclam tissues that are of potential use in predicting new toxin input to the bottom under field conditions. These include: (a) the presence of labile N-sulfocarbamoyl toxins, which if also present in ingested toxic cells will be indicative of very recent exposure to a corresponding bloom, e.g., of *Alexandrium* spp.; (b) the change in the ratio STX/dcSTX over detoxification time in viscera, because dcSTX is a product of metabolic conversion and shows a more pronounced decline over detoxification time in viscera than other tissues (Fig. 9, lower graph); (c) the shift in ratio of α/β epimers (e.g., GTX2/GTX3, dcGTX2/dcGTX3), because epimerization to α isomers occurs in all shellfish tissues, albeit at different rates. Although some internal equilibration can occur within cultured and natural populations of *Alexandrium* cells, dinoflagellates biosynthesize exclusively the β epimers, and therefore high α/β epimeric ratios (> 1:3) are highly rare (Cembella, 1998). The ratio of α/β GTX epimers is also sensitive to PST toxin extraction methods, i.e., subject to physico-chemical conversions (pH, temperature), but with extraction protocols similar to those in the present study, the ratio is typically 2–25x higher than this threshold in PST-producing dinoflagellates (Cembella et al., 1993; Bricelj et al., 1991). Thus, an α/β epimeric ratio ≤0.5 for juvenile clams that detoxify at ~12 °C is strongly indicative of very recent exposure to PST-producing dinoflagellates (within ≤2 weeks). All three parameters listed above are only useful as indicators of relatively recent input of toxin, i.e., in the case of juveniles, occurring within days to a month. In contrast, overall dominance of the tissue toxin profile by decarbamoyl toxins, is expected to indicate the absence of a recent input of PSTs (over the past few months).

S. solidissima vary greatly in body size over their lifespan, attaining up to 22 cm SL (~380 g tissue WW); the minimum commercial size equals 12.7 cm (~80 g). Thus body size is an important factor influencing toxin uptake and loss rates in this species. In controlled laboratory experiments Laby (1997) demonstrated that weight-specific toxin uptake and elimination rates in *S. solidissima* were greater for juvenile clams (27 mm SL, 0.6 g WW of soft tissues) than adults (61 mm SL, 8.1 g WW). This presumably reflects the higher feeding and metabolic rates per unit mass of juveniles relative to adults. Thus the differences in the absolute magnitude of detoxification rates obtained in this study for juveniles cannot be directly extrapolated to commercial-sized animals, and requires allometric scaling. However, the relative differences in detoxification rates among tissue pools and among temperatures are expected to apply to surfclams varying widely in body size.

5. Conclusions

This study provides useful guidance for the development of a toxin kinetic model in *S. solidissima*, providing strong experimental evidence for the need to use a two-compartment model, in which the more toxin-labile tissue pool (here represented by the viscera or non-edible tissues) shows temperature dependence, whereas the other tissue pool (edible tissues) does not. This study also identifies a number of parameters based on the composition of individual toxins that could be used as potential indicators of very recent toxin input in this species.

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