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Effects of the toxic dinoflagellate *Alexandrium monilatum* on survival, grazing and behavioral response of three ecologically important bivalve molluscs

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ABSTRACT

Little is known about interactions between shellfish and the toxic dinoflagellate *Alexandrium monilatum*. Toxic strains produce endotoxins with hemolytic and neurotoxic properties, and have been linked to fish and invertebrate kills. We experimentally assessed the survival, grazing and behavioral responses of three shellfish species to *A. monilatum*. Grazing studies were conducted with two size classes of *Crassostrea virginica*, *Mercenaria mercenaria*, and *Perna viridis*. These species inhabit areas where blooms of *A. monilatum* occur. Clearance rates of each species were depressed when exposed to toxic *A. monilatum* alone or with nontoxic *Pavlova* sp., in comparison to control animals fed only nontoxic algae. Exposure to toxic *A. monilatum* also caused shellfish to decrease shell valve gape. Intact cells of *A. monilatum* were found within shellfish feces, but the cells did not re-establish growing populations following gut passage. Survival of larval *M. mercenaria* and *C. virginica* was also tested when exposed to *A. monilatum* cells. Survival was significantly lower for larvae exposed to sonicated *A. monilatum*, in comparison to control larvae tested with nontoxic *A. tamarense*. Overall, the data indicate that blooms of *A. monilatum* can adversely affect some shellfish species by reducing valve gape and clearance rate, and by inducing larval mortality.

1. Introduction

Outbreaks of harmful algal species have increased in frequency, intensity and geographic distribution, causing public health and economic impacts (Hallegraeff, 1993; Ramsdell et al., 2005). Harmful algae include species that produce toxins or otherwise harm organisms directly or indirectly (see reviews in Shumway, 1990; Burkholder, 1998). "Toxic algae" is a term used in reference to species that produce toxic strains. Such species typically show a range in toxicity including some strains that are apparently unable to express toxicity (see reviews in Burkholder et al., 2005; Burkholder and Glibert, 2006). Nutrients added to coastal regions from cultural eutrophication are one noted cause of increased blooms (Anderson et al., 2002; Glibert et al., 2005), and the movement of shellfish stocks to different areas is one mechanism that may introduce some species of harmful dinoflagellates to new areas, as some are able to pass intact and viable through the shellfish digestive tract (Shumway et al., 1985b; Bricelj et al., 1993; Laabir and Gentien, 1999; Bauder and Cembella, 2000; Springer et al., 2002; Laabir et al., 2007; Hégaret et al., 2008a,b).

Dinoflagellate toxins can contaminate seafood and cause human illness and death when filter-feeding bivalves accumulate and concentrate these toxins that subsequently can be transferred up the food chain (Shumway, 1990; Burkholder, 1998; Landsberg, 2002). Toxin accumulation by shellfish is influenced by grazing rates and behavioral responses. Historically, shellfish were regarded as unaffected vectors of algal toxins, but effects of toxic algae on shellfish behavior, metabolism and survival have increasingly been recognized (Shumway and Cucci, 1987; Shumway and Gainey, 1992). Bivalve molluscs may close shell valves or reduce filtration rate when exposed to toxic dinoflagellates, which decreases exposure to the bloom (e.g. Shumway et al., 1985a; Shumway and Cucci, 1987; Gainey and Shumway, 1988a; Lesser and Shumway, 1993; Lassus et al., 1999; Hégaret et al., 2007). Toxins produced by dinoflagellates have caused shellfish mortalities, and toxic dinoflagellates can also affect shellfish recruitment and survival by causing

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behavioral alterations, depressed feeding, and impaired reproduction and growth (see reviews by Shumway, 1990; Burkholder, 1998; Landsberg, 2002).

The potentially toxic, chain-forming dinoflagellate *Alexandrium monilatum* (Howell) Balech blooms along the east coast of Florida (Howell, 1953; Norris, 1983), north to Chesapeake Bay (Morse, 1947), the Gulf of Mexico (Mississippi Sound, Perry et al., 1979; Texas, Connell and Cross, 1950; Gunter, 1942; Ray and Aldrich, 1967), South America and Central America (Venezuela, Costa Rica, Caribbean Sea, Halim, 1967; Ferraz-Reyes et al., 1985) and the Pacific Ocean off Ecuador (Balech, 1995) (Table 1). Blooms of *A. monilatum* have been associated with mortalities of fish and invertebrates (Table 1). Recently, an *A. monilatum* bloom in the lower York River, Virginia, USA caused mortalities of veined rapa whelks (*Rapana venosa*; Harding et al., 2009). Connell and Cross (1950) described a bloom of *A. monilatum* in Offats Bayou near Galveston, TX, USA, during the summer 1949, and linked sewage pollution to stimulation of the bloom. This bloom occurred immediately after heavy precipitation flushed sewage-polluted surface water into the bayou. Also, areas in which heavy growth of this organism recurred frequently were in wind-sheltered areas of the bayou where effluents from private septic tanks infiltrated the estuary. Based upon the minimum nitrogen cell quota of this species, it has been suggested that high N flux would be required to support development of blooms of *A. monilatum* (Juhl, 2005).

The toxin(s) from *A. monilatum* cause paralysis and mortality in finfish (Gates and Wilson, 1960) and have been shown to be toxic to homeotherms (Erker et al., 1982; Erker et al., 1985). Heating or freezing *A. monilatum* cells increased fish mortality in laboratory experiments, suggesting that this dinoflagellate produces endotoxins released with cell lysis, and maximal toxicity was reported in senescent cultures with high cell autolysis (Aldrich et al., 1967). Ray and Aldrich (1967) found that oysters (*Crassostrea virginica* Gmelin) rarely opened shell valves or filtered when exposed to *A. monilatum*. Polychaetes (*Polydora* sp.) inhabiting the oyster shells, along with fish in separate bioassays, had high mortality rates. Sievers (1969) compared the toxicity of a strain of *A. monilatum* versus a strain of *Karenia brevis* to annelids, crustaceans, molluscs and finfish (sheepshead minnow, *Cyprinodon variegatus* Lacepede). Sheepshead minnows were sensitive to both dinoflagellate species, but mean times to death indicated that they were more sensitive to the *K. brevis* strain than to *A. monilatum*. In contrast, the annelids and molluscs were more sensitive to *A. monilatum* than to *K. brevis*, and the crustaceans were resistant to both species of dinoflagellate tested.

Schmidt and Loeblich (1979) found paralytic shellfish toxins (saxitoxin and gonyautoxins) in laboratory cultures of *A. monilatum*. An extract of *A. monilatum* had neurotoxic and hemolytic properties and was chemically different from saxitoxin and related compounds (Clemons et al., 1980; Bass et al., 1983; Erker et al., 1985). A toxin produced by *A. monilatum* was purified and identified as goniodomin A (Hsia et al., 2005), which also is produced by *Alexandrium pseudogoniaulax* (Murakami et al., 1988). The purified toxin exhibited hemolytic activity (P. Moeller, National Oceanic and Atmospheric Administration – National Ocean Service [NOAA-NOS], Charleston, SC, USA, personal communication, January 2008). Extracts of *A. monilatum* cells were hemolytic to erythrocytes from several mammalian species including humans, and lethal to cockroaches, guppies and mice (Clemons et al., 1980; Bass et al., 1983).

The shellfish species selected for this research are important ecologically and commercially along the East and Gulf Coasts of the U.S. and inhabit areas where blooms of *A. monilatum* have occurred. The objectives of this study were to: (1) examine the effects of *A. monilatum* at bloom density upon clearance rates of eastern oysters (*C. virginica*), northern quahogs (*M. mercenaria* Linnaeus), and green mussels (*P. viridis* Linnaeus); (2) evaluate shellfish behavior (valve gape) in response to *A. monilatum*; (3) assess impacts of *A. monilatum* upon the survival of larval *C. virginica* and *M. mercenaria*; and (4) assess survival of *A. monilatum* after ingestion by shellfish to gain insights into whether or not shellfish may act as vectors for the introduction of *A. monilatum* if transported to new areas.

Table 1
Historic record of blooms of *Alexandrium monilatum* in southeastern and Gulf Coast ecosystems (n.a. = not available; also see Landsberg, 2002; Juhl, 2005).

Location	Date	Reported mortality	Density (Cells L^{-1})	Salinity	Temperature (°C)	Reference
Indian River and Sarasota, FL	August–September 1951	Finfish	n.a.	18–32	30–34	Howell (1953)
Offats Bayou, Galveston, TX	Summer 1949	Finfish, shrimp, crabs	n.a.	n.a.	n.a.	Connell and Cross (1950)
Fort Myers to Naples, FL	1966	Finfish – *Caranx* spp., *Strongylura marina*, *Lagodon rhomboids*	n.a.	>32	>29	Williams and Ingle (1972)
Galveston, TX[a]	1971–1972	Finfish, shellfish, annelids, coelenterates, crustaceans, echinoderms	1.88×10^6 "massive bloom"	30–34	29–32	Wardle et al. (1975)
Indian River, FL	July 1977	n.a.	8.9×10^5	32.0	31.5–32	Norris (1983)
Melbourne Beach, FL	July 1977	n.a.	1.7×10^6	30.5	29.5	Norris (1983)
Port St. John, FL	September 1977	Finfish (thousands)	Bloom	n.a.	n.a.	Norris (1983)
Mississippi Sound (MS-LA)	August 1979	No deaths	1.65×10^7	24–26	30–31	Perry et al. (1979)
Mobile Bay, AL	August 1979	Finfish	n.a.	n.a.	n.a.	Perry et al. (1979)
Pensacola Bay, FL	August 1979	Finfish	3.18×10^7	14	28	Perry et al. (1979)
Indian River, FL	September 1979	n.a.	$<1 \times 10^3$	16–21	24.5–30	Norris (1983)
Gulf of Cariaco, Venezuela	January–February, April–May 1984; January 1985	n.a.	0.1 to 4×10^4	n.a.	n.a.	Ferraz-Reyes et al. (1985)
Coastal MS	1998	Zooplankton, ichthyoplankton	n.a.	n.a.	n.a.	ICES (1999)
York River, VA	September 2007	Veined rapa whelks (*Rapana venosa*)	4.0×10^7	22.4–22.8	27–28	Harding et al. (2009)

[a] 30 species: *Americonuphis magna*, *Anadara brasiliana*, *Anadara ovalis*, *Arenaeus cribarius*, *Bascanichthys scuticaris*, *Bunodosoma cavernata*, *Callinectes sapidus*, *Callinectes similis*, *Clibanarius vittatus*, *Crassostrea virginica*, *Cyprinidon variegates*, *Donax variabilis*, *Emerita benedicti*, *Gobiesox punctulatus*, *Hepatus epheliticus*, *holothuroids*, *Hypleurochilus geminatus*, *Isocheles wurdemanni*, *Mellita quinquesperforata*, *Menippe mercenaria*, *Micropholis atra*, *Nereis* sp., *Oliva sayana*, *Petrolisthes armatus*, *Polinices duplicata*, *Porcellana sayana*, *Spisula solidissima*, *Siphonaria pectinata*, *Terebra cinerea*, *Thais haemastoma*.

2. Materials and methods

2.1. Algal cultures

Toxic *A. monilatum* (strain AMO3; cell length 28–52 μm – from S. Morton, NOAA-NOS, Charleston, SC, USA) was mass-cultured in 10–20-L Nalgene® polycarbonate carboys with L1 medium (Guillard and Hargraves, 1993) at 23 °C on a 12:12 h light/dark (L/D) cycle at ~170 μM photons m^{-2} s^{-1}. The benign alga *Cryptomonas* sp. (clone HP9101; cell length 10–15 μm – from D. Stoecker, Horn Point Environmental Laboratory, University of Maryland, Cambridge, MD, USA), which was used as a food source for control animals, was batch-cultured in 2-L Erlenmeyer flasks with F/2-Si medium (Guillard, 1975) at 22 °C on a 12:12 h L/D cycle at ~50 μM photons m^{-2} s^{-1}. This organism is much smaller than *A. monilatum*, but a nontoxic strain of *A. monilatum* was not available for comparison. As a second food source for control animals, a nontoxic strain of *Alexandrium tamarense* (Lebour) Balech (clone CCMP115, cell length 36–44 μm – from the Provasolli-Guillard Center for the Culture of Marine Phytoplankton [CCMP], Bigelow Laboratory for Ocean Sciences, Bigelow, ME, USA) was included as a control similar in size to *A. monilatum*. Batch cultures of *A. tamarense* were grown in 4-L Erlenmeyer flasks with L1 medium at 23 °C with an 8:16 h L/D cycle at ~115 μM photons m^{-2} s^{-1}. All growth media were prepared with filtered artificial seawater (ASW–Coralife® scientific grade marine salt, mixed to the desired salinity using deionized water; filter pore size 0.45 μm) and sterilized by autoclaving. Cultures were unialgal but contained bacteria.

Sheepshead minnows (*Cyprinodon variegatus*, $n = 3$) were exposed to *A. monilatum* (5.5×10^2 cells ml^{-1}) that were lysed by sonication, in log growth phase, and senescent phase, to verify that the strain was toxic prior to experiments (Table 2). Control fish were maintained in filtered ASW (salinity 30). Sheepshead minnows have been reported to be sensitive to *A. monilatum* toxin(s), and provided an indication of the toxicity of the *A. monilatum* strain used in the experiments (Sievers, 1969). No fish mortalities were observed in ASW or with *A. monilatum* in log growth phase, whereas sonicated and senescent *A. monilatum* caused fish mortality (Table 2). Subcultures of *A. monilatum* and *A. tamarense* were sent to the Marine Biotoxins Program, NOAA-NOS, Charleston, SC, USA for toxin analysis after completion of experiments. Cytotoxicity assays (rat pituitary cell line GH4C1) were conducted on 1 L of each culture following procedures in Hsia et al. (2005). The *A. monilatum* culture was confirmed as toxic whereas, the *A. tamarense* culture was confirmed to be a nontoxic clone by this test.

Cultures of *A. monilatum* in stationary phase were used in the experiments. Prior to use, the cultures were shaken to break up chains (S. Morton, personal communication). The cultures of benign *Cryptomonas* sp. and the nontoxic strain of *A. tamarense* used in the experiments were in log growth phase. Subsamples of each algal culture were preserved with acidic Lugol's solution (Vollenweider, 1974) before each experiment to quantify initial cell densities. The samples were analyzed at 40–400× with a BH2 Olympus light microscope (Olympus Corporation, Melville, NY, USA). Sedgwick–Rafter counting slides were used to quantify *A. monilatum* cells (Kutkuhn, 1958), and Palmer–Maloney counting chambers were used to quantify *A. tamarense* and *Cryptomonas* sp. cells (Throndsen, 1995). Filtered ASW (0.2-μm pore size, salinity 30) was used to adjust algal concentrations to desired initial densities.

Table 2
Bioassays with sheepshead minnows (*Cyprinodon variegatus*).

Treatment ($n = 3$)	% Mortality ($t = 60$ min)	% Mortality ($t = 90$ min)
Control	0	0
Log phase *Alexandrium monilatum*	0	0
Senescent *Alexandrium monilatum*	100	100
Lysed *Alexandrium monilatum*	67	100

2.2. Maintenance of shellfish

Shellfish were obtained from various locations and, based upon local knowledge, were assumed to have had no prior exposure to blooms of *A. monilatum*. Two co-occurring size classes of *P. viridis* (mean shell height ± standard deviation [SD]; 26.7 ± 2.8 mm and 48.9 ± 8.4 mm) assumed to represent different cohorts, were collected off the Gandy Bridge in Tampa Bay, FL. Larval *P. viridis* were not available for testing during this study. Two size classes of *C. virginica* (shell height 29.1 ± 0.9 mm; shell height 69.8 ± 2.3 mm) were obtained from Pemaquid Oyster Company, Waldoboro, ME, USA. Large *M. mercenaria* (shell height 44.1 ± 0.8 mm) were supplied by J&B AquaFood, Jacksonville, NC, USA, and small *M. mercenaria* (shell height 13.3 ± 0.4 mm) were obtained from Millpoint Aquaculture, Core Sound, NC, USA. Reproductive status of the shellfish was not determined. The responses of the larger size class of shellfish were compared to one another and the responses of the smaller size class of shellfish were compared. Larval *C. virginica* were from Narragansett Bay, RI, USA, and larval *M. mercenaria* were obtained from Cherrystone Aquafarms, Cheriton, VA, USA. D-stage larvae (age 10–14 days; mean length, 240 ± 11 μm) were used for experiments immediately upon receipt.

Shellfish were scrubbed to remove attached organisms, and the shells were rinsed with deionized water. The shellfish were acclimated to laboratory conditions at 22–25 °C and salinity 30–32 ASW for at least 1 week prior to experiments in separate 946-L and 492-L recirculating systems. During the acclimation period, shellfish were fed Instant Algae© Shellfish Diet 1800© or Instant Algae© *Pavlova* sp. daily. Shellfish were not fed for 24–48 h prior to experiments to clear the digestive tracts of previously ingested materials.

2.3. Short-term feeding experiments

Experiments to estimate clearance rates were conducted in individual, static systems with two size classes of *C. virginica, M. mercenaria* and *P. viridis*. Each size class was tested separately in short-term trials (1–2 h, $n = 10$, one individual per replicate). Test shellfish were exposed to a bloom density of toxic *A. monilatum* (5.5×10^2 cells ml^{-1}), based upon bloom densities reported in the literature (e.g. Perry et al., 1979; Norris, 1983). As a second treatment, shellfish were exposed to a mixed suspension of toxic *A. monilatum* (5.5×10^2 cells ml^{-1}) and nontoxic *Pavlova* sp. (1×10^4 cells ml^{-1}) to assess whether or not the presence of a benign algal food source would promote higher grazing upon *A. monilatum*. Bricelj et al. (1991) reported that *M. mercenaria* grazed on a toxic strain of *Alexandrium fundyense* (strain GtCA29) only when it was mixed with a nontoxic alga, *Thalassiosira weissflogii*. In the present study, *Pavlova* sp. was used in mixed-food trials rather than *Cryptomonas* sp. because preliminary tests indicated that the cryptophyte lysed when mixed with *A. monilatum* whereas, *Pavlova* sp. cells remained intact. Two sets of control exposures were used: the first set was with the cryptophyte *Cryptomonas* sp. (1×10^4 cells ml^{-1}); and the second was with nontoxic *A. tamarense* (5.5×10^2 cells ml^{-1}). Gentle aeration was used to maintain cells in suspension. Two control containers with empty shells were used to correct for cell settlement during experiments, following Shumway et al. (1985b).

All clearance rates of shellfish were weight-specific, and were estimated using an indirect method wherein depletion of algal cells from the water was quantified over time (Coughlan, 1969). Individual shellfish were placed into separate 1-L glass beakers containing 500 ml of algal suspension, or in separate 2-L plastic buckets containing 1 L of algal suspension, depending upon the size of the individual shellfish being tested. Experiments began when the bivalve mollusc opened its shell valves, or when an extended siphon was observed (*M. mercenaria*).

Shellfish exposed to *A. monilatum* and the *A. monilatum/Pavlova* sp. mixture were allowed to graze for 2 h. Subsamples of the algal suspension (5 ml from each container, $n = 3$, preserved in 0.5% paraformaldehyde) were taken initially and after 2 h. Shellfish fed control algae were maintained for 1 h, with subsamples of the algal suspension (5 ml from each container, $n = 3$; preserved in 0.5% paraformaldehyde) taken initially, at 30 min and 1 h. The control containers with empty shells were sampled along with the containers with live shellfish. Samples were stored in darkness at 4 °C until analysis within 1 month. Cells were enumerated using an EPICS Altra flow cytometer (Coulter Corporation, Miami, FL, USA) equipped with a 488-nm argon laser that was focused to an elliptical point of interrogation (6 μm height × 112 μm width; Parrow et al., 2002). The cells passed through the laser beam in a 100 μm-channel quartz flow cell. The optical alignment and signal stability of the flow cytometer were checked before each use with 10 μm-diameter fluorescent latex microspheres (Coulter Corporation). The instrument was set to analyze chlorophyll *a* fluorescence (>630 nm) and forward-angle light scatter, which records relative particle size. Logarithmic fluorescence versus forward-angle light scatter was displayed graphically using EXPO32 analysis software (Cytometry Systems, Sheffield, UK) and stored in listmode format. The sum of events (number of algal cells) in a sample was determined by defining regions on biplots that identified the algal species based upon these characteristics. The volume of sample analyzed was estimated gravimetrically (Newell et al., 1989). The number of cells ml^{-1} was estimated by dividing the recorded number of events by the total volume analyzed, and rounding to the nearest 10 cells. Samples from each replicate container were counted until all sample counts were within 20% of one another to account for sampling error, and the mean cell concentration ±1 standard error (SE) was reported.

The clearance rate of each individual shellfish was calculated according to Coughlan (1969) as:

$$\text{clearance rate}\,(\text{ml h}^{-1}) = \{(\ln(C_0/C_T) - \ln(C'_0/C'_T)\} \cdot V/T$$

wherein C_0 and C_T were the initial and final cell concentrations during a time interval; C'_0 and C'_T were the initial and final cell concentrations of particles during a time interval in the control vessels that contained the empty shells; V was the volume of food suspension; and T was the time interval between C_0 and C_T or C'_0 and C'_T (h). The term $\ln(C'_0/C'_T)$ was omitted from the equation if the initial (C'_0) and final (C'_T) concentrations in the control vessels were not significantly different during a time interval (Student's *t* test, SAS Institute, Inc. 1999, $\alpha = 0.05$). Clearance rates were estimated from T_o to $T = 30$ min for the control shellfish with benign algae and from T_o to $T = 120$ min for the treatments with shellfish and *A. monilatum*. After experiments, the tissues were removed from the shells and dried at 60 °C for 24 h. The tissue dry weight was used to calculate the weight-specific clearance rate ($ml\ h^{-1}\ g^{-1}$) for each shellfish.

Shellfish that were known to have been disturbed, and animals with a clearance rate of zero, were not included in statistical analyses and were treated as missing data. Cochran–Mantel–Haenszel chi-square test controlling for treatment was used to test for the randomness of the number of replicates missing from the data set for each size class of shellfish species tested (PROC FREQ – CMH; SAS Inc., 1999). It was determined that the number of non-functioning replicates considered missing data was random (CHM; df = 5, $p = 0.3448$). Two-way analysis of variance (ANOVA – GLM; SAS Inc., 1999) was used to assess main and interactive effects of treatment (algal species) and shellfish species upon the response variable (clearance rate) for large and small size class shellfish. A Tukey–Kramer multiple comparisons test (SAS Inc., 1999) was used to determine statistically different pair-wise comparisons ($\alpha = 0.05$).

2.4. Survival

Both size classes of *C. virginica* and *M. mercenaria* survived 24 h of exposure to toxic *A. monilatum*, but *P. viridis* did not. Bioassays with both size classes of *P. viridis* ($n = 10$ each) were conducted to determine the time required for half of the mussels to become moribund (LT_{50}). Experimental conditions were the same as for the short-term feeding experiments, and shellfish were observed hourly. Shellfish were exposed to a bloom density of toxic *A. monilatum* (5.5×10^2 cells ml^{-1}), nontoxic *Cryptomonas* sp. (1×10^4 cells ml^{-1}) or nontoxic *A. tamarense* (5.5×10^2 cells ml^{-1}). Moribund status was defined as loss of valve closure ability, a condition that frequently precedes death (Ray and Aldrich, 1967). A needle was used to prod the mantle edge to check moribund status. Moribund animals were placed in filtered (0.2 μm) ASW for 24 h to monitor possible recovery.

2.5. Fecal analyses

Feces of *C. virginica* and *M. mercenaria* were observed after animals were exposed for 24 h to toxic *A. monilatum* to assess survival of *A. monilatum* cells after ingestion, following Hégaret et al. (2008a,b). Fecal samples of *P. viridis* were checked after 8 h of exposure. Experimental conditions were the same as for the short-term feeding experiments. Shellfish were removed gently from experimental containers after the exposure period and rinsed with deionized water to remove any free-swimming dinoflagellates from the shells. Each shellfish was then placed into a separate container with filtered (0.2-μm pore size; salinity 30) ASW to depurate. A 2-ml sample was taken from each container initially and examined with light microscopy to ensure that no motile dinoflagellates were transferred with the shellfish. Well-formed and consolidated fecal pellets were collected from the containers after 24 h using a Pasteur pipette. The remaining suspension from each replicate container was filtered through a 30-μm mesh sieve to collect any additional feces. Feces were rinsed from the sieve with ASW into 5-ml vials. The shellfish were then transferred to separate containers with filtered ASW to depurate for an additional 24 h, followed by a second collection of feces as described above.

Shellfish feces were examined with light microscopy (Olympus AX70, 40–600×) and photographed (DEI-750 cooled-chip CCD camera, Optronics Engineering, Goleta, CA, USA) to assess whether or not *A. monilatum* cells had passed through the digestive tract intact. Cell viability was tested by inoculating feces collected from each animal after 24 and 48 h of depuration (Bauder and Cembella, 2000). Fecal samples ($n = 10$) were vortex-mixed and 1 ml of each fecal suspension was inoculated into each of two test tubes, one containing 10 ml of L1 growth medium, and one containing 10 ml of natural seawater (NSW, salinity 30; from CCMP, Boothbay Harbor, ME, USA) (Hégaret et al., 2008a). Controls consisted of 1 ml of *A. monilatum* stock culture inoculated into test tubes containing 10 ml of L1 medium ($n = 10$) and into tubes with 10 ml of NSW (salinity 30; $n = 10$). The test tubes were incubated at 23 °C on a 12:12 h L/D cycle at 170 μM photons $m^{-2}\ s^{-1}$. The inoculated fecal subcultures were observed for *A. monilatum* cells at 7-day intervals, including the first week, for 4 weeks and then twice

per month for an additional 2 months using an Olympus CK-40 inverted microscope (40–100×). Subsamples (1.5 ml) from the test tubes with inoculated feces were preserved in acidic Lugol's solution and checked for *A. monilatum* cells after 3 months (Olympus BH-2; 40×). Subsamples taken weekly from the *A. monilatum* controls were preserved in acidic Lugol's solution, and cells were assessed by light microscopy as above.

2.6. Valve gape

The valve gape of the large size class of *C. virginica*, *M. mercenaria* and *P. viridis* (n = 3, 4 individuals per replicate) exposed to toxic *A. monilatum* (5.5×10^2 cells ml^{-1}) versus nontoxic *Pavlova* sp. (5.0×10^4 cells ml^{-1}) was measured as an indicator of avoidance behavior (Shumway and Cucci, 1987; Shumway and Gainey, 1992; Hégaret et al., 2007). Bivalves exposed to low algal concentrations often reduce valve gape (Riisgård et al., 2003). A higher cell density of the control alga was used, therefore, in the valve gape experiments to avoid a major decrease in residual cell concentration attributable to shellfish filtering. A higher cell density of *A. monilatum* was not needed because preliminary clearance rate experiments indicated that residual cell densities of *A. monilatum* still were at >50% of the initial cell density after 2 h of shellfish feeding. Valve gape was measured with optical fibers (data logged once per second) using the methodology and instrument described in Frank (2003) and Frank et al. (2007), except that our system had multiple channels so that up to six animals could be tested at the same time. A Campbell Scientific CR10X data logger (Campbell Scientific, Inc., Logan, UT, USA) recorded the data (voltage output s^{-1}). Voltage was used to estimate valve gape (mm of distance between shells) by plotting the average change in voltage output versus distance (mm increments).

Valve gape experiments were conducted in a container with 8 L of the algal suspension under gentle aeration. Animals were prepared for analysis following Frank et al. (2007), except that Krazy Glue® (Elmer's Products Inc, Columbus, OH, USA) was used to attach the Velcro® (Velcro USA Inc., Manchester, NH, USA) and the optical fibers to each shellfish. Preliminary studies demonstrated that this glue does not adversely affect shellfish and has a short drying time. The voltage output recorded when the shellfish valves were completely closed was used as time zero. All animals were allowed at least 30 min to recover from the aerial exposure (~10 min) experienced during the gluing process. Subsequent opening and closing movements of the shell valves were recorded for at least 2 h. A two-way ANOVA (generalized linear model—GLM; SAS, 1999) was used to assess significant differences between main and interactive effects of shellfish species and algae on valve gape. A Tukey–Kramer multiple comparisons test was used to identify significantly different, pair-wise comparisons (α = 0.05).

2.7. Larval bioassays

The effects of toxic *A. monilatum* upon survival of larval *M. mercenaria* and *C. virginica* were tested using *A. monilatum* that was (1) unconstrained, (2) restricted (held in dialysis tubing), or (3) sonicated. Constrained cells were used to test for the presence of exotoxins from *A. monilatum* that may diffuse through the dialysis tubing, analogous to the approach of Burkholder and Glasgow (1997) and Springer et al. (2002). Previous research indicates that *A. monilatum* produces endotoxins that are released by cell lysis (Aldrich et al., 1967), so the effect of both sonicated and live *A. monilatum* cells on shellfish larvae was tested. The *A. monilatum* populations used in these experiments were in log growth phase to minimize cell autolysis (Aldrich et al., 1967). Controls consisted of larvae exposed to nontoxic *A. tamarense* that was unconstrained, held in dialysis tubing, or sonicated. Larvae were placed into Falcon® polystyrene multi-well plates and observed under a dissection microscope (Olympus SZX12) (n = 10 wells, 25 larvae per well). Only active, apparently healthy larvae were selected for use.

For the unconstrained experiments, culture (*A. monilatum* or *A. tamarense*) was pipetted into each well (n = 10) that contained 5 ml of ASW, for a final concentration of 5.5×10^2 cells ml^{-1}. Suspensions of *A. monilatum* and *A. tamarense* (5.5×10^2 cells ml^{-1}) were each sonicated with a Fisher Scientific 550 Sonic Dismembrator for 60 s bursts for a total of 10 min, chilling at 3 °C between sonications. Suspensions were checked with light microscopy (Olympus IX-70, 40–100×) to ensure that all cells had lysed before use. The suspension (5 ml) then was added to each well (n = 10). Dialysis tubing (molecular weight cut-off 12,000–14,000 Da) was used to prevent direct contact between algae and larvae. Cultures of *A. monilatum* or *A. tamarense* were added to the dialysis tubing for a final concentration of 5.5×10^2 cells ml^{-1} (n = 10; with 5 ml ASW) and the tubing was placed into the wells containing the larvae and 5 ml of ASW.

The well plates with larvae and all controls were placed on a light microscope stage (Olympus CK-40). Mortality (considered as loss of ciliary movement for >1 min, after Springer et al., 2002) was monitored for 2 h to assess survival. The data were not normally distributed and, thus, a Wilcoxon two-sample test (SAS, 1999) was used to determine statistically different, pair-wise comparisons (α = 0.05).

3. Results

3.1. Short-term feeding experiments

3.1.1. Large shellfish size class

Clearance rate was dependent upon algal treatment and varied between shellfish species (two-way ANOVA; algal treatment: df = 3, F = 128.40, $p < 0.0001$; shellfish treatment: df = 2, F = 112.49, $p < 0.0001$). There was also a significant interaction effect (two-way ANOVA; interaction: df = 6, F = 25.42, $p < 0.0001$), which precluded comparisons across the algal species and shellfish species main effects. Large size classes of *C. virginica*, *P. viridis* and *M. mercenaria* fed *A. monilatum* or the *A. monilatum* + *Pavlova* sp. mixture had significantly lower clearance rates than control animals fed *Cryptomonas* sp. or nontoxic *A. tamarense* (Table 3, Fig. 1).

Clearance rates of the large class size of shellfish exposed to *A. monilatum* or the *A. monilatum* + *Pavlova* sp. mixture were

Table 3
Results of multiple comparison tests for the pair-wise effects of algal treatment on clearance rates (ml h^{-1} g^{-1}) of larger size class of *Crassostrea virginica*, *Mercenaria mercenaria* and *Perna viridis* in laboratory experiments (n.s. = not significant).

Shellfish species	*Alexandrium monilatum* versus Mix	*Alexandrium monilatum* versus *Cryptomonas*	*Alexandrium monilatum* versus *Alexandrium tamarense*	*Cryptomonas* versus Mix	*Cryptomonas* versus *Alexandrium tamarense*	*Alexandrium tamarense* versus Mix
Crassostrea virginica	n.s.	$p = 0.0057$	$p = 0.0357$	$p = 0.0024$	n.s	$p = 0.0214$
Perna viridis	n.s.	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0177$	$p < 0.0001$
Mercenaria mercenaria	n.s.	$p < 0.0001$	$p = 0.0128$	$p = 0.0002$	n.s.	$p = 0.0279$

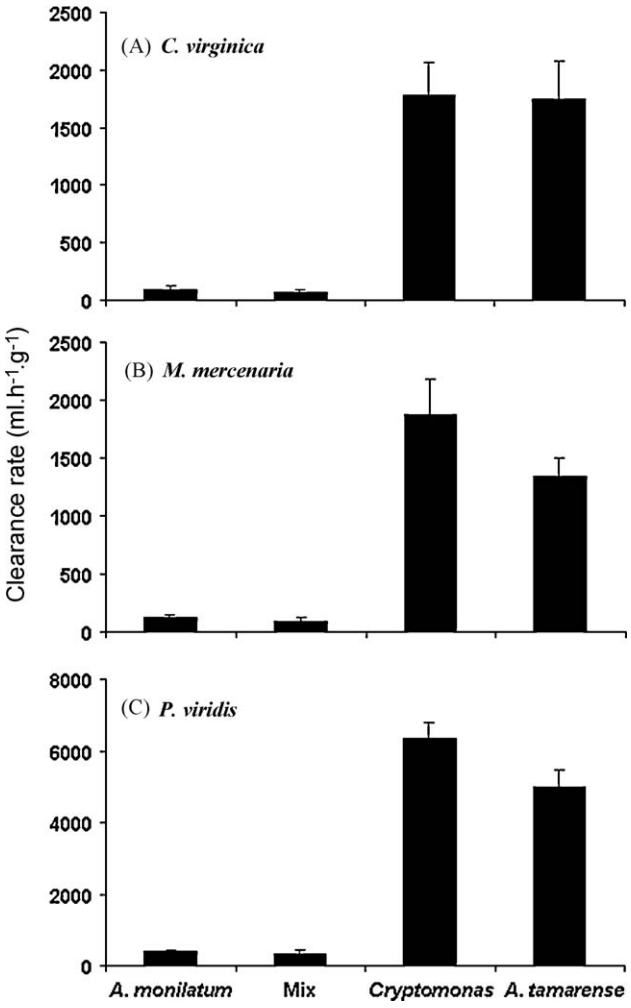

Fig. 1. Weight-specific clearance rates (means ± 1 SE) of large size class shellfish. (A) Large *Crassostrea virginica* exposed to toxic *Alexandrium monilatum* ($n = 5$), *A. monilatum + Pavlova* sp. mixture ($n = 6$), *Cryptomonas* sp. ($n = 7$) and nontoxic *Alexandrium tamarense* ($n = 4$). (B) Large *Mercenaria mercenaria* exposed to toxic *A. monilatum* ($n = 10$), the *A. monilatum + Pavlova* sp. mixture ($n = 7$), *Cryptomonas* sp. ($n = 9$) and nontoxic *A. tamarense* ($n = 9$). (C) Large *Perna viridis* exposed to toxic *A. monilatum* ($n = 8$), the *A. monilatum + Pavlova* sp. mixture ($n = 6$), *Cryptomonas* sp. ($n = 10$) and nontoxic *A. tamarense* ($n = 6$).

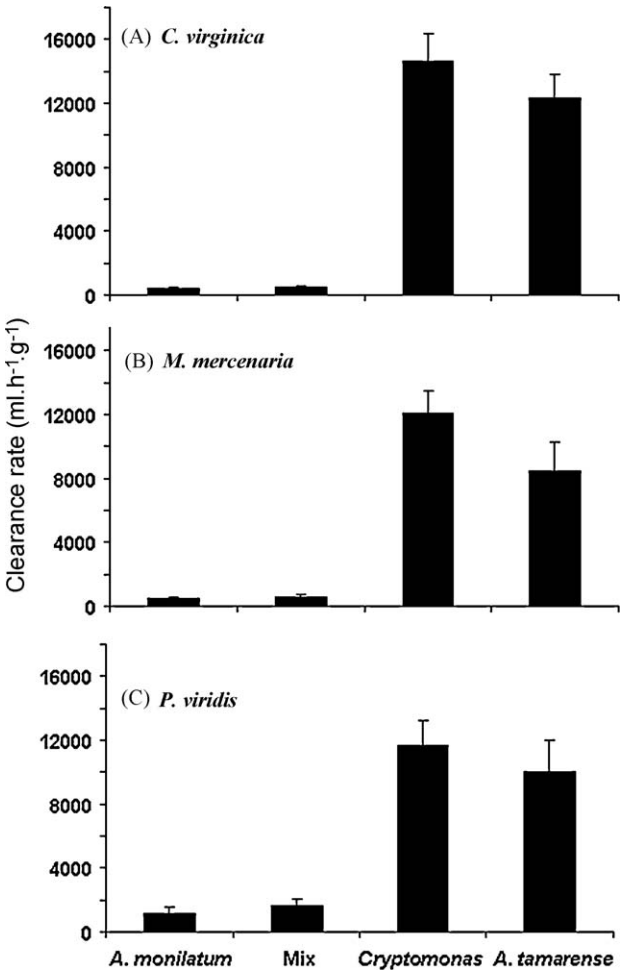

Fig. 2. . Weight-specific clearance rates (means ± 1 SE) of small size class shellfish. (A) Smaller class size *Crassostrea virginica* exposed to toxic *Alexandrium monilatum* ($n = 8$), the *A. monilatum + Pavlova* sp. mixture ($n = 5$), *Cryptomonas* sp. ($n = 7$) and nontoxic *Alexandrium tamarense* ($n = 5$). (B) Small *Mercenaria mercenaria* exposed to toxic *A. monilatum* ($n = 7$), the *A. monilatum + Pavlova* sp. mixture ($n = 6$), *Cryptomonas* sp. ($n = 9$) and nontoxic *A. tamarense* ($n = 8$). (C) Small *Perna viridis* exposed to toxic *A. monilatum* ($n = 7$), the *A. monilatum + Pavlova* sp. mixture ($n = 7$), *Cryptomonas* sp. ($n = 10$) and nontoxic *A. tamarense* ($n = 5$).

comparable for the three shellfish species (Table 3). The larger size class of *P. viridis* fed *Cryptomonas* sp. or nontoxic *A. tamarense* had a higher mean weight-specific clearance rate than *C. virginica* and *M. mercenaria* fed the same control species ($p < 0.0001$). Clearance rates of *C. virginica* and *M. mercenaria* fed *Cryptomonas* or *A. tamarense* were comparable, but *P. viridis* had a lower mean clearance rate when fed *A. tamarense* compared to clearance rates of mussels fed cryptomonads ($p = 0.0177$).

3.1.2. Small size class of shellfish

The smaller size class of *C. virginica*, *M. mercenaria* and *P. viridis* had comparable clearance rates when fed *Cryptomonas* sp. versus nontoxic *A. tamarense*, and comparable clearance rates when fed *A. monilatum* versus the *A. monilatum + Pavlova* sp. mixture (Table 4, Fig. 2). Clearance rates of the smaller size class of shellfish fed *A. monilatum* or the *A. monilatum + Pavlova* sp. mixture were significantly lower than clearance rates of shellfish fed *Cryptomonas* sp. or nontoxic *A. tamarense* (Table 4, Fig. 2). There was a significant effect of algal treatment upon clearance rates of *C. virginica, M. mercenaria* and *P. viridis*, but there was no difference in clearance rates between the shellfish species, and no shellfish species-by-algal treatment interaction (two-way ANOVA; algal treatment: df = 3, $F = 69.97$, $p < 0.0001$; shellfish species: df = 2, $F = 0.42$, $p = 0.6581$; interaction: df = 6, $F = 0.86$, $p = 0.5305$).

Table 4
Results of multiple comparison tests for the pair-wise effects of algal treatment on clearance rates (ml h^{-1} g^{-1}) of the smaller size class of *Crassostrea virginica*, *Mercenaria mercenaria*, and *Perna viridis* in laboratory experiments (n.s. = not significant).

Shellfish species	*Alexandrium monilatum* versus mix	*Alexandrium monilatum* versus *Cryptomonas*	*Alexandrium monilatum* versus *Alexandrium tamarense*	*Cryptomonas* versus mix	*Cryptomonas* versus *Alexandrium tamarense*	*Alexandrium tamarense* versus mix
Crassostrea virginica	n.s.	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	n.s	$p < 0.0001$
Perna viridis	n.s.	$p < 0.0001$	$p = 0.0008$	$p < 0.0001$	n.s	$p = 0.0072$
Mercenaria mercenaria	n.s.	$p < 0.0001$	$p = 0.0015$	$p < 0.0001$	n.s.	$p = 0.0031$

3.2. Survival

Both size classes of *C. virginica* and *M. mercenaria* survived 24 h of exposure to toxic *A. monilatum*, whereas both size classes of *P. viridis* were moribund within 24 h of exposure. Moribund status (defined as loss of valve closure ability) was assessed hourly, and the median time (LT_{50}) was 16 and 10 h, respectively, for the larger and smaller size classes of *P. viridis*. Moribund mussels did not recover when subsequently placed in filtered ASW.

3.3. Fecal analyses

Qualitative analysis of fecal material collected from *C. virginica*, *M. mercenaria*, and *P. viridis* after 24 and 48 h of depuration indicated that *A. monilatum* cells passed intact through the digestive tract of these shellfish species (Fig. 3). Pseudofeces production was not detected during the feeding experiments, but the large *P. viridis* produced copious amounts of mucus containing intact *A. monilatum* cells after 8 h of exposure to *A. monilatum*.

Regular monitoring of the inoculated feces from the smaller class size *C. virginica, M. mercenaria* and *P. viridis* (all three species tested separately) at 7-day intervals using light microscopy did not detect motile *A. monilatum* cells. During the first week of incubation, motile *A. monilatum* cells were found in two of 10 replicates in the NSW medium containing inoculated feces from the large size class of *P. viridis* and *M. mercenaria* collected after 24 h of depuration. Viable cells of *A. monilatum* were not observed from fecal material of *P. viridis* or *M. mercenaria* that was inoculated into L1 medium, or from feces collected after 48 h of depuration. Feces from the large size class of *C. virginica* also yielded motile cells of *A. monilatum* within the first week. Motile cells of *A. monilatum* were observed in the L1 medium with feces from *C. virginica* (four of 10 replicates), and in the NSW with *C. virginica* feces (four of 10 replicates), collected after 24 h of depuration. For feces from large *C. virginica* collected after 48 h of depuration, one replicate in NSW and L1 media contained motile cells of *A. monilatum*. After 2 weeks, one motile cell of *A. monilatum* was found in the NSW medium with inoculated feces from large *M. mercenaria* (one out of 10 replicates) collected after 24 h of depuration. One cell was also observed in the L1 (one out of 10 replicates) medium and NSW (one out of 10 replicates) with feces from *C. virginica* collected after 48 h of depuration. Viable cells of *A. monilatum* were not observed in any of the inoculated fecal cultures after 3 weeks and cells of *A. monilatum* were not detected by microscopic analysis of acidic Lugol's-preserved samples. In contrast, cell production by *A. monilatum* occurred in controls with L1 medium (division rate [k] = 0.19 d^{-1} based upon Guillard, 1973) and NSW medium (k = 0.24 d^{-1}).

3.4. Valve gape

Exposure to toxic *A. monilatum* caused a significant decrease in the mean valve gape of large size class *C. virginica* and *P. viridis* (Fig. 4) relative to animals given nontoxic *Pavlova* sp. (*C. virginica*: $p = 0.0021$; *P. viridis*: $p = 0.0013$). The mean valve gape of large quahogs (*M. mercenaria*) exposed to *A. monilatum* was also lower than that of quahogs given nontoxic *Pavlova* sp. ($p = 0.1024$) (Fig. 4). There was a significant effect of algal treatment on the mean valve gape of large shellfish, but there were no within-treatment differences, that is, no difference in valve gape between shellfish species, and no shellfish species-by-algal treatment interaction (two-way ANOVA; algal treatment: df = 1, $F = 63.28$, $p < 0.0001$; shellfish species: df = 2, $F = 3.37$, $p = 0.0688$; interaction: df = 2, $F = 2.11$, $p = 0.1638$).

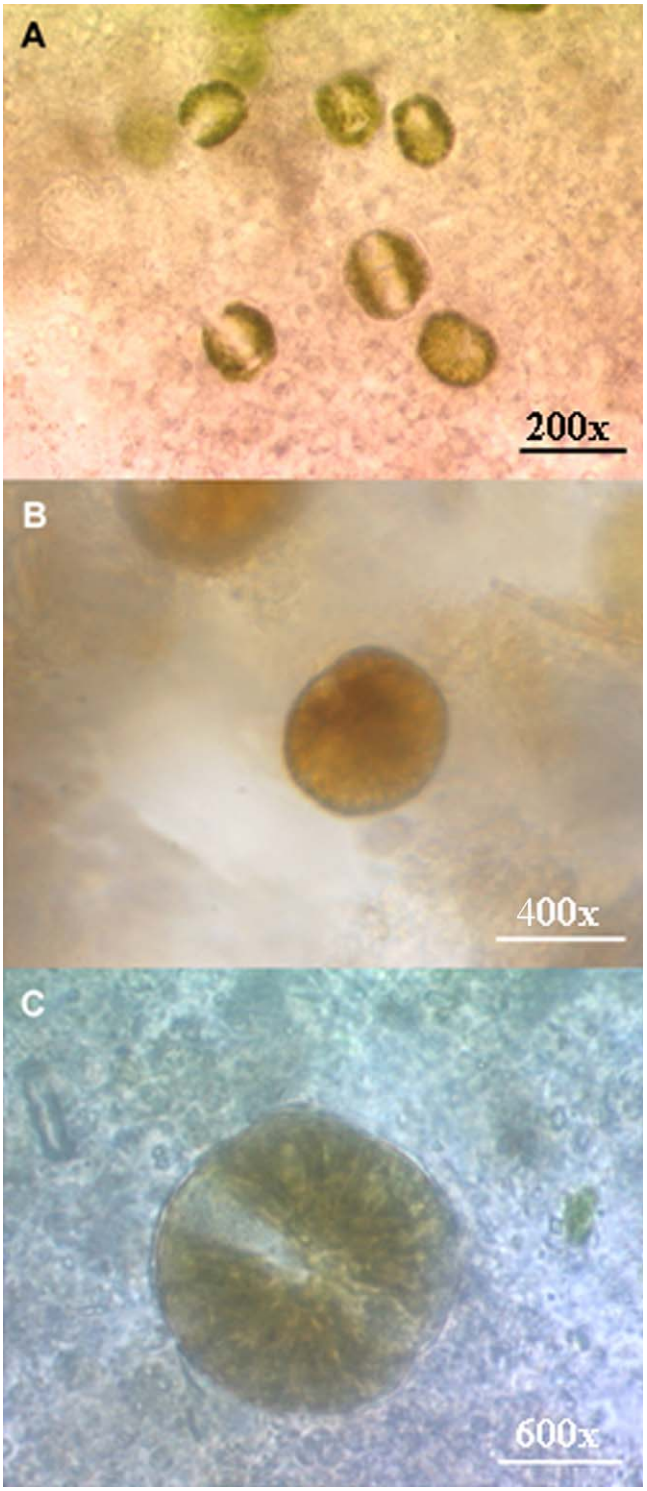

Fig. 3. Intact cells of *Alexandrium monilatum* in feces from post-larval shellfish. (A) Larger size class of *Crassostrea virginica* (feces collected after 48 h of depuration; 200× – scale bar = 50 μm). (B) Large *Mercenaria mercenaria* (feces collected after 24 h of depuration; 400× – scale bar = 20 μm). (C) Larger size class of *Perna viridis* (feces collected after 24 h of depuration; 600× – scale bar = 20 μm).

3.5. Larval bioassays

There was no difference in percent survival of *C. virginica* or *M. mercenaria* larvae exposed to intact or restricted *A. monilatum* and *A. tamarense* (Fig. 5). In contrast, sonicated *A. monilatum* caused a

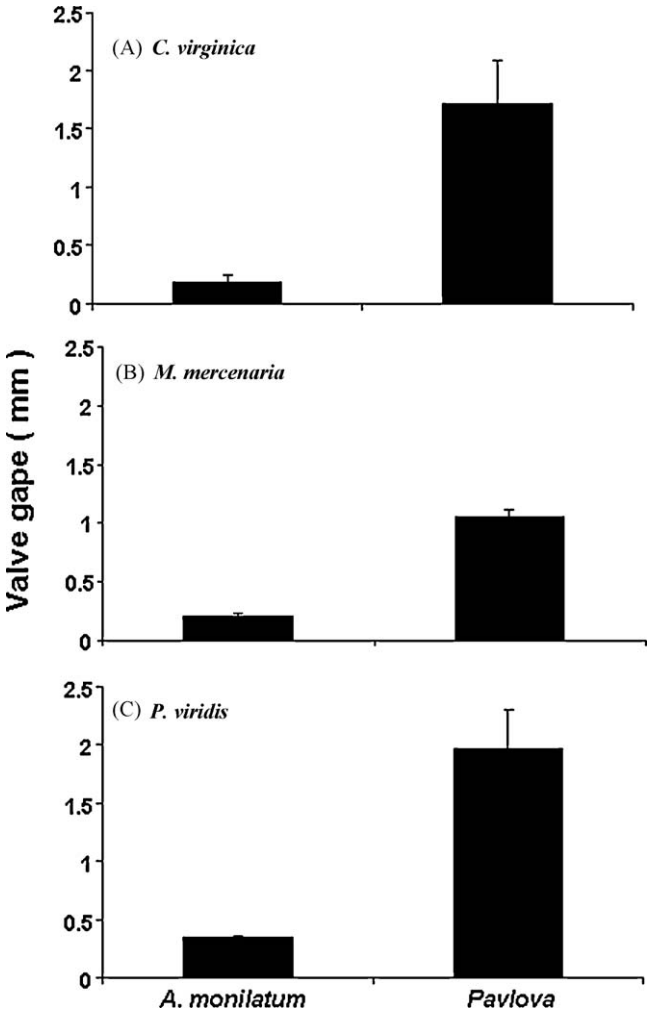

Fig. 4. Valve gape (means ± 1 SE) of the large size class shellfish exposed to toxic *Alexandrium monilatum* versus benign algae (*Pavlova* sp.) for 2 h (n = 3, Tukey–Kramer multiple comparisons test). (A) *Crassostrea virginica* (p = 0.0021); (B) *Mercenaria mercenaria* (p = 0.1024); (C) *Perna viridis* (p = 0.0013).

significant decrease in percent survival of both *C. virginica* and *M. mercenaria* larvae, in comparison to survival with sonicated *A. tamarense* (Wilcoxon two-sample test; *C. virginica*: p = 0.0008; *M. mercenaria*: p = 0.0012). About 90% of the *C. virginica* larvae survived exposure to sonicated *A. monilatum*, whereas only about 38% of the *M. mercenaria* larvae survived.

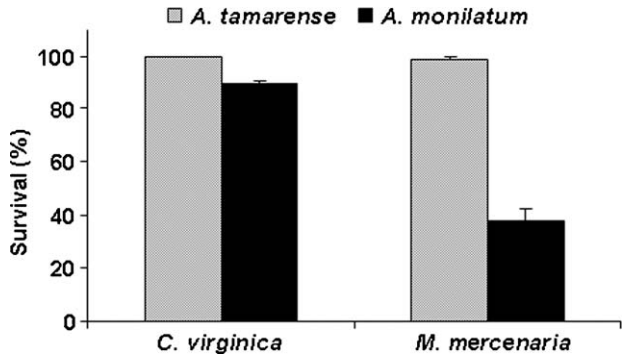

Fig. 5. Survival of larval *Crassostrea virginica* and *Mercenaria mercenaria* exposed to sonicated cells of toxic *Alexandrium monilatum* or nontoxic *Alexandrium tamarense* for 2 h (means ± 1 SE; n = 10).

4. Discussion

This is the first systematic study of the impacts of *A. monilatum* on shellfish. Previous research has demonstrated that bivalve responses to toxic dinoflagellates are species-specific and variable, ranging from avoidance behavior (e.g. valve closure, clearance rate reduction) to normal feeding activity (Shumway and Cucci, 1987; Gainey and Shumway, 1988b). This study documented species-specific responses of *M. mercenaria, C. virginica*, and *P. viridis* to exposure to a toxic strain of *A. monilatum*. In nature, shellfish could be exposed to toxic *A. monilatum* at any stage of their life history, and these experiments suggest that blooms of *A. monilatum* could affect these shellfish species by reducing valve gape and clearance rate, and by inducing mortality in some species.

4.1. Effect of A. monilatum on shellfish clearance rates

Feeding responses of bivalves to harmful algae have been related to the toxicity of the particular dinoflagellate bloom or strain (Bricelj et al., 1991; Springer et al., 2002; Shumway et al., 2006; Hégaret et al., 2007), the amount of toxin accumulated in the bivalves (Bricelj et al., 1991) and the history of exposure to harmful algal blooms (Shumway and Cucci, 1987). Shumway et al. suggested that shellfish with no prior exposure to harmful algae were more sensitive to harmful algae than those exposed on a regular basis (Shumway and Cucci, 1987; Shumway et al., 1987; Gainey and Shumway, 1988b). *Mya arenaria* inhabiting areas with frequent harmful algal blooms were more resistant to paralytic shellfish poisoning (PSP) toxins and accumulated toxins at a high rate (Bricelj et al., 2005). It was concluded that this resistance is attributable to a natural sodium-channel gene mutation that causes a 1000-fold decrease in affinity at the saxitoxin-binding site.

Avoidance behavior effectively isolates shellfish from the external environment and also affects toxin accumulation (Gainey and Shumway, 1988b). Oysters and quahogs exposed to harmful algal blooms generally exhibit lower levels of toxicity than other species tested concurrently, with mussels typically accumulating toxins much more rapidly (Lassus et al., 1989; Bricelj et al., 1990; Shumway, 1990). Previous research has demonstrated that *M. mercenaria* and *C. virginica* exhibit avoidance behavior in response to toxic dinoflagellates, usually as valve closure or reduced filtration (Ray and Aldrich, 1967; Shumway and Cucci, 1987; Bricelj et al., 1990, 1991; Shumway et al., 1990; Hégaret et al., 2007). For example, Shumway and Cucci (1987) found that *M. mercenaria* closed shell valves when exposed to toxic *Protogonyaulax tamarensis* and did not re-open the shells until placed into new seawater. Hégaret et al. (2007) reported that *C. virginica* consistently, and *M. mercenaria* sometimes, closed shell valves when exposed to toxic *A. fundyense*. Northern quahogs also have been noted to burrow in response to harmful algae, with wild populations found at depths of up to 36 cm below the sediment during blooms, as opposed to a normal distribution at about 15 cm (Shumway, 1990). Nerve fibers of *M. mercenaria* apparently are resistant to PSP toxins, but live animals exhibit avoidance behavior that may be mediated by a toxin recognition mechanism (Shumway, 1990; Bricelj et al., 1991).

Clearance rates of various shellfish species are known to be affected by the presence of toxic dinoflagellates. Shumway and Cucci (1987) found that *Ostrea edulis* significantly increased clearance rate when fed toxic *P. tamarensis*, whereas the filtration rate of the eastern oyster, *C. virginica,* was significantly reduced. Hégaret et al. (2007) reported that *Argopecten irradians*, *M. mercenaria, M. arenaria*, and *Mytilus edulis*, but not *C. virginica*, increased clearance rates when fed toxic *A. fundyense*; *A. irradians* and *C. virginica*, but not *M. mercenaria*, *M. arenaria* or *M. edulis*, increased clearance rates when fed the harmful dinoflagellate *Prorocentrum minimum*.

The mechanism for recognition of algal toxins by shellfish is not known. *M. mercenaria* have been described as being less sensitive to saxitoxins than some shellfish species, such as eastern oysters (Twarog and Yamaguchi, 1974). Bricelj et al. (1991) reported evidence of a toxin recognition mechanism in *M. mercenaria*. The adult quahogs ingested and consumed weakly toxic strains of *A. tamarense*, whereas very little of the more toxic *A. fundyense* strain were ingested, and only in the presence of a nontoxic diatom supplement. *Perna canaliculus* exposed to toxic *A. tamarense* had no dramatic physiological effects after short-term feeding, and no mortality after prolonged (2 weeks) exposure (Marsden and Shumway, 1992). Li and Wang (2001) exposed *P. viridis* to toxic and nontoxic strains of *A. tamarense*. The presence of toxin had no impact on feeding rates, indicating that *P. viridis* may be unable to distinguish particles with different toxin contents. It was hypothesized that the feeding rate of this species was not affected either because of the low toxicity of the dinoflagellate clone used in the study, or because of an inherent insensitivity of *P. viridis* clearance rate to toxic algae (Li and Wang, 2001).

It has been reported that *C. virginica* rarely opened or filtered when exposed to *A. monilatum* (Ray and Aldrich, 1967). In our study, all post-larval shellfish filtered *A. monilatum*, but at a significantly lower rate than shellfish fed benign *Cryptomonas* sp. In addition, clearance rates of both size classes of shellfish fed *A. monilatum* were significantly lower than those of shellfish exposed to nontoxic *A. tamarense*. Clearance rates of shellfish fed *Cryptomonas* sp. versus *A. tamarense* were not significantly different, except that the clearance rate of the large size class of *P. viridis* was depressed by about 20% when fed the nontoxic strain of *A. tamarense*. Thus, the nontoxic *A. tamarense* strain generally did not adversely affect clearance rates of the shellfish tested, indicating that the reduction in the clearance rate of shellfish fed *A. monilatum* was not attributable to particle size. Clearance rates of shellfish fed a mixture of toxic *A. monilatum* and nontoxic *Pavlova* sp. were not significantly different from those of bivalves fed toxic *A. monilatum*, so for these shellfish species, the addition of nontoxic algal prey to toxic *A. monilatum* did not result in higher consumption of *A. monilatum*.

Our interpretation of these results is that the depressed clearance rates observed for *C. virginica*, *M. mercenaria*, and *P. viridis* fed toxic *A. monilatum* likely occurred in response to the dinoflagellate toxin(s). The dinoflagellate subcultures used during the short-term grazing experiments were in stationary growth phase to maximize the potential for toxin production. It has been shown that *A. monilatum* produces an endotoxin that is released during cell autolysis, with peak toxicity to fish occurring when *A. monilatum* populations had been declining for a month (Aldrich et al., 1967).

4.2. Shellfish behavior (valve gape) responses to A. monilatum

Clearance rates of bivalve molluscs have been correlated positively with valve gape (Jørgensen and Rissgård, 1988; Jørgensen et al., 1988; Shumway and Gainey, 1992), although it should be noted that valve gape is not a proxy for feeding activity. Reduction in valve gape is often accompanied by retraction of the mantle edges and, where present, siphons, thereby affecting pumping rates (Jørgensen et al., 1988; Jørgensen, 1990). Exposure to toxic *A. monilatum* not only depressed clearance rates of the large size class of *C. virginica, M. mercenaria* and *P. viridis*, but also significantly reduced mean valve gape in comparison to that of control bivalves exposed to nontoxic *Pavlova* sp.

4.3. Impact of A. monilatum on shellfish survival

Sievers (1969) reported that *C. virginica* sustained increased mortality when exposed to toxic *A. monilatum* for 48 h, and naturally occurring blooms of *A. monilatum* have also been associated with mortality of various marine animals (Table 1). An *A. monilatum* bloom in the lower York River, VA occurred in September 2007 and caused mortalities of veined rapa whelks (*R. venosa*; Harding et al., 2009). The rapa whelks showed external signs of stress prior to death including reduced ventilation, inability to attach to hard substrates, periodic pumping of the percular plate, cessation of feeding and increased mucus production. Goniodomin A, a toxin produced by *A. monilatum*, was found within the tissue of rapa whelks and the bivalves attached to them, ranging from 0.02 to 8.39 μg/g tissue. There were no mortalities seen in *C. virginica* or *M. mercenaria* that were in the same flow-through system. However, Ray and Aldrich (1967) observed that *C. virginica* exposed to *A. monilatum* lost valve closure ability, a condition that frequently precedes death. In this study, of the three shellfish species tested, only *P. viridis* were moribund after 24 h of exposure to toxic *A. monilatum*. The larger and smaller size classes of *P. viridis* had an LT_{50} of 16 and 10 h, respectively. The history of exposure to harmful algal blooms can influence the response of shellfish to harmful algae (Shumway and Cucci, 1987). Green mussels naturally occur in the Indo-Pacific (Siddall, 1980) and could be more sensitive to *A. monilatum* because they were only recently introduced to coastal U.S. waters (Benson et al., 2001; Ingrao et al., 2001). It is possible that the green mussel is especially sensitive to exposure to this novel algal toxin; moreover, large mortalities of this species have also been noted during local blooms of another toxic dinoflagellate, *K. Brevis*, in Florida coastal waters (Leverone et al., 2007).

Larval shellfish are known to be especially sensitive to toxic dinoflagellates (Wikfors and Smolowitz, 1995; Yan et al., 2001; Springer et al., 2002; Leverone et al., 2006). In this research, larval *C. virginica* and *M. mercenaria* were sensitive to toxic *A. monilatum*, with larval *M. mercenaria* being more sensitive to *A. monilatum* toxicity (approximately 38% and 90% survival). There was no significant decrease in survival of larval *C. virginica* or *M. mercenaria* exposed to intact or restricted *A. monilatum* versus a nontoxic strain of *A. tamarense*, whereas exposure to sonicated cells of *A. monilatum* significantly depressed survival relative to control larvae. These findings support previous reports of production of endotoxins by *A. monilatum* that are released by cell lysis (Aldrich et al., 1967). The *A. monilatum* cultures used in the experiments with shellfish larvae were in log growth phase to reduce the amount of toxin potentially present from cell autolysis (as suggested by Aldrich et al., 1967 and confirmed in recent work by P. Moeller and coworkers, NOAA-NOS, Charleston, SC, USA; personal communication, January 2008). Thus, as expected, the larvae were not affected by *A. monilatum* until the cells were lysed through sonication.

Under optimal conditions, endotoxins may be released minimally into the surrounding water, but more toxins can be released under stressful conditions or during senescence or collapse of a bloom (Landsberg, 2002). The toxins may confer protective benefit to *A. monilatum* by minimizing both grazing pressure, as indicated in this study, and a competitive advantage over other algae for available resources. Other research described up to 2.5-fold more production of paralytic shellfish toxins in *Alexandrium minutum* exposed to waterborne cues from the copepod grazer, *Acartia tonsa*, in comparison to unexposed control *A. minutum* (Selander et al., 2006). In addition, the induced sub-population was more resistant to further copepod grazing. In this study, *Cryptomonas* sp. lysed when mixed with *A. monilatum*. This adverse effect on some phytoplankton species may enhance nutrient availability for *A. monilatum* in its natural habitat.

4.4. Shellfish as potential vectors of transport: survival of A. monilatum after gut passage

Shellfish form pseudofeces when they encounter high phytoplankton cell densities ($>10^5$ cells ml^{-1}) or when they are stressed

(see review in Morton, 1983). In this study, *A. monilatum* cells were rejected in mucus of large *P. viridis* after exposure to *A. monilatum* for 8 h, suggesting that the shellfish were stressed. Intact *A. monilatum* cells were also found within the feces of all size classes of shellfish species tested, indicating that the shellfish did not digest at least a portion of the ingested cells. Various other potentially toxic dinoflagellate species have been documented to pass intact through the digestive tracts of bivalve molluscs (e.g. Shumway et al., 1985b; Shumway and Cucci, 1987; Bauder and Cembella, 2000; Springer et al., 2002; Hégaret et al., 2007, 2008a,b). For example, based upon inspection of fecal ribbons, Shumway et al. (1985b) reported that *O. edulis* preferentially ingested the dinoflagellate *P. minimum* cells relative to other microalgal species; however, high incidence of fragments and intact cells of *P. minimum* in the fecal ribbons indicated that cells were only partially digested.

Successfully invading species are difficult or impossible to eradicate (Clout and Veitch, 2002); therefore, potential vectors of such species should be considered carefully in efforts to minimize dispersal of algal species. Introduction of harmful bloom species via ship ballast water has received considerable attention (see review Hallegraeff and Bolch, 1992; Hallegraeff, 1993), whereas other potential modes of transfer, by contrast, mostly have been ignored. Given that some harmful dinoflagellate species can survive ingestion, gut passage, and egestion by bivalve molluscs, molluscan shellfish are an obvious potential vector for transfer of harmful algae (Hallegraeff, 1993; Scarratt et al., 1993; Hégaret et al., 2008a,b). For example, a strain of the dinoflagellate *Prorocentrum lima* exhibited cell division following passage through the gut tract of bay scallops (Bauder and Cembella, 2000). Egested cells of a strain of *A. fundyense* were capable of doubling times comparable to those of ungrazed control populations (Bricelj et al., 1993). Strains of *Pfiesteria piscicida* formed temporary cysts when ingested by adult eastern oysters, and within 24 h more than 75% of the cysts tested produced live motile cells (Springer et al., 2002). Laabir and Gentien (1999) found that the toxic, thecate dinoflagellates *A. minutum* and *A. tamarense* were able to pass intact and viable through the digestive tract of *Crassostrea gigas*, but the unarmored dinoflagellate *Karenia mikimotoi* was not. The harmful dinoflagellates *A. fundyense* and *P. minimum* were also able to re-establish growing populations after passage through the digestive tract of several shellfish species (Hégaret et al., 2008a). In the present study, intact *A. monilatum* cells were found within the feces of all shellfish species tested. In addition, culture media inoculated with feces from the large size class of *C. virginica, M. mercenaria* and *P. viridis* contained motile *A. monilatum* cells within the first week, but these cells did not re-establish growing populations.

Walker and Steidinger (1979) reported that *A. monilatum* forms resting cysts in stationary cultures and in nitrogen-deficient medium. The accumulation of benthic resting cysts in an area can act as an inoculum or "seed bed" for subsequent blooms under conducive environmental conditions (Steidinger, 1975; Anderson and Wall, 1978). Benthic resting cysts of *A. monilatum* isolated from Tampa Bay, FL sediments have excysted to produce motile chains (Walker and Steidinger, 1979). Owen and Norris (1982) suggested that *A. monilatum* cysts might have been introduced into new areas through transfer of shellfish from an area that had sustained blooms of *A. monilatum*. In the present study, while some viable cells were seen after short-term collection of fecal material, population-level growth was not observed from the few cells of *A. monilatum* that passed intact through the digestive tracts of shellfish in experimental exposures. These cells were viable for 24 h, supporting other research which suggests that maintaining shellfish out of water for 24 h or in depuration media for 24 h may be a promising mitigation strategy to prevent shellfish-related transport of *A. monilatum* to new areas (Hégaret et al., 2008a,b).

4.5. Population dynamics of *A. monilatum*, and potential impacts on shellfish aquaculture

Little is known about the population dynamics of *A. monilatum* in its natural habitat. Reported blooms of *A. monilatum* in the Gulf of Mexico and along the eastern coast of Florida were frequent 20–50 years ago, along with associated finfish and shellfish kills (Howell, 1953; Gates and Wilson, 1960; Williams and Ingle, 1972; Wardle et al., 1975; Perry et al., 1979; Perry, 1980; Maples et al., 1983; Norris, 1983) (Table 1). Some blooms were linked to stimulation by anthropogenic nutrient enrichment, as mentioned (e.g. Connell and Cross, 1950). Since the 1970s, published data suggest that blooms of *A. monilatum* have occurred sporadically in these areas, possibly because of a lack of systematic sampling. All of the records on *A. monilatum* were compiled from routine monthly sampling during 1989–2002 along a transect extending from Terrebonne Bay to ca. 65 km offshore in Louisiana coastal waters (C transect, Q. Dortch, personal communication, NOAA, Silver Spring, MD, USA; and see Dortch et al., 1997; Thessen et al., 2005). This species was reported in 8 of 13 years, most often in September and October but sometimes also in June, July and December. In addition, records of bloom samples collected by various individuals (Q. Dortch, personal communication) indicate that in September and October of 1995 and 1996, there were numerous reports of bioluminescence and discolored red or brown water in estuarine and coastal waters off Louisiana west of the Mississippi River that subsequently were confirmed to be *A. monilatum* (e.g. September 1995, 6.82 to 8.12×10^3 cells ml^{-1}). Blooms of this dinoflagellate in Virginia have been reported, as well (September 4–10, 2007, 1.2 to 40×10^3 cells ml^{-1}; Harding et al., 2009). Thus, systematic monitoring for *A. monilatum* may show that occurrence is more frequent and widespread than presently recognized.

Areas where blooms of *A. monilatum* historically have been reported coincide with areas increasingly used for shellfish aquaculture and, thus, this harmful alga represents a potential threat to commercial shellfish culture. For example, the Indian River Lagoon of Florida supports an important commercial *M. mercenaria* fishery (Norris, 1983; Vaughan, 1988). This area also has the most productive natural beds of *M. mercenaria* and the highest concentration of active quahog aquaculture leases in Florida (Arnold et al., 2000). The *M. mercenaria* aquaculture industry in Florida has expanded from an essentially non-existent industry in the 1980s to an industry with annual landings approaching $4.6 million US (Arnold et al., 2000). Accordingly, the potential for blooms of *A. monilatum* to impact seafood quality and local fishery-based economies is increasing in some areas. The present study provides technical information on trophic interactions between commercially important shellfish species and the emerging harmful algal bloom species, *A. monilatum*.

5. Conclusions

This study documents responses of three species of shellfish: northern quahogs (*M. mercenaria*), eastern oysters (*C. virginica*), and green mussels (*P. viridis*) exposed experimentally to a toxic strain of the dinoflagellate, *A. monilatum*. All three species showed avoidance behavior by reducing both clearance rate and valve gape when exposed to toxic *A. monilatum*. Based upon survival experiments, the most sensitive species was *P. viridis*; both size classes of this mussel were moribund after 24 h of exposure. Larval *M. mercenaria* and *C. virginica* were sensitive to toxic *A. monilatum*

and larval *M. mercenaria* were more sensitive than larval *C. virginica* (38% and 90% survival, respectively).

Transfer of shellfish from one area to another has been suggested as a potential vector for the transport of harmful algae into new environments (Owen and Norris, 1982; Bricelj et al., 1993; Scarratt et al., 1993; Villa et al., 2001; Lilly et al., 2002; Penna et al., 2005; Hégaret et al., 2008a,b). Intact cells of *A. monilatum* were noted in the feces of all three shellfish species, but apparently were viable only for the first 24 h after gut passage, and were unable to re-establish growing populations. Thus, while shellfish could conceivably be vectors for the dispersal of *A. monilatum*, it is likely that, as shown by Hégaret et al. (2008b) for other species, a period of 24 h out of water or in a depuration situation would mitigate the likelihood of transfer. Overall, the data from this study suggest that blooms of *A. monilatum* could affect the recruitment and survival of some shellfish species by inducing larval mortality and reducing clearance rate and valve gape, thereby affecting food intake. Furthermore, reduced clearance rate and increase in valve closure seen in shellfish exposed to *A. monilatum* may contribute to the development of blooms due to failure of suspension-feeding bivalves to maintain control of the algal population growth (Johnson et al., 2003).

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