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ARTICLE

# Sublethal exposure of eastern oyster *Crassostrea virginica* to the goniodomin-producing dinoflagellate *Alexandrium monilatum*: Fate of toxins, histopathology, and gene expression

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## Abstract

**Objective:** The dinoflagellate *Alexandrium monilatum* forms blooms during summer in tributaries of the lower Chesapeake Bay. Questions persist about the potential for *A. monilatum* to negatively affect aquatic organisms. Its main toxin, goniodomin A (GDA), a polyketide macrolide, has been shown to have adverse effects on animals, for example through cytotoxicity and interaction with actin.

**Methods:** Eastern oysters *Crassostrea virginica* were exposed for 96 h to sublethal concentrations of *A. monilatum* (615 ± 47 cells/mL [average ± SD]; containing mainly intracellular GDA [215 ± 7.15 pg/cell] and to a lesser extent goniodomin B, goniodomin C, and GDA seco-acid as quantified by liquid chromatography coupled to tandem mass spectrometry) or to nontoxic phytoplankton or were unexposed. They were subsequently depurated for 96 h by exposure to nontoxic phytoplankton. Clearance rates were estimated, and oysters were sampled daily and tissue (gill, digestive gland, and remaining tissues) excised for analyses by histopathology, gene expression quantified by quantitative PCR, and goniodomin quantification.

**Result:** A positive clearance rate, no mortality, and no tissue pathologies were observed in oysters exposed to *A. monilatum*. Goniodomin A was detected in gill 6 h after exposure (504 ± 329 µg/kg [average ± SE]) and to a lesser extent in the digestive gland and remaining soft tissues. In the digestive gland, a trend of transformation of GDA to GDA seco-acid was observed. The majority of toxins (≥83%) were depurated after 96 h. Expression of genes involved in oxidative response increased 14-fold after 6 h, and those involved in actin synthesis showed a 27-fold change after 24 h, while expression of apoptosis genes increased 6.9-fold after 96 h compared with the control (eastern oysters exposed to nontoxic phytoplankton).

**Conclusion:** Exposure experiments (nonsublethal or chronic) should be carried out to better assess the threat of this species and toxins for eastern oysters and other marine organisms.

**KEYWORDS**

apoptosis, bivalve mollusks, dinoflagellates, fate of toxins, goniodomin metabolism, goniodomins, oxidative stress, *Alexandrium monilatum*, *Crassostrea virginica*

## INTRODUCTION

Toxic species of the dinoflagellate genus *Alexandrium* have a cosmopolitan distribution and form harmful algal blooms in coastal and estuarine areas (Anderson et al. 2012). Many species produce toxigenic compounds that can accumulate in filter-feeding bivalves, including saxitoxins that are responsible for paralytic shellfish poisoning in humans (Anderson et al. 2012), cyclic imines (e.g., spirolides and gymnodimines that cause neurological symptoms in mice; Zurhelle et al. 2018; Lamas et al. 2021; Ji et al. 2022), unidentified cytotoxic compounds (on protists, grazers, fish, and shellfish) referred to as "bioactive extracellular compounds" (Lassudrie et al. 2016; reviewed in Long et al. 2021), and goniodomins (Murakami et al. 1988).

Goniodomins are polyketide macrolides produced by *A. taylorii*, *A. hiranoi*, *A. pseudogonyaulax*, *A. limii* sp. nov., *A. ogatae* sp. nov., and the chain-forming, bioluminescent species *Alexandrium monilatum* (Balech 1995) (Murakami et al. 1998; Hsia et al. 2006; Zmerli Triki et al. 2016; Tillmann et al. 2020; Abdullah et al. 2023). According to Terao et al. (1989), the dose lethal to 50% of test organisms (LD50) of goniodomin A (GDA) through intraperitoneal injections in mice was $1.2\times10^3$ μg/kg, while pectenotoxin-2 (another phycotoxin produce by some *Dinophysis* spp.; Reguera et al. 2014), which has a similar structure to GDA, had an intraperitoneal LD50 of $2.2\times10^2$ μg/kg (Miles et al. 2004). In addition, studies conducted on rabbit (Furukawa et al. 1993; Yasuda et al. 1998) and human (Mizuno et al. 1998) cells revealed that GDA concentrations in the micromolar range led to modification of the conformation, activity, and content of actin. Similarly, Espiña et al. (2016) reported that GDA and one of its isomers, goniodomin B (GDB), exhibited cytotoxic effects on rat hepatocytes at nanomolar and micromolar concentrations, respectively. However, to date, the effect on humans remains unknown (Anderson et al. 2012) while the effects on marine organisms has only been suspected.

The presence of *A. monilatum* (formerly identified as *Gessnerium mochimaensis* and *Gonyaulax monilata*) has been documented in several tropical and subtropical climate locations. In the Western Hemisphere, it was observed off the coasts of Venezuela (Halim 1967; Ferraz-Reyes et al. 1985), Costa Rica (Viquez and Hargraves 1995), and the United States in the Gulf of Mexico (Connell and Cross 1950); along the Atlantic coast of Florida (Howell 1953); and in the York River, a tributary of the lower Chesapeake Bay (Mackiernan 1968). In addition, *A. monilatum* also has been reported in the western Black Sea (Moncheva et al. 2001) and the southwestern region of India (Sanilkumar et al. 2009). Since 2007, large, dense, patchy blooms of *A. monilatum* have been encountered near annually in late summer in the southern region of the Chesapeake Bay, particularly in the York River basin (Wolny et al. 2020). During blooms, the concentration of this species can reach as high as 100,000 cells/mL (Wolny et al. 2020). In addition, recent studies revealed the presence of GDA in different locations within the lower Chesapeake Bay (Onofrio et al. 2021; Pease et al. 2023). The toxin was found in dissolved form (i.e., using SPATT [Solid Phase Adsorption Toxin Tracking]) during the summer 2017 bloom season and at relatively high concentrations compared with other algal toxins (e.g., dinophysistoxin-1, azaspiracid-2, or domoic acid) outside the blooming period of *A. monilatum* (Onofrio et al. 2021). Pease et al. (2023) detected low amounts of GDA (0.45 μg/kg) in adult eastern oysters *Crassostrea virginica* (Gmelin, 1791) deployed in cages in the lower Chesapeake Bay in early summer 2019 and 2020, while no *A. monilatum* cells were detected by quantitative PCR.

Blooms of *A. monilatum* have been associated with mortality of aquatic organisms, including a variety of finfish species, since the early 1950s in Texas (Connell and Cross 1950; Wardle 1975; Perry et al. 1979), Alabama (Perry et al. 1979), and Florida (Howell 1953; Williams and Ingle 1972; Norris 1983) (reviewed in May et al. [2010] and Burkholder et al. [2018]). These blooms have been found to have negative effects on eastern oyster and on veined rapa whelk *Rapana venosa* held in flow-through tanks fed with York River water. Specifically, reports have indicated a decrease in ventilation and an increase in the production of mucus, epithelial erosion,

**Impact statement**

This study highlighted accumulation and depuration of goniodomins (polyketide macrolides) in tissues of eastern oysters exposed to the dinoflagellate *Alexandrium monilatum* without mortality or pathological changes. This sublethal exposure increased the expression of genes involved in apoptosis, oxidative stress responses, and cellular structure. This work revealed that *A. monilatum* and its toxins could be a potential emerging threat for eastern oyster, especially as the number of goniodomin-producing species is increasing worldwide.

and mortality (Harding et al. 2009; Pease 2016). Thus, the presence of *A. monilatum* and one of its toxins in the lower Chesapeake Bay may suggest a potential regional risk.

Laboratory exposure studies of marine organisms to cultures of *A. monilatum* have corroborated the in situ observations (reviewed in Table 1). Rosa et al. (2013) observed mortality (up to 50%) in ascidians exposed for 24 h to 550 cells/mL of *A. monilatum* (strain AM-02). Mortality of larvae, juvenile, and adult finfish has been documented upon exposure to *A. monilatum* cultures containing 100–1000 cells/mL for a period of hours to days (Gates and Wilson 1960; Aldrich et al. 1967; Sievers 1969; Ray and Aldrich 1976; May et al. 2010). Moreover, similar exposure (i.e., concentrations and durations) with adult eastern oysters resulted in a decrease in the clearance rate, a decrease in valve gape, shell closure, epithelial necrosis, gill erosion, and ultimately death (Ray and Aldrich 1976; May et al. 2010; Pease 2016). Gates and Wilson (1960) observed an increase in toxicity when the dinoflagellate cells were disrupted, suggesting a stronger effect when intracellular metabolites were released. Notably, most of the negative effects have been observed using lysed cells or cultures in the senescent growth phase (Table 1), even at low cellular concentrations, which suggests a potential leakage and an increase in extracellular metabolite concentrations, including GDA and its degradation products (e.g., GDA seco-acid [GDA-sa]) (Table 1). Goniodomin A has been shown to accumulate in laboratory-held mollusks (veined rapa whelks and their shell epibionts, including mussels and oysters) exposed for several days to bloom waters containing *A. monilatum* (40,000 cells/mL) up to 8770 µg/kg in the gastropod and was likely responsible for the observed adverse effects reported by Harding et al. (2009).

There is little information on the direct effect of *A. monilatum*, and the potential accumulation pathway of its toxins, on farmed shellfish species, such as eastern oyster. Therefore, this study evaluated the in vitro effect of a sublethal concentration of *A. monilatum* on the mortality, clearance rate, histopathology, and gene expressions of triploid eastern oyster, as well as on the fate of toxins (i.e., uptake, compartmentalization, accumulation, and depuration), during 96 h of exposure (fed daily) and 96 h of depuration.

# METHODS

## Culture conditions

A monoclonal nonaxenic *Alexandrium monilatum* culture (Amon07) was established from a water sample collected from the York River, Virginia (37°14′40.7″N, 76°30′05.0″W) during a bloom in 2007. The haptophytes *Tisochrysis lutea* (T-iso, CCMP1324) and *Pavlova pinguis* (CCMP609), the chlorophyte *Tetraselmis chui* (CCMP908), and the diatom *Chaetoceros muelleri* (CCMP1316) were obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota. Cultures were grown in L1 without silicate (L1-Si) (Guillard and Hargraves 1993) and enriched with silicate for the diatom species; made with 0.22-µm-filtered natural seawater obtained from Wachapreague, Virginia, at a salinity of 33‰; and diluted in MilliQ water to a salinity of 20‰. Cultures were kept in glass flasks at 20°C in a light : dark cycle of 12 h light : 12 h dark at a light intensity with an average ± SD of 210 ± 21 µmol photons $m^{-2} s^{-1}$. Three days prior to the onset of the experiment, the *A. monilatum* culture was concentrated using its phototactic ability (Juhl 2005) and 90% of the supernatant was removed and replaced with fresh L1-Si medium to eliminate the majority of extracellular toxins and other potential bioactive compounds in the aged growth medium, which were previously hypothesized to cause lethal effects in aquatic organisms (Table 1).

## Maintenance of eastern oysters

Adult triploid eastern oysters (market size, average ± SD shell height and width = 75 ± 6.5 and 51 ± 6.8 mm, respectively; $n = 96$) were provided by the Virginia Institute of Marine Science's Aquaculture Genetics and Breeding Technology Center. Eastern oysters were cleaned, rinsed, and then placed in 36-L glass tanks containing 30 L of aerated 20‰ York River water (5 µm filtered). The average ± SD water temperature over the course of the experiment was 21 ± 1°C. The eastern oysters were acclimated for 5 days before experimental exposure.

## Experimental design

A total of 36 adult eastern oysters per tank were randomly placed into three independent aerated 36-L glass tanks in a final volume of 5 L of York River water (20‰; 5 µm filtered) and were subjected to three different exposure treatments for a duration of 96 h. The three tanks of eastern oysters were exposed to the following: tank 1 = *A. monilatum* at a concentration of 615 ± 47 cells/mL (average ± SD density calculated over the 4 days of exposure), tank 2 = an equal mixture of four nontoxic phytoplankton species (*T. lutea*, *P. pingus*, *T. chui*, and *C. muelleri*) at an average ± SD concentration of 13,925 ± 4635 cells/mL, and tank 3 = unexposed (i.e., unfed). Every 24 h, the

**TABLE 1** Effects observed during in vitro or laboratory experiments performed on marine animals exposed to cultures of *A. monilatum* (adapted and updated from May et al. 2010 and Burkholder et al. 2018).

| Exposure organism | Life stage | Stressor | Concentration (cells/mL) | Exposure time/process | Effects (concentrations in cells/mL) | Reference |
|---|---|---|---|---|---|---|
| Finfish (Striped Mullet *Mugil cephalus*) | Juveniles | *Alexandrium monilatum* (culture, lysed cells, and supernatant) | 1400 | Hours/once | Paralysis and mortality (100% after ≈0.5 h in fish exposed to frozen and heated cells and after 1–4 h to raw cultures). No effect of supernatant | Gates and Wilson 1960 |
| Finfish (Guppy *Poecilia reticulata*) | Juveniles | *Alexandrium monilatum* (cultures in exponential and decay growth phases) | 1000–2000 | 7 days/once | Mortality (2 h of survival when exposed to culture [2000] in late exponential growth phase and 1 h when exposed to culture in decay [1000] growth phase) | Aldrich et al. 1967 |
| Finfish (Sheepshead Minnow *Cyprinodon variegatus*), Mollusk (eastern oyster *Crassostrea virginica*, hooked mussel *Brachidontes recurvus* [also known as *Ischadium recurvum*], ivory barnacle *Amphibalanus eburneus*), Crustacean (flatback mud crab *Eurypanopeus depressus*, daggerblade grass shrimp *Palaemon* [also *Palaemontes*] *pugio*), Annelida (pile worm *Alitta succinea*, *Polydora websteri*) | Adults or juveniles | *Alexandrium monilatum* (cultures in stationary or senescent growth phases) | 70–1200 | 48 h/once | Mortality in Sheepshead Minnow (100% at 600), in eastern oyster (80% at 1000), in hooked mussel (100% at 900), in daggerblade grass shrimp (20% at 700), in pile worm (100% at 700), and in *P. websteri* ("many" at 600) | Sievers 1969 |
| Mollusk (eastern oyster) | Adults | *Alexandrium monilatum* (likely[a] stationary or senescent phases) | ≈1000 | 24 h/once | Shell closure | Ray and Aldrich 1976 |
| Finfish (Gulf Killifish *Fundulus grandis*) | Adults | *Alexandrium monilatum* | ≈1000 | Hours | Mortality (100% after 9 h) | |

(Continues)

**TABLE 1** (Continued)

| Exposure organism | Life stage | Stressor | Concentration (cells/mL) | Exposure time/ process | Effects (concentrations in cells/mL) | Reference |
|---|---|---|---|---|---|---|
| Mollusk (eastern oyster, Asian green mussel *Perna viridis*, and hard clam [or nothern quahog] *Mercenaria mercenaria*) | Adults | *Alexandrium monilatum*, strain AM-03 (cultures in senescent growth phase) | 550 | Hours to days/once | Decrease in clearance rate and valve gape of all species (1–2 h). Mortality (60% after 24 h) in Asian green mussel | May et al. 2010 |
| Mollusk (eastern oyster and hard clam) | Larvae (D-shaped, 10–14 d) | *Alexandrium monilatum*, strain AM-03 (culture, constrained culture in dialysis tubing, and lysed cells in exponential growth phase) | 550 | 2 h/once | Mortality when exposed to lysed cells (10% and 62% for eastern oyster and hard clam, respectively) | |
| Finfish (Sheepshead Minnow) | Larvae | *Alexandrium monilatum*, strain AM-03 (culture in exponential and in senescent growth phases and lysed cells) | 550 | 1–1.5 h/once | Mortality (100% after 90 min) except in larvae exposed to exponentially growing culture | |
| Ascidians (leathery sea squirt *Styela clava*, sea grape *Molgula manhattensis*, *Ciona intestinalis*, *Botrylloides violaceus*, *Didemnum vexillum*, and golden star tunicate *Botryllus schlosseri*) | Adults | *Alexandrium monilatum*, strain AM-02 (culture in exponential growth phase) | 550 | 24 h | Mortality in golden star tunicate (50%), sea grape (26%), *D. vexillum*, and leathery sea squirt (16%) | Rosa et al. 2013 |
| Mollusk (eastern oyster) | Adult | *Alexandrium monilatum* Amon07 (culture and lysed cells) | 100–7600 | 12–96 h/once | Mortality when exposed to culture (17–66% after 60–96 h at 1000 and 84% after 96 h at 2000) and lysed cells (16–66% after 24–96 h at 5000). Epithelial necrosis and erosion in gill and mantle at each exposure conditions | Pease 2016 |
| Mollusk (eastern oyster) | Adult | *Alexandrium monilatum* Amon07 (culture, concentrated and 90% of the medium was replaced with fresh medium prior to the experiment) | 600 | 96 h/every 24 h | Accumulation of goniodomins in eastern oyster tissues, transformation, and depuration. Overexpression of genes involved in cellular structure and physiological process: oxidative stress, inflammatory response, and apoptosis | This study |

[a]Two- to 10-week-old cultures (grown at 25°C) of *A. monilatum* used by Ray and Aldrich 1976 were likely in stationary or senescent growth phases.

tanks were subjected to a replacement process involving the replacement of water and algae while eastern oysters were randomly relocated within rinsed tanks. Following the 96 h of exposure, all eastern oysters and tanks were rinsed and the oysters were allowed to depurate for a further 96 h in aerated York River water. Eastern oysters previously exposed to *A. monilatum* or the nontoxic phytoplankton species were provided with an equal mixture of the nontoxic phytoplankton at an average ± SD of 16,950 ± 2540 cells/mL every 24 h. The unexposed treatment was continued for the period of depuration. Water samples (1 mL) were collected every 24 h before and after water and phytoplankton renewal for subsequent enumeration of algae cells and to calculate clearance rate and observation of feces or pseudofeces (see below). At the beginning of the exposure ($t = 0$ h), $n = 6$ eastern oysters per tank were randomly marked and monitored for mortality throughout the experiment. After 0, 6, 24, and 96 h of exposure and 24 and 96 h of depuration, $n = 6$ eastern oysters per tank were sampled for histology and subsequent toxin and gene expression analyses. Sampled eastern oysters were immediately substituted with marked individuals (held under the same laboratory conditions and unexposed) in order to maintain the same number of eastern oysters ($n = 36$) and tank conditions in each treatment.

## Cell counting, clearance rate calculation, biovolume measurement, and feces or pseudofeces observation

Fixed phytoplankton samples in Lugol's solution (1% final concentration) were enumerated using a Nageotte cell counting chamber and an Olympus IX51 inverted light microscope. Biovolume of *A. monilatum* was assessed by measuring height and diameter of more than 30 individual cells that were calculated as rotational ellipsoids (Olenina et al. 2006).

The calculation of the clearance rate of eastern oysters (mL/hour/individual) was carried out based on measurement of depletion of algal cells over time in a closed system (Coughlan 1969):

$$\text{Clearance rate} = \frac{v}{t \times n} \times \ln\left(\frac{\text{Conc}_0}{\text{Conc}_t}\right),$$

where $v$ is the volume of water (mL), $t$ is the time (h), $n$ is the number of eastern oysters, and $\text{Conc}_0$ and $\text{Conc}_t$ are the initial algae concentration (cells/mL) at time 0 and $t$, respectively. Results from a preliminary experiment revealed negligible cell settlement; thus, no correction of the clearance rate was required.

Fecal materials were observed using a light microscope, and the presence and/or absence of motile and/or immotile *A. monilatum* cells was assessed.

## Oyster processing

At 0, 6, 24, and 96 h of exposure and at 24 and 96 h of depuration, $n = 6$ eastern oysters were removed from each tank treatment. Removed eastern oysters were measured, (shell height and width), weighed, then shucked. An approximate 5-mm cross section of whole oyster tissue (which included gills, mantle, gonads, and digestive glands) was resected and preserved in Davidson's Fixative (Shaw and Battle 1957) for 48 h, then transferred to 70% ethanol for subsequent processing for paraffin histology using standard methods. The remaining soft tissues (mostly composed of mantle) were stored at −20°C. A small subsample of gill and digestive gland tissues (≈50 mg) was collected and stored in DNA/RNA Shield (Zymo Research, Tustin, California) for subsequent gene expression analyses. The remaining gill, digestive gland, and soft tissues were stored separately at −20°C for subsequent toxin quantification (see below).

## Histopathology

Six-micron tissue sections stained with hematoxylin and eosin and placed on glass slides were evaluated on an Olympus BX51 light microscope, which was equipped with an Olympus DP73 digital camera for image capture using cellSens imaging software. Slides were examined at 200–400× magnification for signs of pathology, including hemocyte infiltration into different tissues or organs, edema, and disruption or erosion of epithelia that could potentially be associated with exposure to algal toxins or other environmental stressors (Pease 2016; Guévélou et al. 2019).

## Extraction and analysis of goniodomins

### Chemicals and solvents

For extraction, HPLC-grade (≥99.9%) methanol (MeOH) and hexane were purchased from Fisher Scientific (Waltham, Massachusetts) while Milli-Q water was supplied by a Milli-Q integral 3 system (Millipore, Saint-Quentin-Yvelines, France). For the preparation of

mobile phases, Milli-Q water and HPLC-grade MeOH (CHROMASOLV, Honeywell, France), acetonitrile (CHROMASOLV, Honeywell, Germany), formic acid (CHROMANORM, VWR Chemicals, UK), and ammonium formate (10 M in $H_2O$; BioUltra, Sigma Aldrich, Switzerland) were used. The GDA standard was provided by T. M. Harris as a dry residue in a sealed ampoule. The residue was solubilized extemporaneously in MeOH at a concentration of 10 µg/mL to avoid interconversions of GDA.

## Extraction

Toxin extraction was adapted from Gaillard et al. (2023) and Smith et al. (2018) for the *A. monilatum* cell pellet (intracellular toxins) and culture supernatant (extracellular toxins) and from a European Union harmonized standard operating procedure (European Union Reference Laboratory for Marine Biotoxins 2015) for eastern oyster tissues. Briefly, the inoculum culture of *A. monilatum* was subsampled ($n = 3$, 1 mL) and gently centrifuged (2000 × *g* at 4°C for 10 min) to sediment the *A. monilatum* cells. The remaining supernatant was carefully removed, and both the cell pellet and supernatant were stored at −20°C until extraction. The cell pellets were extracted in 1.5 mL of MeOH using a vortex mixer and bath sonication (25 kHz at <20°C for 15 min) and centrifuged (3234 × *g* at 4°C for 15 min) to concentrate the cellular debris. The methanolic fractions were filtered onto 0.22-µm PTFE syringe filters (Millipore, Sigma, Burlington, Massachusetts). The culture supernatant samples were extracted using a 60-mg Oasis HLB solid phase extraction cartridge (Waters, Milford, Massachusetts).

The frozen individual eastern oyster tissues (i.e., gill, digestive gland, and remaining tissues; $n = 6$) were thawed and homogenized on ice using a homogenizer (Tissue Tearor) at 19,000 rpm for 30 s. Afterward, 0.5 g of homogenized tissues were transferred into a 15-mL conical polypropylene tube, extracted twice with 2.25 mL of MeOH, and centrifuged (3234 × *g* at 4°C for 10 min), and then 2.5 mL of the methanolic extracts were transferred into scintillation vials and washed with 5 mL of hexane (vortexed and centrifuged [2000 × *g* at 4°C for 10 min]). The extracts were filtered onto 0.22-µm PTFE syringe filters (Millipore, Sigma, Burlington, Massachusetts) and stored in glass vials at −20°C.

The samples were dried on ice for approximately 12 h and shipped to the METALG laboratory (IFREMER, Nantes, France) in 1.5-mL glass vials for toxin analysis performed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS).

## Liquid chromatography

Separation was performed using a Nexera XR UFLC system (Shimadzu, Tokyo, Japan), on a Kinetex C18 column (Phenomenex, 100 mm × 2.1 mm, 2.6 µm, 100 Å) equipped with a precolumn (Phenomenex, SecurityGuard ULTRA C18 for 2.1-mm columns), both maintained at 40°C. The elution gradient started with 2 min at 85% eluent A (water, 20 mM formic acid, 1 mM ammonium formate) and 15% eluent B (95% acetonitrile, 20 mM formic acid, 1 mM ammonium formate), followed by a linear increase of B to 100% in 7 min, held for 3 min before going back to initial conditions in 0.5 min and then 2 min equilibration. The flow rate was 0.3 mL/min, and the injection volume was 5 µL.

## Mass spectrometry

The 4000-QTRAP hybrid triple quadrupole mass spectrometer (Sciex) was used in positive electrospray ionization and multiple-reaction monitoring mode. Both ammonium ($[M + NH_4]^+$) and sodium ($[M + Na]^+$) adducts were monitored (Table 2). The instrumental parameters were optimized by infusion and flow injection analysis using the GDA standard: curtain gas = 25 psi, collision gas = high, ion spray voltage = 5000 V, temperature = 450°C, and nebulizer/auxiliary gas = 40/45 psi.

## Identification of goniodomin analogues

In the absence of standards, a mixture of goniodomins (including GDB and GDC) was obtained from interconversion of GDA using the acidic conditions reported by Harris et al. (2021). This allowed us to identify the retention times of congeners B and C. To confirm the identity of the peaks, enhanced product ion coupled to tandem mass spectrometry (EPI-MS/MS) spectra as well as the elution order of goniodomins were compared to those found in the literature. In addition, the transition *m/z* 831.4 > 423.1 of the disodium adduct, characteristic of the seco-acid form of GDA, allowed the discrimination between GDC and GDA-sa (Harris et al. 2023). The composition of the 423-fragment ion can be confidently attributed to $C_{20}H_{25}Na_2O_7^+$ resulting from a retro-Diels-Alder cleavage of the D-ring, producing the proximal daughter ion C1-C16 (*m/z* 423).

## Quantification of goniodomins

The dry residue of each sample was solubilized in 100-µL MeOH before liquid LC–MS/MS analysis. Samples were

**TABLE 2** Transitions ($m/z$ of precursor and product ions, collision energy) monitored for the LC–MS/MS analysis of goniodomin A (GDA), goniodomin B (GDB), goniodomin C (GDC), and 34-desmethyl-GDA, including seco-acid congeners (GDA-sa and GDC-sa).

| Adduct | Toxins | Precursor ion ($m/z$) | Product ions ($m/z$) | Collision energy (eV) |
|---|---|---|---|---|
| $NH_4^+$ | GDA/GDB | 786.5 | 607.2 | 39 |
| | | 786.5 | 733.4[a] | 35 |
| | | 786.5 | 139.0 | 53 |
| | GDC/GDA-sa | 804.5 | 607.5 | 39 |
| | | 804.5 | 751.5[a] | 35 |
| | | 804.5 | 139.5 | 53 |
| | GDC-sa | 822.5 | 733.5[a] | 35 |
| | | 822.5 | 139.5 | 53 |
| | 34-desmethyl-GDA | 772.5 | 607.5 | 39 |
| | | 772.5 | 719.5[a] | 35 |
| | | 772.5 | 125.4 | 53 |
| $Na^+$ | GDA/GDB | 791.4 | 413.2 | 47 |
| | | 791.4 | 609.3[a] | 51 |
| | | 791.4 | 747.3 | 67 |
| | GDC/GDA-sa | 809.5 | 765.5[a] | 54 |
| | | 809.5 | 747.5 | 68 |
| $2Na^+$ | GDA-sa | 831.4 | 423.1 | 47 |

[a]Transitions used for quantification.

quantified using a seven-point external GDA calibration curve (0.5, 2, 10, 20, 100, 200, and 400 ng/mL). All other analogues, when present, were quantified as GDA equivalent, assuming equal molar response factors between analogues.

### Characterization of the analytical method: Recovery of GDA in different matrices

The recovery of GDA was calculated using blank matrices ($n = 3$) spiked with a GDA standard solution prior to extraction. Eastern oyster tissues (i.e., gill, digestive gland, and remaining tissues) dissected from uncontaminated animals were spiked with GDA at 400 µg/kg (wet weight) and extracted as described above. A strain of *A. minutum* was used as the blank algal matrix, in the absence of a non-GDA-producing strain of *A. monilatum*. The cell pellet (about 500,000 cells) was spiked to a final concentration of 20 ng/mL and extracted as aforementioned. Finally, the L1-Si culture medium was used as the extracellular (supernatant) blank matrix. The 1-mL sample was spiked with GDA to a final concentration of 100 ng/mL and extracted on the solid phase extraction cartridge (Smith et al. 2018). In addition, nonspiked control samples were processed for each matrix to ensure that there was no contamination of control samples initially or during handling.

### Characterization of the analytical method: Sensitivity in the different matrices

The instrumental sensitivity was determined for each matrix by injecting methanol extract spiked with 1–10 ng/mL of GDA. The limit of detection (LOD) and limit of quantification (LOQ) were defined as the lowest concentration giving a signal-to-noise ratio of 3 and 10, respectively.

## Gene expression

Samples of gill and digestive gland ($n = 6$ per time point and treatment) stored at −20°C in DNA/RNA Shield representing the six replicates were randomly pooled, two per pool, to reduce variability and the number of samples processed so that $n = 3$ for each tank and sampling time point. Tissue samples were treated with 100 µg/mL of proteinase K solution for 1 h at 37°C and for 15 min at 60°C with shaking (360 oscillations per minute) followed by extraction of RNA using a MagMAX Viral RNA Isolation kit (Ambion, Austin, Texas) following the manufacturer's protocol. An in-column DNase step was performed using a ZR-Duet DNA/RNA MiniPrep Plus kit (Zymo Research, Tustin, California) according to the manufacturer's instructions. The RNA purity and concentration were analyzed using both NanoDrop 2000 and Qubit spectrophotometers. The cDNA was synthesized by reverse transcription of 500 ng of total RNA using an Omniscript Reverse Transcription kit (Qiagen, Hilden, Germany) following the

manufacturer's protocol. Quantitative PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System. Each reaction consisted of 3.82 μL of PCR-grade water, 5 μL of PowerUp SYBR Green master mix (Thermo Fisher, Waltham, Massachusetts), 1 μL of cDNA template, and 0.9-μM primers (Integrated DNA Technologies, Coralville, Iowa). The transcript levels of seven genes (Table 3), involved in various physiological processes, and one housekeeping gene were studied: anti-apoptotic protein (*aapo*), actin (*actin*), allantoicase (*allan*), cadherin (*cad*), caspase-1 (*casp1*), glutathione peroxidase (*gpx*), glutathione reductase (*gr*), and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) as the housekeeping gene. Preliminary studies indicated that *gapdh* was the most stable housekeeping gene, in comparison to the other housekeeping genes that were tested; *elongation factor 1-alpha* and *18S ribosomal RNA* (data not shown). The cycling protocol consisted of an initial denaturation at 95°C for 20 s, followed by 40 cycles of denaturing at 95°C for 3 s and annealing/extension at 60°C for 30 s; then, a melting curve analysis was performed and consisted of a denaturation at 95°C for 15 s, followed by an annealing step for 15 s at 60°C.

## Data and statistical analyses

The relative quantification of gene transcripts was calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001):

$$C_T = \text{threshold cycle}$$

$$\Delta C_T = C_T\,(\text{target gene}) - C_T\,(\text{housekeeping gene})$$

$$\Delta\Delta C_T = \Delta C_T\,(\text{treatment sample}) - \Delta C_T\,(\text{untreated control}),$$

where the housekeeping gene was *gapdh* and untreated control was the eastern oysters exposed to nontoxic phytoplankton.

Statistical analyses for clearance rate and gene expression were performed on RStudio (version 2023.06.0). After checking the assumptions of independence (Durbin–Watson test), homoscedasticity (Bartlett test), and normality (Shapiro–Wilk test) of the residuals, a one-way ANOVA followed by a Tukey post hoc test were computed. Otherwise a Kruskal–Wallis test followed by a Conover test were performed. Differences were considered statistically significant when $p < 0.05$.

# RESULTS

## Eastern oyster mortality and clearance rate

Throughout the duration of the experiment, no mortalities were observed in any eastern oysters from the three treatments. The eastern oysters exposed to *A. monilatum* and nontoxic phytoplankton exhibited positive and comparable ($p > 0.05$) average ± SD clearance rates of 46 ± 12 and 36 ± 4 mL/h/individual, respectively. Fecal materials (feces and pseudofeces) from eastern oysters exposed to *A. monilatum* contained both immotile and motile *A. monilatum* cells and were observed throughout the 96-h-exposure period but not after 24 h of depuration. Cells of *T. lutea*, *P. pingus*, or *T. chuii* (we were unable to distinguish between taxa) and *C. muelleri* were observed in fecal materials during exposure to the nontoxic phytoplankton treatment and also during the depuration phase.

## Histology

Microscopic evaluations of eastern oyster gill and digestive gland tissues from the three treatments revealed no obvious signs of pathology, with tissue and organ structure appearing normal (Figures S1 and S2 in the Supplement available in the online version of this article) and the eastern oysters examined appearing to be in uniformly good health.

## Toxin analysis

### Characterization of the analytical method

The extraction method proved highly effective in recovering GDA from eastern oyster tissues and from algal cells (i.e., the intracellular toxin fraction), with a yield ranging from 87% to 95% (Table 4). The HLB-SPE (hydrophilic-lipophilic-balanced solid phase extraction) recovery of GDA from the extracellular fraction (i.e., dissolved GDA in algal culture medium) was lower (60%) but still considered acceptable.

The sensitivity of the LC–MS/MS method for detecting and quantifying GDA (Table 5) was identical for the three different eastern oyster tissues tested (gill, digestive gland, and remaining tissues), with a limit of detection (LOD) and limit of quantification (LOQ) of 1.8 and 5.4 μg/kg, respectively. In the microalgae pellet, the LOD and LOQ were 0.60 and 1.8 femtograms (fg) per cell. In the supernatant, the LOD and LOQ were 0.40 and 1.2 ng/mL, respectively.

### Toxins in *A. monilatum*

The goniodomin profile of the York River 2007 isolate of *A. monilatum* (Amon07) contained an intracellular average ± SD primarily of GDA (215 ± 7.15 pg/cell), equivalent to 3.6 ± 0.12 fg/μm$^3$, and approximately 16-, 38-,

**TABLE 3** Information on genes amplified by quantitative PCR, including gene name, abbreviation, GenBank accession number, function, and forward and reverse primer sequences used for quantitative PCR and reference.

| Gene name | Abbreviation | GenBank accession | Function | Primer sequences | Reference |
|---|---|---|---|---|---|
| *Anti-apoptotic protein* | *aapo* | XM_022477915 | Apoptosis | Fw 5′-AAAATGAACCGGCAACTCGC-3′<br>Rev 5′-TTCTGCCATCTGCCGAACTT-3′ | Jones et al. (2019) |
| *Actin* | *actin* | AF026063 | Cell structure, cell motility, cell division | Fw 5′-TACTCTTTCACCACCACAGCCG-3′<br>Rev 5′-TAGAGATGAGGATGAAGCAGCAG-3′ | Romero Geraldo et al. (2020) |
| *Allantoicase* | *allan* | XM_034472631 | Purine cycling, metabolism | Fw 5′-TGGAAAGAACTGGTCCCAAG-3′<br>Rev 5′-GTCTGGCTATGCCACCATCT-3′ | Mat et al. (2018) |
| *Cadherin* | *cad* | XM_022480422 | Cell adhesion, ion binding, and membrane transport; role in morphogenesis | Fw 5′-GGCGGGTTGTTCTTTGTGAC-3′<br>Rev 5′-AACAACAGCTACGTACGGGG-3′ | Jones et al. (2019) |
| *Caspase-1* | *casp1* | HQ425703 | Inflammatory response and apoptosis | Fw 5′-CTGAACGAGCGGAATGGCA-3′<br>Rev 5′-CCTGACCTTTCTGTAGTGTA-3′ | Qu et al. (2014) |
| *Glutathione peroxidase* | *gpx* | EF692639 | Oxidative stress | Fw 5′-GACCGTGGAACCAATGGACATC-3′<br>Rev 5′-GTTGGATTCGGACACAGATAGGG-3′ | Mat et al. (2013) |
| *Glutathione reductase* | *gr* | CU685269 | Detoxification process | Fw 5′-TTCGCCCTGCTGCTATGG-3′<br>Rev 5′-TTGCCCTGGGAGATGTTTG-3′ | Fabioux et al. (2015) |
| *Glyceraldehyde 3-phosphate dehydrogenase* | *gapdh* | AJ544886 | Catalyzation in glycolysis | Fw 5′-GGAGACAAGCGAAGCAGCAT-3′<br>Rev 5′-CACAAAATTGTCATTCAAGGCAAT-3′ | Romero Geraldo et al. (2020) |

**TABLE 4** Recoveries of goniodomin A (GDA) in spiked blank eastern oyster tissues and phytoplankton matrices ($n = 3$).

| | Oyster gill | Oyster digestive gland | Oyster remaining tissues | Phytoplankton pellet (intracellular) | Phytoplankton supernatant (extracellular) |
|---|---|---|---|---|---|
| Recovery of GDA | 87% | 90% | 88% | 95% | 60% |

**TABLE 5** Limit of detection (LOD) and limit of quantification (LOQ) of the LC–MS/MS method for goniodomin A (GDA) ($n = 3$).

| | Oyster gill | Oyster digestive gland | Oyster remaining tissues | Phytoplankton pellet (intracellular) | Phytoplankton supernatant (extracellular) |
|---|---|---|---|---|---|
| LOD | 1.8 µg/kg | 1.8 µg/kg | 1.8 µg/kg | 0.60 fg/cell | 0.40 ng/mL |
| LOQ | 5.4 µg/kg | 5.4 µg/kg | 5.4 µg/kg | 1.8 fg/cell | 1.2 ng/mL |

**TABLE 6** Average intracellular (pg/cell), intracellular per cell biovolume (fg/µm$^3$), extracellular (equivalent pg/cell and ng/mL), and extracellular in tank (calculated ng/mL) concentrations of goniodomin A (GDA), goniodomin B (GDB), goniodomin C (GDC), and goniodomin A seco-acid (GDA-sa) in the inoculum of *A. monilatum* (Amon07). Values are expressed as average ± SD ($n = 3$). Abbreviation: LOD = limit of detection. Examples of chromatograms can be found in Figure S3 in the Supplement available in the online version of this article.

| ***A. monilatum* (Amon07)** | **GDA** | **GDB** | **GDC** | **GDA-sa** |
|---|---|---|---|---|
| Intracellular (pg/cell) | 215 ± 7.15 | 13 ± 1.1 | 5.6 ± 0.39 | 11 ± 2.0 |
| Intracellular per cell biovolume (fg/µm$^3$) | 3.6 ± 0.12 | 0.22 ± 0.018 | 0.094 ± 0.0066 | 0.18 ± 0.034 |
| Extracellular (equivalent pg/cell) | 0.17 ± 0.10 | <LOD | <LOD | 5.0 ± 0.63 |
| Extracellular (ng/mL) | 0.51 ± 0.32 | <LOD | <LOD | 7.7 ± 0.95 |

and 20-fold less of GDB, GDC, and GDA-sa, respectively (Table 6). Only 2% of the total toxins were detected in the extracellular fraction, and it was dominated by GDA-sa (5.0 ± 0.63 equivalent pg/cell; Table 6).

## Fate of toxins in exposed eastern oysters

No toxins were detected in any of the treatments or tissues sampled at $t = 0$ h. Six hours after the initial exposure to an average ± SD of 660 ± 100 cells/mL of *A. monilatum*, GDA, GDB, GDC, and GDA-sa were detected in gill, digestive gland, and the remaining tissues of eastern oyster, with some variability between individuals (Figure 1A–C). Goniodomin A accumulated in gill to the highest concentration detected of all the forms of goniodomins measured in tissues, with an average ± SE maximum concentration of 504 ± 329 µg/kg, while much lower amounts of GDB, GDC, and GDA-sa were found in the gill at the same time point (i.e., 33 ± 21, 21 ± 13, and 4.2 ± 2.8 µg/kg, respectively) (Figure 1A). In comparison to gill, the concentration of GDA in the digestive gland and remaining tissues was much lower at this 6-h time point (21 and 4 times less GDA, respectively) (Figure 1B,C). One day following the initial exposure ($t = 24$ h, *A. monilatum* concentration on average ± SD of 607 ± 90 cells/mL), an apparent decrease (threefold to eightfold) in the concentrations of GDA, GDB, and GDC was observed in both the gill and remaining tissues while the concentration of GDA-sa remained relatively stable (Figure 1A,C). In contrast, a trend of accumulating GDA and GDA-sa in the digestive gland was observed between 6 and 24 h (two and four times greater, respectively) while the amounts of GDB and GDC detected remained relatively level (Figure 1B). After 96 h of exposure (*A. monilatum* concentration on average ± SD of 553 ± 81 cells/mL), no obvious trend of accumulation of GDB, GDC, and GDA-sa was observed in the gill or remaining tissues while the average ± SE GDA level in gill remained high (59 ± 4.5 µg/kg; Figure 1A). However, at the same time point, GDA-sa appeared to accumulate in the digestive gland, reaching an average ± SE of 46 ± 13 µg/kg compared with 2.6 ± 1.9 µg/kg after 6 h of exposure (Figure 1B).

After 96 h of exposure to *A. monilatum*, the eastern oysters were allowed to depurate by exposure to nontoxic phytoplankton at an average ± SD of 16,950 ± 2540 cells/mL for an additional 96 h. After 24 h of depuration, the GDA levels in gill tissues decreased to 3% of what was detected in those tissues after 96 h of exposure (Figure 1A) while 17% and <LOD (<1.8 µg/kg) of the toxin were detected in the remaining tissues and the digestive gland, respectively, after 96 h of depuration (Figure 1B,C). After 96 h of depuration, GDA was below the <LOD (<1.8 µg/kg) in all tissues

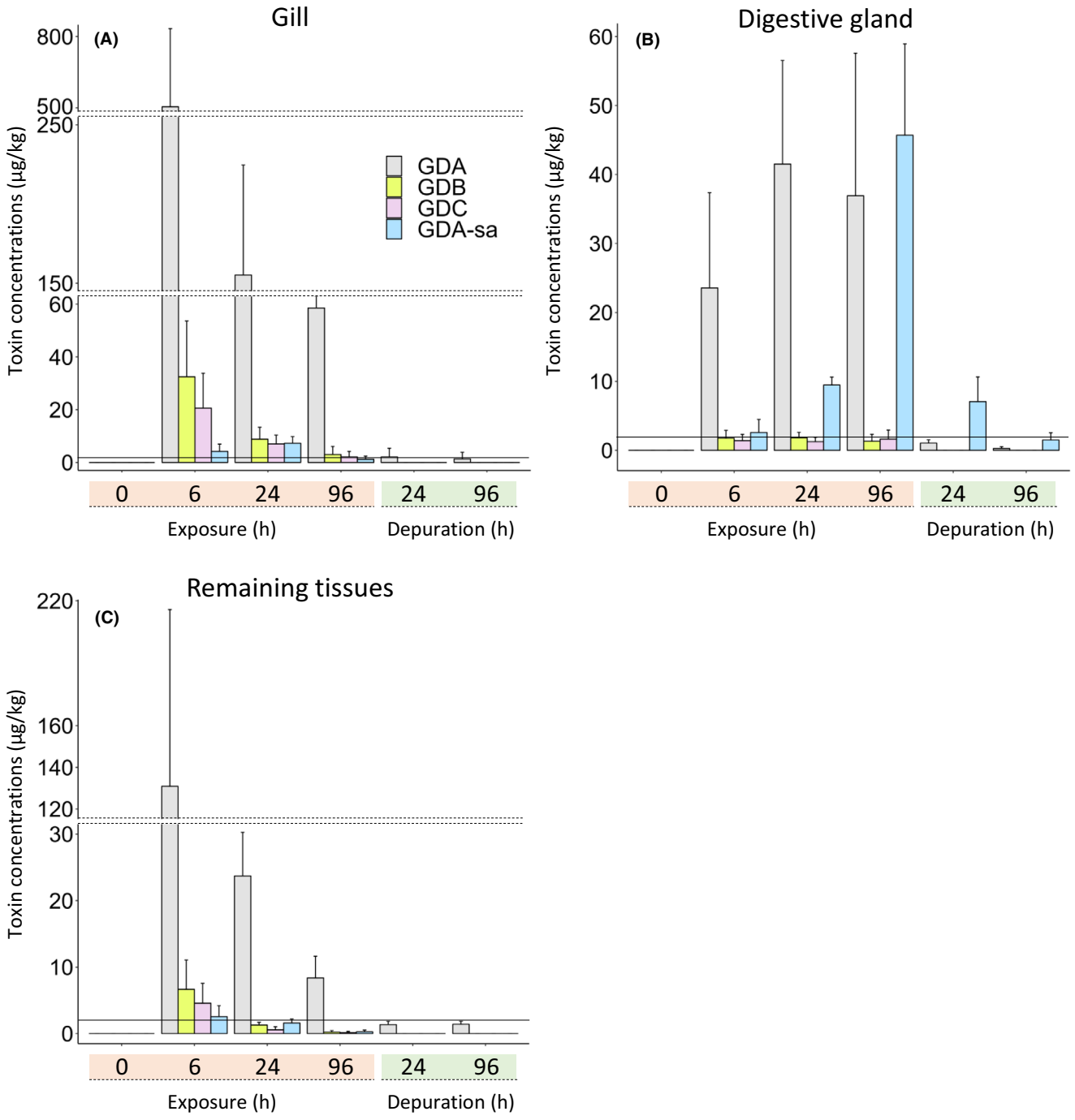


**FIGURE 1** Concentration (µg/kg) of goniodomin A (GDA), goniodomin B (GDB), goniodomin C (GDC), and goniodomin A seco-acid (GDA-sa) in eastern oyster (A) gill, (B) digestive gland, and (C) remaining tissues during 96 h of exposure to an average ± SD of 615 ± 47 cells/mL of *Alexandrium monilatum* (Amon07) followed by 96 h of depuration (eastern oysters exposed to a mixture of nontoxic phytoplankton). Data ($n = 6$) are average ± SE. The horizontal plain bar represents the limit of detection of GDA of 1.8 µg/kg. In some cases, the average of the six replicates were less than the limit of detection. Note that there are different scales for the *y*-axes and discontinuous scales for panels (A) and (C).

tested (Figure 1A–C). Goniodomin B and C levels were also rapidly undetectable in all tissues after 24 h of depuration (Figure 1A–C). Similar results were observed with GDA-sa not detected in the gill and remaining tissues, but 15% and 3% of the toxin remained in the digestive gland after 24 h and 96 h of depuration, respectively, compared with the peak after 96 h of exposure (Figure 1B).

## Gene expression

The $C_T$ (threshold cycle) values of the housekeeping gene (*gapdh*) were similar between gill and digestive gland samples and between treatments, as well as the exposure and the depuration phases ($p > 0.05$). Data were therefore normalized to *gapdh*, and the unexposed treatment was

used as an untreated control. Six hours after the initial exposure to *A. monilatum*, the *actin* gene(s) was significantly ($p < 0.01$) overexpressed in the digestive gland tissues of exposed eastern oysters compared with the same tissues in the unexposed treatment, which were 4.5 ± 0.31 (average ± SD) versus 2.0 ± 0.27 and compared with gill (3.6-fold) (Figure 2A). At the same time point, a substantial and significant increase ($p < 0.05$) in the expression level of the *gpx* gene in samples of gill and digestive gland (27-fold and 2.8-fold, respectively) from eastern oysters exposed to *A. monilatum* was observed compared to the same tissues from the unexposed treatment. Additionally, the expression level of the *gpx* gene in the gill was ≃4 times higher than in the digestive gland ($p < 0.05$), with an average ± SD relative quantification of 14 ± 2.9 (Figure 2A). Similarly, the average fold change of the *gr* gene expression was significantly higher ($p < 0.05$) in samples of gill exposed to *A. monilatum* compared with those from the unexposed treatment. Additionally, a nonsignificant trend of overexpression of the *allan* and *casp1* genes was observed under the same conditions at the 6-h time point (Figure 2A). After 24 h following the initial exposure to *A. monilatum*, a significant upregulation of the *actin* gene(s) was observed in samples of gill (27 ± 3.7-fold change) when compared to the digestive gland and to the gill in the unexposed treatment ($p < 0.01$) (Figure 2B). A nonsignificant increase in expression of the *cad* gene was observed in samples of digestive gland from eastern oysters exposed to *A. monilatum* compared with the same tissue in the unexposed treatment (average ± SD = 11 ± 6.9-fold change) (Figure 2B). On the last day of exposure ($t = 96$ h), a significant overexpression of *aapo* ($p < 0.05$), *actin* ($p < 0.01$), *gpx* ($p < 0.01$), and *gr* ($p < 0.01$) genes was observed in samples of digestive gland from eastern oysters exposed to *A. monilatum* compared with the same tissue in the unexposed treatment (average ± SD, with fold changes of 6.9 ± 2.2, 1.6 ± 0.18, 2.9 ± 0.18, and 4.5 ± 0.18, respectively), as well as a nonsignificant trend of higher expression of the *allan* gene (5.4 ± 2.3-fold change) (Figure 2C). During the depuration phase (Figure 2D,E), only the *aapo* gene was significantly overexpressed in samples of gill from eastern oysters exposed to *A. monilatum* compared with the unexposed treatment after 24 h (Figure 2D). The level of expression of other genes remained constitutive while the level of transcription of several genes was not detected (e.g., *casp1*), even in the untreated control (*gr*).

## DISCUSSION

The present study provides insight into the fate of toxins and sublethal effects of *A. monilatum* on eastern oyster, a molluscan species with both ecological and commercial significance. Recurrent blooms of this dinoflagellate species have been observed in several river estuaries that feed into the lower Chesapeake Bay since 2007. Previous studies have shown that *A. monilatum* can have negative effects upon eastern oyster (reviewed in Table 1), but the involvement of goniodomins and their effects and fate remain unclear. To date, six species of *Alexandrium* are considered GDA producers, including *A. monilatum* (Hsia et al. 2006); *A. hiranoi* (Murakami et al. 1998); two recently described species from Malaysia, *A. limii* and *A. ogatae* (Abdullah et al. 2023); as well as two species from the Mediterranean Sea, *A. taylorii* (Tillmann et al. 2020) and *A. pseudogonyaulax* (Zmerli Triki et al. 2016), reinforcing that GDA may be emerging in Europe and in Southeast Asia. This study aimed at determining the direct effects of exposure to a low concentration of an exponentially growing culture of *A. monilatum* on the clearance rate, mortality, histology, fate of toxins, and gene expression of eastern oyster over a 96-h exposure and 96-h depuration experiment.

Among the four goniodomins detected in the culture of *A. monilatum* (Amon07) (i.e., GDA and the congeners GDB, GDC, and GDA-sa), only 2% of the toxins were found in the extracellular compartment, which was dominated by GDA-sa. *Alexandrium monilatum* cells contained a high concentration (average ± SD = 215 ± 7.15 pg/cell) of GDA and lower concentrations of GDB, GDC, and GDA-sa (13 ± 1.1, 5.6 ± 0.39, and 11 ± 2.0 pg/cell, respectively). The concentrations of goniodomins in this study are different from that of by Hintze (2021), who using the same strain reported eightfold reduction of intracellular GDA (27 pg/cell), two- and fivefold higher GDB and GDC levels, and fivefold less GDA-sa. The difference in goniodomin concentrations observed between the two laboratories may be explained by the difference in culture methods (e.g., different culture media), difference in extraction and analytical methods, and the use of different GDA standards.

In the current study, no mortality was observed in eastern oysters exposed to *A. monilatum* for 96 h. Eastern oysters exposed to *A. monilatum* displayed a positive clearance rate that was similar to the clearance rate in eastern oysters exposed to a nontoxic phytoplankton species. No obvious pathologies were observed in eastern oysters exposed to *A. monilatum*, the nontoxic phytoplankton or in the unexposed group.

Similarly to this study, May et al. (2010) observed the presence of motile *A. monilatum* cells in the feces of adult eastern oyster exposed for hours to days to a single diet of 550 cells/mL of a culture in decay growth phase (strain AM-03). Moreover, the authors observed a decrease in clearance rate and valve gape indicating an avoidance behavior (Shumway and Cucci 1987; May et al. 2010).

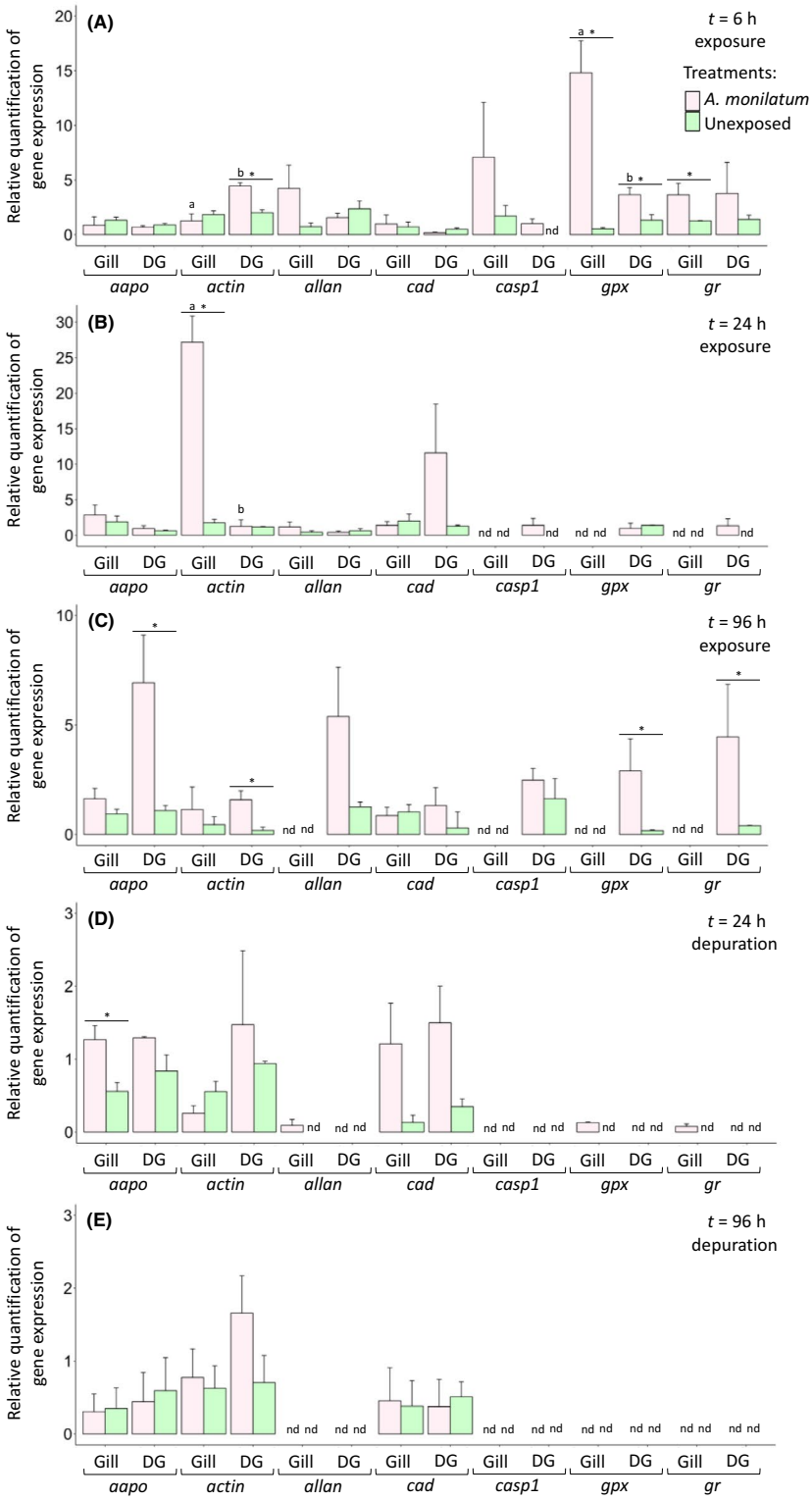


**FIGURE 2** Relative quantification of gene expression ($2^{-\Delta\Delta C_T}$ method; Livak and Schmittgen 2001) in eastern oyster gill (Gill) and digestive gland (DG) after (A) 6 h, (B) 24 h, and (C) 96 h of exposure to *A. monilatum* (Amon07; 615 ± 47 cells/mL) or no exposure, followed by (D) 24 h and (E) 96 h of depuration (exposed to a mixture of nontoxic phytoplankton, except for the unexposed treatment that remained unfed). Data ($n = 3$) are average ± SD, normalized to glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), with the treatment eastern oysters exposed to nontoxic phytoplankton as a control. Expression of each gene was compared between treatments (exposed to *A. monilatum* or unexposed) and between tissue compartments (gill and digestive gland) two by two, and differences ($p$-value ≤ 0.05) were marked with an asterisk or with different letters, respectively. When $C_T$ (threshold cycle) was higher than 40, the relative quantification of gene expression was written as "nd" (not detected). Note the different scales for the $y$-axes. Abbreviations are as follows: anti-apoptotic protein (*aapo*), actin (*actin*), allantoicase (*allan*), cadherin (*cad*), caspase-1 (*casp1*), glutathione peroxidase (*gpx*), and glutathione reductase (*gr*).

That behavior could be explained by the use of *A. monilatum* culture in stationary growth phase (shaken prior to the experiment) (May et al. 2010). Several exposure studies using *A. monilatum* cultures from late exponential to decay growth phases (from 550 to 2000 cells/mL) showed mortality in juvenile and adult fish (Striped Mullet, Guppy, Sheepshead Minnow, and Gulf Killifish) and bivalves (eastern oyster, hooked mussel, and Asian green mussel) (Table 1; Gates and Wilson 1960; Aldrich et al. 1967; Sievers 1969; Ray and Aldrich 1976; May et al. 2010). In contrast, the eastern oysters exposed to *A. monilatum* (Amon07) in this study showed low or no avoidance behavior. The *A. monilatum* culture in this experiment was maintained in exponential growth phase when used in exposure experiments. Additionally, efforts were made to minimize the concentration of extracellular metabolites (i.e., including goniodomins) in the supernatant (2% of the total toxins; Table 6) and was likely more representative of an ecological exposure (i.e., no or low concentration of extracellular metabolites in culture medium). The absence of these metabolites is hypothesized to have contributed to the absence of any apparent physiological effect in the current study.

The presence of toxins in the samples of eastern oyster gill, digestive gland, and remaining tissues corroborated the observation of no or low avoidance response by the animal and suggested that the accumulated toxins in eastern oysters originated mainly from *A. monilatum* cells and to a much lesser extent from the extracellular compartment. Overall, the clearance rate in exposed eastern oysters was positive, but some variability at the individual level should be expected, as has been reported for other bivalves exposed to paralytic shellfish toxin producers (Haberkorn et al. 2011; Mat et al. 2013). Those observations may support the interindividual variability in toxin concentrations in this study, particularly in the gill.

Notably, 6 h after the initial exposure to *A. monilatum*, the GDA concentration in the gill was high (504 ± 329 μg/kg) and decreased after 24 h (threefold less) and 96 h (eightfold less). A similar pattern was noted in the remaining tissues, with a decrease from 131 ± 85.0 after 6 h to 8.38 ± 3.26 μg/kg after 96 h. Concentrations of GDA in the digestive gland remained relatively low during the exposure phase; however, there was a general trend toward accumulation. One possible hypothesis explaining the dynamic of higher accumulation of toxins in gill and remaining tissues compared with the digestive gland could be that the toxins were actively or passively (via cell lysis or leakage) released by the *A. monilatum* cells during the transport of water from the siphon to their pallial organs (mantle, gills, and labial palps) (Bayne 2017) and remained surrounding these organs. This hypothesis is strengthened by the fact that *A. monilatum* cells are particularly fragile (authors' personal observation). Harding et al. (2009) found higher amounts of GDA in the exterior foot tissues of veined rapa whelks, with 770–8770 μg/kg compared to 30–200 μg/kg of the toxin in the interior foot tissue samples (only in two of five individuals), suggesting that direct exposure to the surrounding water containing *A. monilatum* led to higher accumulation of the toxin. However, this pattern differs from Jauffrais et al. (2013), who found higher concentrations of the lipophilic azaspiracids in gill and remaining tissues of blue mussel *Mytilus edulis* exposed to the dissolved form of the toxins while the toxins were mainly found in digestive gland when the animals were exposed to cells (i.e., the dinoflagellate *Azadinium spinosum*). A second hypothesis could be that goniodomins have a higher affinity for the gill and remaining tissues than digestive gland. However, this hypothesis needs further testing as previous studies on other lipophilic toxins, such as pectenotoxin (in the surf clam *Mesodesma donacium*; Blanco et al. 2018) or okadaic acid (in the clam *Anomalocardia flexuosa*; Leite et al. 2021), or even polar toxins such as tetrodotoxin (in Pacific oyster C*rassostrea gigas*; Dhanji-Rapkova et al. 2021) showed a higher concentration in the digestive gland compared with other tissues. Overall, in this work, the dynamic of goniodomins in gill and remaining tissues remain unclear, and the pattern of toxin distribution in exposed oysters should be explored further. The accumulation of the toxin, and thus the potential negative effects, may be different according to the route of exposure. Therefore it is important to examine the effects on oysters of dissolved GDA using a purified standard versus *A. monilatum* cells as has been done for other toxic species and their toxins (Jauffrais et al. 2013; Blanco 2018).

Over the time course of the exposure study, the accumulated GDA in the digestive gland appeared to show a trend of transformation to GDA-sa. This transformation, which results from the cleavage of the ester linkage under mild or relatively alkaline conditions, such as in sea water or in culture media (pH 8.2; Harris et al. 2023), also happened after at least 24 h in this study in the slightly acidic pH of the digestive gland (minimum pH 6; Blanco et al. 2018). A similar observation with pectenotoxin was also reported in the surf clam exposed to *Dinophysis acuminata*, with a 10- to 20-fold higher concentration of the seco-acid product of PTX-2 found in their digestive gland (Blanco et al. 2018). Likewise, New Zealand scallops *Pecten novaezelandiae* exposed to *D. acuta* (Suzuki et al. 2001) behaved similarly. The seco-acid may also result from an enzymatic activity (i.e., esterase). Indeed, MacKenzie et al. (2012) isolated and described an esterase from the green-lipped mussel *Perna canaliculus* with the ability to hydrolyze pectenotoxin-2 and -1. In addition, esterification of other lipophilic

toxins have been observed in bivalve's digestive glands, such as okadaic acid in Mediterranean mussel *Mytilus galloprovincialis* (Rossignoli et al. 2011), pinnatoxin-G and portimine-A (produced by *Vulcanodinium rugosum*) in a mussel species not specified (Aráoz et al. 2020), or gymnodimine-A in Mediterranean mussel (Ji et al. 2020). Those studies highlighted potential depuration strategies, which could include an esterification step, within the animal's systems when exposed to these lipophilic toxic compounds. The transformation kinetics of GDA to GDA-sa requires further investigation, especially between different tissues (e.g., digestive gland versus gill and remaining tissues where no apparent transformations were observed) and in different compartments (sea water versus shellfish tissues). The latter is important because disparities in the lifetime of GDA exist between different studies (Hintze 2021; Onofrio et al. 2020). The eastern oysters exposed to *A. monilatum* in this study completed the depuration of GDB and GDC after 24 h, while traces of GDA-sa and GDA (<LOD) remained in the digestive gland after 96 h of depuration. This study is the first to provide information about timing, compartmentalization, and eventual fate of goniodomins in eastern oyster exposed to a low concentration of *A. monilatum*.

The gene expression analysis in this study revealed an overexpression of actin coding gene(s) during exposure in both gill and digestive gland. A nonsignificant overexpression of *cadherin* (*cad*) gene was observed in the digestive gland of eastern oysters when *actin* expression was at its highest. Cadherin proteins are actin-dependent cell-to-cell adhesion molecules and function by interacting with the actomyosin cytoskeleton and have a role in morphogenesis and homeostasis (Oda et al. 2002; Mège and Ishiyama 2017). Similarly, a difference in *actin* gene expression was observed in Pacific oyster exposed to *A. minutum* cultures producing paralytic shellfish toxin or bioactive extracellular compounds (Mat et al. 2018). Overexpression of *cadherin* gene was also observed in eastern oyster challenged with the pathogenic bacteria *Roseovarius crassostreae* (McDowell et al. 2014). It remains to be determined whether the effects of *A. monilatum* and the goniodomins on eastern oyster *actin* and *cadherin* expression in this study were indirect (nonspecific) or direct. Furthermore, the apoptosis process may have been initiated in the eastern oysters exposed to *A. monilatum* in this study. Indeed, the gene coding for the anti-apoptotic (*aapo*) protein (protein-NR13 member of the Bcl-2 family; Medhioub et al. 2013) was overexpressed at the end of the exposure phase in the digestive gland and early in the depuration phase in the gill, and *caspase-1* (*casp*) was overexpressed at the beginning of the exposure phase in gill during this current study. The pro- and anti-apoptotic proteins are used as a signal in the cell to trigger or end apoptosis, which can be executed by caspases (O'Brien and Kirby 2008), such as the caspase-1 protein, which is directly involved in apoptosis as an executioner and plays a role in the inflammatory response (Qu et al. 2014). However, in the current study, pathological evaluation of gill and digestive gland tissues did not reveal any obvious apoptotic cells; thus, 96 h of exposure to a sublethal concentration of *A. monilatum* may only have initiated the apoptosis process in these eastern oysters. Finally, in this study, the transcript levels of *glutathione peroxidase* (*gpx*) and *glutathione reductase* (*gr*) in eastern oysters exposed to *A. monilatum* were over- and underexpressed, suggesting an oxidative stress in the eastern oyster tissues analyzed. The exposure to xenobiotics such as toxins can enhance the production of reactive oxygen species in organisms and lead to cell and tissue damage, such as lipid peroxidation, DNA damage, or immune dysfunction (Wheeler et al. 1990; Lesser 2006). One of the major antioxidant defenses in organisms is based on the induction or inhibition after stress of the glutathione peroxidase and reductase cycling (Cossu et al. 1997). Glutathione peroxidase catalyzes the transformation of peroxides and superoxide to nontoxic forms, while the glutathione reductase reduces oxidized glutathione to glutathione, used as a substrate by glutathione peroxidase (Wheeler et al. 1990). In this study, an overexpression was observed in both the gill and the digestive gland 6 h after the initial exposure to *A. monilatum*, and then the expression decreased after 24 h and increased at 96 h in the digestive gland, followed by a decrease in both tissues during the depuration phase. In the digestive gland of Mediterranean mussels exposed to *A. pacificum* (formerly the paralytic shellfish toxin producer *A. tamarense*), the activity of antioxidant enzymes, particularly glutathione peroxidase, exhibited two consequent typical bell-shaped patterns explained by the depletion and regeneration of glutathione (Qiu et al. 2013), which is consistent with the findings of the present study and suggested that the *gpx* and *gr* genes were expressed to reduce reactive oxygen species in oyster tissues. Similarly, Fabioux et al. (2015) observed a higher expression of *gr* in Pacific oyster exposed to *A. minutum* (paralytic shellfish toxin producer) and suggested an antioxidant reaction of the animals. Overall, modification in the expression of genes associated with physiological processes, including actin synthesis, oxidative stress response, apoptosis, and cell adhesion were observed in this study, indicating that eastern oysters experienced physiological stress as a result of exposure to *A. monilatum* (or its toxin), even at sublethal cell concentrations for 4 days. Finally, the role of additional bioactive extracellular compounds from *A. monilatum* should be explored. These compounds from *A. minutum* have been shown to cause negative effects in oysters (e.g., modification of valve activity or decreasing

fertilization in Pacific oyster; Castrec et al. 2018, 2019), while Tillmann et al. (2020) observed a lytic activity of another GDA producer (i.e., *A. taylorii*) on protists, suggesting that bioactive extracellular compounds may also exist in *A. monilatum* (authors' unpublished observations).

The environmental concentrations of *A. monilatum* in summer in tributaries of the lower Chesapeake Bay are in the range of the exposure concentration used in this study but can reach 100,000 cells/mL and last for several weeks to a month (Mulholland et al. 2018; Wolny et al. 2020), suggesting a potential threat for filter-feeding organisms, such as eastern oyster (Pease 2021; Pease et al. 2023). In addition, while toxin depuration occurred in 96 h, the amount of accumulated toxins and the depuration time could be more important when exposed to higher *A. monilatum* concentrations and could induce physiological stress and pathologies, as observed in Pease (2016). Further investigations on the direct and indirect effects of *A. monilatum* on eastern oyster would be beneficial for shellfish aquaculture industries in the region but also in the Mediterranean basin, where GDA and its producers were observed in areas of aquaculture (Zmerli Triki et al. 2015, 2016; Tillmann et al. 2020), and more recently in the Southeast Asian region (Abdullah et al. 2023).

## CONCLUSION

The present study showed that exposure of eastern oysters to an exponentially growing culture of *A. monilatum* at a sublethal concentration did not cause any notable changes on the host physiology and histologic presentation of host tissues. Goniodomins (GDA, GDB, GDC, and GDA-sa) were detected in gill, digestive gland, and remaining tissues of exposed eastern oysters and were mostly depurated after 96 h of depuration. Exposure to *A. monilatum* induced modification of the expression of genes involved in important physiological responses (i.e., apoptosis, oxidative stress responses, cellular structure, and general metabolism). Whether the negative effects could be attributed to *A. monilatum* cells and/or to the uptake and the accumulation of goniodomins in tissues should be explored in future studies. For instance, the effect of purified goniodomin or of unknown bioactive extracellular compounds on aquatic organisms has not been investigated. In addition, reports and field observations show that *A. monilatum* blooms recurred since 2007 in the lower Chesapeake Bay (Wolny et al. 2020), and goniodomins may potentially emerge in Europe and Southeast Asia (Moncheva et al. 2001; Espiña et al. 2016; Zmerli Triki et al. 2016; Tillmann et al. 2020; Abdullah et al. 2023); thus, the potential of goniodomins and goniodomin producers to cause negative effects on aquatic organisms should be considered, especially in coastal areas where shellfish and finfish farming activities are located.

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## CONFLICT OF INTEREST STATEMENT

Authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available from the corresponding author upon request.

## ETHICS STATEMENT

There were no ethical guidelines applicable to this study.

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## SUPPORTING INFORMATION

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