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# Effects of toxic *Alexandrium tamarense* on behavior, hemocyte responses and development of brown ring disease in Manila clams

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ABSTRACT: In this study of short-term exposure of *Ruditapes philippinarum* from Brittany, France, to an *Alexandrium tamarense* isolate that produces high concentrations of paralytic shellfish toxins (PSTs), the effects of the isolate on clam fitness, toxin uptake, and the response of hemocytes (responsible for internal defense in bivalves) were examined. Adults exhibited individual variability in resistance to the effects of PSTs, measured by their burrowing capacity. If genetically based, this may (1) indicate that resistance is under natural selection and (2) provide a new model to advance our understanding of the molecular basis for PST resistance in bivalves, so far determined only in *Mya arenaria*. Juvenile clams were more vulnerable to PSTs than adults, experiencing 100% burrowing incapacitation within 1 d of exposure to *A. tamarense*. They also experienced growth suppression and high mortalities following 4 d of toxification. Juveniles challenged with *Vibrio tapetis*, the cause of brown ring disease (BRD), and exposed to *A. tamarense*, developed significantly fewer BRD symptoms relative to controls fed non-toxic algae, but suffered higher mortality. Adult clams exposed to *A. tamarense* showed a significant increase in hemocyte concentrations and a small, but significant, decrease in phagocytic activity, and no effect on hemocyte viability or other functional parameters. We speculate that the inhibitory effects on BRD progression may be attributable to toxicity of PSTs to *V. tapetis,* inability of juveniles to activate the shell conchiolin-deposition response, and/or an overall increase in phagocytic cells induced by *A. tamarense*. Harmful algae and pathogens may thus interact and modulate the effects of disease in bivalve populations.

KEY WORDS: *Ruditapes philippinarum* · Paralytic shellfish toxins · *Vibrio* · Burrowing · Hemocytes

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## INTRODUCTION

The Japanese littleneck or Manila clam *Venerupis* (= *Ruditapes* = *Tapes*) *philippinarum* (Adams & Reeves, 1850), a native of the western Pacific, is a major commercial species worldwide, especially in Canada, the USA, France, the north Adriatic lagoons in Italy, Great Britain, Japan, China, and South Korea (Goulletquer 1997). It was deliberately introduced to France from the Pacific coast of North America (Puget Sound) in the early 1970s, and in France, naturalized populations are found on both Atlantic and Mediterranean coasts, especially in the Gulf of Morbihan (southern Brittany) and Arcachon Bay (SW France) (Flassch & Leborgne 1992, Dang et al. 2008). Manila clams occur in eutrophic, sheltered environments, in a wide range of sediments, where they burrow only to shallow depths (≤10 cm) and attain a maximum shell length (SL) of ~65 mm (Paillard et al. 2008).

The Atlantic coast of France has been affected since the late 1980s by blooms of the paralytic shellfish toxin (PST) producer *Alexandrium minutum*, especially along the NW coast of Brittany (Réseau National de Surveillance du Phytoplancton et des Phycotoxines

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[REPHY]; www.ifremer.fr/envlit/surveillance/rephy.htm). Since the late 1990s, *A. catenella* has occurred in the Thau Lagoon on the Mediterranean coast (Lilly et al. 2002) and has expanded along the NW Mediterranean coast (Vila et al. 2001). Prior studies on laboratory exposure of Manila clams to PST-producing *Alexandrium* spp. have been limited to the use of very low-toxicity strains, including an *A. tamarense* isolate from the South China Sea characterized by the predominance of low-potency N-sulfocarbamoyl toxins (toxicity = 3.2 pg saxitoxin equivalents [STXeq] cell$^{-1}$) (Li & Wang 2001, Li et al. 2002), an *A. minutum* isolate from Morlaix Bay, Brittany (2.4 pg STXeq cell$^{-1}$) (Bougrier et al. 2001) that produces almost exclusively gonyautoxins (GTXs) (Lassus et al. 1994), and an *A. catenella* isolate from the Thau Lagoon (5 to 15 pg STXeq cell$^{-1}$) (Hégaret et al. 2007a).

Manila clams can accumulate relatively high levels of PSTs in the natural environment, attaining maximum toxicities of 2000 to 6100 µg STXeq 100 g$^{-1}$ wet weight of whole tissues (reviewed by Bricelj & Shumway 1998). These levels are an order of magnitude lower than those accumulated by mussels *Mytilus edulis* and scallops *Pecten maximum*, but much higher than those of oysters *Crassostrea gigas* (Lassus et al. 1989) and more comparable to those attained by the softshell clam *Mya arenaria* (Bricelj & Shumway 1998).

Paralytic shellfish toxins act by blocking voltage-gated sodium ($Na^+$) channels in excitable nerve and muscle cells (Catterall 2000). Exposure to toxic *Alexandrium* spp. thus causes paralysis of the foot and pallial musculature in sensitive *Mya arenaria*, leading to burrowing incapacitation (MacQuarrie & Bricelj 2008, Bricelj et al. 2010), which may increase predatory risk in nature. In this bivalve species, pronounced individual differences in the capacity to accumulate PSTs and in the resistance to the effects of these neurotoxins have been attributed to a single point mutation that encodes for a single amino acid substitution in Domain II of the pore region of the $Na^+$ channel protein (Bricelj et al. 2005). Clams with a history of exposure to PSTs have a higher proportion of resistant individuals compared to unexposed populations, based on the effect of PSTs on burrowing behavior, *in vitro* nerve resistance to STX, and genotype, indicating that PSTs can select for resistance in natural populations (Bricelj et al. 2004, Connell et al. 2007). We hypothesize that individual variation in resistance to PSTs may also occur in *Ruditapes philippinarum*, a species that, like *M. arenaria,* is widely distributed in coastal waters with varying exposure to PSTs, thus providing an alternate model species in which to investigate the molecular/genetic basis for resistance to PSTs among bivalve molluscs.

A number of previous studies have documented effects of bacterial pathogens and protozoan parasites (e.g. Oubella et al. 1993, Goedken et al. 2005, Allam & Ford 2006), and harmful algal blooms (HABs) (e.g. Hégaret & Wikfors 2005) on hemocyte parameters and other defense-related factors in bivalves. Blooms of toxic microalgae can, however, co-occur with bivalve bacterial and protistan pathogens, yet few studies have examined their interactive effects on bivalve populations (Hégaret et al. 2007a, da Silva et al. 2008). In Europe, the Manila clam is affected by brown ring disease (BRD) caused by the bacterium *Vibrio tapetis*, which results in characteristic brown conchiolin deposits on the inner surface of the shells and which can lead to heavy mortalities of juveniles and adults, especially in cultured populations (Paillard & Maes 1990, Paillard et al. 2004). In NW France, blooms of *Alexandrium minutum* typically occur in the summer. Heavy clam mortalities associated with BRD generally occur in the winter and spring, resulting from the development of disease during the summer and fall. Thus, interactive effects between PSTs and the progression of BRD could occur in nature.

The main objectives of this study were thus to determine under controlled laboratory conditions (1) the potential interactive effect on the development of BRD of exposing juvenile *Ruditapes philippinarum* to toxic *Alexandrium* spp. cells after challenge by *Vibrio tapetis*, (2) the susceptibility of juvenile and adult Manila clams to exposure to toxigenic *A. tamarense* as measured by the effects on burrowing behavior, and (3) the effect of exposure to *Alexandrium* spp. on the concentrations and functions of hemocytes extracted from hemolymph of adults, given that hemocytes play a key role in both cellular and humoral defense in bivalve molluscs (Cheng 1996). An important additional objective of this study was to assess the individual variability in susceptibility to PSTs in an adult Manila clam population from the coast of Brittany, France, which is known to experience blooms of *Alexandrium* spp. of relatively low toxicity compared to those prevalent in the NW Pacific coast of North America. Finally, these laboratory experiments allowed determination of the short-term capacity for accumulation and biotransformation of individual PSTs in tissues of Manila clams following exposure to a highly toxic *A. tamarense* isolate. Biotransformation of low-potency toxins in dinoflagellate cells to more potent PSTs in bivalve tissues is important to determine, as it can potentially lead to increased shellfish toxicity.

## MATERIALS AND METHODS

All experiments were conducted at the Laboratoire des Sciences de L'Environnement Marin (LEMAR), Institut Universitaire Européen de la Mer (IUEM), Brittany, France.

**Microalgal and bacterial cultures.** Non-axenic stock cultures of *Alexandrium tamarense* (strain PR18b) isolated from the estuary of the Gulf of St. Lawrence, Canada were air-shipped from the Institute for Marine Biosciences, National Research Council (IMB/NRC), Canada, to France. This strain was selected because there is already considerable information available on the *in vivo* responses of other bivalve species, including *Mya arenaria*, to this strain (e.g. Bricelj & Shumway 1998, Bricelj et al. 2005, 2010), thus allowing interspecies comparisons. Its mean equivalent spherical diameter (ESD) as determined with a Beckman-Coulter Multisizer was 30.1 µm. Algae were batch cultured non-axenically at 14°C and 35 salinity with a 14 h light:10 h dark cycle, in 2 l glass flasks without aeration or in 10 l round-bottom, aerated flasks, using an L1 medium modified by the addition of $NH_4Cl$ at a final concentration of 50 µM (Guillard & Hargraves 1993). Seawater for the smaller flasks was 0.22 µm filtered and sterilized by microwaving using a protocol adapted from Keller et al. (1988). The nutrients were autoclaved separately before addition to the medium under a laminar hood and the final pH checked to ensure that it remained below 8. Non-toxic flagellates *Pavlova* spp. and *Isochrysis galbana* (strain T-iso) were mass cultured in f/2 medium (Guillard & Ryther 1962) with aeration and under continuous light. All cultures were harvested in late exponential growth phase.

*Vibrio tapetis* (strain CECT4600) isolated from *Ruditapes philippinarum* in Landéda, northern Brittany, France (Paillard & Maes 1990) was grown on ZoBell's 2216 agar — 1 l distilled water, 15 g agar (AES), 30 g sea salts (Sigma), 4 g peptone (AES), 1 g yeast extract (AES) and 0.1 g $FePO_4$ (Merck) — at 18°C for 72 h. Before *in vivo* inoculation experiments or *in vitro* hemocyte challenge, a *V. tapetis* suspension was prepared in sterile seawater (SSW) and the concentration adjusted to $5 \times 10^8$ bacteria $ml^{-1}$, based on spectrophotometric determination of bacterial density at 490 nm.

**Expt 1: exposure of juvenile clams to toxic *Alexandrium tamarense* and *Vibrio tapetis*.** Juvenile Manila clams were obtained from the Satmar, Marennes-Oléron nursery at an initial mean shell length (SL) of 12.5 mm (±0.10 SE, range = 11.2 to 13.8 mm, n = 50 clams) on February 12, 2007. They were acclimated at LEMAR at 16°C and 35 salinity and kept on a maintenance diet of *Pavlova* spp. and/or *Isochrysis galbana* (strain T-iso), without sediment.

All juvenile clams were inoculated 2 d after their arrival (Day 0 of the experiment), one-half with 50 µl of *Vibrio tapetis* suspension in SSW ($2.5 \times 10^7$ bacteria per individual) and the other half with the same volume of 0.22 µm filtered seawater (FSW controls) following a protocol by Paillard & Maes (1990) (Fig. 1). Prior to injection, the clams were kept out of water overnight and covered with wet paper towels to stimulate rapid opening upon re-immersion and thus to facilitate injection. A few minutes prior to inoculation they were placed in a shallow tray with seawater and algae. As soon as an individual opened its valves, the bacterial inoculum or FSW was injected into its pallial cavity with a 25-gauge 5/8″ needle attached to a 1 ml tuberculin syringe. Clams were again kept out of water under moist towels until the following morning, when they were transferred to 6 ~15 l aerated static tanks, 3 with *V. tapetis*-inoculated clams and 3 with FSW controls, for a post-inoculation recovery period (Fig. 1). The tanks contained unfiltered, ambient seawater at salinity 35 and were held in a temperature-controlled room at 16°C. Water in these tanks was changed daily, at which time dead clams were removed and recorded. Clams were batch-fed once a day by adding *Pavlova* spp. or *Isochrysis galbana* to achieve a total cell density of ~60 000 cells $ml^{-1}$ (maintenance diet). All water and materials used for *V. tapetis*-treated animals were chlorinated overnight prior to release. An initial sample of 30 clams was taken on Day 6 post-inoculation for baseline determination of dry tissue weight (DW) after oven-drying at 60°C for 24 h.

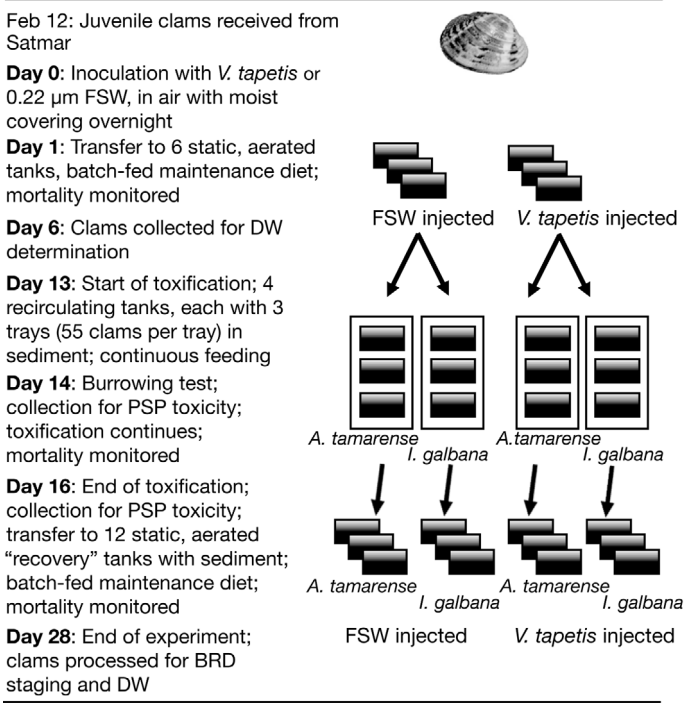


Fig. 1. *Ruditapes philippinarum.* Chronology of experimental manipulations of juvenile Manila clams in Expt 1

Clams experienced some mortalities in the days following inoculation, mainly in *Vibrio tapetis*-inoculated batches and primarily during the first 5 d post-inoculation (total losses were <1.2% in controls and <21% in *V. tapetis*-injected clams by Day 9 post-inoculation). Mortalities resulting from this initial handling/injection had ceased by Day 11 post-inoculation.

On Day 13 post-inoculation, clams were transferred to 4 aerated 45 l tanks, each fitted with an externally mounted, low-pressure recirculating pump (Super King, Danner Mfg) that maintains algae in suspension but does not damage *Alexandrium* spp. cells. Two of these tanks received toxic *Alexandrium tamarense* maintained at a constant cell density of 50 cells $ml^{-1}$ and 2 control tanks received *Isochrysis galbana* to maintain a volume equivalent cell concentration (~33 000 cells $ml^{-1}$) via continuous delivery from stock cultures with a Masterflex peristaltic pump. The resulting 4 treatments were as follows (Fig. 1): (1) FSW-injected and exposed to *A. tamarense*; (2) *Vibrio tapetis*-injected and exposed to *A. tamarense*; (3) *V. tapetis*-injected and fed *I. galbana*; and (4) FSW-injected and fed *I. galbana.* Treatment 4 was considered the control (no bacteria/no toxic algae).

Within each of the 4 tanks, clams were held in 3 separate trays (n = 55 clams per tray) filled with a 6 to 7 cm deep layer of sediment (white quartz sand, Zolux®; ~0.5 mm grain size) into which the clams were allowed to burrow prior to toxification (Fig. 1). The *Alexandrium tamarense* exposure concentration was selected based on prior experiments with juvenile (4 mm) *Mya arenaria*, in which 100 cells $ml^{-1}$ caused severe mortalities, 10 cells $ml^{-1}$ had no effect on survival or growth, although it affected motility, and 50 cells $ml^{-1}$ caused 15% mortalities by the end of 2 wk and severe growth inhibition (Bricelj et al. 2010). Cell densities of *A. tamarense* in the experimental tanks were determined microscopically and those of *Isochrysis galbana* were determined with an electronic Coulter counter.

**Burrowing test.** Burrowing capacity as a measure of susceptibility to PSTs was determined beginning on Day 14 after 24 h exposure to toxic cells (Fig. 1). Clams in all groups that had completely burrowed prior to toxification were removed from the sediment and placed on the surface in the same tanks, and the number of burrowed and non-burrowed clams was determined at the end of 2 h. This period was based on preliminary trials showing that nearly all untreated clams would reburrow within 2 h after being removed from sediment. The criterion for considering a clam to be burrowed was that its umbo be under the sediment. Toxification was continued during and after the burrowing test for a total of 4 d. At the end of the burrowing test, a subsample of clams (burrowed and non-burrowed) from toxified and control tanks was frozen at –80°C for toxin analysis.

**Progression of BRD.** At the end of toxification (Day 16 post-inoculation), clams from the 4 treatments were divided, according to treatment, among 12 tanks (15 l) containing fresh seawater and sediment, and batch-fed a maintenance diet of *Isochrysis galbana*, as previously, until the end of the experiment on Day 28 post-inoculation (Fig. 1). The end point of the experiment was selected based on previous findings that the % BRD prevalence in inoculated Manila clams reached 90% by Day 28 post-inoculation at 12°C (Paillard & Maes 1990).

Daily observations were conducted to determine mortalities and the seawater in the tanks changed at the same time; dead clams were removed and preserved in 70% ethanol to assess BRD status. Clams were also sampled at the end of the experiment to determine the oven-dried DW of soft tissues (n = 30 per treatment) and BRD status. Shells of the clams that died prior to the end of the study as well as those of survivors were observed under a dissecting microscope to determine the total BRD prevalence and the distribution of disease stages. The BRD symptoms were categorized on the 0 to 7 scoring system of Paillard & Maes (1994), but combined into 4 conchiolin deposit groups (CDG0, CDGI [stages 1 to 2], CDGII [stage 3], CDGIII [stages 4 to 7]) (Paillard et al. 2004) for statistical analysis to avoid categories with <5 values. These groups reflect none, very light, intermediate, and moderate-heavy conchiolin deposits, respectively. The instantaneous daily growth coefficient ($k$) was calculated from $k = [(\ln DW_f - \ln DW_o)/t] \times 100$, where $t$ = time interval in d, and $DW_f$ and $DW_o$ are final and initial values determined on Day 28 and Day 6 post-inoculation, respectively.

**Expt 2: exposure of adult clams to toxic *Alexandrium tamarense*.** Adult clams were collected manually from an intertidal population at Tascon in the Gulf of Morbihan, Brittany, on January 4, 2007. Only burrowed clams were collected, as those lying at the sediment surface tend to have higher prevalence and severity of BRD than burrowed ones (Paillard & Maes 1994). Experimental clams ranged in SL from 30.7 to 42.1 mm, averaging 35.9 mm (SE = 0.23); mean DW of soft tissues = 307.1 mg (SE = 71.8). They were initially held at LEMAR in flow-through Bay of Brest seawater at ambient temperature of 12 to 13°C, and then transferred to the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER) hatchery at Argenton, where they were acclimated to the experimental temperature (16°C) and fed an optimum ration of a mixed microalgal diet for 1 wk prior to the start of the experiment.

**Burrowing and toxin accumulation.** Experimental clams were divided among 6 aerated aquaria (45 l) (16 to 20 individually numbered clams per aquarium), containing the same type of sediment as used for juvenile

clams and using the same recirculating pumps. All clams burrowed before toxification. Three aquaria received *Alexandrium tamarense* harvested at ~8000 cells $ml^{-1}$ to attain an experimental concentration of 100 cells $ml^{-1}$ and 3 received *Isochrysis galbana* stock cultures at a volume-equivalent concentration. A constant concentration was maintained via continuous delivery from stock cultures with a Masterflex peristaltic pump.

After 16 h of exposure to the toxic or non-toxic diet, all clams were removed from the sediment and laid horizontally on the substrate in their respective aquaria to determine the number of burrowed (umbo covered by sediment) and non-burrowed individuals at 30 min and then at hourly intervals over a total period of 12 h.

**Hemocyte responses.** At the end of the burrowing test, hemolymph (~600 µl) was collected from the posterior adductor muscle of each treated and control clam using a 25-gauge needle attached to a 1 ml syringe. The sample was first examined microscopically to verify the presence of hemocytes and lack of debris, and stored temporarily in a 1.5 ml microcentrifuge tube on ice to retard cell aggregation. The hemolymph was then filtered through 80 µm mesh to eliminate cellular aggregates before analysis on a FACScalibur flow cytometer (Becton-Dickinson). The following parameters were measured following established methods (Delaporte et al. 2003): (1) Total hemocyte concentration (THC); (2) concentration of granulocytes; (3) concentration of hyalinocytes; and (4) % viability/mortality by staining of live and dead hemocytes, respectively. Additionally, the following functional parameters were measured: (5) % phagocytosis, as measured by the number of cells containing ≥3 fluorescent latex beads (2 µm diameter) after 2 h incubation at 18°C; (6) phagocytosis capacity as measured by the mean number of beads per phagocytically active hemocyte; and (7) total oxidative activity, measured using the fluorescent probe 2′7′-dichlorofluorescein diacetate (DCFH-DA). The latter indicates the oxidative stress of the cells and/or their potential to kill non-self, engulfed particles by the production of reactive oxygen species. Hemocyte adhesion in the presence of *Vibrio tapetis*, a measure of the toxicity of the bacterium to host cells, was compared to controls in FSW (as per Choquet et al. 2003). In order to obtain sufficient numbers of hemocytes for the assay, it was necessary to pool hemolymph from 4 to 6 clams, which were separated according to treatment and whether they were burrowers or non-burrowers. Following hemolymph sampling, all clams were analyzed for SL, air-dried shell weight, and wet soft tissue weight. Clam soft tissues were then frozen at −80°C for subsequent determination of individual condition index (CI) and toxin concentration. Soft tissue DW was determined after lyophilization of frozen tissues for 48 h. The individual CI of burrowers and non-burrowers was determined as CI = (DW tissues/shell weight) in g × 100. Shells of all clams were examined to assess their BRD status.

**Toxin analysis.** Toxin extracts of clam tissues and *Alexandrium tamarense* cells were obtained following previously described methods (MacQuarrie & Bricelj 2008). Tissues of adult clams (n = 46 from the toxic treatment, also used to determine the CI) were analysed individually. Pooled juveniles (4 to 6 samples of n = 2 to 10 clams from each of the FSW- and *Vibrio tapetis*-injected groups exposed to toxic algae) were sampled on Day 14 post-inoculation, at the end of the burrowing assay, and at the end of toxification on Day 16 post-inoculation. The frozen tissues were lyophilized and air-shipped for analysis to IMB/NRC, where they were weighed, pulverized, and extracted in 0.1 mol $l^{-1}$ acetic acid (HOAc) with a Polytron homogenizer, over an ice bath. Only 2 to 3 samples from the non-*A. tamarense* treated clams were similarly analyzed to confirm that they contained no detectable toxin levels. *Alexandrium tamarense* cells ($1 \times 10^6$ cells) were extracted in 3 ml of 0.03 mol $l^{-1}$ HOAc to maintain the integrity of individual toxins, using a Vibracell ultrasonicator. Toxin analysis by high-performance liquid chromatography with fluorescence detection (HPLC-FD) was performed following methods of Oshima (1995) with minor modifications. Individual toxins were quantified with certified external standards provided by IMB's certified reference materials program (CRMP). Concentrations of individual toxins were converted to STXeq using toxin-specific conversion factors (mouse units [MU] $\mu mol^{-1}$) and a conversion factor of 0.23 µg STXeq $MU^{-1}$ (Cembella et al. 1993). Individual toxicities were summed up to yield total toxicity in µg STXeq 100 $g^{-1}$ tissue wet weight.

**Statistical analysis.** All percentages were arcsine transformed prior to analysis to normalize the data. In the juvenile clam experiment, it was obvious without statistical analysis, that certain treatments were different from others. In these cases (% burrowing, % mortality, and % in each CDG), statistical analysis was reserved for differences that were not obvious. Percent burrowing and mortality were compared using a *t*-test for samples with equal variances, after confirming that there were no significant differences in variances. Survival trajectories were also compared using a stratified log-rank test for equality of survivor functions (STATA software). The distribution of CDGs was compared between treatments using a Pearson's $\chi$-squared test. The instantaneous growth coefficients in the 4 treatments were compared by 1-way ANOVA followed by Tukey's multiple comparisons. The CI of burrowed and non-burrowed adult clams in the toxic treatment

was compared by ANOVA following verification that variances were homogeneous. To calculate the percent molar contribution of each toxin group to the total, the 2 epimers were added (C1 + C2, and GTX2 + GTX3) since some epimerization may occur during extraction.

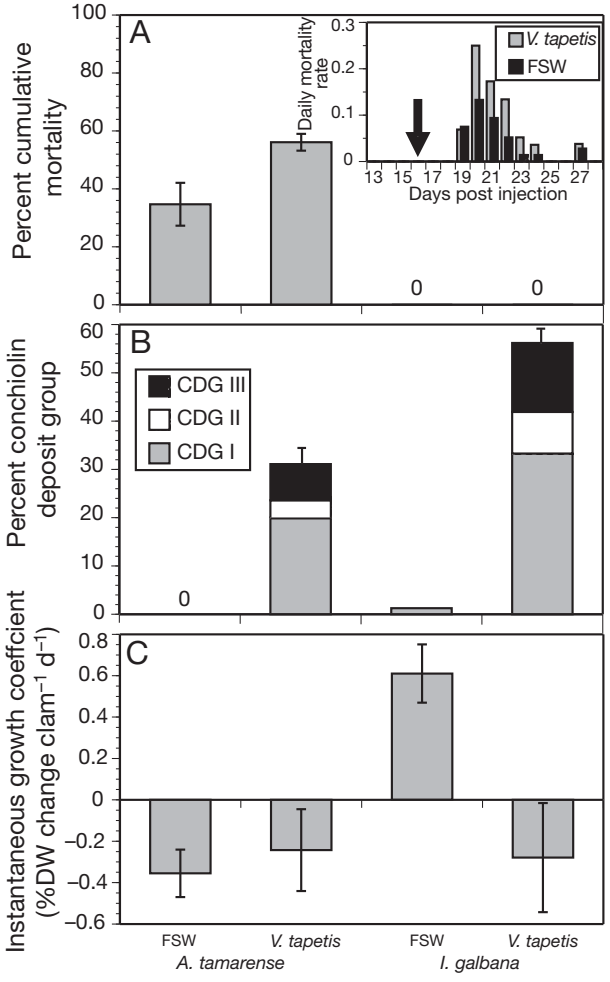


Fig. 2. *Ruditapes philippinarum.* A test of the combined effects of exposure to *Vibrio tapetis* and *Alexandrium tamarense* on juvenile Manila clams (Expt 1; see Fig. 1): (A) Mean percent cumulative mortality at the end of the experiment (inset shows the temporal progression of mortalities during Days 13 through 16 and after toxification; the arrow marks the end of toxification); (B) Mean percent prevalence of brown ring disease (BRD) stages; and (C) Instantaneous daily growth coefficient (*k*) in terms of dry weight of soft tissues of juvenile Manila clams between Days 6 and 28 post-injection (see Fig. 1); initial mean DW = 19.71 mg (±0.63 SE). Error bars in (A) and (C) represent the mean (±SE) of 3 observations, 55 clams in each in A and 10 in C. BRD stages in (B) were scored from 1 to 7 according to Paillard & Maes (1994) and grouped by severity as conchiolin deposit groups (CDG) I, II, and III (see 'Hemocyte response' in 'Materials and methods'). No BRD stage 7 clams were found in this study. The total BRD prevalence is the mean (±SE) of 3 observations. In (B) for *the A. tamarense* diet, n = 66 for filtered seawater (FSW) and 53 for *V. tapetis*; for the *Isochrysis galbana* diet, n = 80 for filtered seawater (FSW) and 105 for *V. tapetis*

Hemocyte parameters were compared by ANOVA if their variances were homogeneous (Bartlett's test); otherwise, they were analysed using the Kruskal-Wallis non-parametric test. For all tests, differences were considered significant at $p < 0.05$.

## RESULTS

### Expt 1: juvenile Manila clams

This experiment compared mortalities, BRD prevalence and growth (Fig. 2), and burrowing behavior (Fig. 3) between single and dual treatments of toxic algae and *Vibrio tapetis*.

#### Mortality

Cumulative mortalities occurring between the start of toxification and the end of the experiment (28 d post-inoculation) were observed only in juvenile clams

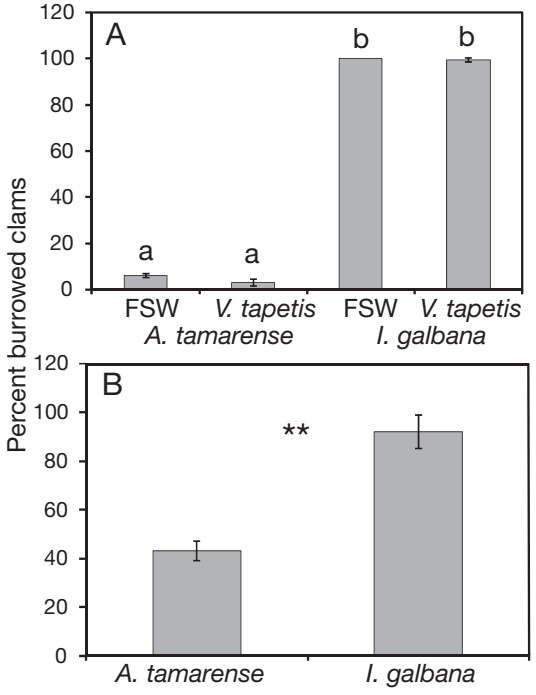


Fig. 3. *Ruditapes philippinarum.* (A) Percent of juvenile clams (12 mm SL; Expt 1) that burrowed within 2 h following 24 h exposure to either *Isochrysis galbana* (clone T-iso) or toxic *Alexandrium tamarense* (mean ± SE of 3 observations, n = 55 clams each). Clams were injected with filtered seawater (FSW = controls) or *Vibrio tapetis* 2 wk before toxification began (see Fig. 1). Different letters indicate significantly different results ($p \leq 0.05$). (B) Percent of test population of adult Manila clams (36 mm SL) that burrowed within 5 h following 16 h exposure to either *I. galbana* (clone T-iso) or toxic *A. tamarense* (Expt 2) (mean ±SE of 3 observations, 20 clams each except for 1 *A. tamarense* tank with 16 clams). **$0.01 < p \leq 0.05$

fed *Alexandrium tamarense* (Fig. 2A), but differed significantly between FSW- and *Vibrio tapetis*-injected clams (34.7 and 56.1%, respectively; $p = 0.03$). Nevertheless, daily mortalities for these 2 groups followed the same pattern. They were greatest 4 d following removal from the toxic diet and declined gradually thereafter (inset in Fig. 2A). Survivorship trajectories were also found to be significantly different between these 2 treatments (stratified log-rank test for equality of survivor functions; $p < 0.001$). In contrast, clams fed the *Isochrysis galbana* diet, whether injected with FSW or *V. tapetis*, showed 100% survival throughout the same period.

### Burrowing

Severe and significant burrowing incapacitation (>93% non burrowers) after 24 h toxification was observed only in treatments in which juveniles were fed toxic *Alexandrium tamarense* cells, whereas 100% of clams fed non-toxic algae (whether injected with FSW or *Vibrio tapetis*) reburrowed within 2 h (Fig. 3A). Thus, *in vivo* challenge with pathogenic *V. tapetis* did not affect burrowing behavior 2 wk post-inoculation.

### BRD prevalence

The total prevalence of BRD (sum of CDG I, II, and III) in juvenile clams at Day 28 post-inoculation was highest (56%) in clams challenged with *Vibrio tapetis* and fed the control algae, and was significantly different from *V. tapetis*-challenged clams (32%) fed toxic algae ($p = 0.017$) (Fig. 2B). Among control clams injected with FSW, only 1 (FSW and non-toxic algae), which had a stage-2 deposit, showed evidence of BRD. There was also a significant difference in the CDG distribution between the 2 *V. tapetis*-injected treatments ($p = 0.039$). The incidence of clams with more advanced stages of CDG (CDGII and CDGIII) was higher in *V. tapetis*-challenged clams fed the control *Isochrysis galbana* diet, than in those fed toxic algae (Fig. 2B).

The clams injected with *Vibrio tapetis* and fed toxic algae had significantly lower BRD prevalence at the end of the experiment than those fed the nontoxic algae, but they also had much greater mortality (Fig. 2A,B). We considered the possibility that, during the experiment, BRD-affected clams in the toxic-algae-fed group died in greater numbers than the controls, thereby disproportionately reducing prevalence in the final sample. Therefore, we compared the BRD prevalence and severity of clams that died during the experiment with that of live clams at the end of the experiment. A total of 21% of the *V. tapetis*-injected clams that died during the study had BRD symptoms, and of these, only 15% were in stages >2. This compares to 38% prevalence and 20% in stages >2, respectively, in the survivors. Even if the toxified clams had survived and the prevalence and severity of BRD had increased during the final week of the study, it is unlikely that it would have increased enough to account for the differential between the toxified and nontoxified clams at the final sampling.

### Growth

The instantaneous daily growth of soft tissues ($k$), calculated between Day 6 and Day 28 post-inoculation, was positive only in the control treatment (FSW-injected clams fed non-toxic algae), and significantly different from those of all other treatments ($p \leq 0.01$) (Fig. 2C). The $k$ values of all other treatments were negative, indicative of tissue weight loss, and not significantly different from each other. Growth reflects only the 4 d period of continuous feeding, since before and after that clams were fed a maintenance diet insufficient to support growth.

## Expt 2: adult Manila clams

This trial was designed to identify the potential individual variation in the susceptibility to PSTs of adult clams, as measured by their burrowing capacity, and to determine their hemocyte responses following short-term exposure to a high-toxicity dinoflagellate strain.

### Burrowing

Exposure for 16 h to a highly toxic *Alexandrium tamarense* strain resulted in a significant reduction in the percentage of adults that were capable of reburrowing relative to clams exposed to the non-toxic control diet of *Isochrysis galbana* ($p < 0.01$, Fig. 3B). There was, however, considerable variability among individuals of the same population in the susceptibility to PSTs, as measured by this behavioral index. In contrast to 100% burrowing of the non-toxified juveniles, only 87% of the adults in control tanks had burrowed after 2 h (vs. 38% on average in the toxic treatment). The burrowing response did not reach asymptotic values until 5 h of observation, when on average 42 (SE = 5.4) and 92% (SE = 7.4) of the test population had burrowed in the toxic and non-toxic diets, respectively (Fig. 3A). The condition index (CI) of clams exposed to toxic algae, which averaged 4.15 (SE = 0.25) and 3.71 (SE = 0.85) in burrowers and non-

burrowers, respectively, did not differ significantly between these 2 groups (ANOVA, p = 0.16). This confirms that burrowing incapacitation was a response to PST exposure, and not attributable to low nutritional condition.

### Effects on hemocytes

There were no significant differences in any of the hemocyte parameters measured between burrowers and non-burrowers fed either diet (Table 1). Therefore values were pooled for all clams within each diet for analysis of treatment effect. Compared to controls, short-term exposure to highly toxic *Alexandrium tamarense* cells resulted in a significant increase in total hemocyte concentration (THC), mainly due to an increase in granulocyte concentration, and a highly significant negative, although small effect on the hemocytes' phagocytic activity and capacity, but no effect on hemocyte viability or oxidative activity (Table 1). Pooled samples indicated that adhesion of clam hemocytes after *in vitro* exposure to *Vibrio tapetis* was not significantly affected by toxic algae and was statistically similar in burrowers and non-burrowers (ANOVA, p = 0.32 and p = 0.98, respectively).

The prevalence of BRD was 12.9% in clams from the toxified treatment and 16.7% in control tanks, and most were in CDGII. Therefore neither CI nor BRD status could explain the observed differences in hemocyte concentrations between the toxic treatment and controls.

### Toxin accumulation and biotransformation

We here determined the toxicities attained by juveniles and adult clams following short-term exposure to a highly toxic diet, and the capacity for biotransformation of individual PSTs present in the diet. The toxicity of *Alexandrium tamarense* (strain PR18b) averaged 67.0 and 104.8 pg STXeq cell$^{-1}$ during the juvenile and adult experiments, respectively. Juvenile and adult clams accumulated PSTs at a comparable rate of 302 and 362 µg STXeq h$^{-1}$ respectively, although the former were exposed to one-half the *A. tamarense* concentration and a lower mean cell toxicity. Adult burrowers attained a higher mean toxicity than non-burrowers, 6018 (SE = 383; n = 24), and 5528 µg STXeq 100 g$^{-1}$ (SE = 427; n = 21), respectively, but the difference was not statistically significant (ANOVA, p = 0.39) over this short period of toxin exposure.

The molar composition of individual toxins in the dinoflagellate cells was dominated by the more labile, but low-potency sulfocarbamoyl toxins C1+2 (66.2%), followed by the high-potency carbamate toxins NEO (21.0%), and STX (8.4%), and a smaller contribution of GTX2+3 of intermediate potency (3.7%) (Fig. 4). Although the epimers C1, C2, and GTX3, GTX2 were pooled for the statistical analysis below, C2 and GTX3 remained the dominant epimers in clam tissues during this relatively short period (16 to 24 h) of toxification. Thus, the C2:C1 molar ratio averaged 3.74 and 4.59 in juveniles and adults, respectively, and the ratio GTX3:GTX2 averaged 4.33 and 4.43 in juveniles and adults, respectively.

Table 1. Results of flow cytometric analysis of hemocytes from adult Manila clams following 16 h exposure to toxic *Alexandrium tamarense* (treated, T) or *Isochrysis galbana* (control, C) — means (SE). Percent phagocytosis = percent hemocytes containing ≥3 beads; phagocytosis capacity = number of beads per phagocytically active hemocyte; oxidative activity = fluorescence level of dichlorofluorescein diacetate (DCFH-DA) in arbitrary units. Significant differences are **bold**, and the direction of the effect is given in parentheses

| Variable | Treated (SE)[a]<br>Control (SE)[a] | Treated (T)[a] vs. control (C)<br>Test | <br>p |
|---|---|---|---|
| Total hemocytes ml$^{-1}$ (THC) | $9.00 \times 10^5$ ($1.13 \times 10^5$)<br>$6.07 \times 10^5$ ($6.4 \times 10^4$) | Kruskal-Wallis | **0.018** (T > C) |
| Granulocytes ml$^{-1}$ | $2.78 \times 10^5$ ($4.02 \times 10^4$)<br>$1.42 \times 10^5$ ($2.07 \times 10^4$) | Kruskal-Wallis | **0.0037** (T > C) |
| Hyalinocytes ml$^{-1}$ | $6.23 \times 10^5$ ($7.82 \times 10^4$)<br>$4.65 \times 10^5$ ($4.88 \times 10^4$) | Kruskal-Wallis | 0.052 |
| Percent hemocyte mortality | 3.04 (0.44)<br>3.16 (0.54) | ANOVA | 0.90 |
| Percent phagocytosis | 36.53 (1.84)<br>42.92 (1.50) | ANOVA | **0.011** (T < C) |
| Phagocytosis capacity | 7.81 (0.26)<br>8.79 (0.25) | ANOVA | **0.004** (T < C) |
| Oxidative activity | 41.42 (2.31)<br>41.83 (2.42) | ANOVA | 0.90 |

[a]Burrowers and non-burrowers combined

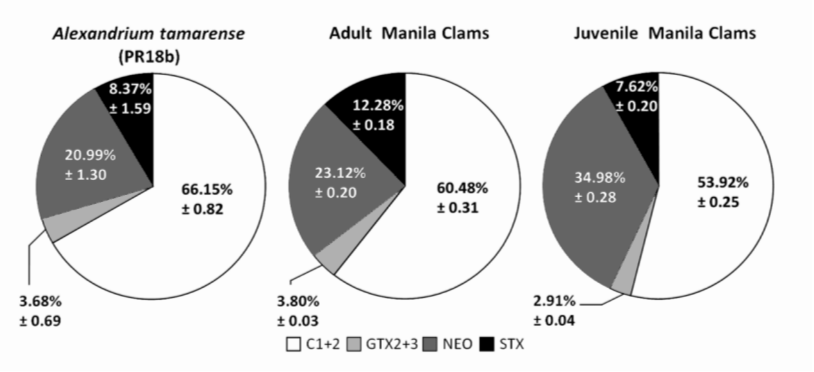


Fig. 4. *Ruditapes philippinarum*. Percent molar contribution (mean ±SE) of paralytic shellfish toxins (PSTs) in *Alexandrium tamarense* (strain PR18b) cells (n = 8), and adult and juvenile Manila clam tissues (46 clams and 10 samples of 2 to 10 pooled juveniles, respectively) after ~1 d of exposure to *A. tamarense* (epimers are combined). C1+2 = sulfocarbamoyl toxins C1 + C2; GTX2+3 = gonyautoxins GTX2 + GTX3; NEO = neosaxitoxin; STX = saxitoxin. Toxins that made a negligible contribution to the total toxin in clam tissues (<0.2%), namely dcGTX3 and GTX5 (= B1), are not shown although included in the totals; GTX1, GTX4, and dcGTX2 were not detected in clam tissues. Strain PR18b contained trace amounts of GTX4 and dcGTX3, but no detectable levels of GTX1, GTX2, GTX5, or dcGTX2. The ranking of potencies as determined by the mouse bioassay is: STX > NEO > GTXs > C toxins (see Fig. 3 in Bricelj & Shumway 1998)

Over 16 h of toxification, adult Manila clams showed a significant decrease to 60.5% in the contribution of C1+2 ($p < 0.001$), a significant increase in that of NEO ($p < 0.01$) and STX ($p < 0.001$) to 23.1 and 12.3%, respectively, and no significant change in GTXs ($p = 0.67$) compared to the *Alexandrium tamarense* cells (Fig. 4). Values for all individuals were pooled for this analysis, since there was no difference in the total tissue toxin composition of burrowing and non-burrowing adult clams ($p > 0.23$ for all toxins). There was also no difference between the toxin composition of *A. tamarense*-exposed juveniles injected with *Vibrio tapetis* and FSW controls (the sample size of non-burrowers was too small to provide a reliable comparison with burrowers), so data were pooled for comparison with adults in Fig. 4. After 1 d of toxification, juveniles showed an even more pronounced decrease in the contribution of C1+2 (to 53.9%) and increase in NEO (to 35.0%) relative to *A. tamarense* cells (Fig. 4). The toxin composition of adults and juveniles differed significantly for all toxins ($p < 0.001$), with juveniles showing a greater reduction of C1+2 and greater increase in NEO than adults. Thus, the molar ratio of N-sulfocarbamoyl to carbamate toxins, which has been proposed as an index of the degree of toxin conversion (Cembella et al. 1993), was 2.25 in *A. tamarense* and was reduced to 1.71 in adults and 1.26 in juveniles. Only trace concentrations of dcGTX3 were detected in Manila clam tissues, and were also detected in trace amounts in *A. tamarense* (strain Pr18b), indicating that Manila clams are unable to perform rapid decarbamoylation of sulfocarbamoyl toxins to dcGTXs.

## DISCUSSION

### Effects of PSTs on burrowing capacity of *Ruditapes philippinarum*

This study is the first to determine the effects of laboratory exposure of *Ruditapes philippinarum* to a highly toxic PST-producing *Alexandrium* spp., as all prior studies used isolates of relatively low toxicity. Burrowing incapacitation within ≤1 d of toxin exposure was observed in both juveniles and adults, but the percentage of affected clams was greater among the former and incapacitation occurred sooner even though they were exposed to one-half the *A. tamarense* concentration and a 36% lower mean cell toxicity. Juveniles were exposed for 24 versus 16 h in adults (Fig. 3), but longer exposure of adults would not be expected to alter their burrowing results given that the percentage of clams burrowing became asymptotic within 5 h of toxin exposure. No adults died during 16 h of PST exposure. This agrees with prior findings that mortalities of subadult *Mya arenaria* (33 to 47 mm SL) typically started only after ~1 to 2 wk of exposure to 100 cells $ml^{-1}$ of the same isolate (MacQuarrie & Bricelj 2008). Greater vulnerability to PSTs of small clams was also demonstrated when burrowing and survival were compared between *Mya arenaria* ~12 mm and 35 mm SL exposed to comparable toxin concentrations (Bricelj et al. 2010). Burrowing capacity is a critically important fitness parameter that is likely under strong natural selection, especially in juvenile bivalves before they attain a size refuge from predators.

In the field, the prevalence of BRD is greater in Manila clams collected at the surface than in those that remained burrowed (Paillard et al. 1994), suggesting that BRD may impair burrowing capacity or prevent maintenance of a normal position in sediment. In the present study, however, the ability to reburrow was not compromised in juvenile clams 2 wk following *Vibrio* inoculation, and no mortalities were induced by exposure to *Vibrio* alone. It is possible that negative effects on burrowing are associated only with more severely diseased clams.

A key finding of our study is that the burrowing assay allowed identification of toxin-resistant and sensitive adults within the same, Gulf of Morbihan, source population (45% burrowers and 54% non-burrowers, respectively, following correction for 8% of clams that did not burrow in the controls). This area typically experiences relatively low maximum *Alexandrium*

*minutum* concentrations (<1 cell $ml^{-1}$) compared to other sites along the northern French coast where maxima of up to 22 800 and 5800 cells $ml^{-1}$ have been reported (Morlaix and the Abers Finistères, respectively; REPHY). The toxin concentration was comparable in adult burrowers and non-burrowers, indicating that the former were able to tolerate a high toxin body burden while retaining the ability to burrow. In Atlantic *Mya arenaria* populations, the prevalence of resistant individuals as measured by the burrowing response following laboratory exposure to toxic *A. tamarense*, was correlated with that measured by *in vitro* nerve resistance to STX (Bricelj et al. 2004). Earlier studies that ranked the nerve susceptibility of North American bivalve species to STX did not include *Ruditapes philippinarum* (Twarog et al. 1972, Kvitek & Beitler 1991). However, if the individual variability in burrowing response shown in this study can be confirmed in future studies using the *in vitro* nerve assay, as was done in *M. arenaria* (Bricelj et al. 2005), *R. philippinarum* will provide a valuable, alternate model species in which to investigate the molecular/genetic basis for resistance to PSTs in bivalves.

### Toxin accumulation and biotransformation

In this study adult, commercial-sized *Ruditapes philippinarum* attained relatively high toxicities, up to ~6000 µg STXeq 100 $g^{-1}$, following only brief (16 h) exposure to a highly toxic *Alexandrium tamarense* isolate. This value exceeds the regulatory level for safe harvesting by ~2 orders of magnitude, and is comparable to the maximum (6100 µg STXeq 100 $g^{-1}$) reported for Manila clams in the field (British Columbia, Canada) (reviewed by Bricelj & Shumway 1998). Thus, Manila clams could potentially affect public health in areas where they are exposed to high *Alexandrium* spp. concentrations and/or cell toxicities. *Mya arenaria* from a predominantly resistant population, as determined by the burrowing assay, exhibited an 8-fold higher PST uptake rate than clams from a mostly sensitive population over 2 wk of toxification (MacQuarrie & Bricelj 2008). The fact that no difference was detected between adult burrowers and non-burrowers in our study is attributed to the short duration of toxin exposure.

Differences in the PST composition between bivalve tissues and the dinoflagellates they ingest are commonly observed due to metabolic/chemical transformation and/or differential retention/elimination, but the magnitude and rate of these changes varies greatly among species (Cembella et al. 1993, Bricelj & Shumway 1998). A reduction in C toxins and increase in NEO in clam tissues relative to *Alexandrium tamarense* were the 2 most salient changes documented in this study (Fig. 4). The greater changes observed in juvenile clams, evidenced by the greater reduction in C toxins, are consistent with their more rapid metabolism per unit body mass relative to adults. Conversion of the more labile C toxins, however, would be expected to result in an increase in GTXs and in STX, rather than in NEO, according to the pathways: C1+2 → GTX2+3 → STX, due to enzymatic hydrolysis of N-sulfocarbamoyl toxins followed by reductive conversion of GTXs (Bricelj & Shumway 1998). The anomalous changes observed (increase in NEO) could result from differential retention of individual toxins, e.g. more rapid elimination of GTXs and/or selective retention of NEO. It is difficult to compare our toxin composition results with prior studies in Manila clams (Lassus et al. 1989, 1994, Samsur et al. 2006) in which tissues were extracted in strong acid (0.1 HCl) using the AOAC method, which is known to affect the integrity of individual PSTs. Overall, our results indicate that *Ruditapes philippinarum* resembles *Mya arenaria* in its relatively limited capacity for toxin conversion, demonstrated both *in vivo* (Bricelj & Shumway 1998) and *in vitro* (Fast et al. 2006).

Manila clams were not capable of rapid (24 h) decarbamoylation of PSTs. In contrast, decarbamoyl (dc) toxins are produced preferentially and very rapidly (within a few h of feeding on toxic cells) from N-sulfocarbamoyl toxins, as well as secondarily and at a slower rate from their corresponding carbamate toxins in some bivalves such as surf clams *Spisula solidissima* (Cembella et al. 1993, Bricelj & Shumway 1998). Rapid decarbamoylation of C toxins to dcGTXs (up to 90% conversion within 4 h in the digestive gland), and slower decarbamoylation of GTXs to dcGTXs, was demonstrated *in vitro* in littleneck clams *Protothaca staminea*, in which the reaction appears to be enzymatically mediated (Sullivan et al. 1983, Fast et al. 2006).

### Effects of PSTs on Manila clam hemocytes

No adverse, direct effects on hemocytes were previously found by *in vitro* exposure of *Ruditapes philippinarum* hemolymph to extracts of *Alexandrium tamarense* (strain PR18b) containing high concentrations of PSTs, although a non-PST producing *Alexandrium* strain elicited strong adverse effects (Ford et al. 2008). Similarly, no effects of *in vivo* exposure to toxic *Alexandrium* spp. on hemocytes were found in oysters *Crassostrea virginica* and *C. gigas*, although they attained only low toxicities (<130 µg STXeq 100 $g^{-1}$) (Hégaret et al. 2007b). In the present study, in which *R. philippinarum* attained much higher toxicities, a significant, deleterious effect of *A. tamarense* on hemocyte function was found only for percent phagocytosis (15% reduction,

$p \leq 0.05$) and phagocytic capacity (11% reduction, $p \leq 0.01$). Furthermore, exposure to *A. tamarense* led to a significant, 1.5-fold increase in total hemocyte concentrations (THC) ($p \leq 0.05$), and a doubling of granulocytes. The latter are the most powerfully phagocytic cells in *Ruditapes* spp. and thus considered most effective in cellular defense (López et al 1997, Donaghy et al. 2009). Toxified clams showed a 26% increase in the total number of phagocytic hemocytes (THC × percent phagocytosis) relative to control clams.

In contrast to our results, Galimany et al. (2008a) documented a significant reduction in the number of circulating eosinophilic hemocytes of *Mytilus edulis*, exposed for 9 d to a low-toxicity *Alexandrium fundyense* isolate (0.43 pg STXeq cell$^{-1}$) relative to controls fed non-toxic *Rhodomonas* spp. Histology indicated that this depletion was associated with hemocyte migration into the alimentary canal via diapadesis. Given the results of Ford et al. (2008), however, it cannot be ruled out that these observations are related to a non-PST toxic metabolite. Exposure of mussels to the toxic dinoflagellate *Prorocentrum minimum* also showed migration of hemocytes to the alimentary canal, yet there were no detectable effects on hemocyte concentration or functions (Galimany et al. 2008b). Exposure of *Ruditapes philippinarum* to *P. minimum* for 6 d led to a significant (17%) reduction in phagocytosis, but no effects on hemocyte mortality and concentration, reactive oxygen species (ROS) production, or agglutination activity, yet it was associated with histological abnormalities (myopathy) in the foot and adductor muscle (Hégaret et al. 2009).

The increase in hemocyte concentrations detected in our study following short-term (16 h), acute exposure of adult Manila clams to a highly-toxic dinoflagellate cannot be clearly associated with an increased immune response (Adamo 2004), as it could reflect differences in algal diets (Delaporte et al. 2003) or redistribution of hemocytes between tissues and the circulatory system resulting from PST-induced paralysis. Our interpretation of *in vitro* trials using the same toxic isolate (Ford et al 2008) and the hemocytic analysis from this study is that short-term (~1 d) exposure to highly toxic *Alexandrium tamarense* (strain Pr18b) does not compromise hemocyte numbers or function, as measured by most of the parameters tested, although it was sufficient to induce foot paralysis and, thus, burrowing incapacitation. This suggests that behavioral responses are more sensitive, and may respond more rapidly, than hemocyte functional responses to the detrimental effects of PSTs. For example, highly reproducible behavioral effects of exposure to *A. minutum* were documented in *Crassostrea gigas*, as measured by valve activity, including a significant increase in the number of valve microclosures within 1 d of exposure to the toxic diet (Tran et al. 2010). In their study, oysters were exposed to 4.7 ng STXeq ml$^{-1}$, a value comparable to that (3.4 ng STXeq ml$^{-1}$) maintained in our juvenile experiment.

### Single vs. interactive effects of *Alexandrium tamarense* and *Vibrio tapetis* in juvenile Manila clams

Juvenile clams from the high-toxicity *Alexandrium tamarense* treatment experienced tissue weight loss and high mortalities, unlike those fed the control diet (Fig. 2A,C). Juvenile Manila clams showed a significant reduction in soft-tissue growth when fed a unialgal diet of a weakly toxic *A. tamarense* strain (ATCI01, 3.2 pg STXeq cell$^{-1}$) for 15 d relative to controls fed *Thalassiosira pseudonana*, although they suffered no mortalities (Li et al. 2002). In their study, scope for growth (SFG) of adult Manila clams, calculated from physiological rates, was also sharply reduced when this toxic strain was offered in increasing proportions in a mixed diet relative to a diatom control. In the unialgal toxic diet, both absorption efficiency and clearance rate (CR) were significantly reduced, and SFG became negative. Thus, negative growth of *A. tamarense*-exposed juvenile clams in our study is likely, at least partly due to inhibition of clam feeding and absorption, although the high mortalities and the burrowing incapacitation observed also point to toxin-induced effects unrelated to nutritional deficiency. MacQuarrie & Bricelj (2008) suggested that high mortalities of *Mya arenaria* juveniles exposed to the same PR18b strain used in our study might be caused by hypoxia of the pallial cavity resulting from toxin-induced paralysis and lack of irrigation. Whatever the underlying mechanism, Manila clams are clearly much more sensitive to the effects of PSTs than mussels, and more comparable to softshell clams. Thus, a unialgal diet of *A. tamarense* (toxicity = 9.5 pg STXeq cell$^{-1}$) supported positive growth of *Mytilus edulis* juveniles, although 26% lower than those on a diatom diet, but no mortalities (Bricelj et al. 1993), and yielded comparable growth to diatom controls in *Perna viridis* juveniles fed strain ATCI01 (Li et al. 2002).

In the present study, *Vibrio tapetis*-injected juveniles fed the control diet for 4 d followed by 12 d on a maintenance diet also exhibited weight loss, whereas those in the control treatment showed positive growth. Plana & Le Pennec (1991) found that *V. tapetis*-injected juvenile Manila clams showed adverse changes in the morphology of the digestive tubules relative to FSW controls in both fed and starved treatments 30 d post-inoculation. The reduction in percent of absorptive stages 1+2 (from 92 to 52% in fed clams) and increase

in degenerative stages 3+4 (from 8 to 47%), were comparable in clams with both early and advanced stages of BRD, and were similar to those associated with starvation. Juveniles injected with *V. tapetis* also exhibited a significantly lower tissue DW compared to controls after 30 d of starvation, which was attributed to loss of glycogen reserves (Plana et al. 1996). Feeding resumption from Day 30 to 50, however, led to an increase in glycogen only in control clams injected with FSW. Glycogen content by the end of the feeding period was inversely related to the severity of BRD symptoms of *V. tapetis*-injected clams, indicating that BRD caused irreversible changes in the digestive gland's ability to store carbohydrate reserves, although clams recovered from visible signs of BRD. We thus suggest that tissue weight loss of infected juveniles in the present study, despite *ad libitum* food availability over 4 d, was likely a direct effect of *V. tapetis*-induced atrophy of the digestive gland and thus impaired absorption, although CR may also have been compromised. Prior studies confirm that naturally BRD-affected adult *Ruditapes philippinarum* experienced a decrease in CI and glycogen (Goulletquer et al. 1989, Paillard 1992). Furthermore, the CI, CR, and feeding time of field-infected adults were reduced relative to asymptomatic clams, although this effect was significant only for individuals with advanced stages of BRD (CDG ≥ 4 to 5) (Flye-Sainte Marie et al. 2007). BRD severity was also negatively correlated with respiration rate, but examination of these authors' data suggests that the rate of this decline with increasing CDG was less pronounced than that of CR.

Our results both supported and rejected our hypothesis that combined exposure to *Vibrio tapetis* and toxic algae would aggravate the effects of either of these alone on the progression of BRD. The clams subjected to the dual challenge experienced much higher mortality than those fed the nontoxic diet, which had no deaths, and somewhat higher mortality than those exposed to the toxic diet but injected with FSW (Fig. 2A). Yet, this dual exposure to both *Alexandrium tamarense* and *V. tapetis* was associated with lower BRD prevalence by the end of the experiment than the *Isochrysis galbana* + *V. tapetis* treatment. This result could not be explained by higher mortality of symptomatic clams in the dual treatment, suggesting that the toxic algal exposure may have slowed the progression of BRD (Fig. 2B). Several mechanisms can be invoked to explain this outcome. First, *A. tamarense* may be directly toxic to *V. tapetis.* Second, the overall negative effect of toxic algal exposure on metabolism, as indicated by the loss of tissue weight, could have inhibited the clams' capacity to produce the conchiolin deposit. This may have been exacerbated if hypoxia occurred in the pallial cavity, as suggested for *M. arenaria* (MacQuarrie & Bricelj 2008). A related finding was reported for juvenile *Crassostrea virginica* injected with the bacterial agent of Roseobacter oyster disease (ROD, also known as juvenile oyster disease, JOD). Juveniles <10 mm often died before they were able to form the characteristic conchiolin ring on the inner shell, whereas larger juveniles did produce the characteristic conchiolin response (Maloy et al. 2007). For both BRD and ROD, the bacterial agents are often found trapped between layers of the conchiolin deposit and under shell layers following repair, suggesting that it may be a defense mechanism (Paillard et al. 1994). Thus, the failure of juvenile Manila clams to produce conchiolin may be a measure of the failure of a defense system that led to high mortality.

The possibility that exposure to PSTs resulted in selective or earlier mortality of clams with BRD signs was excluded by determining the CDG stage of dead clams throughout the course of the experiment (see 'BRD prevalence' in 'Results'). It remains possible, however, that exposure to *Alexandrium tamarense* stimulated the clams' cellular response against *Vibrio tapetis*. Allam et al. (2001) found a positive correlation between the concentration of phagocytically active cells in both hemolymph and extrapallial fluid with resistance to BRD in experimentally injected *Ruditapes* spp. We can only speculate on a potential role of hemocytes in suppressing BRD prevalence in juveniles exposed to *A. tamarense*, since the 26% increase in the number of phagocytic hemocytes was determined in adults, and following only short-term exposure to *A. tamarense*.

There are parallels between the results of our study and a prior study in which exposure of *Ruditapes philippinarum* to *Karenia selliformis*, a dinoflagellate with strong hemolytic action and producer of gymnodimine, reduced the prevalence of *Perkinsus olseni* in infected clams (da Silva et al. 2008). Exposure to *K. selliformis* had no effect on the clams' hemocyte concentration, percent mortality, percent phagocytosis, or ROS production, although it caused a significant decrease in mean hemocyte size and percent apoptotic cells. Intact *K. sellifomis* cells, however, induced increased mortality of *P. olseni in vitro*, indicating that direct toxicity to the parasite, rather than a change in the host's hemocyte concentrations or function, was responsible for the transient abatement of parasite prevalence and intensity of infection. Similarly, *in vitro* exposure to both *P. minimum* culture medium and whole cells resulted in a significant increase in the mortality of *P. olseni* trophozoites (Hégaret et al. 2009). Therefore, additional work is needed on the dose-dependent effects of toxic algae on bivalve pathogens, so far examined for very few toxic species, to better understand their combined role in the natural environment.

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