

Diarrheic toxins in field-sampled and cultivated *Dinophysis* spp. cells from southern Brazil

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Abstract In southern Brazil, mixotrophic dinoflagellates belonging to the *Dinophysis acuminata* complex have recently been involved in diarrheic shellfish poisoning episodes through the production of lipophilic toxins such as okadaic acid (OA) and dinophysistoxin-1 (DTX-1). The present investigation used a combination of laboratory cultures and field surveys at three large estuarine systems in that region to examine toxin retention in *Dinophysis* spp. cells under optimum or growth-limiting conditions. This study represents the first successful culture of a *Dinophysis* isolate from the Atlantic South America region. Starved *D. acuminata* complex cells reached 5.6-fold higher cellular OA quotas (up to 18 pg cell⁻¹) than *Mesodinium rubrum*-fed cultures 20 days after inoculation in the laboratory. Moreover, in field samples, light-limited cells at the bottom of a stratified water column were less abundant, yet 6.6- to 11-fold more toxic (up to 26.4 pg OA and 1.7 pg DTX-1 cell⁻¹) than those located at the illuminated surface. This phenomenon of toxin retention by slow-dividing cells may partially explain the enormous variation in cell toxin quota found within *Dinophysis* spp. populations from a single location, and it may have serious implications for cell count-based monitoring program in bivalve aquaculture areas. In fact, only low to moderate OA levels were detected in the digestive glands of oysters *Crassostrea* spp. (up to 17.8 ng g⁻¹) and the guts and livers of filter-feeding fish (44.7 ng g⁻¹) during the present study, despite the relatively high *Dinophysis* cell densities (up to 19,500 cells L⁻¹) found in the field.

Keywords Toxin accumulation · Mixotrophy · Batch culture · Growth-limiting conditions · Diarrheic shellfish poisoning · Harmful algal bloom

Introduction

More than 100 species have been assigned to the dinoflagellate genus *Dinophysis* (Gómez 2005), but only 12 are known to produce the diarrheic toxins okadaic acid (OA) and dinophysistoxins (DTXs), as well as other lipophilic toxins, such as pectenotoxins (PTXs) (revised in Reguera et al. 2012b). To date, seven of these 12 species have been found to be associated with diarrheic shellfish poisoning (DSP) outbreaks: *Dinophysis acuminata*, *Dinophysis acuta*, *Dinophysis caudata*, *Dinophysis fortii*, *Dinophysis miles*, *Dinophysis ovum*, and *Dinophysis sacculus*. Additionally, DTX-1 has been found in a single sample of *Dinophysis rotundata*, *Dinophysis tripos*, and *Dinophysis* (= *Phalacroma*) *mitra* cells collected with a micropipette from field samples (Lee 1989), but no DSP cases have been assigned to these species in other occasions when they were the only potentially toxic species present in the water (Caroppo et al. 1999; Pazos et al. 2010). Furthermore, *Dinophysis infundibulus* produces toxins, but only in laboratory cultures (Johansen 2008; Suzuki et al. 2009), and *Dinophysis norvegica* has been found to be related to toxic events in eastern Canada (Subba Rao et al. 1993), although it is not considered a threat in Japan and Scandinavia. Overall, the *D. acuminata* complex—*D. acuminata*, *D. ovum*, *D. sacculus*—is the most widespread group in coastal areas with freshwater inputs (Zingone and Larsen 2011) and is responsible for most intoxication episodes.

Okadaic acid and its derivatives, the DTXs, are acidic polyethers that may inhibit protein phosphatase, causing diarrhea and other gastrointestinal symptoms in humans

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(Yasumoto et al. 1985). The toxicity of PTXs to humans is uncertain because these polyether-lactones are hepatotoxic to mice by intra-peritoneal injection but not by oral administration (Miles et al. 2004). Since the original 1976–1977 DSP episodes in northeast Japan (Yasumoto et al. 1978, 1980), hundreds of people worldwide have been poisoned following consumption of contaminated bivalve mollusks. The severity of each outbreak depends upon the *Dinophysis* cell abundance that the bivalves are exposed to, as well as the toxin level (i.e., quota) contained in those cells. Cell densities as low as 100–200 cells L⁻¹ may be enough to cause DSP outbreaks (Yasumoto et al. 1985) if the cell toxin quota is high.

Production and retention (i.e., accumulation) of toxins by *Dinophysis* cells are regulated by a combination of genetic and environmental factors (Cembella and John 2006). As a result, there are large inter- and intra-specific variations in the toxin profiles (e.g., Fux et al. 2011) and toxin cell quotas (Reguera and Pizarro 2008) of strains from different locations. Toxin quotas and profiles may even vary over a single light–dark cycle in field *Dinophysis* populations (Pizarro et al. 2008; Fux et al. 2010), with the maximum toxin quota found during the mid-dark period for *D. acuta* (Fux et al. 2010). In addition, one order of magnitude variations in the cellular toxin quota have been reported for natural *D. acuta* populations over the course of their growing season, with maximum values found during their stationary phase (Pizarro et al. 2009), when cell division ceases due to growth-limiting conditions.

Following the first successful cultivation of *D. acuminata* in the laboratory by Park et al. (2006), other researchers have been able to investigate toxin production in various *Dinophysis* species under more controlled conditions (Kamiyama and Suzuki 2009; Hackett et al. 2009; Kamiyama et al. 2010; Tong et al. 2011; Smith et al. 2012; Nielsen et al. 2012 for *D. acuminata*; Nagai et al. 2011 for *D. acuminata* and *D. fortii*; Rodríguez et al. 2012 for *D. tripos*; Nielsen et al. 2013 for *D. acuta*). As observed in field studies, the toxin content in laboratory cultures was consistently higher in the stationary growth phase than in the exponential phase, which may be due to an imbalance between toxin production and cell division in the different growth phases (Tong et al. 2011).

Despite a recent DSP episode associated with a *D. cf. acuminata* bloom in southern Brazil that poisoned more than 150 people (Proença et al. 2007) and despite the shellfish harvest bans that have been issued every year following that outbreak, there is no information on the variability of the toxin cell quota in *Dinophysis* spp. from that area. The present study combines results from field surveys and laboratory cultures to address this issue, including a comparative investigation of the toxin contents in both field-sampled and laboratory-cultivated cells of the

D. acuminata complex under either optimum (i.e., availability of both light and prey) or growth-limiting conditions.

Methods

Field sampling

Sampling campaigns were performed from January 2011 to May 2012 in Paranaguá Bay (PB), Guaratuba Bay (GB), and Babitonga Bay (BB), the largest estuarine systems in the states of Paraná and Santa Catarina, southern Brazil (Fig. 1).

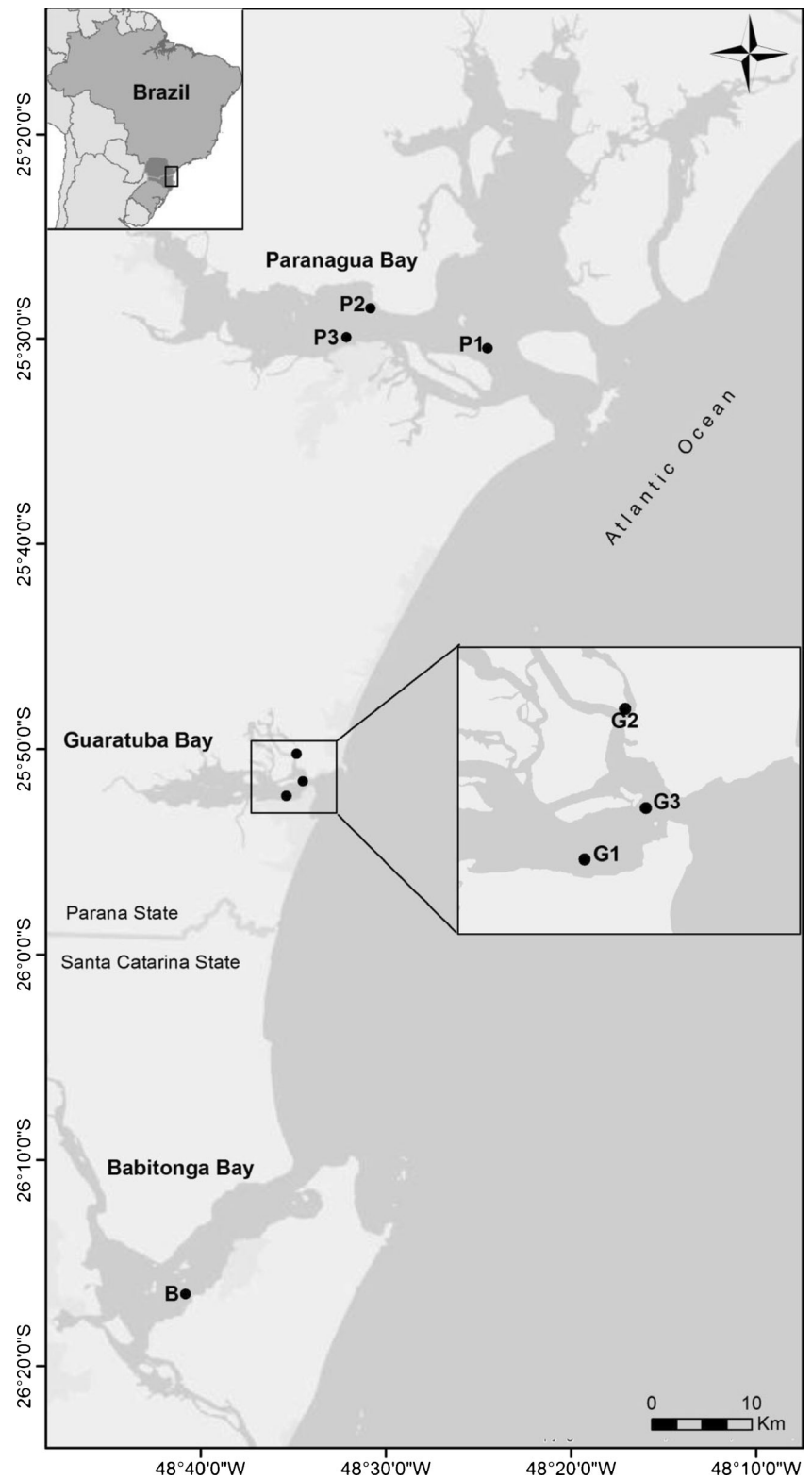
At each sampling location (Table 1), water transparency was assessed with a Secchi disk, and water temperature and salinity were measured in situ at the surface and 0.5 m above the bottom. Water samples were collected from both depths with a Van Dorn bottle for dissolved inorganic nitrogen (DIN) analysis on a spectrophotometer (Shimadzu UV-1601PC; Japan). In addition, two 80–350-mL aliquots of seawater sampled from each depth were gently passed through glass microfiber filters (24 mm diameter, 1.2 µm particle retention; Whatman GF/C) for toxin analysis of retained cells, as described below, and 25–50-mL aliquots of Lugol-fixed samples were settled on Utermöhl chambers for cell counting under a microscope (Zeiss AxioVert.A1; Germany). The entire chamber was inspected for the presence of *Dinophysis* spp. cells at a detection limit of 20–40 cells L⁻¹.

Upon the detection of high *D. acuminata* complex cell densities in PB on January 4, 2011, exemplars of oysters (*Crassostrea brasiliiana*, *Crassostrea rizophorae*) and suspensivorous fish (*Cetengraulis edentulis*, *Mugil* sp.) were obtained within 6–24 h from local fishing markets. Samples from selected digestive tissues were removed for both toxicity assessment by mouse bioassay and diarrhetic toxin analysis by liquid chromatography with mass spectrometry detection (LC–MS/MS).

Cultures

A culture of the cryptophyte *Teleaulax amphioxieia* (GenBank number AB364287), originally isolated from Inokushi Bay, Japan (34°47'N, 131°53'E), in February 2007 (Nishitani et al. 2008), was generously donated by Dr. S. Nagai (NRIFS, Japan) and maintained in the laboratory in f/2 medium (Guillard 1975) at 22±1 °C, 30 PSU, 120 µmol photons m⁻² s⁻¹, under a 12:12 h light–dark cycle. A strain of the ciliate *Mesodinium (Myrionecta) rubrum* was isolated from GB, Brazil (25°51'30.8"N, 48°34'30.0"W), in early September 2011 and maintained under the culture conditions described above, with the periodic addition (every 3–5 days) of *T. amphioxieia* cells at a 10:1 (prey/predator) ratio. Finally, strains of the *D. acuminata* complex and *D. caudata* were isolated in late September 2011 from PB (25°28'26.0"N,

Fig. 1 The study area in southern Brazil, showing the locations of the sampling sites in Paranaguá Bay (P1–P3), Guaratuba Bay (G1–G3), and Babitonga Bay (B)



48°30'32.5"W) and GB, Brazil (25°50'14.8"N, 48°34'48.5" W), respectively. Cultures of these dinoflagellates were established in the laboratory under the same conditions, with the periodic addition of *M. rubrum* cells at a 3:1 (prey/predator) ratio.

Growth and toxin production by cells belonging to the *D. acuminata* complex were evaluated in the presence or the absence of its prey, *M. rubrum* (fed and starved cultures, respectively). In each case, cells of the *D. acuminata* complex were placed in triplicate wells containing 4.5 mL of culture

Table 1 Geographic location and physicochemical characteristics of the water collected from different sampling sites and depths at Paranaguá Bay (P1–P3), Guaratuba Bay (G1–G3), and Babitonga Bay (B), southern Brazil

| Site | Location | Date | Time | Depth (m) | Temp. (°C) | Sal. | Dissolved inorganic nitrogen (μM) | | |
|------|----------------------------|--------------|-------|-----------|------------|------|--|---------|---------|
| | | | | | | | Ammonium | Nitrate | Nitrite |
| P1 | 25°30'27.9"N; 48°24'30.9"W | Jan 5, 2011 | 12:05 | 0.5 | 30 | 24 | 3.557 | 4.467 | 0.780 |
| | | | 12:10 | 14.0 | 29 | 28 | 8.963 | 2.915 | 0.434 |
| P2 | 25°28'26.0"N; 48°30'32.5"W | Jan 5, 2011 | 09:45 | 0.5 | 30 | 23 | 7.355 | 4.096 | 0.805 |
| | | | 09:50 | 8.0 | 30 | 24 | 8.621 | 5.325 | 0.762 |
| P3 | 25°30'06.5"N; 48°32'02.0"W | Jan 5, 2011 | 11:25 | 0.5 | 30 | 23 | 11.068 | 5.740 | 0.970 |
| | | | 11:30 | 9.0 | 29 | 26 | 9.573 | 5.277 | 1.105 |
| G1 | 25°52'18.1"N; 48°35'22.4"W | Aug 24, 2011 | 10:05 | 0.5 | 16 | 14 | 11.215 | 1.844 | 0.394 |
| | | | 10:10 | 5.5 | 17 | 23 | 9.775 | 1.619 | 0.391 |
| G2 | 25°50'14.8"N; 48°34'48.5"W | Aug 24, 2011 | 11:50 | 0.5 | 16 | 19 | 9.128 | 1.816 | 0.308 |
| | | | 11:55 | 2.0 | 17 | 27 | 10.540 | 2.085 | 0.404 |
| G3 | 25°51'30.8"N; 48°34'30.0"W | Sep 15, 2011 | 13:05 | 0.5 | 19 | 25 | 9.566 | 2.581 | 0.195 |
| | | | 13:10 | 10.0 | 19 | 28 | 10.493 | 2.345 | 0.141 |
| B | 26°16'52.0"N; 48°40'38.3"W | May 21, 2012 | 14:30 | 0.5 | 22 | 24 | n.d. | n.d. | n.d. |
| | | | 14:35 | 2.0 | 22 | 25 | n.d. | n.d. | n.d. |

Samples were taken from the sub-surface or 0.5 m above the bottom
nd not determined

medium at an initial density of 50 cells mL^{-1} . Fed cultures received 100 *M. rubrum* cells mL^{-1} every 3 days for 9 days, and then every 4 days for the following 11 days. A third group (control), composed of only *M. rubrum* cells at an initial density of 100 cells mL^{-1} , was also set up in triplicate and received periodic additions of *M. rubrum* cells as described above. Food addition was obtained by adding around 100 μL (66–131 μL) of *M. rubrum* concentrated culture (2,500–4,000 cells mL^{-1}) to each sampling unit. This caused a periodic dilution effect on the *Dinophysis* culture, which was minimum considering the volume of culture added. Even so, the calculated dilution rate was taken into account when calculating the *Dinophysis* growth rate.

From each sampling unit, 0.5 mL samples were periodically collected for *D. acuminata* complex and/or *M. rubrum* cell counting on a Sedgewick-Rafter chamber. At the end of the 20-day trial, the remaining volume (1.5–2.5 mL per well) was collected, and the cells were gently retained in glass microfiber filters (24 mm diameter, 1.2 μm particle retention; Whatman GF/C) for toxin analysis. In addition, toxin production was investigated in a single 10-mL aliquot of a *M. rubrum*-fed experimental culture of *D. caudata*, sampled 25 days after inoculation.

Toxin analysis

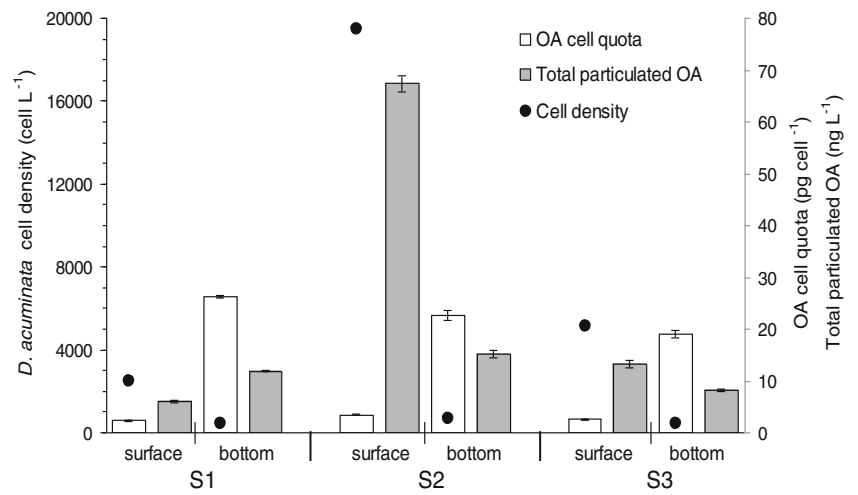
The filters containing *Dinophysis* spp. cells from both cultures and field samples were disrupted using a sonic dismembrator (Cole Parmer CPX130; USA) for 1 min at 50 % duty cycle,

after the addition of 100 % methanol (1.2 mL per filter). The extract was then re-filtered using syringe filters (13 mm diameter, 0.2 μm particle retention; Waters Acrodisc) prior to the LC analysis.

Samples of selected tissues from oysters or fish were homogenized using a Turrax homogenizer (Marconi MA-102; Brazil) and were then separated into two aliquots. The first one (15 g) was extracted in acetone–ether, evaporated at 40 °C, and re-suspended in 1 % Tween solution for assessment of toxicity via a mouse bioassay (Yasumoto et al. 1978). The second aliquot (2 g) was suspended in 20 mL of 100 % methanol (JT Baker; USA) and filtered using syringe filters prior to injection into the LC system.

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) toxin analysis was carried out 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 (50 mm \times 4.6 mm I.D., 1.8 μm ; Phenomenex; 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^{-1} flow rate. Diarrhetic toxins were detected using selected reaction monitoring (SRM) with the ion source in negative mode. The following SRM transitions were monitored: m/z 817.5 \rightarrow 255.1 and 817.5 \rightarrow 113.0 for DTX-1; m/z 803.5 \rightarrow 255.1, and m/z 803.5 \rightarrow 113.0 for OA and DTX-2. Okadaic acid concentrations were calculated from a calibration curve made of

Fig. 2 *D. acuminata* complex cell density, intracellular okadaic acid (OA) quota, and total particulate OA concentration in the water at different sampling sites and depths at Paranaguá Bay, southern Brazil (January 5, 2011). Error bars represent the standard deviation obtained from different sample aliquots ($n=2$) filtered from each location/depth for cellular toxin determination



serial dilutions (50–0.78 ng mL⁻¹) of the reference standard (National Research Council; Halifax, Canada) in deionized water. The concentration of DTX-1 was estimated from the OA calibration curve, after corrections to account for the difference in molecular weights.

Results

Field samples

Samples of *Dinophysis* spp. for counting and diarrhetic toxin determination were obtained at a broad water temperature range, varying from 16 °C in August (winter) to 30 °C in January (summer). Salinity ranged from 14 to 28 psu, and the values were consistently higher near the bottom than at the

surface at the same locations, indicative of water stratification, especially in GB (Table 1). Conversely, there was no clear relationship between the sampling depth and the concentration of DIN, which ranged from 3.6 to 11.2 μM for ammonium, 1.6 to 5.7 μM for nitrate, and 0.1 to 1.1 μM for nitrite. The ammonium concentrations were generally higher in PB, whereas the nitrate and nitrite levels were higher in GB (Table 1). The euphotic zone depth, as assessed by a Secchi disk, was generally shallow, limited to the upper 3 m in BB, 3.6–4.8 m in GB, and 4.5–6.0 m in PB, while the total depths varied from 8 to 14 m.

In PB, *D. acuminata* complex was the only group of *Dinophysis* species observed, with cell densities ranging from 0.4–0.7 × 10³ cells L⁻¹ near the bottom to 2.5–19.5 × 10³ cells L⁻¹ at the surface. In contrast to the cell density, the cellular toxin contents were 6.6- to 11-fold higher near the bottom

Table 2 *Dinophysis* spp. cell density and cellular toxin content (picograms per cell) for different sampling sites and depths at Paranaguá Bay (P1–P3), Guaratuba Bay (G1–G3), and Babitonga Bay (B), southern Brazil

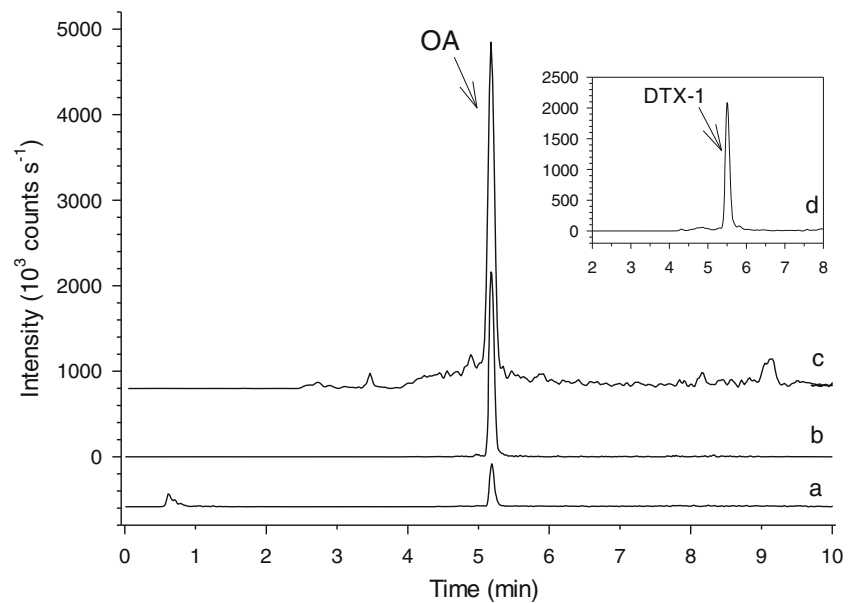
| Site | Depth | Cell density (cells L ⁻¹) | | | Toxin content (pg cell ⁻¹) | |
|------|-----------------|---------------------------------------|-------------------|------------------|--|------------------------|
| | | <i>D. acuminata</i> complex | <i>D. caudata</i> | <i>D. tripos</i> | OA | DTX-1 |
| P1 | sf | 2,488 | | | 2.4 | n.d. |
| | bt | 450 | | | 26.4 | n.d. |
| P2 | sf | 19,467 | | | 3.5 | n.d. |
| | bt | 667 | | | 22.7 | n.d. |
| P3 | sf | 5,150 | | | 2.6 | n.d. |
| | bt | 433 | | | 19.0 | n.d. |
| G1 | sf ^a | 2,500 | 100 | 150 | 4.5–6.7 ^b | <d.l.–1.7 ^b |
| G2 | sf ^a | 4,100 | 200 | 300 | 5.4 | 1.2 |
| G3 | sf ^a | 5,000 | 2,500 | | 5.8 | <d.l. |
| B | sf ^a | 2,500 | | | 4.5–6.8 ^b | 0.6–0.7 ^b |

OA okadaic acid; DTX-1 dinophysistoxin-1; sf surface; bt bottom; dl detection limit; nd not determined

^aNo sample available at the bottom

^bRange obtained from different sample aliquots ($n=2$) filtered for cellular toxin determination

Fig. 3 LC-MS chromatograms at m/z 803.5–255.1 of **a** *D. acuminata* complex culture containing 2.85 ng okadaic acid (OA) mL^{-1} ; **b** an OA calibration solution at 12.5 ng mL^{-1} ; and **c** a field sample containing 133.3 ng OA mL^{-1} . The insert shows the LC-MS chromatogram **d** at m/z 817.5–255.1 of “c”, showing a peak at 5.5 min related to the presence of dinophysistoxin-1 (DTX-1). Conditions, 2.5 μm C18 column (50 mm \times 2.0 mm I.D.) at 35 $^{\circ}\text{C}$; 0.75 mL min^{-1} of aqueous methanol with 0.1 % formic acid (gradient elution as described in “Materials and methods”)



(19.0–26.4 pg OA cell^{-1}) than at the surface (2.4–3.5 pg OA cell^{-1}) at the same locations (Fig. 2, Table 2). As a result, even though only a few hundred cells were present near the bottom at those sampling sites, the total particulate OA concentrations were, in some cases, similar or higher than those at the surface, where thousands of *D. acuminata* complex cells were present (Fig. 2). No other toxic phytoplankton species were found. Diatoms, primarily *Skeletonema costatum*, *Nitzschia* spp., *Odontella* spp., and *Pleurosigma* spp., were dominant in all samples, with other dinoflagellates such as *Scropsiella trochoidea*, *Prorocentrum minimum*, and *Ceratium furca* being relatively more abundant at the surface. *M. rubrum*, the ciliate that *Dinophysis* spp. prey upon, was present at all sampling

locations and was also more abundant at the surface (up to 6.6×10^3 cells L^{-1}).

In GB, the abundance of *D. acuminata* complex cells at the surface was lower ($2.5\text{--}5.0 \times 10^3$ cells L^{-1}) than those measured in PB, although *D. caudata* ($0.1\text{--}2.5 \times 10^3$ cells L^{-1}) and/or *D. tripos* ($0.2\text{--}0.3 \times 10^3$ cells L^{-1}) also co-occurred in all samples. For this location, the toxin content was calculated for all *Dinophysis* cells combined, and it ranged from 4.5 to 6.7 pg OA cell^{-1} (Table 2). In BB, both the abundance (2.5×10^3 cells L^{-1}) and the toxin content (4.5–6.8 pg OA cell^{-1}) of cells belonging to the *D. acuminata* complex were very similar to the findings for GB, although no other toxic *Dinophysis* species were found at BB.

Table 3 Filter-feeders sampled from Paranaguá Bay during a period of relatively high abundance of *D. acuminata* complex cells in the water column: toxin burden (nanograms OA per gram) and acute toxic effect as assessed by mouse bioassay (MB)

| Filter-feeder | Tissue | Date | Toxin level (ng g^{-1}) | | Toxicity (MB) |
|---|-----------------|--------------|-----------------------------------|-------|---------------|
| | | | OA | DTX-1 | |
| <i>C. edentulus</i> (fish) ^a | Guts+liver | Jan. 5, 2011 | 44.7 | <d.l. | Negative |
| <i>Mugil</i> sp. (fish) ^b | Guts+liver | Jan. 5, 2011 | n.d. | n.d. | Negative |
| <i>Crassostrea</i> spp. (oyster) ^c | Digestive gland | Jan. 5, 2011 | 17.8 | <d.l. | Negative |
| <i>Crassostrea</i> spp. (oyster) ^d | Digestive gland | Jan. 6, 2011 | <d.l. – 14.7 | <d.l. | Negative |

d.l. detection limit, n.d. not determined

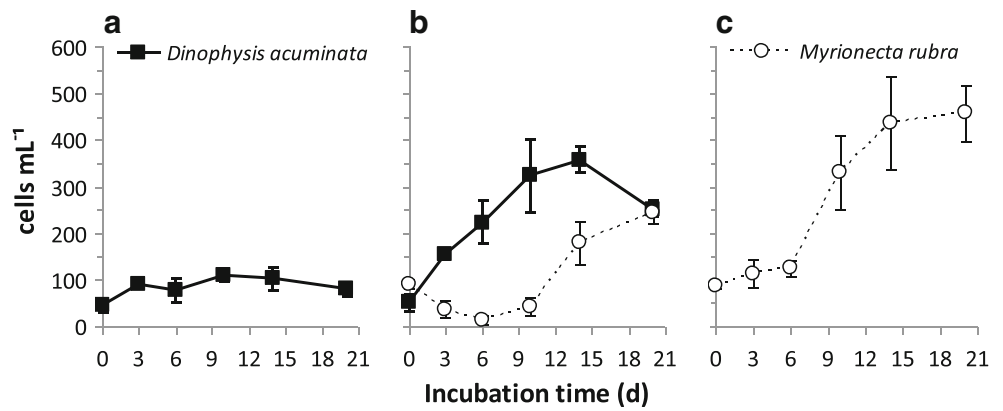
^a $n=1$ pool of 3 individuals

^b $n=1$ individual

^c $n=1$ pool of 12 individuals

^d $n=3$ pools of 9–14 individuals

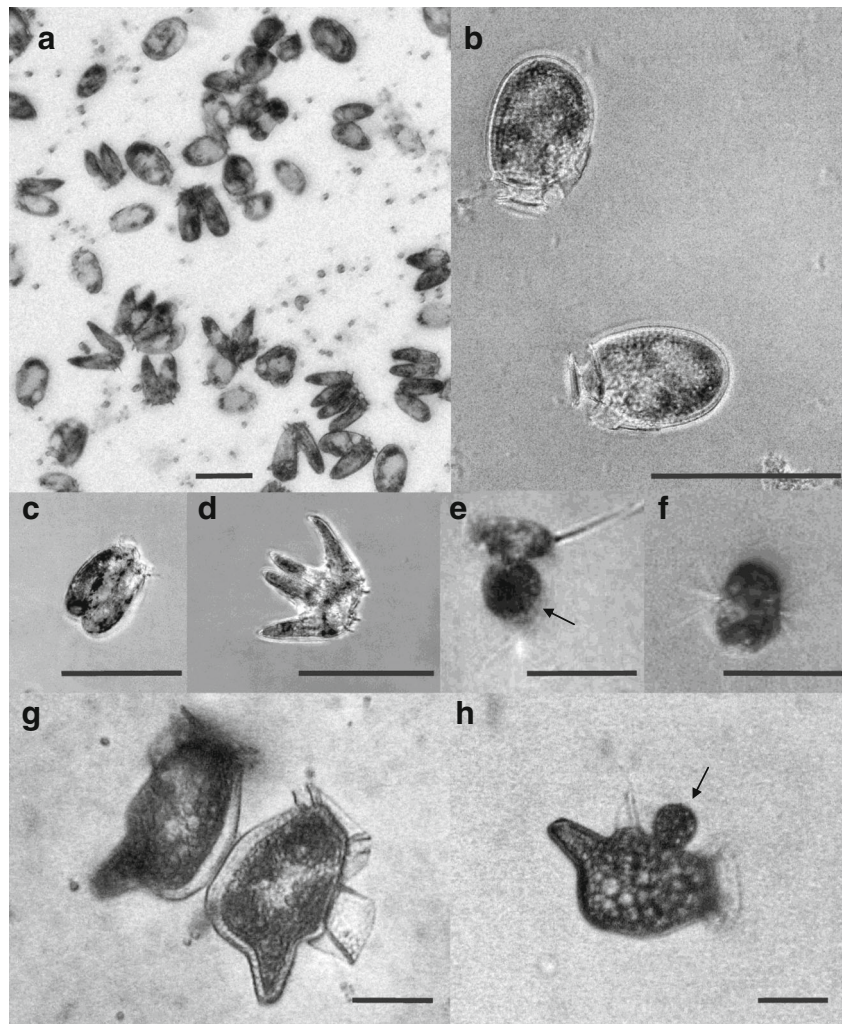
Fig. 4 Cell density of *D. acuminata* complex in **a** starved or **b** *M. rubrum*-fed cultures. The cell density of *M. rubrum* is also shown for cultures in the **b** presence or **c** absence of *D. acuminata* complex cells. Error bars = standard deviation for different cultures ($n=3$)



In addition to OA, DTX-1 was also detected in plankton samples from GB and BB, at levels ranging from non-detectable to 1.7 pg cell⁻¹ (Fig. 3, Table 2). Unfortunately, the presence of DTX-1 could not be examined in plankton samples from PB due to analytical constraints (i.e., data loss).

In addition, OA, but not DTX-1, was simultaneously found at moderate levels in the digestive glands of oysters (up to 17.8 ng g⁻¹), as well as in the guts and livers of filter-feeding fish (44.7 ng g⁻¹) sampled from PB (Table 3). Extracts from such samples, however, exhibited no acute toxic effect as assessed by mouse bioassay.

Fig. 5 Photomicrographs of cultivated cells: **a–e** *D. acuminata* complex, **f** *M. rubrum*, **g, h** *D. caudata*. **b** End of the growth trial, showing the high cell density attained. **c, g** Cell division by binary fission. **d** Tetrads formed by incomplete sequential vegetative divisions. **e, h** Cells actively feeding after the capture of *M. rubrum* (arrows), which is shown in its natural form in **(f)**. Scale bar = 50 μm



Cultures

In the culture experiments, the initial *D. acuminata* complex cell density (50 ± 18 cells mL^{-1}) in the presence of its prey, *M. rubrum*, was similar ($p > 0.05$) to that of the starved cultures (43 ± 14 cells mL^{-1}). Similarly, the initial *M. rubrum* cell densities were comparable in both the presence (88 ± 10 cells mL^{-1}) and absence of *D. acuminata* complex cells (85 ± 6 cells mL^{-1}) (Fig. 4).

Over the course of the growth experiment, the *D. acuminata* complex cell density increased only in the presence of its prey, reaching a maximum of 357 cells mL^{-1} 14 days after inoculation. *D. acuminata* complex cells divided exponentially ($\mu = 0.19$ day $^{-1}$) during the first 10 days of culture, while the *M. rubrum* cell density remained low despite periodic additions of 100 *M. rubrum* cells mL^{-1} (Fig. 4b). In contrast, in the absence of its consumer, *M. rubrum* attained a density of 329 ± 80 cells mL^{-1} after 10 days and a maximum density of 436 ± 99 cells mL^{-1} 14 days following culture inoculation (Fig. 4c).

Dividing *D. acuminata* complex cells (Fig. 5) were frequently observed in the *M. rubrum*-fed cultures (up to 12 % of the cells after 10 days) but were rarely observed in starved cultures. Additionally, actively feeding *D. acuminata* complex cells were also found in the fed cultures, and the formation of tetrads (i.e., four parallel, dividing cells in the shape of a Japanese fan) was sporadically observed in our maintenance cultures of dinoflagellates assigned to the *D. acuminata* complex (Fig. 5).

By the end of the growth trial (after 20 days), the OA content of starved *D. acuminata* cells (18.0 pg cell $^{-1}$) was 5.6-fold higher than that of *M. rubrum*-fed cultures (3.2 pg cell $^{-1}$). DTXs were not detected in the cultures, and the presence of PTX-2 was not examined. Additionally, a culture of *D. caudata* was preliminary established (maximum cell density = 375 cells mL^{-1}) using *M. rubrum* as prey, but no detectable levels of OA or DTXs were found. The presence of PTXs was not examined for *D. caudata* as well.

Discussion

The present study is the first to document successful cultures of *Dinophysis* spp.—*D. caudata* and *D. acuminata* complex—from eastern South America. Previously, Fux et al. (2011) had grown a strain of the *D. acuminata* complex isolated from Chile, western South America, using an Antarctic *M. rubrum* strain as the prey at 6 °C. In contrast, we used a local *M. rubrum* isolate as the prey and temperatures representative of field conditions (22 ± 1 °C). Current morphological and DNA sequencing data are not sufficient to resolve the taxonomy of the “*D. acuminata* complex” for

both Chilean (Fux et al. 2011) and Brazilian (C Odebrecht, pers. comm.) strains.

Our *D. caudata* strain (DCT-BR1) attained a maximum density of 375 cells mL^{-1} after 25 days of incubation, whereas our *D. acuminata* complex strain (DAC-BR1) yielded a maximum of 357 ± 27 cells mL^{-1} after 14 days of incubation. Maximum cell densities were generally lower than those previously reported for *D. acuminata*, which ranged from 167 to 11,000 cells mL^{-1} (Park et al. 2006; Kim et al. 2008; Kamiyama and Suzuki 2009; Riisgaard and Hansen 2009; Kamiyama et al. 2010; Nagai et al. 2011; Nielsen et al. 2012), and for *D. caudata* (5,200 cells mL^{-1} ; Nishitani et al. 2008), most likely because of the lower amounts of *M. rubrum* offered as prey during our experiments (periodic additions of 0.5–2.0 *M. rubrum* cells *Dinophysis* cell $^{-1}$) compared with those used at the beginning of the culture incubations in other studies (e.g., up to ~60–70 *M. rubrum* cells *Dinophysis* cell $^{-1}$) (Riisgaard and Hansen 2009; Nagai et al. 2011).

The exponential growth rates attained by our Brazilian *D. acuminata* complex strain, ranging from 0.25 day $^{-1}$ (0–6 days) to 0.72 day $^{-1}$ (0–3 day), are comparable to those reported for strains from Denmark (up to 0.45 day $^{-1}$) and Japan (0.70–0.82 day $^{-1}$) grown with a similar irradiance and light–dark cycle (Riisgaard and Hansen 2009; Nagai et al. 2011) but lower than those obtained by Kim et al. (2008) and Park et al. (2006) under continuous light (0.91–0.95 day $^{-1}$). In addition to continuous light, increased temperature (Kamiyama et al. 2010) and increased prey availability (Kim et al. 2008; Kamiyama and Suzuki 2009; Riisgaard and Hansen 2009) also support higher *D. acuminata* growth rates in culture. Therefore, *D. acuminata* may be classified as an obligate mixotroph, requiring both prey and light for growth and long-term survival (Kim et al. 2008).

During the maintenance of our *D. acuminata* complex culture, we observed the formation of triads and tetrads (i.e., stacks of three or four dividing cells, respectively), which appeared more frequently in the late stationary/decline growth phase (Fig. 5d). Such malformations, which had never been reported for cells belonging to this taxonomic complex but were previously observed in cultures of *D. caudata* (Nishitani et al. 2008), *D. fortii* (Nagai et al. 2008), and *D. infundibulus* (Nishitani et al. 2008), resemble a common feature observed in aged cultures of the diatom *Pseudo-nitzschia multiseriis* (L.L. Mafra Jr. and S.S. Bates, pers. comm.) and may be interpreted as abnormally incomplete successive cell divisions. To our knowledge, there is no report of these “stacked” cells in natural populations of *Dinophysis* spp.

In the present study, *D. caudata* cells did not contain detectable amounts of diarrhetic toxins after 25 days of incubation. There is no report of toxin production by cultivated *D. caudata* to date, but the toxin content in cells from field samples ranged from non-detectable to 56.5 pg OA cell $^{-1}$ and

54 pg DTX-1 cell⁻¹ (Fernandez et al. 2001; Marasigan et al. 2001). Additionally, *D. caudata* is considered the main causative agent of DSP outbreaks in Singapore (Holmes et al. 1999). The lack of toxin retention in *D. caudata* cells in the present study may be related to the advanced age of our culture, which had already entered the decline growth phase by the time the sample was collected for toxin analysis. As much as 80–90 % of the toxin produced by *D. acuminata* and *D. acuta* cultures, for instance, may be excreted to the surrounding medium in late stationary phase (Nagai et al. 2011; Nielsen et al. 2013). Unfortunately, we were not able to measure toxins in the dissolved fraction or over the entire *D. caudata* growth cycle before we lost this culture. Thus, the capacity of *D. caudata* from southern Brazil to produce diarrhetic toxins remains uncertain and will be the subject of further investigations.

In contrast, the intracellular okadaic acid concentrations reported herein for both natural populations (2.4–26.4 pg cell⁻¹) and a local isolate (3.2–18.0 pg cell⁻¹) of the *D. acuminata* complex are comparable to values reported elsewhere, confirming *D. acuminata* complex as a major threat to the fast-growing local shellfishery aquaculture industry. Cells from southern Brazil attained OA contents similar to or higher than those of isolates from northeast USA (0.01–0.44 pg cell⁻¹; Hackett et al. 2009; Tong et al. 2011; Smith et al. 2012) and Japan (0.0–12.2 pg cell⁻¹; Kamiyama and Suzuki 2009; Nagai et al. 2011), as well as field populations from the south coast of South Korea (0.4–0.9 pg cell⁻¹; Kim et al. 2010), northern France (1.6 pg cell⁻¹; Lee 1989), Sweden (0.1–16.6 pg cell⁻¹; Johansson et al. 1996; Lindahl et al. 2007), the west coast of South Africa (7.8 pg cell⁻¹; Pitcher et al. 2011), and Atlantic Canada (25.5 pg cell⁻¹; Cembella 1989). Other regions historically affected by *D. acuminata* blooms have experienced even higher yet fairly variable cellular OA levels, such as northwest Spain (1–37 pg cell⁻¹; Blanco et al. 1995), Denmark (0–40 pg cell⁻¹; Andersen et al. 1996), and southern France (0–158 pg cell⁻¹; Marcaillou et al. 2005).

The highest toxin contents measured from both cultures and natural populations of the *D. acuminata* complex in this study were associated with growth-limiting conditions. For instance, *D. acuminata* complex cells sampled at depths below the euphotic zone of all sampling sites in PB were 5.5- to 29-fold less abundant but 6.5- to 11-fold more toxic than those collected at the surface. This result may suggest that *D. acuminata* complex cells near the bottom of these stratified water columns were experiencing much slower cell division rates and therefore accumulating greater intracellular amounts of toxin over time. Since the cellular toxin levels in the present study were not significantly related to salinity ($r^2=0.26$), temperature ($r^2=0.13$), or the DIN concentration ($r^2=0.01$ – 0.08), slower growth was most likely the combined result of light limitation and lower food availability at the bottom

(max. 550 *M. rubrum* cells L⁻¹) compared with the illuminated surface (max. 6,630 *M. rubrum* cells L⁻¹). Cellular toxin quotas in *D. acuminata* complex cells from PB were thus inversely related to *M. rubrum* availability ($r^2=0.73$), whereas the cell densities of both organisms were directly related ($r^2=0.95$). Even though these paired data were only available for a limited number of samples ($n=6$), the difference in cellular toxin quotas between *Dinophysis* cells sampled at the bottom and the surface was very pronounced and consistent across different areas of PB.

A similar inverse relationship between cell density and toxin content has already been described for co-occurring *D. acuminata* and *D. acuta* populations in two Swedish fjords (Lindahl et al. 2007). The authors found higher cell abundances and lower toxin cell quotas at the pycnocline of the most enclosed fjord relative to its DIN-depleted surface layer and to the whole water column of the most turbulent fjord. Lindahl et al. (2007) hypothesized that, given a supposed allelopathic role of OA for non-DST-producing algal species (Windust et al. 1996), the observed pattern could be related to a putative chemical signal system that induces *Dinophysis* density-dependent toxin production (i.e., fewer cells would need to be more toxic to produce the same allelopathic effect as more abundant populations). This mechanism remains to be demonstrated.

Data from this and other recent culture studies may shed light on this subject. Our starved *D. acuminata* complex cultures had 5.6-fold higher cellular OA content than *M. rubrum*-fed cells under the same conditions, and this higher level appears to be at least partially related to the faster cell division rates attained by the latter. Starved cells experienced, on average, only 1.5 divisions over the entire incubation period, whereas *M. rubrum*-fed cells completed >3 successive divisions over the same period, which suggests a process of toxin dilution in the fast-growing cells. In fact, such a simple explanatory mechanism is supported by the finding that newly divided *D. acuta* cells, picked from field samples, contained nearly half the toxin content of the cells they originated from (Johansen 2008) and by the finding of an increase in the toxin cell quota for both *D. acuminata* and *D. acuta* cultures after starvation (Nielsen et al. 2012, 2013). Therefore, the imbalance between toxin production and the cell division rate, which changes over the growth cycle, may be the main factor explaining the ~2- to 5-fold higher OA and DTX-1 contents measured during the stationary growth phase, relative to those detected during exponential growth (Table 4), as calculated from batch cultures of *D. acuminata* (Kamiyama et al. 2010; Nagai et al. 2011; Tong et al. 2011; Nielsen et al. 2012), *D. acuta* (Nielsen et al. 2013), and *D. fortii* (Nagai et al. 2011).

The uncoupling of cell density and toxin content described herein will be ultimately reflected in the lack of a relationship between the *Dinophysis* cell density and toxin accumulation

Table 4 Intracellular content of lipophilic toxins (picograms per cell) in cultivated *Dinophysis* spp. strains in the exponential and/or stationary growth phase, indicating a general increase in the latter growth phase as demonstrated by the mean stationary/exponential ratios for each toxin

| Species | Exponential phase | | | Stationary phase | | | Stat./expon. ratio | | | |
|---------------------|-------------------|-----------|---------|------------------|---------|--------|--------------------|-------|-------|---------------------------------------|
| | OA | DTX-1 | PTX-2 | OA | DTX-1 | PTX-2 | OA | DTX-1 | PTX-2 | Ref. |
| <i>D. acuminata</i> | 0.01–0.02 | 0.08 | 14–15 | 0.02–0.05 | 0.32 | 12–14 | 2.3 | 4.0 | 0.9 | Tong et al. (2011) ^a |
| | 0.02 | 0.02 | 20 | | | | | | | Hackett et al. 2009 ^b |
| | 2.1–4.7 | 0.2–1.1 | 34–50 | 8.1–12 | 2.1–3.5 | 48–67 | 3.0 | 4.3 | 1.4 | Nagai et al. 2011 ^c |
| | 4.0–10 | 0.1–0.3 | 28–77 | 17–29 | 0.5–1.1 | 90–120 | 3.3 | 4.0 | 2.0 | Kamiyama et al. 2010 ^d |
| | | | 3.7–7.1 | | | 11–20 | | | 2.9 | Nielsen et al. 2012 ^e |
| | | | <d.l. | 2.5–4.8 | 15 | | | | | Kamiyama and Suzuki 2009 ^c |
| <i>D. acuta</i> | 2.2–3.0 | | 40–62 | 10–17 | | 93–131 | 5.2 | | 2.2 | Nielsen et al. 2013 ^f |
| <i>D. fortii</i> | 1.2–1.5 | <d.l.–0.3 | 62–114 | 2.0–8.6 | 0.1–0.4 | 88–162 | 3.9 | 1.7 | 1.4 | Nagai et al. 2011 ^c |
| <i>D. tripos</i> | <d.l. | <d.l. | 179–232 | | | | | | | Rodríguez et al. 2012 ^b |

dl detection limit, nd not determined

^a Cells in the exponential phase at 284 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ and the stationary phase at 65 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$

^b Late exponential growth phase

^c Stationary phase prior to *M. rubrum* re-addition (12–24 days)

^d Data shown for cells incubated at 22 °C; a similar increase in toxin content at 14 and 18 °C was observed, but not at 10 °C

^e Data shown for cells acclimated to 15 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$; a similar increase in toxin content was observed at 130 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$

^f Data shown for cells acclimated to 130 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$; a similar increase in toxin content was observed at 15 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$

by shellfish in aquaculture sites. Whereas the occurrence of moderate- to high-density populations of *Dinophysis* spp. is not necessarily linked to high toxin levels in co-occurring bivalves (Dahl and Johannessen 2001), very low cell densities (<500 cells L^{-1}) may be sufficient to cause mollusk contamination above the regulatory limit and may trigger DSP outbreaks (Yasumoto et al. 1985), especially if moderate to high amounts of OA and/or DTX (i.e., >1 pg OA eq. cell⁻¹) are accumulated in the cells of the causative *Dinophysis* species (Reguera et al. 2012b). In contrast, DSP episodes are less severe when PTX is the dominant or the sole toxin involved, such as in western Japan (Suzuki and Mitsuya 2001), north-east USA (Hackett et al. 2009), and Galicia, Spain (Pazos et al. 2010; Rodríguez et al. 2012).

Both the toxin content (2.4–26.4 pg OA cell⁻¹) and the cell density (430–19,500 cells L^{-1}) measured for populations of the *D. acuminata* complex in southern Brazil during the present study represent serious health risks for shellfish consumers. Nonetheless, the toxin levels measured concurrently in oysters from Paranaguá Bay [0–17.8 ng g⁻¹ of digestive glands (DG)] were much lower than the regulatory limit for human consumption (160 ng g⁻¹ of edible tissues). Oysters, however, are not the best indicator of DSP risk, as they may accumulate up to two orders of magnitude lower OA levels than mussels (Vale and Sampayo 2002; Lindegarth et al. 2009; Kim et al. 2010). For instance, mussels can accumulate OA levels as high as 3,222 ng g⁻¹ of edible tissues when exposed to 400–10,000 *D. acuminata* cells L^{-1} (Reizopoulou et al. 2008), 3,670 ng g⁻¹ of DG after 5 weeks of exposure to 650–

3,000 *D. acuminata* cells L^{-1} (Lindegarth et al. 2007), or even 14,000 ng g⁻¹ of DG when only smaller exposed individuals are considered (Duinker et al. 2007). Mussels are not extensively farmed in Paranaguá Bay, but the mangrove mussel *Mytella guyanensis* is regularly harvested from natural beds and consumed by the local population without regular toxin assessment. Moreover, our LC-MS/MS procedure did not include a hydrolysis step, which may have underestimated the total toxin content in our bivalve samples. After ingestion by shellfish, diarrhetic toxins are rapidly converted into fatty-acid esters, which are usually less toxic than the toxins originally produced by the dinoflagellates but may still account for most of the overall toxicity in those organisms (reviewed in Munday 2013). A proper investigation of toxin ester profiles in Brazilian commonly farmed bivalves exposed to toxic *Dinophysis* cells is currently in progress.

Following recent episodes of human intoxication, shellfish harvest bans started to be issued during blooms of the *D. acuminata* complex in the state of Santa Catarina, southern Brazil (Proença et al. 2007). As a consequence, in 2012, Brazilian authorities created a National Program on Mollusk Safety, which will, as in other countries, use cell counts as an alert to perform regulatory mouse bioassays and LC-MS/MS analyses of different toxin groups. However, as observed in this and other studies (e.g., Pizarro et al. 2008), there may exist large intra-specific variations in toxin contents among *Dinophysis* cells from a single geographic region, making DSP outbreaks difficult to predict based on cell counts (Marcaillou et al. 2005). Bivalve selective feeding (Mafra

et al. 2010), aggregation of cells in thin layers (Pizarro et al. 2008), and the proportion of toxic cells in relation to the total food available for filter-feeders (Jørgesen and Andersen 2007; Reguera et al. 2012a) may also help explain the frequent mismatches between cell abundance in the water column and shellfish toxicity.

Okadaic acid was also detected at low levels (44 ng g^{-1}) in a pool of liver and digestive tract tissues from the filter-feeder fish *Cetengraulis edentulus* in Paranaguá Bay. This result, along with other rare reports of OA detection in marine animals other than bivalves, including the polychaete *Sabella spallanzanii* ($0.9\text{--}37 \text{ ng g}^{-1}$), ascidians ($32\text{--}340 \text{ ng g}^{-1}$) (Reizopoulou et al. 2008), the crabs *Carcinus maenas* and *Polybius henslowi* ($17\text{--}322 \text{ ng OA eq. g}^{-1}$) (Vale and Sampayo 2002) and mussel-feeder flounders (222 ng g^{-1}) (Sipiä et al. 2000), demonstrates that bivalve mollusks may not be the only vector for human intoxication by DSTs. Furthermore, the possible presence of DST-esters in fish tissues, which would aggravate the risks of DSP by fish consumption, remains to be investigated.

Altogether, our results from field surveys and laboratory cultures indicate that toxin retention by *D. acuminata* complex cells may be substantially greater at growth-limiting conditions. Growth limitation by either light or prey availability resulted in less abundant but much more toxic *D. acuminata* complex cells both in the field and in small-scale cultures, which may have serious implications for cell count-based monitoring programs in bivalve aquaculture areas.

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