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ORIGINAL ARTICLE

Environment

Differential okadaic acid accumulation and detoxification by oysters and mussels during natural and simulated *Dinophysis* blooms

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Abstract A bloom of *D. acuminata* complex was detected in early March 2008 in the shellfish farming area of Baía Sul, southern Brazil. Cell density reached up to 4.7×10^4 cells/L at the surface by March 31st, quickly decreasing ($<4.0 \times 10^3$ cells/L) 1–2 weeks later as salinity increased (>32) in all sampling stations. On average, commercial-sized mussels, *Perna perna*, accumulated 11-fold higher okadaic acid (OA) levels in the digestive glands (DG) (maximum 2422 ng/g) compared to those of oysters, *Crassostrea gigas* (maximum 271 ng/g). Moreover, 50 % of the mussel DG extracts tested ($n = 42$) produced acute toxic effects as assessed by mouse bioassay, whereas all oyster samples produced negative results ($n = 17$). In parallel, juvenile oysters, *Crassostrea brasiliana* and *C. gigas*, and juvenile mussels, *P. perna*, exposed to a laboratory-simulated *D. acuminata* complex bloom (1350–13,750 cells/L) accumulated 7.5, 23.4, and 51.1 ng OA/g in whole bodies (22.7, 66.1, and 183.3 ng OA/g in visceral tissues), respectively. Toxin levels in the whole bodies dropped to similarly low average levels in all bivalve species (3.6, 3.7, and 1.3 ng/g, respectively) after 168 h of depuration. Mussels, therefore, detoxified OA at faster rates (0.023/h) than oysters *C. gigas* (0.010/h) and *C. brasiliana* (0.004/h).

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Introduction

Bivalve aquaculture has become a major economic activity in many coastal regions worldwide. Contamination of bivalve mollusks by phytoplankton toxins via suspension-feeding on toxic cells, however, poses a constant threat for the consumer's safety and the economic stability of this activity, as harvesting bans need to be occasionally issued to prevent intoxication episodes in humans.

One of the most common types of intoxication, diarrhetic shellfish poisoning (DSP), is characterized in humans by gastrointestinal symptoms such as diarrhea, vomiting, nausea, and abdominal pain [1], which can persist for up to three days. It is caused by the consumption of bivalves containing high concentrations of okadaic acid (OA) and/or its analogs, the dinophysistoxins (DTX1 and DTX2), as well as their acylated forms (DTX3 group) and diol ester derivatives (DTX4 and DTX5). Gastroenteritis symptoms from DSP have been reported in humans following the ingestion of shellfish containing as little as 48–75 µg OA [2, 3].

DSP episodes have been registered worldwide, mainly in areas with significant shellfish exploitation. Some places such as Japan, southern Chile, and northwestern Europe, including Spain, Portugal, Scotland, Ireland, France, Sweden, and Norway can be considered global hotspots due to the high incidence of DSP outbreaks and/or prolonged harvesting bans. Other places, such as the Mediterranean, China, Korea, southern Australia, North America, and southern Brazil are also subjected to *Dinophysis* spp. blooms and have recently experienced periodic

contamination of bivalves and even human poisoning episodes (reviewed in Reguera et al. [4]).

In southern Brazil, more specifically, in the state of Santa Catarina, diarrhetic shellfish toxins (DSTs) have been regularly detected by HPLC and mouse bioassay since 1996, when monitoring programs for toxins and harmful algae in shellfish farming areas were initiated [5]. In January 2007, a major bloom of *Dinophysis* cf. *acuminata* (up to 52,000 cells/L) in Tijucas Bay, Santa Catarina, was related to the contamination of *Perna perna* mussels with DST levels above the regulatory limit (RL) for human consumption, i.e., 160 ng OA eq./g of shellfish meat, or 2000 ng OA eq./g of hepatopancreas (HP), as assessed by mouse bioassay [6]. The episode led to >150 notified cases of human poisoning before a 23-day harvesting ban was issued due to the contamination of mussels and, to a lesser extent, oysters [6].

The amount of toxin accumulated by different bivalve species depends upon their ingestion rates of toxic cells, which, in turn, is a function of their particle capture efficiency, clearance rates, and capacity for selective feeding, as well as processes regulating toxin assimilation or elimination, such as digestion (i.e. absorption efficiency), affinity for the toxic compounds, toxin transformation (i.e. metabolism and conjugation), and excretion [7]. For instance, transformation of DSTs in bivalves occurs in the presence of specific enzymes during extra- and probably intra-cellular digestion (reviewed in Reguera et al. [4]). Thus, transformation of DSTs can be either intensified or limited in certain bivalve species [8, 9], and this affects their detoxification rates and, ultimately, their capacities for toxin accumulation during prolonged exposure to toxic phytoplankton cells.

Several field studies have suggested marked species-specific differences in DST accumulation among commercial bivalves. For instance, oysters do not always accumulate DSTs, and when they do, toxin levels are at least ten times lower than those of co-occurring mussels [10–15]. Accordingly, during previous *D. acuminata* complex blooms in southern Brazil, the mussel *P. perna* has consistently accumulated higher OA levels compared to those of oysters, *Crassostrea gigas*, which rarely attain the RL, even though they are mostly co-occurring and similarly cultivated (i.e. suspended in long lines) species. Even so, in most countries, harvesting bans are regularly issued for all bivalves farmed or harvested within an affected area.

In order to evaluate species-specific actions during future harvesting closures for OA-contaminated bivalves, this study compares toxin accumulation and detoxification by mussels (*P. perna*) and oysters (*C. gigas* and *C. brasiliana*) during both natural and laboratory-simulated *D. acuminata* complex blooms. Controlled laboratory experiments were designed to assure that all bivalves were strictly exposed to the same cell densities and environment conditions during both toxin uptake and depuration phases, which may not always occur in the field, where slight differences in depth and location of the long lines that sustain each bivalve species may result in differential exposure to the patchy *Dinophysis* populations. In addition, the native mangrove oyster, *C. brasiliana*, which has been increasingly cultivated in southern Brazil, is not farmed or harvested in the same geographic locations as the other two species.

Materials and methods

Field sampling

South Bay ("Baía Sul") is a sheltered and narrow area located in southern Brazil between Santa Catarina Island and the continent (Fig. 1). Several bivalve mollusk farms are established along the coast in this region, mostly using the long line method to cultivate Pacific oysters *Crassostrea gigas* and brown mussels *Perna perna* all year round. Bivalves are suspended 1.5–3 m from the surface in this shallow and well-mixed water body. Water temperature usually varies from 17 to 28 °C during the year and salinity typically ranges from 24 to 35. Other bivalve species such as the clam *Anomalocardia brasiliana* are also present, but they are commercialized in much smaller amounts.

Five sites in South Bay under a regular harmful algal and toxin monitoring program were sampled every 15 days by local authorities. During the *D. acuminata* complex bloom that occurred in late summer/early autumn 2008, sampling frequency was intensified in order to track closely the DST contamination levels in bivalves and prevent poisoning outbreaks. Four sampling sites were selected to evaluate the simultaneous accumulation of diarrheic toxins in *C. gigas* and *P. perna*: (#1) Caieira da Baía Sul; (#2) Costeira do Ribeirão; (#3) Freguesia do Ribeirão; and (#4) Praia do Cedro (Fig. 1).

Water samples from each sampling site were collected at the surface with a bucket. Aliquots (200 mL) were fixed with 1 % lugol solution for counting the main phytoplankton taxa. Subsamples of 20–50 mL were settled in Utermöhl chambers and at least 200 phytoplankton cells were counted per sample. *Dinophysis* cells were observed under the light microscope (Zeiss® Axiovert.A1), and randomly selected individuals ($n = 90$) were measured using a coupled camera (Zeiss® AxioCam ERc5 s) and the image processing software AxioVision 4. Cell features, shape, and measurements were compared to the literature [16–18] for taxonomic identification at species level. Salinity was determined prior to sample preservation using a portable refractometer (American Optical, USA) with ± 1 PSU

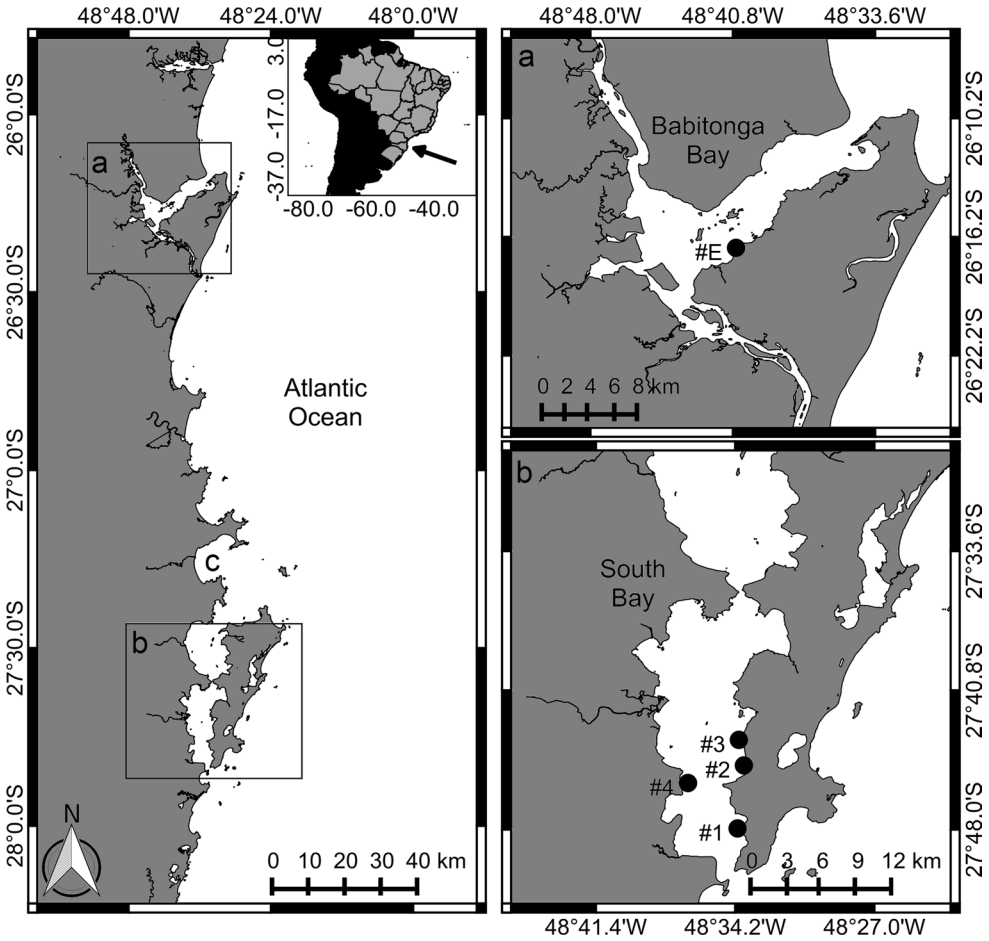

Fig. 1 Map of Santa Catarina State, southern Brazil, showing the location of the sampling sites in South Bay (*b*) where the 2008 bloom of *Dinophysis acuminata* complex was recorded, the place in Babitonga Bay (*a*) where cells used in the toxin accumulation experiment were collected, and Tijucas Bay (*c*), where a major bloom was recorded in 2007 [6]

resolution and water temperature was determined in situ using a regular field thermometer (0–40 ± 0.1 °C).

Additionally, bivalves were collected from the same sampling sites and transported in thermal boxes to the laboratory. Each sample was composed of about 60 commercial-sized individuals of either *P. perna* or *C. gigas*. Bivalves were washed, opened, and their HP were dissected and stored in a freezer until toxin analysis.

Table 1 Mean wet weight (g) of visceral (VC) and other, non-visceral (NV) soft tissues; percent of VC weight in relation to the total body weight; and mean shell height among "*n*" bivalves sampled during the toxin accumulation experiment

Species	VC weight (g)	NV weight (g)	VC (%)	Shell height (mm)	*n*
Crassostrea gigas	0.07	0.17	29.5	34.27	100
C. brasiliana	0.19	0.40	31.4	29.17	100
Perna perna	0.10	0.31	23.4	31.99	50

Toxin accumulation experiment

Juvenile (3–4 months old) oysters (*Crassostrea gigas* and *C. brasiliana*) and mussels (*Perna perna*) were obtained from growers in Florianópolis and São Francisco do Sul, Santa Catarina State, Brazil, and brought to the laboratory where they were maintained in aerated 200-L tanks, containing 140 L of filtered seawater (Cuno® membrane filter, 1 µm pore size) at 22 ± 1 °C and salinity of 30 ± 2. They were placed in floating, perforated trays and received a non-toxic, mixed algal diet (*Tetraselmis suecica*, *Isochrysis galbana*, and *Chaetoceros muelleri*) until the day of the experiment. Ammonium concentration and pH were monitored daily, water was partially replaced (70 % of the total volume) twice a week, and the tanks were completely sanitized every 1–2 weeks to assure a good water quality. For the toxin accumulation experiment, 106 similar-sized individuals of *C. brasiliana*, 106 of *C. gigas*, and 54 of *P. perna* were selected (Table 1). Juvenile bivalves are easier to maintain in the laboratory, consume fewer cells during the experiment, excrete smaller ammonium amounts, and are much less prompt to lose biomass due to spawning, yet they exhibit feeding responses comparable to adult

individuals because their feeding organs and alimentary tracts are already fully developed and functional [19] at the size and age used in the present study.

The experiment was divided into two sequential phases: (1) "Uptake", when bivalves were exposed to a toxic diet containing *D. acuminata* complex cells for 24 h; and (2) "Depuration", when bivalves were fed a monospecific, non-toxic diet (average ~100,000 *T. suecica* cells/L) for an additional 168-h period. The toxic diet was obtained from multiple phytoplankton net (20 μm mesh size) trawls at Babitonga Bay, São Francisco do Sul (#E, Fig. 1), followed by sieving through a zooplankton net (60 μm mesh size) to remove larger particles. The concentrated material (mostly 20–60 μm particles) was placed into four 20-L carboys containing filtered local seawater at 28 salinity. The presence of *D. acuminata* complex cells was verified in situ, and the carboys were immediately transported to the laboratory. The concentrated suspension contained 41,100–86,600 *D. acuminata* complex cells/L.

In the laboratory, 20 L of the concentrated suspension was added to a tank containing 100 L of filtered seawater (salinity 30) to make up the toxic diet (initial abundance ~14,000 *D. acuminata* complex cells/L). The tank was kept at the conditions described for the maintenance period, and the Uptake phase started when the bivalves, which had been acclimated for 24 h on a ~100,000 *T. suecica* cells/L diet, were transferred to the tank containing the toxic diet. Over the following 24 h, the remaining volume of the concentrated suspension (60 L) was constantly added to the tank using a peristaltic pump, as well as by periodical refills with a beaker, to sustain a roughly constant cell density. Bivalve samples consisting of two mussels or four oysters of each species were collected in triplicate after 5, 9, 14, and 24 h of exposure to the toxic diet. In addition, initial samples (0 h) for each bivalve species were taken prior to their contact with the toxic cells.

Following the initial 24 h period, the remaining bivalves were removed from the tank, rinsed with filtered seawater, and reallocated to a second tank, where they received the non-toxic depuration diet for a week. Similarly, samples were taken in triplicate throughout the depuration period, after 3, 10, 48, and 168 h of exposure to the non-toxic diet. Sampled bivalves were externally wiped, their shells were opened and the tissues were dissected into two fractions: visceral (VC) tissues, including the HP; and non-visceral (NV), soft tissues. Shell height was measured and both tissue fractions were individually weighed. Samples, composed of combined tissues from two mussels or four oysters of each species, were placed in centrifuge tubes and kept frozen until toxin analysis.

During both Uptake and Depuration phases, periodical 10 mL water samples were taken from the experimental tank and preserved in 1 % lugol for cell counts using a Sedgewick-Rafter chamber. Additionally, 40 mL samples were collected from the carboys containing the concentrated suspension and gently passed through microfiber glass filters (Whatman GF/C, Buckinhamshire, UK) for analysis of intracellular toxin content in *D. acuminata* complex cells.

Toxin analysis

Samples from the bloom

Two to three grams of HP from both *C. gigas* and *P. perna* samples were homogenized with 10 mL of 100 % methanol using an Ika Ultra-Turrax® disperser. Tissue extracts were prepared by washing HP homogenates (2–3 g) twice with 20 mL methanol. Tissue extracts were then centrifuged for 3 min at 600×*g* and filtered over 0.22 μm nylon filters (Millipore, Bedford, MA, USA).

Toxin analysis was conducted by liquid chromatography-tandem mass spectrometry (LC–MS/MS) using an Agilent 1200 series (USA) LC system coupled to a 3200AB Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems, USA) equipped with a TurboSpray interface. Chromatographic separations were performed on a C-18 column (Zorbax Eclipse XDB-C18, 50 × 4.6 mm I.D., 1.8 μm; Agilent, USA) using 10 % acetonitrile (A) and 90 % acetonitrile (B) as the mobile phase in a binary system with a linear gradient elution of 10–100 % B in 10-min runs at 35 °C and a 0.75 mL/min flow rate. The presence of dinophysistoxins (DTXs) and okadaic acid (OA) was investigated using selected reaction monitoring (SRM) with the ion source in negative mode. The following SRM transitions monitored: *m/z* 817.5 → 255.1 and *m/z* 817.5 → 113.0 for DTX-1; *m/z* 803.5 → 255.1, and *m/z* 803.5 → 113.0 for OA and DTX-2; and the following source parameters were used: source temperature = 650 °C, ionspray voltage = 4500 V, declustering potential = 120 V, entrance potential = 10 V, and cell exit potential = 2 V. Collision cell entrance potential and collision energy were, respectively, 28 V and 72 eV for *m/z* 817.5 → 255.1 and *m/z* 803.5 → 255.1, or 41 V and 82 eV for *m/z* 817.5 → 113.0 and *m/z* 803.5 → 113.0. The dwell time was set at 75 ms. Okadaic acid concentrations were calculated from the calibration curve made of serial dilutions (50–0.78 ng/mL) of the reference standard (National Research Council, Halifax, NS, Canada) in 100 % methanol.

In parallel to LC–MS/MS analysis, toxicity of the samples was assessed by mouse bioassay according to Yasumoto et al. [1]. Briefly, 20 g of HP was extracted with 100 mL of 100 % acetone. The extract was passed through a paper filter and then dried using a rotary evaporator at 54 °C and 340 mbar; the residue was re-suspended in 4 mL

of 1 % Tween® solution. Aliquots (1 mL) were injected intraperitoneally in three male Swiss mice (17–20 g each), and the result was considered positive when at least two out of three mice died within 24 h.

Samples from the experiment

After thawing, 100 % methanol (JT Baker, USA) was added to the bivalve tissue samples in a 9 mL:1 g ratio. Tissues were then disrupted using a sonic dismembrator (Cole Parmer CPX130; USA) for 3 min, applying pulses of 3 s with 1-s intervals, at 70 % amplitude. Extracts were then centrifuged for 3 min at 600×*g*. Aliquots (0.25 mL) were collected from the supernatant and passed through centrifuge filters (Millipore Ultrafree-MC, Durapore PVDF, 0.45 µm porosity) at 9000×*g* for 1 min to remove possible debris. In addition, filters containing *D. acuminata* complex cells (toxic diet samples) were added to 4 mL of 100 % methanol and processed as previously described for bivalve tissues. All samples were completely evaporated with nitrogen and then reconstituted with the same volume of 100 % methanol prior to toxin analysis.

LC–MS/MS analysis of toxins was carried out according to the method of Suzuki et al. [20], with a slight modification—the use of multiple reaction monitoring (MRM) instead of selected ion monitoring (SIM). A model 1100 liquid chromatograph (Agilent, Palo Alto, CA, USA) was coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer Q Trap™ (PE-SCIEX, Thornhill, ON, Canada). Separations were performed on Quicksilver cartridge columns (50 mm × 2.1 mm id) packed with 3 µm Hypersil-BDS-C8 (Keystone Scientific, Bellefonte, PA, USA) maintained at 20 °C. Both eluents A (water) and B (acetonitrile:water/95:5) contained 2 mM ammonium formate and 50 mM formic acid [21, 22]. Linear gradient elution from 20 to 100 % B was performed over 10 min and then held at 100 % B for 15 min, followed by re-equilibration with 20 % B (13 min). Flow rate was 0.2 mL/min and the injection volume was 5 µL. The LC effluent was introduced into a TurboIonSpray interface without splitting. High-purity air heated to 500 °C was used as the nebulizing gas. The following SRM transitions were monitored: *m/z* 817.5 → 255.3 for DTX-1; *m/z* 803.5 → 255.3 for OA and DTX-2, *m/z* 1041.6 → 255.3 for 7-o-acyl OA, *m/z* 1055.7 → 255.3 for 7-o-acyl DTX-1, *m/z* 857.5 → 137.2 for PTX-2, *m/z* 875.5 → 137.2 for PTX-2 seco acid, *m/z* 873.5 → 137.2 for PTX-1, and *m/z* 887.5 → 519.4 for PTX-6, with OA, DTX-1 and PTX-2 concentrations calculated from the calibration curve as previously described, using reference standards available at FRA-NRIFS, Yokohama, Japan.

Results

Field sampling

Dinophysis cells measured (mean ± SD) 37.3 ± 2.2 × 25.1 ± 2.0 × 8.7 ± 0.9 µm (length × width × cingulum width) and presented morphology characteristic of those described for *D. acuminata* [16–18]. However, current morphological and DNA sequencing data are not sufficient to resolve the taxonomy of the "*Dinophysis acuminata* complex" for either Chilean [23] and Brazilian (C. Odebrecht, personal communication) strains, especially considering the resemblance between *D. acuminata* and *D. ovum*. We thus considered our cells as belonging to the *D. acuminata* complex in the present study.

The bloom of *D. acuminata* complex reported at South Bay in 2008 started in early March, with cell counts rising over approximately 30 days from non-observed to the maximum of 46,196 cells/L at site #1 on April 2nd (Fig. 2). After that, cell density of *D. acuminata* complex at the surface quickly decreased (<4000 cells/L) as the salinity increased (>32). The trend of the bloom decay following an increase in salinity was also observed at all other sampling sites (Fig. 2). The time lag between the maximum value of cell count and the maximum value of salinity varied from 1 to 2 weeks, depending on the sampling site and frequency. During the bloom development, water temperature (not shown) varied irregularly between 23 and 27 °C, depending on the sampling location and date, and no clear relationship with *D. acuminata* complex cell density could be established. Cells of *D. acuminata* complex were not detected after April 28th in the whole region (Fig. 2), when the water temperature had dropped to <23 °C.

Okadaic acid was the only diarrheic toxin detected during the bloom. Although bivalves accumulated relatively high levels of OA, toxin contents in bivalves were not directly related to *D. acuminata* complex cell density in the water. At sampling sites #1 and #4, maximum OA levels in HP of both mussels (Fig. 2) and oysters (not shown) were detected about 2 weeks after the peak in *D. acuminata* complex cell density, coinciding with the period of maximum salinity. No clear trend was determined for the other two sampling sites (Fig. 2).

Taking all samples together ($n = 17$ for oysters and 42 for mussels), the mean OA concentration during the outbreak was approximately 100 ng/g of HP for *C. gigas*, with a maximum of 271 ng/g recorded on April 7th at site #4, and 500 ng/g of HP for *P. perna*, with a maximum of 2422 ng/g registered on April 16th at site #1 (Table 2). Considering a historical (i.e. obtained over 8 years of monitoring data) average conversion factor of HP to whole flesh meat of 6.7 and 10 for adult *P. perna* and *C. gigas* from that

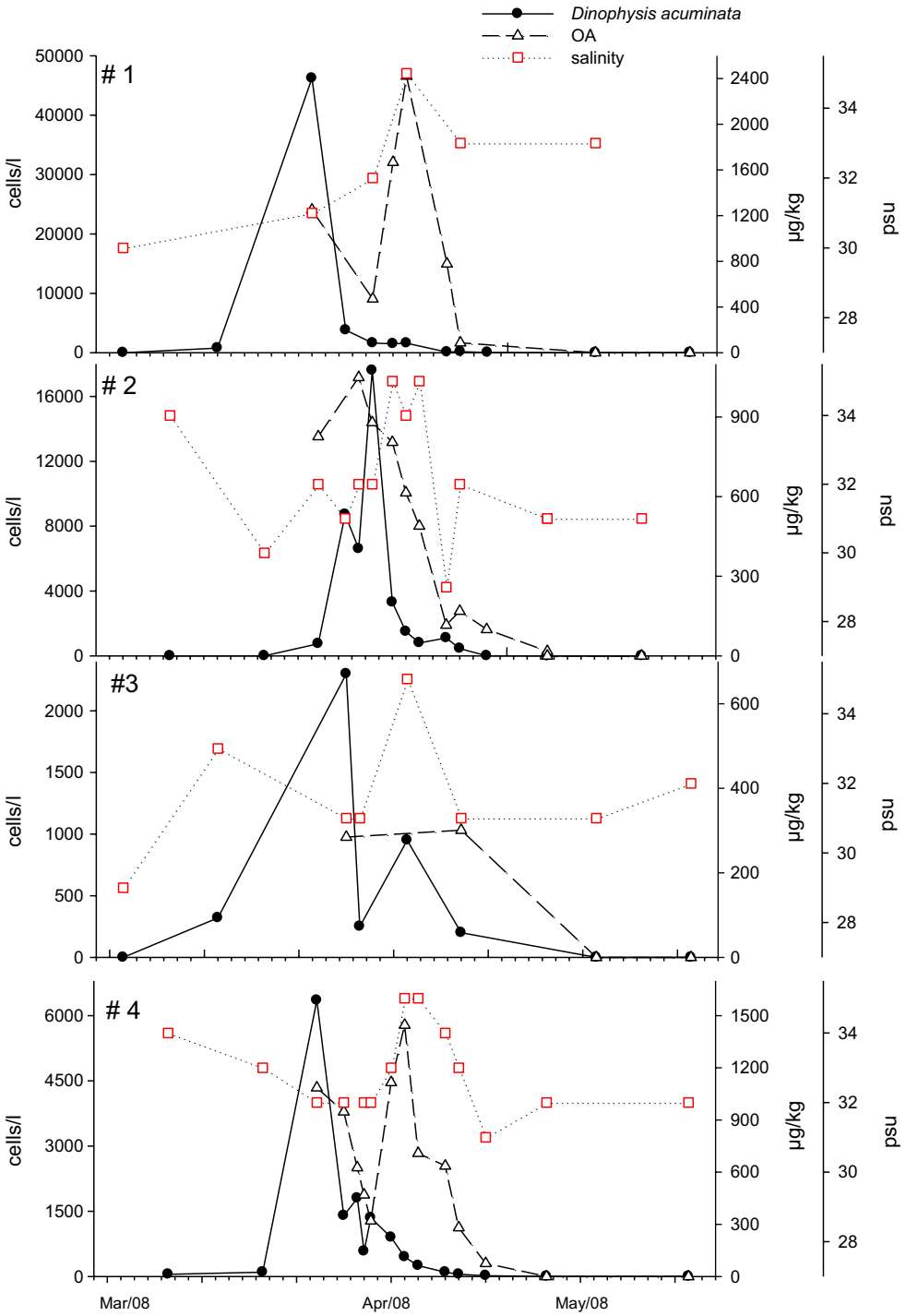

Fig. 2 Cell density (cells/L) of *Dinophysis acuminata* complex, concentration of okadaic acid (OA, µg/kg) in hepatopancreas of *Perna perna* mussels, and salinity at four sampling sites in South Bay, Santa Catarina, Brazil: (*#1*) Caieira da Baía Sul; (*#2*) Costeira do Ribeirão; (*#3*) Freguesia do Ribeirão; (*#4*) Praia do Cedro, from March 3rd to May 26th, 2008

region, respectively, the maximum toxin levels in whole bodies would be around 27 ng/g for oysters and 363 ng/g for mussels. Okadaic acid concentrations were consistently higher in mussels than oysters sampled simultaneously from the same site, although the interspecific difference varied considerably, from 3.5 to 25.4 times (Table 2). On average, OA levels in HP of mussels were 11-fold higher than those of oysters. In addition, 50 % of the extracts from samples containing mussel HP produced acute toxic effect as assessed by mouse bioassay, whereas oyster samples produced only negative results.

Toxin accumulation experiment

Juvenile bivalves used during the toxin accumulation experiment exhibited mean shell height of 29 mm

Table 2 Salinity; cell density (cells/L) of the total phytoplankton assemblage (Tot. Phyto) and of *Dinophysis acuminata* complex cells (*D. acum*); relative abundance of *D. acuminata* complex cells (*%D. acum*); mouse bioassay (MBA) results for diarrheic toxins, expressed as negative (Neg.) or positive (Pos.); and okadaic acid concentration in hepatopancreas (OA HP) of *Crassostrea gigas* (*C.g*) and *Perna perna* (*P.p*); as well as the ratio between the OA levels measured in hepatopancreas of *P. perna* and *C. gigas* (*P.p*:*C.g* ratio), sampled simultaneously at four sampling sites in South Bay Santa Catarina, Brazil: (#1) Caieira da Baía Sul; (#2) Costeira do Ribeirão; (#3) Freguesia do Ribeirão; (#4) Praia do Cedro

Site	Date	Salinity	Cell density (cells/L)			MBA		OA HP		
			D. acum	Tot. Phyto	*% D. acum*	*C.g*	*P.p*	*C.g*	*P.p*	*P.p*:*C.g* ratio
#1	Apr. 11, 2008	32	1620	690,143	0.23	Neg.	Pos.	32	471	14.5
#1	Apr. 14, 2008	34	1500	156,304	0.96	Neg.	Pos.	116	1667	14.4
#1	Apr. 16, 2008	35	1600	126,246	1.27	Neg.	Pos.	95	2422	25.4
#2	Apr. 09, 2008	32	6600	579,528	1.14	Neg.	Pos.	81	1049	13.0
#2	Apr. 11, 2008	32	17,600	512,197	3.44	Neg.	Pos.	68	879	13.0
#2	Apr. 14, 2008	35	3300	504,983	0.65	Neg.	Pos.	75	805	10.8
#2	Apr. 16, 2008	34	1500	45,689	3.28	Neg.	Pos.	201	615	3.1
#3	Apr. 16, 2008	35	950	78,152	1.22	Neg.	Pos.	132	713	5.4
#4	Apr. 07, 2008	32	1400	293,371	0.48	Neg.	Pos.	271	947	3.5
#4	Apr. 09, 2008		1800	1,202,340	0.15	Neg.	Pos.	133	626	4.7
#4	Apr. 11, 2008	32	1350	589,147	0.23	Neg.	Pos.	93	320	3.5
#4	Apr. 14, 2008	33	900	192,374	0.47	Neg.	Pos.	102	1116	10.9
#4	Apr. 16, 2008	35	450	102,199	0.44	Neg.	Neg.	63	1446	22.8
Mean		33.4	3121	390,206	1.07			112	1006	11.2
Standard deviation		1.4	4621	332,280	1.08			63	566	7.2
Maximum		35.0	17,600	1,202,340	3.44			271	2422	25.4

(*C. brasiliana*), 32 mm (*Perna perna*), and 34 mm (*C. gigas*). Mean wet weight of *C. brasiliana* was about 2.5 and 1.4 times greater than those of *C. gigas* and *P. perna*, respectively. The fraction composed of visceral tissues corresponded to 23.4 % of the total soft tissue weight for mussels and 29.5–31.4 % for oysters (Table 1).

Cell density of *D. acuminata* complex in the experimental tank fluctuated between 1350 and 13,750 cells/L during the first 24 h of the toxin accumulation experiment (Uptake phase), as a result of the balance between bivalve feeding and cell replenishment over time. Time-averaged cumulative cell density gradually dropped from 8900 cells/L during the first 2 h of exposure to 5300 cells/L by the end of the Uptake phase (Fig. 3). Okadaic acid was the only toxin detected in these cells (2.7–5.3 pg/cell). During the following 7 days (Depuration phase), bivalves were exposed to the non-toxic *T. suecica* diet at 102,300 ± 24,300 cells/L (average ± standard error, SE).

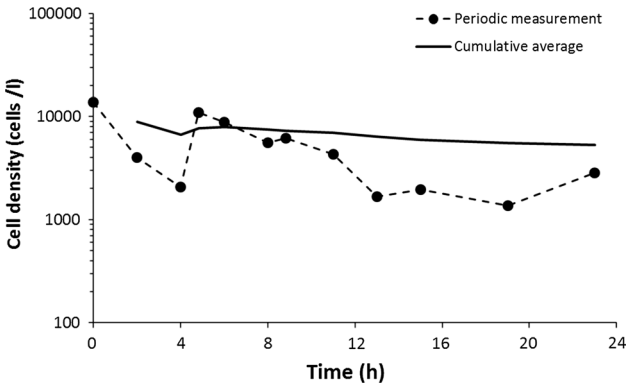

Fig. 3 Abundance of *Dinophysis acuminata* complex cells offered to juvenile individuals of *Crassostrea gigas*, *C. brasiliana*, and *Perna perna* during the first 24 h (Uptake phase) of the toxin accumulation experiment

After 24 h of exposure to the toxic diet, the concentration of accumulated OA in the whole body varied significantly ($p < 0.001$, 2-way ANOVA) among bivalve species, reaching average levels of 7.5, 23.4, and 51.1 ng/g for *C. brasiliana*, *C. gigas*, and *P. perna*, respectively (Fig. 4). Toxin levels decreased only slightly or even continued to increase over the first 10 h of depuration, reaching average levels of 10.2, 16.8, and 51.6 ng/g for the same species, respectively. After the entire 168 h depuration period, OA levels had dropped to similarly low average levels (3.6–3.7 ng/g) in both oyster species and close to zero (1.3 ng/g) in mussels (Fig. 4). Therefore, mussels detoxified OA at faster rates (0.023/h) than oysters *C. gigas* (0.010/h) and *C. brasiliana* (0.004/h) (Fig. 5), especially in the VS tissue fraction. In fact, for all bivalve species, OA levels in the VS had decreased since the beginning of the depuration period,

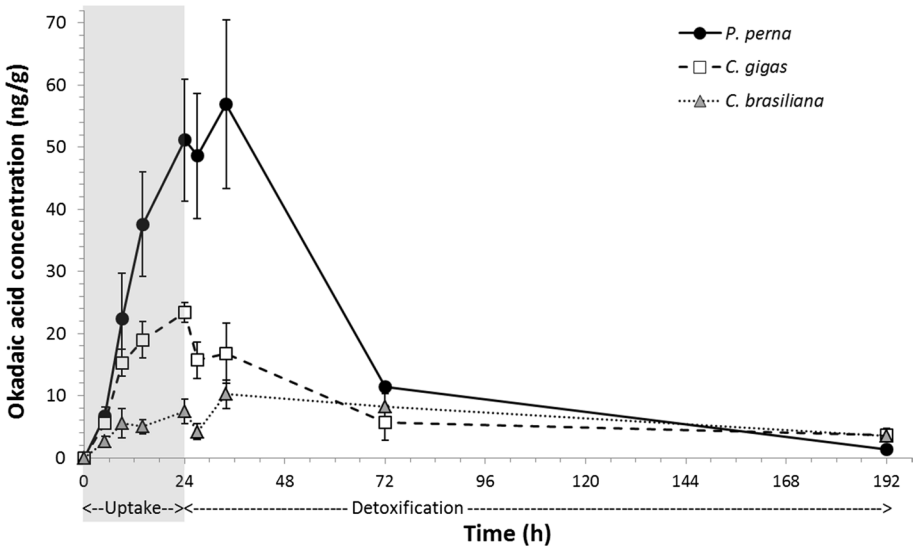

Fig. 4 Time course of the okadaic acid concentration (OA, mean ± standard error) in whole bodies of mussels (*Perna perna*) and oysters (*Crassostrea gigas* and *C. brasiliana*) during a laboratory experiment. Bivalves were exposed to a plankton suspension containing 1350–13,750 *Dinophysis acuminata* complex cells/L (1.7–3.3 pg/cell) for 24 h (uptake) followed by 168 h (detoxification) on 102,300 ± 24,300 *Tetraselmis suecica* cells/l

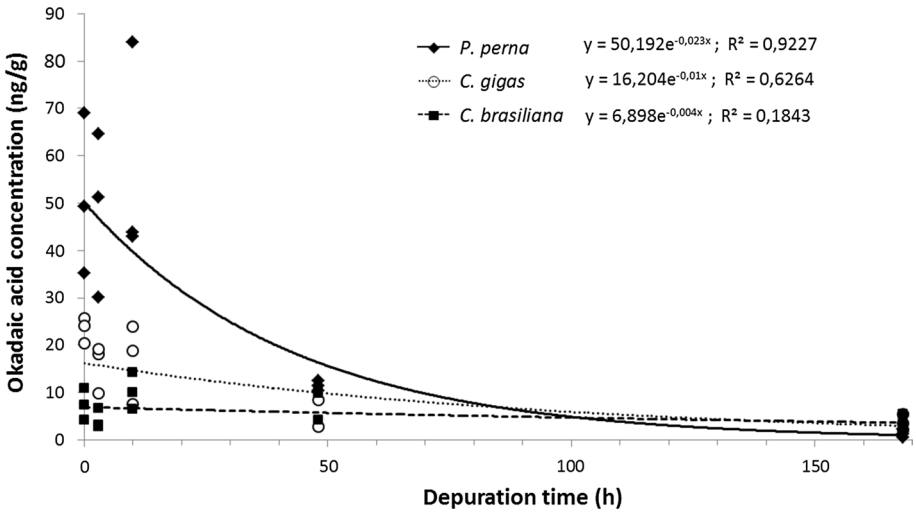

Fig. 5 Time course of the okadaic acid concentration (OA, mean ± standard error) in whole bodies of mussels (*Perna perna*) and oysters (*Crassostrea gigas* and *C. brasiliana*) during the detoxification phase of a laboratory experiment, fitted to exponential curves. Bivalves previously exposed to a plankton assemblage containing 1350–13,750 *Dinophysis acuminata* complex cells/L (1.7–3.3 pg/cell) received a non-toxic diet (102,300 ± 24,300 *Tetraselmis suecica* cells/L) for 168 h

while they remained constant or even increased during the first 10 h of depuration in the NV tissues (Fig. 6). No other toxin was detected in any of the bivalve species during both uptake and depuration periods.

Discussion

This is the first report of simultaneous uptake of diarrhetic shellfish toxin (DST) from *Dinophysis* cells by multiple bivalve species under controlled laboratory conditions. When exposed to *Dinophysis acuminata* complex cells in laboratory-simulated blooms, *Perna perna* mussels accumulated considerably higher okadaic acid (OA) levels than the oysters *Crassostrea brasiliana* and *Crassostrea gigas*. This supported what we observed during a natural bloom of *Dinophysis acuminata* complex in Santa Catarina State, southern Brazil, when mussels accumulated 3–25 times higher OA levels than co-occurring oysters. Interestingly, mussels also exhibited faster toxin elimination rates at the laboratory, as discussed below.

The factors affecting the initiation, maintenance, and termination of *Dinophysis* blooms are not fully elucidated. In all sampling sites of South Bay, Santa Catarina, cells belonging to the *D. acuminata* complex quickly attained high abundances (up to 4.7×10^4 cells/L), then dropped to non-detectable levels just a few weeks later when the salinity had increased from 30–31 to 34–35. This relationship contrasts with the findings of Kim et al. [14], who reported an increase in *D. acuminata* cell density as salinity decreased from 25 to 20 in Jinhae Bay, Republic of Korea. It is apparently also in disagreement with the general pattern observed over 8 years of monitoring in Aveiro Lagoon, Portugal, where cell abundance of *Dinophysis* spp. is usually inversely related to rainfall, and thus, directly related to salinity levels [24]. In Aveiro, a semi-closed coastal lagoon,

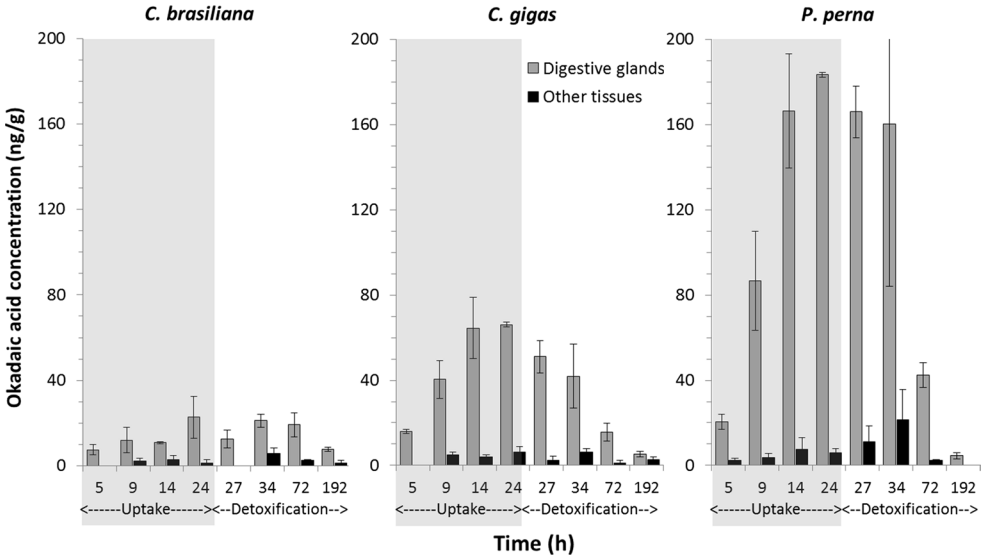

Fig. 6 Time course of the okadaic acid concentrations (OA, mean ± standard error) in visceral and non-visceral tissues of mussels (*Perna perna*) and oysters (*Crassostrea gigas* and *C. brasiliana*) during a laboratory experiment. Bivalves were exposed to a plankton assemblage containing 1350–13,750 *Dinophysis acuminata* complex cells/L (1.7–3.3 pg/cell) for 24 h (accumulation) followed by 168 h (detoxification) on 102,300 ± 24,300 *Tetraselmis suecica* cells/L

increased river discharge disrupts the thermohaline stratification of the water column, which in drier periods favors the growth of several dinoflagellates, mainly *Dinophysis* spp. [24]. South Bay, in contrast, is an open system with a broad connection to the sea, where high salinities (~35) are generally related to the incursion of continental shelf water masses (Mizuta et al. [25] and references therein), which may wash *Dinophysis* cells out and/or increase the turbulence, preventing stratification inside the bay.

Several studies have reported maximum *Dinophysis* cell densities at the sub-surface, associated with a persistent thermohaline stratification of the water column (e.g. [15, 26–29]). In marine systems intermittently subject to conditions of intensified stratification, such as the southern Benguela area in South Africa, these events support high abundances of *Dinophysis* (10^5 cells/L) at the sub-surface, which drop markedly during periods of vertical mixing [15]. During the present investigation, the South Bay area was subject to the passage of cold fronts associated with southern winds, a condition that enhances the turbulence inside the bay [25], probably explaining the quick decrease in *D. acuminata* complex abundance observed in early April 2008. These cold front systems also affected the water temperature at the surface, which oscillated up and down from March to early April 2008 and thus exhibited no clear relationship with the onset and development of the bloom in our study. From late April on, the temperature dropped to >23 °C and no *D. acuminata* complex cells were found in South Bay until late June 2008. Although water temperature may have some influence on the seasonal variability of *Dinophysis* spp. abundance, especially in temperate areas, thermohaline stratification seems to be much more relevant for bloom initiation and maintenance (e.g. [29–32]).

Maintenance of toxic blooms of the mixotrophic dinoflagellate *Dinophysis* spp. also depends upon the availability of prey such as the kleptoplastidic ciliate *Mesodinium rubrum*. For instance, a bloom of *Dinophysis* cf. *ovum*, recorded with an automated high-quality Imaging FlowCytobot (IFCB) imaging system in the Mission-Aransas estuary (TX, USA), reached its abundance peak 3–4 weeks after a short-lived bloom of *M. rubrum* [33]. *M. rubrum* is usually present in the water when *D. acuminata* complex reaches cell abundances >1,000 cells/L in southern Brazil [34], but we cannot construct any relationship between *M. rubrum* cell density and the onset of the present *D. acuminata* complex bloom in South Bay due to the low sampling frequency enforced prior to bloom initiation. The roles of prey availability and water column stratification on the formation of *Dinophysis* blooms in southern Brazil will be addressed at proper temporal and spatial scales in further investigations.

Over the course of the relatively dense *D. acuminata* complex bloom reported herein, brown mussels *P. perna* accumulated OA concentrations twice as high as the regulatory limit (RL) of 160 ng/g of shellfish meat for human consumption. Shellfish harvesting and selling was banned for 19 days and no cases of DSP were officially reported during this episode. The maximum OA body burden determined for *P. perna* was similar to those reported in mussels *Choromytilus meridionalis* during two *D. acuminata*-dominated blooms (92–267 and 20–430 ng/g) off the west and southwest coast of South Africa (max. 5.7×10^5 and 8.3×10^4 cells/L, respectively) [15, 28]. However, OA levels in *P. perna* were lower than those detected in *Mytilus edulis* (30–820 ng/g) during a monitoring program in Sweden [35], and in *Perna viridis* (1120 ng/g) exposed to a mixed bloom containing highly toxic cells of *Dinophysis*

caudata (max. 2.8 × 10^3 cells/L, 7.9–56.5 pg OA/cell) and *D. miles* (max. 5.0 × 10^3 cells/L, 5.7–25.0 pg OA/cell) in Sapian Bay, the Philippines [36]. Cells of *D. acuminata* complex, the most common bloom-forming species in southern Brazil, usually exhibit limited toxicity (2.4–6.8 pg OA/cell), although other species with larger and possibly more toxic cells such as *D. tripos* may co-occur in the area [34]. Additionally, *D.* cf. *acuminata* occasionally occurs at very high cell densities (e.g. 7.0 × 10^5 cells/L) and causes recurrent shellfish closures in Santa Catarina State [37], indicating that DSP is a constant threat in southern Brazil. In fact, >150 cases of human poisoning associated with the consumption of contaminated bivalves, mainly *P. perna*, were recorded in 2007 during a bloom of *D.* cf. *acuminata* (up to 5.2 × 10^4 cells/L) in South Bay and Tijucas Bay, Santa Catarina (Fig. 1) [6].

During the 2008 bloom, OA levels in *P. perna* were three to 25 times greater than those measured in the co-occurring oyster *C. gigas*, with OA levels depending on the site and date of sampling (Table 2). Toxin levels that accumulated in whole oysters (max. ~27 ng OA/g) were always well below the RL, as assessed both by HPLC and mouse bioassay. Differential OA accumulation was further confirmed by our laboratory investigation in which *P. perna* accumulated toxin levels 2.2 times higher than those of *C. gigas* and 6.8 times higher than those of mangrove oysters *C. brasiliana* at the end of the 24-h toxin uptake period. Similarly, *Mytilus galloprovincialis* mussels contained 4.4–18-fold higher OAeq. concentrations than *C. gigas* in a Tunisian coastal lagoon during the peak of a *Dinophysis sacculus* bloom [13], and *C. meridionalis* mussels accumulated 20-fold greater DST concentrations than those of *C. gigas* during a dense bloom dominated by *D. acuminata* in South Africa [15]. More strikingly, *M. edulis* mussels accumulated 120 times greater amounts of OA than co-occurring oysters, *Ostrea edulis*, after 4 weeks of exposure to a natural community of *Dinophysis* spp. in Sweden [12]. Mussels also exhibit higher capacity for DST accumulation relative to co-occurring species of clams and/or scallops, in addition to oysters (Table 3). For instance, *M. galloprovincialis* mussels accumulated OA concentrations up to 13-fold higher than those of *Pactinopecten yessoensis* scallops in Japan [38], and up to 40-, 50-, and 350-fold higher than those of *Flexopecten proteus* scallops and the clams *Chlamys varia* and *Venus verrucosa,* respectively, in Greece [39].

In bivalves, inter-specific differences in toxin accumulation may arise from a differential capacity to select food particles in a mixed suspension, either selectively ingesting or preferentially rejecting toxic cells, as documented for *Crassostrea virginica* oysters feeding upon domoic acid-producing *Pseudo-nitzschia multiseries* cells in simulated mixed suspensions with flagellates [40]. In that case, oysters preferentially rejected *P. multiseries* cells in pseudofeces, especially longer cells, which explained their consistently lower domoic acid levels relative to those of mussels [41]. Conversely, Sidari et al. [42], analyzing the stomach content of *M. galloprovincialis* during a DSP outbreak, suggested that mussels may preferentially select and ingest dinoflagellate cells over diatoms in a mixed suspension, and that such selection is particularly noticeable for *Dinophysis* spp., whose cells also seemed to be digested more efficiently than other dinoflagellates. This would explain their high capacity for DST uptake. Selective rejection of *Dinophysis* cells in pseudofeces has not been investigated in any bivalve so far. Besides ingestion and digestion of toxic cells, differential capacities for retention, and transformation and/or elimination of individual toxins can also explain inter-specific differences in toxin accumulation by bivalves [7].

Bivalve tissues may exhibit varying affinities for algal toxins, resulting in very contrasting toxin concentrations as a result of either short or very long retention periods. For instance, PSP and ASP toxins may persist for months or years in the siphon of butter clams, *Saxidomus giganteus* [43], and in non-visceral tissues of razor clams, *Siliqua patula* [44], respectively. DSTs are largely accumulated in the HP of bivalves, notably mussels, where they may attain concentrations >10,000 ng/g, as reported for *M. galloprovincialis* during a *D. acuminata* bloom in the Ría de Vigo [45], and >14,000 ng/g, as measured in 1-year-old *M. edulis* in western Norway [46]. In the present study, commercial-sized *P. perna* accumulated up to 2412 ng OA/g in their HPs during the bloom in South Bay. Mussels are extremely tolerant of the cytotoxic effects of OA, which may, in part, be related to the uptake and storage of OA within their lysosomal system [35]. Some studies attempted to make a connection between the high capacity for DST accumulation in the bivalve HP and its relatively high lipid content [47], but this is still controversial. Madigan et al. [48] suggested that a difference in lipid content was responsible for the higher DST accumulation in *C. gigas* relative to king scallops, *Pecten fumatus*, during a *D. acuminata* bloom in southern Australia. Conversely, there was no clear relationship between toxin concentration and lipid content of *M. edulis* mussels during either toxin uptake or elimination [49].

Only trace levels of DSTs have been found in non-visceral tissues of different mussel species. The contribution of non-visceral tissues to the total toxin burden ranged from 3 to 6 % in *M. galloprovincialis* (<1 % when calculated from concentrated extracts; [50]), and from 9 to 12.5 % in *M. edulis* [51, 52]; however, it may be greater in other bivalve species such as the scallops *Argopecten irradians* (23 % [53]) and *Pecten fumatus* (78 % [48]). In our experiment, OA levels in non-visceral tissues were still undetectable in all bivalves after the initial 5 h of toxin uptake; however, their contribution to the total toxin burden

Table 3 Maximum okadaic acid (OA) levels reported in hepatopancreas (HP) and/or whole bodies of mussels (ms), oysters (oy), clams (cl), scallops (sc), and cockles (ck) sampled simultaneously during natural or simulated (laboratory) blooms of *Dinophysis* spp.

Species	OA conc. (ng g^{-1})		Location	Toxic exposure	Source
	HP	WB			
Perna perna (ms)	2422.4	363.3[a]	S Brazil	*D. acuminata* complex bloom; 450–47,000 cells L^{-1}	Present study
Crassostrea gigas (oy)	270.7	27.1[a]			
P. perna (ms)	188.3	51.1	S Brazil	24 h laboratory exposure to *D. acuminata* complex; 1370–13,750 cells L^{-1}	Present study
C. gigas (oy)	66.1	23.4			
C. brasiliana (oy)	22.7	7.5			
Mitylus galloprovincialis (ms)	168.0		S South Korea	*D. acuminata* bloom; 200–1000 cells L^{-1}	[14]
C. gigas (oy)	<DL				
M. edulis (ms)	3670.0		W Sweden	*Dinophysis* spp. bloom (dominated by *D. acuta*); 650–3000 cells L^{-1}	[12]
Ostrea edulis (oy)	30.0				
Pinna bicolor (cl)		51.0[b]	S Australia	*D. acuminata* bloom; max. 20,800 cells L^{-1}	[42]
C. gigas (oy)		23.0[b]			
Pecten fumatus (sc)		18.0[b]			
M. galloprovincialis (ms)		102.0[c]	NE Tunisia	Moderate cell densities of *D. sacculus*; max. 420 cells L^{-1}	[13]
C. gigas (oy)		14.5[c]			
M. edulis (ms)		160.0	W Sweden	Bivalves sampled from several locations	[11]
O. edulis (oy)		<DL			
Cerastoderma edule (ck)					
Choromytilus meridionalis (ms)		267.0	SW South Africa	*D. acuminata*-dominated bloom; max. 570,000 cells L^{-1}	[15]
C. gigas (oy)		12.0			
M. edulis (ms)		2310.0[d]	Portugal	*D. acuminata* bloom; max. 6000 *D. acuminata* cells L^{-1}, 80 *D. acuta* cells L^{-1}	[3]
Crassostrea japonica (oy)		50.0[d]			
M. galloprovincialis (ms)		2123.4[e]	Greece	*D. acuminata* bloom; 600–10,700 cells L^{-1}	[33]
M. galloprovincialis (ms)		3222.2[f]			
Modiolus barbatus (ms)		647.8			
Flexopecten proteus (sc)		148.9			
Chlamys varia (cl)		80.4			
Venus verrucosa (cl)		37.9			

DL detection limit

[A] Estimated by applying average conversion factors of DG to whole flesh meat

[b] Free-OA only

[c] OA equivalent, as measured by PP2A assay

[d] Total OA in edible tissues

[e] In suspended rafts

[f] On the sediment below the rafts

increased to 9–13 % in *P. perna*, 16–23 % in *C. gigas*, and 23–34 % in *C. brasiliana* during the rest of the 24-h uptake period. At this point, part of the toxin load may reach other tissues and organs, whose relative contribution to the total toxin burden will depend on their molecular affinity, as well as toxin transformation and elimination (i.e. detoxification) rates. As observed for *A. irradians* scallops after 48-h exposure to DST-producing *Prorocentrum lima* cells, the rapid toxin accumulation upon initial contact with toxic cells contrasted with a low overall toxin assimilation efficiency (<1 %) during long-term (14 day) exposure [53], suggesting that most of the toxin body burden may be associated with intact or recently digested cells, primarily confined to the bivalve digestive tract during the first hours of toxin uptake. The fact that 7-*O*-acyl OA (usually the main OA degradation product in bivalves) was not found in our samples also supports this suggestion.

In a previous study, Bauder et al. [53] investigated DST loss in the scallop *A. irradians* and estimated slower detoxification rates in the viscera (0.0035/h) than in gonads (0.021/h) and other non-visceral tissues, including the adductor muscle (0.028/h). Conversely, in our experiment,

detoxification of OA was faster in the viscera compared to non-visceral tissues of all bivalves. During the first 48 h of depuration, OA levels quickly decreased in the viscera of *P. perna* and *C. gigas*, while the toxin burden in non-visceral tissues of these species, as well as in both tissue compartments of *C. brasiliana*, slightly increased over the same period. A similar increase in OA concentrations was also observed during early depuration of *M. galloprovincialis* under controlled conditions; the authors suggested that hydrolysis of conjugated forms was probably the cause [50]. However, no derivatives or conjugated forms of OA were found in our bivalve samples as analyzed by LC–MS/MS. Alternatively, such increase in OA levels in non-visceral tissues during early depuration may suggest a two-compartment detoxification process with transfer of toxin from viscera to other tissues, as calculated for domoic acid-contaminated *C. virginica* and *M. edulis* [54]. Even though some studies find a better fit by using more complex two-compartment models characterized by faster detoxification rates at early depuration stage [35, 53, 55, 56], in most cases toxin loss can be adequately described by calculating a constant detoxification rate (i.e. single-compartment model) ([45, 49, 57, 58], present study). The importance of a second toxin compartment is generally very small and would not justify the great increase in model complexity [45].

Toxin half-life was estimated as ~1 day for *P. perna*, ~2 days for *C. gigas*, and ~5 days for *C. virginica* in our study, which is much shorter than other values previously reported in laboratory depuration studies for *M. edulis* (8–45 days [35, 49, 59]) and *M. galloprovincialis* (3–12 days [45, 56, 57, 60]). Nevertheless, our values were calculated from juvenile individuals maintained at a constant temperature of 22 °C; detoxification of DST in bivalves is expected to be directly affected by water temperature due to its general effect on basal metabolic rates in poikilothermic organisms (reviewed in Shumway et al. [61]). In fact, *M. edulis* mussels eliminated OA at significantly faster rates when kept at 24 °C, relative to those maintained at 18 °C [49]. Moreover, the toxin half-life increased from ~8 days at those temperatures to 32 and 50 days when naturally contaminated individuals of the same species were allowed to detoxify at 10 or 5–8 °C, respectively [35, 46]. Therefore, since aquaculture sites in Santa Catarina may experience water temperatures ranging from 16 to 30 °C, and DSP outbreaks may occur at any time in the year [37], the short OA half-life values calculated herein may be even briefer in the field, at least from mid-spring to mid-autumn, when water temperature is >22 °C.

The availability of non-toxic food during depuration may also affect DST detoxification, probably due to increasing digestive activity and metabolic fecal losses [60, 62], as suggested by field studies (e.g. [60, 63, 64]), but not confirmed under laboratory conditions [35, 49]. In this respect, our laboratory conditions (i.e. availability of non-toxic food throughout the depuration period) represent roughly what bivalves would experience in the field. Finally, juvenile bivalves may exhibit faster detoxification rates than those estimated in the laboratory from commercial-sized individuals. Juvenile oysters, *C. virginica*, for instance, showed faster detoxification of domoic acid relative to two size classes of adults, probably due to their higher metabolic rates; *M. edulis* mussels, in contrast, did not experience the same effect [54].

As indicated here for okadaic acid and in previous studies for domoic acid [54], accumulation and detoxification of algal toxins varies at a great extent in different bivalve species. Implementation of a species-specific management strategy during toxic blooms would reduce the economic impact of blanket harvesting closures for all commercial bivalve species and deserves further consideration and testing under varying field conditions.

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Conflict of interest The authors declare no conflict of interest.

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