ABSTRACT

PATE, SUSAN ELIZABETH. Impacts of the toxic dinoflagellate *Alexandrium monilatum* on three ecologically important shellfish species. (Under the direction of Dr. JoAnn M. Burkholder.)

Little is known about interactions between shellfish and *Alexandrium monilatum* (Howell) Balech, a toxigenic dinoflagellate that forms blooms mostly in the Gulf of Mexico. Toxic *A. monilatum* produces endotoxins with hemolytic and neurotoxic properties, and has been linked to major fish and invertebrate kills. The responses of three ecologically important shellfish species to *A. monilatum* (toxic strain AMO3) were experimentally assessed. In the first set of experiments, grazing studies were conducted with adult and juvenile eastern oysters (C*rassostrea virginica* Gmelin), northern quahogs (*Mercenaria mercenaria* Linnaeus), and green mussels (*Perna viridis* Linnaeus), which inhabit areas where *A. monilatum* blooms occur. Clearance rates of each shellfish species were depressed when exposed to toxic *A. monilatum* (bloom density of ~5.5 x 10^2 cells mL^{-1}) alone or with nontoxic Instant Algae® *Pavlova*, in comparison to clearance rates of control animals fed benign cryptophyte algae. There was also a reduction in the clearance rate of adult and juvenile *C. virginica*, *P. viridis*, and *M. mercenaria* exposed to *A. monilatum*, in comparison to control animals fed a nontoxic strain of a dinoflagellate of similar size, *Alexandrium tamarense* (Lebour) Balech (clone CCMP115). Exposure to toxic *A. monilatum* also caused adult *P. viridis*, *C. virginica* and *M. mercenaria* to decrease their valve gape. Intact *A. monilatum* cells were found within shellfish feces, but *A. monilatum* cells did not divide following passage through the gut. In the second set of experiments, survival of larval *M. mercenaria* and *C. virginica* was tested when the larvae were exposed to *A. monilatum* as intact cells, cells held in dialysis tubing, or

sonicated cells. Survival of larvae was significantly less when exposed to sonicated *A. monilatum*, in comparison to survival of control larvae that were tested with nontoxic *A. tamarense*. Overall, these data indicate that *A. monilatum* blooms can adversely affect survival of some shellfish species by reducing clearance rate and valve gape, affecting food intake, and inducing larval mortality.

IMPACTS OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM MONILATUM* ON THREE ECOLOGICALLY IMPORTANT SHELLFISH SPECIES

by

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BIOGRAPHY

Susan Elizabeth Pate was created by Phillip Pate and Ivey Strickland in Zebulon, NC in 1978 and made her first appearance on Tuesday June 26, 1979. She grew up on a tobacco farm in Zebulon, NC with her sister Jennifer and numerous family pets, including Bo Bo, Beep Beep, Napolean, Colors, Baby, Aquarius, Skeet, Samantha and Toonsis a.k.a Gray Baby. To most people's surprise, she had a very entertaining and interesting childhood in Zebulon, NC. She basically grew up at her parent's video store, where she learned a lot about movies and pop culture. Even though she loves television, movies and music very much, she has always had a love for science and nature.

Her family would go on family vacations in N.C. where they camped all over the state, from the mountains to the coast. She learned a lot about N.C. flora and fauna on these trips. Like many other young people, Susan thought that she wanted to be a veterinarian because she loves animals and science. At the time, she didn't understand that you don't have to be a doctor to be a scientist. She soon discovered that she is too empathetic to be a veterinarian and this was not the right career choice for her.

Susan has always been very passionate about school and had her own drive to succeed. She was very successful in all of her years in school and knew that she wanted to be a scientist at an early age. She took an AP Biology class in high school that would influence her life in several ways. First, it influenced her to major in Biology and not pre-vet in college and made her realize that she had a gift for science. Secondly, she met her future husband, Steven May, on a class camping trip to the mountains. After high school, Susan attended the University of N.C. at Wilmington to study Biology and Chemistry. This was a good choice on her part because UNCW allowed her to grow up and become

the independent woman that she is now. At UNCW, she met Tara Williams in her genetics class who became her best friend and later introduced her to Dr. JoAnn Burkholder.

At UNCW, she also had the opportunity to work on juvenile blue crab research. Her honor's thesis was on the permeability of juvenile blue crab gills. This research experience made her realize that she wanted to go to graduate school and study ecology. Tara and Susan took a class called global environmental problems their senior year at UNCW. During this class, Susan learned about toxic algae and shellfish. She quickly became very interested in the subject and was lucky enough to be able to tag along with Tara while she met with JoAnn Burkholder about possibly attending graduate school. During this meeting, Susan realized that she wanted to study toxic algae and shellfish at the Center for Applied Aquatic Ecology. She was accepted and given the opportunity to work on this thesis.

At graduate school, Susan met several really good friends, including one of her best friends Hayley Skelton. As you can see, science not only was a good career choice for Susan, but it also brought several important people into her life. Susan plans to continue a career in research after graduation.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

1. INTRODUCTION

Outbreaks of some 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 are known to produce toxins or to harm organisms directly or indirectly, such as causing anoxia or mechanical damage to gills (see review in Shumway 1990). “Toxic algae” is a term used in reference to species that have toxic strains. Such species typically show a range in toxicity including some strains that are apparently unable to express toxicity (see review in Burkholder et al. 2001). Nutrients added to coastal regions from cultural eutrophication have stimulated blooms of some harmful algal species (see review in Anderson et al. 2002). The movement of shellfish stocks to different areas is another mechanism that may have promoted this increase (Hallegraeff 1993), since some species of harmful dinoflagellates 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, Hégaret et al. in prep.).

Dinoflagellate toxins can contaminate seafood and cause human illness and death (see reviews in Burkholder 1998, Landsberg 2002) when filter-feeding bivalves accumulate and concentrate toxins that can be transmitted up the food chain (Shumway 1990). Toxin accumulation by shellfish is influenced by their grazing rates and behavioral responses. Historically, shellfish were regarded as unaffected vectors of algal toxins, but effects of toxic algae on their behavior, metabolism and survival increasingly have been recognized (see review in Shumway and Gainey 1992). Bivalves often reduce their filtration rate and increase their valve closure when exposed to toxic dinoflagellates, which effectively decreases their exposure to the bloom (Shumway et al. 1985a, Shumway and Cucci 1987, Gainey and

Shumway 1988a, Shumway 1990, Lesser and Shumway 1993, Laabir and Gentien 1999, Lassus et al. 1999, Matsuyama et al. 2001). Toxins and other bioactive substances produced by dinoflagellates have caused shellfish mortalities, and toxic dinoflagellates can also affect shellfish recruitment and survival indirectly by causing behavioral alterations, depressed feeding, and impaired reproduction and growth (see reviews in Shumway 1990, Burkholder 1998, Landsberg 2002).

The toxic, chain-forming dinoflagellate *Alexandrium monilatum* 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-Gunter 1942, Connell and Cross 1950, Ray and Aldrich 1967), Venezuela (Caribbean Sea-Halim 1967, Ferraz-Reyes 1985), and the Pacific Ocean off Ecuador (Balech 1995) (Table 1). Blooms of *A. monilatum* have been associated with mortalities of fish and invertebrates in Florida (Howell 1953, Williams and Ingle 1972, Perry et al. 1979, Norris 1983), Alabama (Perry et al. 1979), coastal Mississippi (ICES 1999), and Texas (Gunter 1942, Connell and Cross 1950, Gates and Wilson 1960, Wardle et al. 1975) (Table 1). Connell and Cross (1950) described a bloom of *A. monilatum* during the summer of 1949 in Offats Bayou near Galveston, Texas, and linked sewage pollution to stimulation of the bloom. This bloom, the most widespread and destructive on record in this area, 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 existed. Based on the minimum N cell quota of this species, it has been suggested that high N flux would be required to support development of *A. monilatum* blooms (Juhl 2005).

Table 1. Documented blooms of *Alexandrium monilatum* and associated mortalities of aquatic organisms

Location	Year(s)	Mortalities	Bloom densities (cells · mL^{-1})	Reference
Galveston, Texas, USA	1930s-1949	Fish, shrimp, oysters, crabs	Not reported	Gunter (1942)
Galveston, Texas, USA	1949	Fish	Not reported	Connell and Cross (1950)
Indian River, Florida, USA	1951	Few fish	Not reported	Howell (1953)
Sarasota, Florida, USA	1951	None reported	Not reported	Howell (1953)
Galveston, Texas, USA	1955	Fish	5.0×10^2 - 1.0×10^3	Gates and Wilson (1960)
Fort Myers to Naples, Florida, USA	1966	Jack (*Caranx* sp.) needlefish (*Strongylura marina* (Walbaum)), pinfish (*Lagodon rhomboids* (Linnaeus))	Not reported	Williams and Ingle (1972)
Galveston, Texas, USA	1971-1972	*Bunodosoma cavernata* (Bosc); *Americonuphis magna*; (Andrews); *Nereis* sp.; *Polinices duplicata* (Say); *Thais haemastoma* (Linnè); *Terebra cinerea* (Born); *Anadara ovalis* (Bruguière); *Anadara brasiliana* (Lamarck); *Donax variabilis* (Say); *Spisula solidissima* (Dillwyn); *Oliva sayana* (Ravenel); *Siphonaria pectinata* (Linnaeus); *Crassostrea virginica* (Gmelin); *Emerita benedicti* (Schmitt); *Arenaeus cribarius* (Lamarck); *Clibanarius vittatus* (Bosc); *Isocheles wurdemanni* (Stimpson); *Hepatus epheliticus* (Linnaeus); *Porcellana sayana* (Leach); *Callinectes sapidus* (Rathbun); *Menippe mercenaria* (Say); *Petrolisthes armatus* (Gibbes); *Callinectes similis* (Williams); *Micropholis atra* (Stimpson); *Mellita quinquesperforata* (Leske); "*Holothuroids*"; *Hypleurochilus geminatus* (Wood); *Bascanichthys scuticaris*, (Goode & Bean); *Gobiesox punctulatus* (Poey)	1.88×10^3	Wardle et al. (1975)

Table 1. (cont'd.)

Location	Year(s)	Mortalities	Bloom densities (cells · mL^{-1})	Reference
Mississippi Sound, USA	1979	None reported	1.65×10^4	Perry et al. (1979)
Pensacola Bay, Florida, USA	1979	Fish	3.18×10^4	Perry et al. (1979)
Mobile Bay, Alabama, USA	1979	Fish	Not reported	Perry et al. (1979)
Indian River, Florida, USA	1977	None reported	8.9×10^2	Norris (1983)
Melbourne Beach, Florida, USA	1977	None reported	1.7×10^3	Norris (1983)
Port St. John, Florida, USA	1977	Thousands of fish	Not reported	Norris (1983)
Indian River, Florida, USA	1979	None reported	Not reported	Norris (1983)
The Gulf of Cariaco, Venezuela	1984	None reported	$40 - 1 \times 10^2$	Ferraz-Reyes (1985)
Mississippi Coast, USA	1998	Zooplankton and ichthyoplankton	Not reported	ICES (1999)

Toxic strains of *Alexandrium monilatum* produce a toxin(s) that causes paralysis and mortality in fish (Gates and Wilson 1960). Heating or freezing the cells has increased fish mortality in laboratory experiments, suggesting that this dinoflagellate produces an endotoxin that is released with cell lysis, and maximal toxicity has been reported in senescent cultures with high cell autolysis (Aldrich et al. 1967). Ray and Aldrich (1967) found that oysters (*Crassostrea virginica*) rarely opened or filtered when exposed to *A. monilatum*. Polychaetes (*Polydora*) inhabiting the oyster shells, along with fish in separate bioassays, had high mortality rates. The authors cautioned that similar work testing the effects of larger doses and various ages of cultures would be required to assess the full range of impacts of *A. monilatum*. Seivers (1969) compared the toxicity of one strain of *A. monilatum* versus one strain of *Karenia brevis* (Davis) Hansen et Moestrup 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 tested *K. brevis* strains than to *A. monilatum*. In contrast, the annelids and molluscs were more sensitive to the tested strains of *A. monilatum* than to *K. brevis*, and the crustaceans were resistant to both dinoflagellates.

Schmidt and Loeblich (1979) found that in laboratory cultures, paralytic shellfish poison toxins (saxitoxin and gonyautoxins) were present. A toxic extract from *A. monilatum* was isolated and reported to exhibit neurotoxic and hemolytic properties and to be chemically different from saxitoxin and related compounds (Clemons et al. 1980, Bass et al. 1983, Erker et al. 1985). Crude phenol-water extracts of *A. monilatum* were toxic to CD-1 mice (Erker et al. 1982). As a function of dose, the extract, when administered by intraperitoneal administration to male CD-1 mice, produced transient piloerection, sedation,

ataxia, labored breathing, cyanosis, convulsions, loss of reflexes and death, with a LD_{50} of 2.3 mg $\cdot$ kg^{-1} (Erker et al. 1982). Mice that survived for 24 h after exposure to the extract lost weight and ate and drank less. Gross pathology analyses showed congestion and/or hemorrhage in the stomach wall, edema, and congestion in the mesenteric blood vessels. Control mice did not display any signs of toxicity (Erker et al. 1982). The author concluded that *A. monilatum* is toxic to homeotherms. Erker et al. (1985) prepared an *A. monilatum* extract excluding saxitoxins, gonyautoxins and structurally related toxins. The extract contained a water-soluble glycosidic substance that caused toxic affects (sedation, abdominal constriction, fecal clumping in perianal area, ataxia, tremors, cyanosis, loss of reflexes, convulsions and death, with an LD_{50} of 2.28 mg $\cdot$ kg^{-1} and median lethal oral dose of 6.73 mg $\cdot$ kg^{-1}) in CD-1 mice when administered orally and by intraperitoneal injection.

Recently, a toxin produced by *Alexandrium monilatum* was purified and identified (Hsia et al. 2005) as goniodomin A, which is also produced by *Alexandrium pseudogoniaulax* (Biecheler) Horoguchi. Goniodomin A is an antifungal polyether macrolide that inhibits cell division in fertilized sea urchins (Murakami et al. 1988, Abe et al. 2002). It has also been found to be antiangiogenic in endothelial cells by inhibiting actin reorganization and affecting actomyosin ATPase activity (Furukawa et al. 1993, Yasuda et al. 1998, Matsunaga et al. 1999, Abe et al. 2002). Goniodomin A has caused perihepatitis, non-fatty vacuoles in the hepatocytes, central necrosis of the liver and massive necrosis of lymphocytes in the cortical layer of the thymus in male ICR mice (Terao et al. 1989). It is not yet known whether this toxin also exhibits hemolytic activity, but extracts of *A. monilatum* cells were hemolytic to erythrocytes from several mammalian species, including humans, and lethal to cockroaches, guppies and mice (Clemmons et al. 1980, Bass et al. 1982).

Interactions between shellfish and *Alexandrium monilatum* are poorly understood. Shellfish are an important link between the pelagic and benthic food webs because, through suspension- and deposit-feeding activities, they can cycle large amounts of particulate matter within the environment (see review in Ward and Shumway 2004). Shellfish interact with coastal water columns by removing phytoplankton, depositing feces and pseudofeces, and cycling dissolved nutrients (Prins et al. 1998). Shellfish fecal material provides food for benthic organisms and shellfish populations can mediate the flux of organic material to the benthos (Newell 1988). Bivalve larval stages also provide food for larval and early juvenile fishes (Pattillo et al. 1997). Bivalve molluscs can act as a natural control against the adverse effects of eutrophication in estuaries by filtering both inorganic and organic particles limiting turbidity and phytoplankton blooms (Kennedy 1991).

The objectives of this study were to (i) examine the effects of *Alexandrium monilatum* at bloom density on clearance rates of juvenile and adult eastern oysters (*Crassostrea virginica*), green mussels (*Perna viridis*), and northern quahogs (*Mercenaria mercenaria*); (ii) evaluate adult shellfish behavior (valve gape) in response to *A. monilatum*; (iii) assess impacts of *A. monilatum* on the survival of larval *C. virginica* and *M. mercenaria*; and (iv) assess survival of *A. monilatum* after ingestion by adult and juvenile shellfish, to gain insights as to whether these shellfish can act as vectors for the introduction of *A. monilatum* if transported to new areas. The shellfish species selected for this research are ecologically and commercially important along the east and gulf coasts of the United States and inhabit areas where *A. monilatum* blooms have occurred. Northern quahogs and eastern oysters are a food source for a variety of bottom feeding fish and invertebrate species, and their larval stages provide food for larval and early juvenile fishes (Burrell 1986, Pattillo et

al. 1997). Eastern oysters provide reef habitat for several organisms in the Gulf of Mexico (Wells 1967). Green mussels recently were introduced to the Gulf of Mexico (Ingrao et al. 2001). This species has an extensive capacity for larval and adult dispersal, early maturity, rapid growth rate and high productivity (Ingrao et al. 2001).

MATERIALS AND METHODS

2.1. Algal cultures

Experiments were conducted at the Center for Applied Aquatic Ecology, Raleigh, NC. Toxic *Alexandrium monilatum* (strain AMO3; cell length 28-52 µm – from S. Morton, National Oceanic and Atmospheric Administration – National Ocean Service [NOAA-NOS], Charleston, SC) was mass-cultured in 10-20 L Nalgene® polycarbonate carboys with L1 media (Guillard and Hargraves 1993). It was grown at 23ºC on a 12:12 h light/dark (L/D) cycle at ~170 µE $s^{-1}m^{-2}$. The benign alga *Cryptomonas* sp. (clone HP9101; cell length 10-15 µm – from D. Stoecker, Horn Point Environmental Laboratory, University of Maryland, Cambridge, MD), which was used as a food source for control animals, was batch-cultured in 2-L Erlenmeyer flasks with F/2 media (Guillard 1975) at 22°C on a 12 h:12 h L/D cycle at ~50 µE s^{-1} m^{-2}. 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* (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) was included due to its similarity in size to *A. monilatum*. Batch cultures of *A. tamarense* were grown in 4-L Erlenmeyer flasks with L1 media at 23ºC with 8 h:16 h L/D cycle at ~115 µE m^{-2} s^{-1}. All growth media were prepared with 30-ppt filtered artificial seawater (ASW as 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 and non-axenic.

Juvenile sheepshead minnows (*Cyprinodon variegates,* n=3) were exposed to *Alexandrium monilatum* that were lysed by sonication, *A. monilatum* in log growth phase and

senescent *A. monilatum* to verify that the strain was toxic prior to experimentation (Table 2). Control fish were in filtered 30 ppt ASW. This finfish species is sensitive to *A. monilatum* toxin(s) and provided an indication of the relative toxicity of the *A. monilatum* strain used in the experiments (Sievers 1969). No *C. variegates* mortalities were observed in ASW or with *A. monilatum* in log growth phase. However, sonicated and senescent *A. monilatum* caused 100% fish mortality. The data suggest that the *A. monilatum* strain used in these experiments produces an endotoxin that is released upon cell lysis.

Sub-cultures of *Alexandrium monilatum* and *Alexandrium tamarense* were also sent to the Marine Biotoxins Program, NOAA-NOS, Charleston, SC for toxin analysis after the completion of the 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 to be toxic, whereas the *A. tamarense* culture was evaluated to be a nontoxic clone.

Cultures of *Alexandrium monilatum* in stationary phase were used in the experiments. Prior to use, the cultures were shaken vigorously to break up any long cell chains, this method has been successful with other alga species (G Wikfors, Northeast Fisheries Science Center, NOAA, Milford, CT, personal communication). Cultures of *Cryptomonas* sp. and *Alexandrium tamarense* in log growth phase were used in experiments. Subsamples of each algal culture were preserved with acidic Lugol's solution (Vollenweider 1974) before each experiment to quantify initial (T_o) cell densities. The samples were analyzed at 40-400x with a BH2 Olympus light microscope (Olympus Corporation, Melville, NY). 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 (Throndson 1995). Filtered (0.2-µm) 30 ppt ASW was used to adjust algal concentrations to desired initial densities.

Table 2. Bioassays with juvenile sheepshead minnows (*Cyprinodon variegates*).

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

Juvenile and adult shellfish (for sizes see Tables 3, 4) were obtained from various locations, and based on local knowledge, were assumed to have had no prior exposure to *Alexandrium monilatum* blooms. Juvenile and adult *Perna viridis* were collected off the Gandy Bridge in Tampa Bay, FL by Mr. Jonathon Fajans (University of Florida, Gainesville, FL). Juvenile and adult *Crassostrea virginica* were obtained from Pemaquid Oyster Company, Waldoboro, ME. J and B AquaFood, Jacksonville, NC supplied adult *Mercenaria mercenaria*, and juveniles were obtained from Millpoint Aquaculture, Core Sound, NC. Mr. Dale Leavitt supplied *C. virginica* larvae (Roger Williams University, Bristol, RI), and *M. mercenaria* larvae were obtained from Cherrystone Aquafarms, Cheriton, VA. D-stage larvae (age 10 to 14 days; mean length 240 ± 11 μm) were used immediately upon receiving them for experimentation. Larval *Perna viridis* were not available for testing during this study.

Upon arrival, juvenile and adult shellfish were scrubbed to remove epiphytes and other organisms, and the shells were rinsed with deionized water. Adult and juvenile shellfish were acclimated to laboratory conditions at 22-25ºC and in 30-32 ppt ASW for at least one week prior to experimentation in separate 946 L (250 gallon) and 492 L (130 gallon) re-circulating systems. Physical and chemical variables (dissolved oxygen, buffering capacity, general hardness, pH, salinity, temperature, nitrate/nitrite, ammonium) were monitored to ensure that environmental conditions were suitable for shellfish maintenance (Lawson 1995). The 946 L tanks contained a biological filtration system that consisted of a bio-tower with bubble-washed beads fed at a constant flow of 11 $L \cdot min^{-1}$. The water was also pre-filtered with tangential-injection protein skimmers and sponge filters to remove organics and reduce accumulation of

Table 3. Shell height and dry weight of juvenile shellfish used in the experiments (means ± 1 standard deviation [SD]).

Shellfish species	Shell height (mm)	Dry weight (g)
Perna viridis	26.7 ± 2.8	0.06 ± 0.02
Mercenaria mercenaria	13.3 ± 0.4	0.04 ± 0.006
Crassostrea virginica	29.1 ± 0.9	0.06 ± 0.01

Table 4. Shell height and dry weight of adult shellfish used in the experiments (means ± 1 SD).

Shellfish species	Shell height (mm)	Dry weight (g)
Perna viridis	48.9 ± 8.4	0.3 ± 0.09
Mercenaria mercenaria	44.1 ± 0.8	0.8 ± 0.06
Crassostrea virginica	69.8 ± 2.3	1.3 ± 0.2

particulates and dissolved organic substances within the tower. A canister filter (Ocean Clear J319) fed at a constant flow of 15 L · min^{-1} was used in the 492 L system, along with a tangential-injection protein skimmer and sponge filter.

During the acclimation period, shellfish were fed Instant Algae© Shellfish Diet 1800© (consisting of a mixture of nontoxic algae as 20% nontoxic *Pavlova* sp. (haptophyte, cell length 4 - 8 µm), 25% *Isochrysis* sp. (haptophyte, cell length 4 - 8 µm), 20% *Tetraselmis* sp. (chlorophyte, cell length 7 - 10 µm), and 35% *Thalassiosira weissflogii* (Grunow) Fryxle et Hasle (ochrophyte diatom, cell length 10-20 µm); sum of cell concentration from all species = ~ 2 x 10^9 cell mL^{-1}) or Instant Algae© *Pavlova* (cell concentration = ~ 3.3 x 10^9 cell mL^{-1}) daily, using an amount known to provide nearly optimum food conversion for the number of shellfish in the systems (Instant Algae© shellfish feed calculator http://www.reed-mariculture.com/shellfish/shellfish.xls based on Wikfors et al. 1999). Shellfish were not fed for 24 - 48 h prior to experiments in order to clear their gut tracts of previously ingested materials.

2.3. Short-term feeding experiments

Experiments to estimate clearance rates were conducted in individual static systems with juvenile and adult *Perna viridis*, *Crassostrea virginica* and *Mercenaria mercenaria*, with adult and juvenile species tested separately in short-term trials (1 - 2 h; n = 10, 1 animal per replicate; Table 5). The experiments were conducted in an environmental incubator (Thermoforma, Marietta, OH) at 22ºC at ~ 60 µE m^{-2} s^{-1}. Test animals were exposed to a bloom density of toxic *Alexandrium monilatum* (5.5 x 10^2 cells · mL^{-1}), based on bloom densities reported in the literature (Ferraz-Reyes 1985, Perry et al. 1979, Norris 1983, Wardle et al. 1975, Gates and Wilson 1960, Williams and Ingle 1972) (Table 1). As a second treatment, shellfish were fed a mixed suspension of toxic *A. monilatum* (5.5 x 10^2 cells · mL^{-1})

Table 5. Cell concentrations (cells · mL^{-1}) for algal treatments used in clearance rate experiments (means ± 1 SD).

Toxic *Alexandrium monilatum*	**Nontoxic *Alexandrium tamarense***	***Cryptomonas* sp.**	**Toxic *Alexandrium monilatum/ Pavlova* mix**
520 ± 110	500 ± 110	11,040 ± 930	540 ± 120 / 10,840 ± 1,180

and nontoxic Instant Algae® *Pavlova* (1 x 10^4 cells · mL^{-1}) to assess whether the presence of a benign algal food source would promote higher grazing of *A. monilatum.* Bricelj et al. (1991) found that *M. mercenaria* grazed on a highly toxic strain of *Alexandrium fundyense* Balech (strain GtCA29) only when mixed with nontoxic *Thalassiosira wessflogii*. In the present study, *Pavlova* was used in the mixed-food trials rather than *Cryptomonas* sp. because preliminary tests indicated that the cryptophyte lysed when mixed with *A. monilatum*, whereas *Pavlova* cells remained intact.

Two sets of control animals were used: the first set was fed the cryptophyte *Cryptomonas* sp. at 1 x 10^4 cells · mL^{-1}; the second was fed nontoxic *Alexandrium tamarense* at 5.5 x 10^2 cells · mL^{-1}. 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). Pseudofeces can confound estimates for clearance rates because cells are removed from the water column, but not actually ingested (Hildreth 1980). Thus, < 1.5 x 10^4 cells · mL^{-1} were used in these experiments in an attempt to minimize production of pseudofeces. Gentle aeration was used to maintain cells in suspension, which was shown to be successful in preliminary trials. Two additional control containers were included for each trial that consisted of empty shells and the same quantity of the algal treatment being tested, to correct for settlement during the experiments (Shumway et al. 1985b).

Clearance rates of juvenile and adult shellfish were estimated using an indirect method wherein depletion of algal cells from the water column is quantified over time (Coughlan 1969). Individual juvenile shellfish were placed into separate 1 L glass beakers each containing 500 mL of algal suspension; adults were placed into separate 2 L plastic buckets each containing 1 L of algal suspension. Experiments began (T_o) when the animal opened its shell

valves, or when siphon extension was observed (*Mercenaria mercenaria*). Low particle concentrations can make the clearance rate of shellfish difficult to estimate (Widdows 1985). Therefore, a relatively large volume of suspension was used to estimate clearance rates so that the residual particle concentrations did not decline to less than 50% of the initial concentration (Shumway et al. 1985b; Shumway and Cucci 1987).

The duration of feeding experiments was based on preliminary studies and gave shellfish ample time to graze prey without a major decrease in residual cell concentration. Shellfish exposed to *Alexandrium monilatum* and the *A. monilatum/Pavlova* mixture were allowed to graze for 2 h. Sub-samples of the algal suspension (5 mL from each container, n = 3) were taken initially and after 2 h. Each sub-sample was centrifuged at 4,000 RPM for 7 min, re-suspended, and preserved in 0.5% paraformaldehyde (final concentration; Troussellier et al. 1993). Centrifugation caused minimal cell loss and ensured that long chains of *A. monilatum* were broken up before analysis in order to prevent clogging the flow cytometer. Controls were maintained for 1 h, with sub-samples of the algal suspension (5 mL from each container, n = 3; preserved in 0.5% paraformaldehyde) taken initially and at 30 min and 1 h. Samples were held in darkness at 4ºC until analysis within one month.

Cells were enumerated using an EPICS Altra flow cytometer (Coulter Corporation, Miami, FL) equipped with a 488-nm argon laser that was focused to an elliptical point of interrogation (6 µm height x 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 10µm-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 signals was displayed graphically using EXPO32 analysis software (Cytometry Systems, Sheffield, UK) and stored in listmode format. The events (number of algal cells) in a sample were determined by defining regions on the graph that represented the algal species based on these characteristics. The volume of sample analyzed was estimated gravimetrically (Newell et al. 1989). The cell number $\cdot$ mL^{-1} was calculated by dividing the recorded number of events by the total volume analyzed. Samples (taken in triplicate) from each replicate container were counted until each sample count was within 20% of one another, to account for sampling error, and the mean cell concentration $\pm$ 1 standard error (SE) was reported.

The clearance rate of individual animals was calculated using the equation from Coughlan (1969) as:

$$\text{clearance rate } (mL \cdot h^{-1}) = \{(\ln (C_0/C_T) - \ln (C_0'/C_T')\} \cdot V/T$$

wherein C_0 and C_t were the cell concentrations at the initial and final cell concentrations, respectively, during a time interval; C_0' and C_T' were the initial and final cell concentrations, respectively, of particles during a time interval in the control vessels; 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; differences considered significant at $p < 0.05$). Clearance rates were estimated from T_o to T = 30 min for the controls and from 0 to 2 h for the treatments with shellfish and *Alexandrium monilatum*. During these time intervals as mentioned, residual particle concentrations did not decline below 50% of the initial concentration.

Clearance rates of shellfish vary with body size, so shellfish dry weight was used to standardize clearance rate (Jørgensen 1990). After experiments, the bivalves were dissected and the tissues dried at 60ºC for 24 h. The tissue dry weight was used to calculate the weight-specific clearance rate ($mL \cdot h^{-1} \cdot g^{-1}$) for each animal. 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 adult and juvenile shellfish species tested (PROC FREQ – CMH; SAS Institute, Inc. 1999). It was determined that the number of non-functioning replicates considered missing data was random (CHM; df = 5, p = 0.3448). Two two-way analysis of variances (ANOVA - GLM; SAS Institute, Inc. 1999) were used to assess main and interactive effects of treatment (algal species, Table 5) and shellfish species (Tables 3, 4) on the response variable (clearance rate) for juvenile and adult shellfish. A three-way analysis of variance was not used because there was a significant three-way interaction. A Tukey-Kramer multiple comparisons test (SAS Institute, Inc. 1999) was used to determine statistically different pair-wise comparisons (α = 0.05).

2.4. Survival

Adult and juvenile *Crassostrea virginica* and *Mercenaria mercenaria* survived 24 h of exposure to toxic *Alexandrium monilatum*, but *Perna viridis* did not. Bioassays with adult and juvenile *P. viridis* were conducted to determine the time required for half of the mussels to become moribund (LT_{50}). Individual adult *P. viridis* (n = 10) were placed into separate 2 L containers containing 1 L of *A. monilatum* at bloom concentrations as above. Individual juvenile *P. viridis* (n = 10) were exposed to 500 mL of *A. monilatum* in separate 1 L glass

beakers. Other experimental conditions were the same as for the short-term feeding experiments. Shellfish were observed hourly. Moribund status was defined as loss of valve closure ability, a condition that usually precedes death (Ray and Aldrich 1967). A needle was used to prod the mantle cavity to check moribund status.

2.5. Fecal analyses

Feces of juvenile and adult *Crassostrea virginica* and *Mercenaria mercenaria* were checked after animals were exposed for 24 h to toxic *Alexandrium monilatum* (initial concentration, 5.5×10^2 cells $\cdot$ mL^{-1}) to assess survival of *A. monilatum* cells after ingestion. Feces of juvenile and adult *Perna viridis* were checked after animals were exposed to *A. monilatum* for 8 h; the shorter exposure period was required because green mussels were moribund after 24 h of exposure to *A. monilatum* (see section 2.4.). Experimental conditions were the same as for the short-term feeding experiments. Animals were gently removed from experimental containers after the exposure period and rinsed with de-ionized water to remove any free-swimming dinoflagellates from their shells. Each animal was then placed into a separate container with filtered (0.2 µm) ASW to depurate, a 2 ml sample was taken from each container to make sure no motile dioflagellates were transferred with the shellfish. After 24 h, pseudofeces were collected. Pseudofeces were distinguished from feces by their amorphous appearance (loose, without defined shape); feces are more well formed and consolidated (Ma et al. 1999). The remaining suspension from each replicate container was filtered separately through a 30-µm-mesh sieve to collect feces. Feces were rinsed from the sieve with ASW into 5-mL vials. The animals were then transferred to separate containers with filtered ASW to depurate for an additional 24 h, followed by collection of feces and/or pseudofeces as described above.

Shellfish feces were examined under light microscopy (Olympus AX70, 40-600x) and photographed (DEI-750 cooled-chip CCD camera, Optronics Engineering, Goleta, CA) to assess whether *Alexandrium monilatum* cells had passed through the digestive tract intact. Cell viability was tested by inoculating feces from each animal collected after 24 h and 48 h of depuration (Bauder and Cembella 2000). Fecal samples (n = 10) were vortex-mixed and 1 mL of each fecal solution was inoculated into a test tube containing 10 mL of L1 growth media. Each solution with feces was also inoculated (1 mL) into 10 mL of 30-ppt natural seawater (seawater from CCMP, Boothbay Harbor, ME). Two sets of controls consisted of 1 mL of *A. monilatum* stock culture inoculated into test tubes containing 10 mL of L1 media (n = 10), and into tubes with 10 mL of 30-ppt natural seawater (n = 10). The test tubes were held at 23ºC on a 12:12 h L/D cycle at 170 μE m^{-2} s^{-1}. The inoculated feces were checked for *A. monilatum* cells at 7-day intervals, including the first week, for 4 weeks using an Olympus CK-40 inverted microscope (40-100x). 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 4 weeks (Olympus BH-2; 40x). Subsamples taken weekly from the *A. monilatum* controls were preserved in acidic Lugol's solution and cells were quantified by light microscopy as above.

2.6. Valve gape

The valve gape of adult *Perna viridis*, *Crassostrea virginica*, and *Mercenaria mercenaria* (n = 3, 4 animals per replicate trail) exposed to toxic *Alexandrium monilatum* (5.5 x 10^2 cells · mL^{-1}) versus nontoxic Instant Algae® *Pavlova* (5.0 x 10^4 cells · mL^{-1}) was measured as an indicator of avoidance behavior (see review in Shumway and Gainey 1992). Bivalves exposed to low algal concentrations often reduce their valve gape (Riigård et al. 2003). To avoid a major decrease in residual cell concentration due to shellfish feeding, a

higher concentration of the control alga was used in the valve gape experiments. A higher concentration of *A. monilatum* was not needed because preliminary clearance rate tests indicated residual cell concentration of *A. monilatum* do not fall below 50% the initial concentration after 2 h of shellfish feeding. Valve gape was measured with optical fibers using the methodology and instrument described in Frank et al. (2003) (Figure 1). A Campbell Scientific CR10X data logger (Campbell Scientific, Inc., Logan, UT) recorded the data (voltage output s^{-1}).

The valve gape sensor was calibrated before experiments by recording the voltage output when the optical fibers were moved in mm increments (Frank et al. 2003). Voltage was used to estimate valve gape (mm distance between shells) by plotting the average change in voltage output versus distance (mm increments). Light decayed exponentially with distance, so the following equation was used to determine the slope of the line:

$$y = b \cdot e^{mx},$$

where y = voltage output, b = voltage output equal to zero valve gape (y intercept), x = distance between fiber ends in mm, and m = slope of the calibration line. By solving for x, the following equation was used to estimate valve gape:

$$x = \{\ln(y/b)\} \cdot m^{-1}$$

The experiments were conducted in a container with 8 L of the algal suspension under gentle aeration, at 22ºC. Animals were prepared for analysis following Frank et al. (2003), except that Krazy Glue® (Elmor's Products Inc, Columbus, OH) was used to attach the Velcro® (Velcro USA Inc., Manchester, NH) 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 of shellfish with closed valves was recorded at T_o. All animals were allowed at

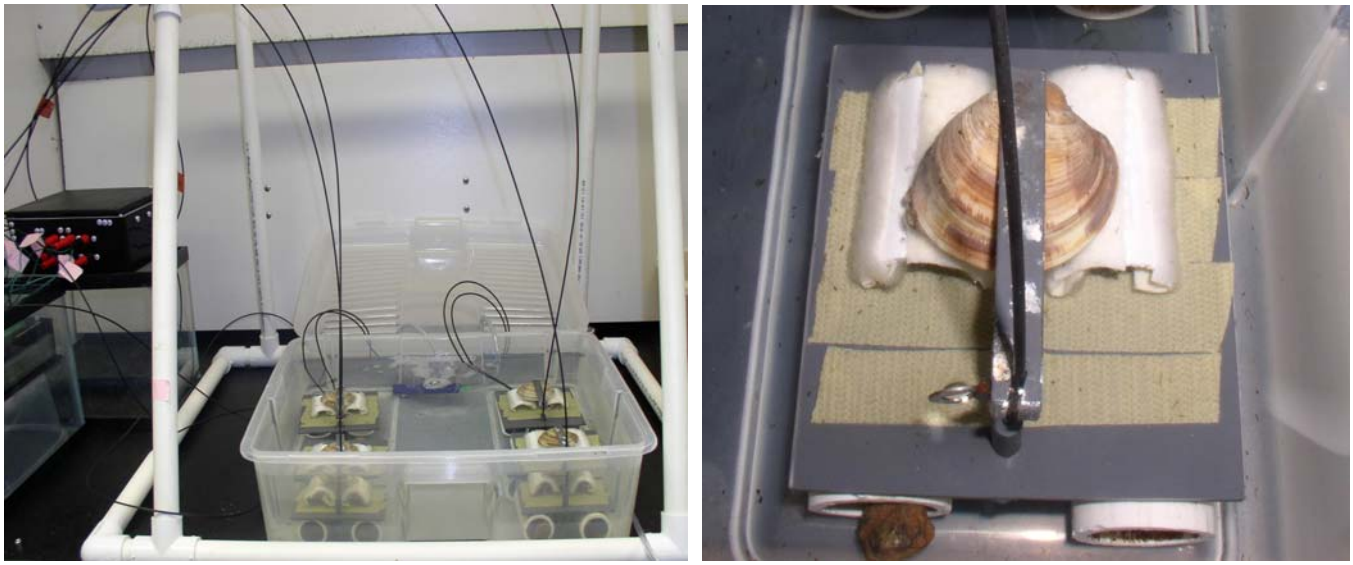

Figure 1. Adult *Mercenaria mercenaria* exposed to toxic *Alexandrium monilatum,* with fiber optical cable attached to valve to record shell valve movements.

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. Mean valve gape of shellfish after 2 h was reported, $\pm$ 1 SE. A two-way ANOVA (GLM; SAS Institute, Inc. 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 ($\alpha = 0.05$).

2.7. Larval bioassays

The effect of toxic *Alexandrium monilatum* on survival of *Mercenaria mercenaria* and *Crassostrea virginica* larvae was tested using *A. monilatum* that was (i) unconstrained, (ii) restricted (held in dialysis tubing), or (iii) sonicated. Constrained cells were used to test for the presence of exotoxins from *A. monilatum* that may diffuse through the dialysis tubing and cause mortality, 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). Therefore, the effect of sonicated and live *A. monilatum* cells on shellfish 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 *Alexandrium tamarense* that was unconstrained, held in dialysis tubing, or sonicated. Larvae were placed into Falcon® polystyrene multi-well plates 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 (*Alexandrium monilatum* or *Alexandrium tamarense*) was pipetted into each well (n = 10) that contained 5 mL of ASW, for a final concentration of 5.5×10^2 cells $\cdot$ mL^{-1}. Suspensions of *A. monilatum* and *A. tamarense* (5.5 x

10^2 cells · mL^{-1}) were each sonicated with a Fisher Scientific 550 Sonic Dismembrator for 60 s at a time for a total of 10 min, chilling at 3ºC between sonications. The suspensions were checked under a microscope (Olympus IX-70, 40-100x) to ensure that all cells had lysed before use. Five mL of the suspension was added to each well (n = 10). Dialysis tubing (molecular weight cut-off 12,000 to 14,000 Da, Fisher Scientific International Inc., Hampton, NH) was used to prevent direct contact of the algae and larvae. Cultures of *A. monilatum* and *A. tamarense* were added to the dialysis tubing for a final concentration of 5.5 x 10^2 cells · mL^{-1} (n =10; with 5 ml ASW).

The well plates with larvae and all controls were placed on a light microscope stage (Olympus CK-40). Mortality (loss of ciliary movement for > 1 min; 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 Institute, Inc. 1999) was used to determine statistically different pair-wise comparisons ($\alpha = 0.05$).

3. RESULTS

3.1. Short-term feeding experiments

3.1.a. Adult shellfish. There was a significant effect of algal treatment and shellfish species on clearance rates of adult shellfish (two-way ANOVA; algal treatment: $df = 3$, $F = 123.07$, $p < 0.0001$; shellfish treatment: $df = 2$, $F = 113.50$, $p < 0.0001$). There was also a significant interaction effect (two-way ANOVA; interaction: $df = 6$, $F = 25.21$, $p < 0.0001$), which precluded comparisons across the algal species and shellfish species main effects. Adult *Crassostrea virginica* and adult *Perna viridis* fed *Alexandrium monilatum* or the *A. monilatum*/*Pavlova* mixture had significantly lower clearance rates than control animals fed *Cryptomonas* sp. or nontoxic *Alexandrium tamarense* (Table 6, Figs. 2, 3). The interaction effect was due to differences in the clearance rate of *Mercenaria mercenaria* fed *A. tamarense* compared to the other two shellfish species. Clearance rates of *M. mercenaria* fed *A. monilatum* or the *A. monilatum/Pavlova* mixture were significantly lower than clearance rates of quahogs fed *Cryptomonas* sp. However, there was no significant difference in the clearance rates of *M. mercenaria* fed *A. monilatum* or *A. monilatum/Pavlova* mixture compared to the *A. tamarense* treatment (Table 6, Fig. 4).

Clearance rates of adult animals exposed to *Alexandrium monilatum* or the *A. monilatum/Pavlova* mixture were comparable among the three shellfish species (Table 6). Adult *Perna viridis* fed *Cryptomonas* sp. or nontoxic *Alexandrium tamarense* had higher clearance rates than adult *Crassostrea virginica* and *Mercenaria mercenaria* fed the same control species ($p < 0.0001$). Clearance rates of adult *C. virginica* and *M. mercenaria* fed *Cryptomonas* or *A. tamarense* were comparable, but adult *P. viridis* had lower clearance rates

Table 6. Results of multiple comparison tests for the pair-wise effects of algal treatment on clearance rates (ml h^{-1} g^{-1}) of adult shellfish in laboratory experiments. n.s. = not significant

Shellfish species	***Alexandrium monilatum* versus Mix**	***Alexandrium monilatum* versus *Cryptomonas***	***Alexandrium monilatum* versus *Alexandrium tamarense***	**Mix versus *Cryptomonas***	**Mix versus *Alexandrium tamarense***	***Cryptomonas* versus *Alexandrium tamarense***
Crassostrea virginica	n.s.	$p = 0.0070$	$p = 0.0409$	$p = 0.0030$	$p = 0.0250$	n.s.
Perna viridis	n.s.	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0208$
Mercenaria mercenaria	n.s.	$p < 0.0001$	n.s.	$p = 0.0003$	n.s.	n.s.

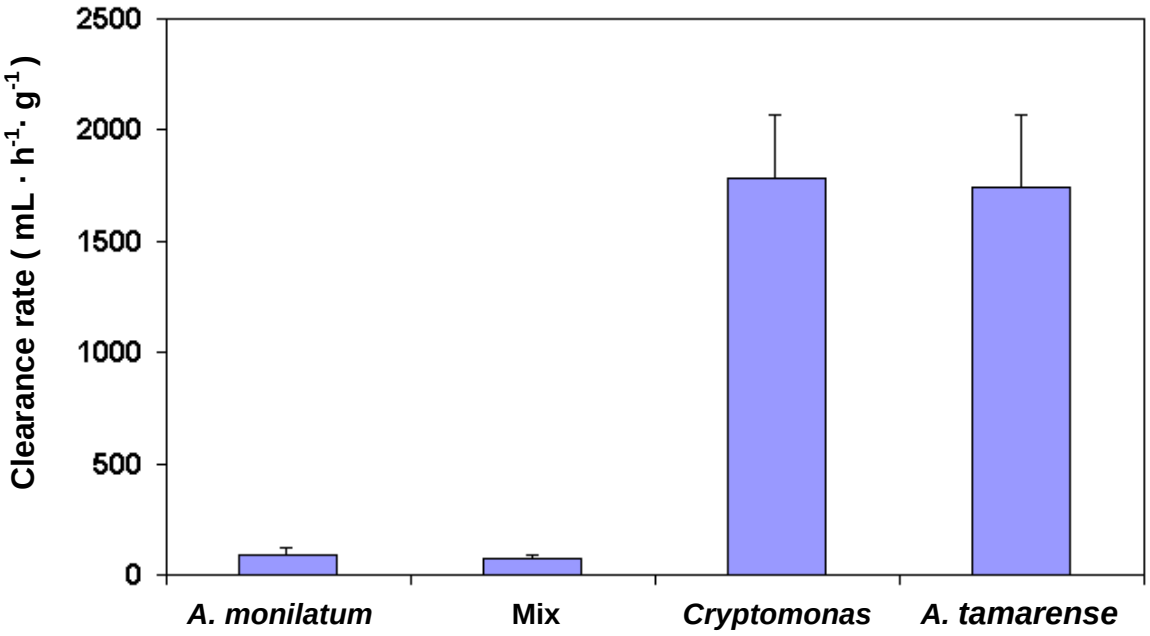

Figure 2. Mean weight-specific clearance rates (± 1 SE) of adult *Crassostrea virginica* exposed to toxic *Alexandrium monilatum* (n = 5), *A. monilatum*/*Pavlova* mix (n = 6), *Cryptomonas* sp. (n = 7), and nontoxic *Alexandrium tamarense* (n = 4).

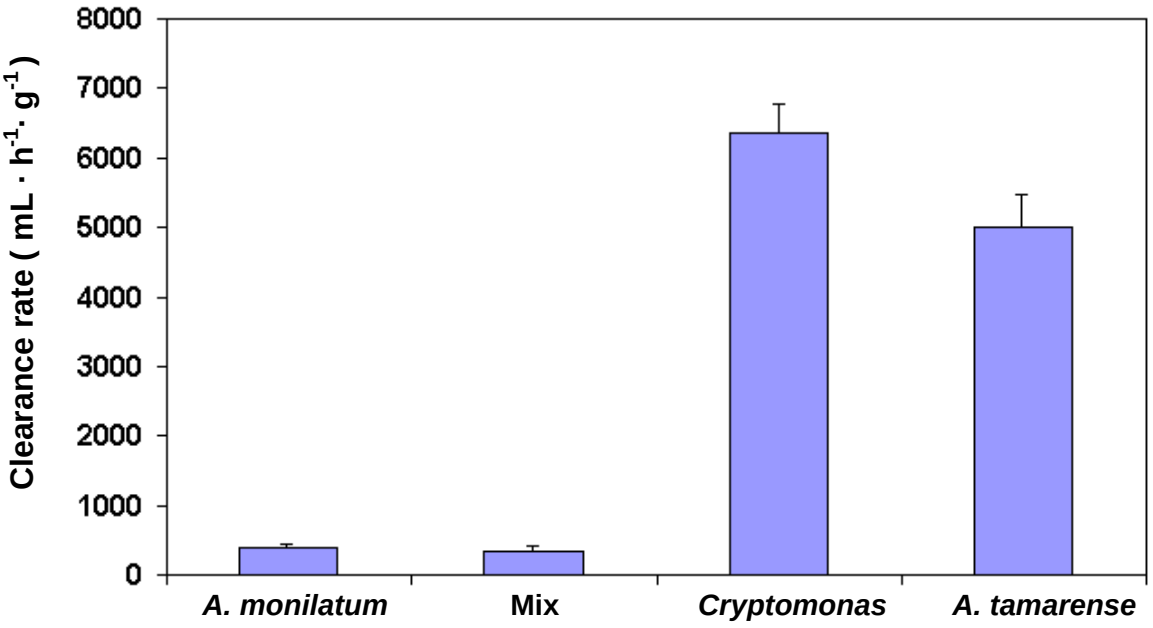

Figure 3. Mean weight-specific clearance rates (± 1 SE) of adult *Perna viridis* exposed to toxic *Alexandrium monilatum* (n = 8), *A. monilatum*/*Pavlova* mix (n = 6), *Cryptomonas* sp. (n = 10) and nontoxic *Alexandrium tamarense* (n = 6).

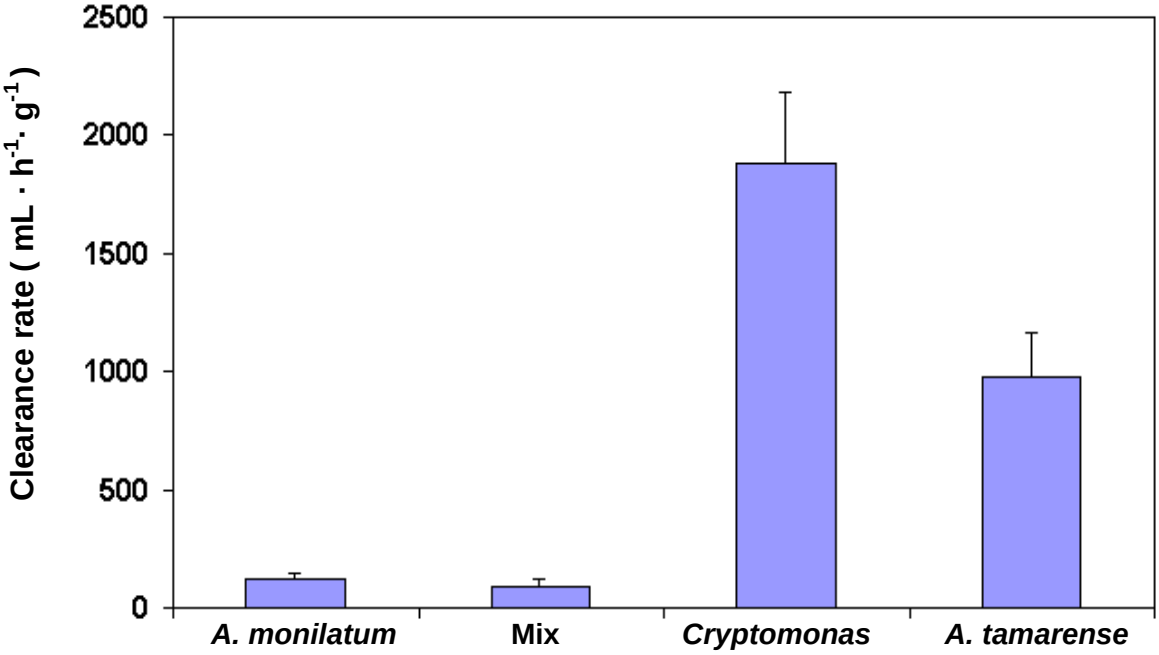

Figure 4. Mean weight specific clearance rate (± 1 SE) of adult *Mercenaria mercenaria* exposed to toxic *Alexandrium monilatum* (n = 10), *A. monilatum*/*Pavlova* mix (n = 7), *Cryptomonas* sp. (n = 9) and nontoxic *Alexandrium tamarense* (n = 5).

when fed *A. tamarense* compared to clearance rates of mussels fed cryptomonads ($p = 0.021$).

3.1.b. Juvenile shellfish. There was a significant effect of algal treatment on clearance rates of juvenile shellfish, but there was no difference in clearance rates among shellfish species, nor a 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: $df = 6$, $F = 0.86$, $p = 0.5305$). Juveniles of all three species had comparable clearance rates when fed *Cryptomonas* sp. versus nontoxic *Alexandrium tamarense*, and comparable clearance rates when fed *Alexandrium monilatum* versus the *A. monilatum*/*Pavlova* mixture (Table 7, Figs. 5-7). Clearance rates of juveniles fed *A. monilatum* or *A. monilatum*/*Pavlova* were significantly lower than clearance rates of animals fed *Cryptomonas* or nontoxic *A. tamarense* (Table 7, Figs. 5-7).

3.2. Survival

Adult and juvenile *Crassostrea virginica* and *Mercenaria mercenaria* survived 24 h of exposure to toxic *Alexandrium monilatum*, but adult and juvenile *Perna 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 h and 10 h, respectively, for adult and juvenile *P. viridis*.

3.3. Fecal analyses

Qualitative analysis of fecal material collected from *Crassostrea virginica*, *Perna viridis*, and *Mercenaria mercenaria* after 24 h and 48 h of depuration indicated that *Alexandrium monilatum* cells were able to pass intact through the digestive tract of these

shellfish species (Figs. 8-10). Pseudofeces production was not detected during the feeding experiments, but adult *P. viridis* produced copious amounts of pseudofeces after 8 h of exposure to *A. monilatum*. The pseudofeces also contained intact *A. monilatum* cells (Fig. 11).

Checks of the inoculated feces at 7-day intervals using light microscopy did not detect *Alexandrium monilatum* cells from fecal material of juvenile shellfish (all three species), nor from adult *Mercenaria mercenaria*. During the first week of incubation, in 2 of 10 replicates, *A. monilatum* cells were found swimming in the NSW media containing inoculated feces from adult *Perna viridis* collected after 24 h of depuration. No cells were observed from *P. viridis* fecal material that was inoculated into L1 media, or from feces that were collected after 48 h of depuration. Feces from adult *Crassostrea virginica* also yielded swimming *A. monilatum* cells within the first week. Cells were observed in the L1 media with *C. virginica* feces collected after 24 h of depuration (2 of 10 replicates) and NSW with *C. virginica* feces collected after 24 h of depuration (2 of 10 replicates). For adult *C. virginica* feces collected after 48 h of depuration, 1 replicate in NSW media contained swimming *A. monilatum* cells.

No *Alexandrium monilatum* cells were observed in any of the inoculated feces cultures, however, after 2-4 weeks, and microscopic analysis of acidic Lugol's-preserved samples did not detect *A. monilatum* cells. In contrast, *A. monilatum* cell production occurred in controls with L1 media (division rate (k) = 0.19 d^{-1} based on Guillard 1973; Fig. 12) and NSW media (k = 0.24 d^{-1}; Fig. 13).

Table 7. Results of multiple comparison tests for the pair-wise effects of algal treatment on clearance rates (ml h^{-1} g^{-1}) of juvenile shellfish in laboratory experiments. n.s. = not significant

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

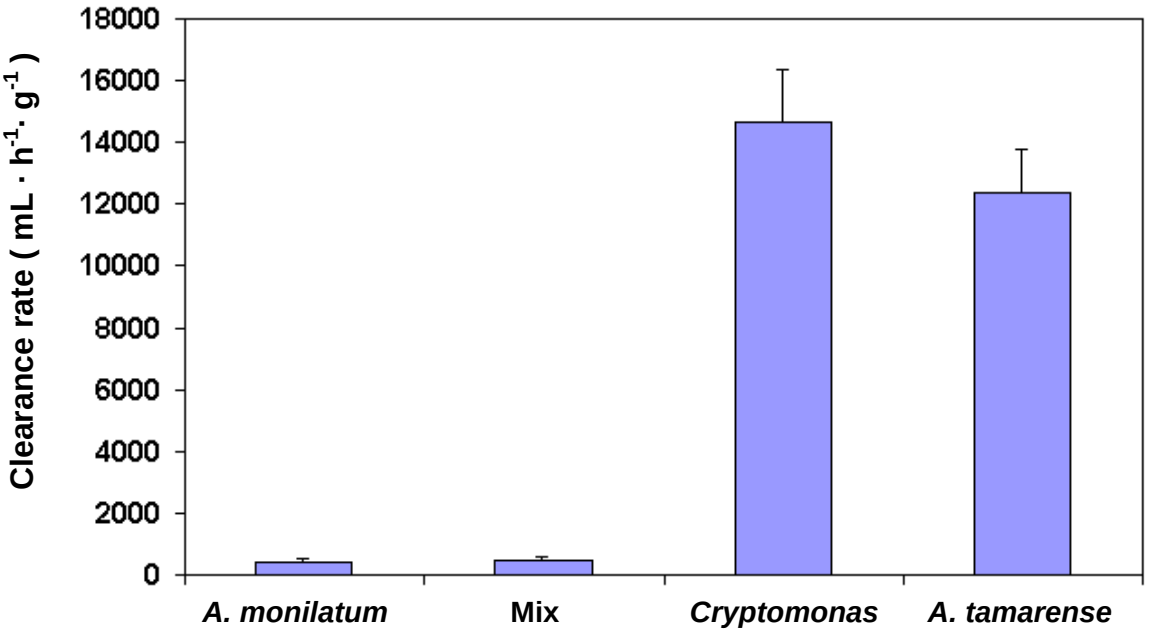

Figure 5. Mean weight-specific clearance rates (± 1 SE) of juvenile *Crassostrea virginica* exposed to toxic *Alexandrium monilatum* (n = 8), *A. monilatum*/*Pavlova* mix (n = 5), *Cryptomonas* sp. (n = 7) and nontoxic *Alexandrium tamarense* (n = 5).

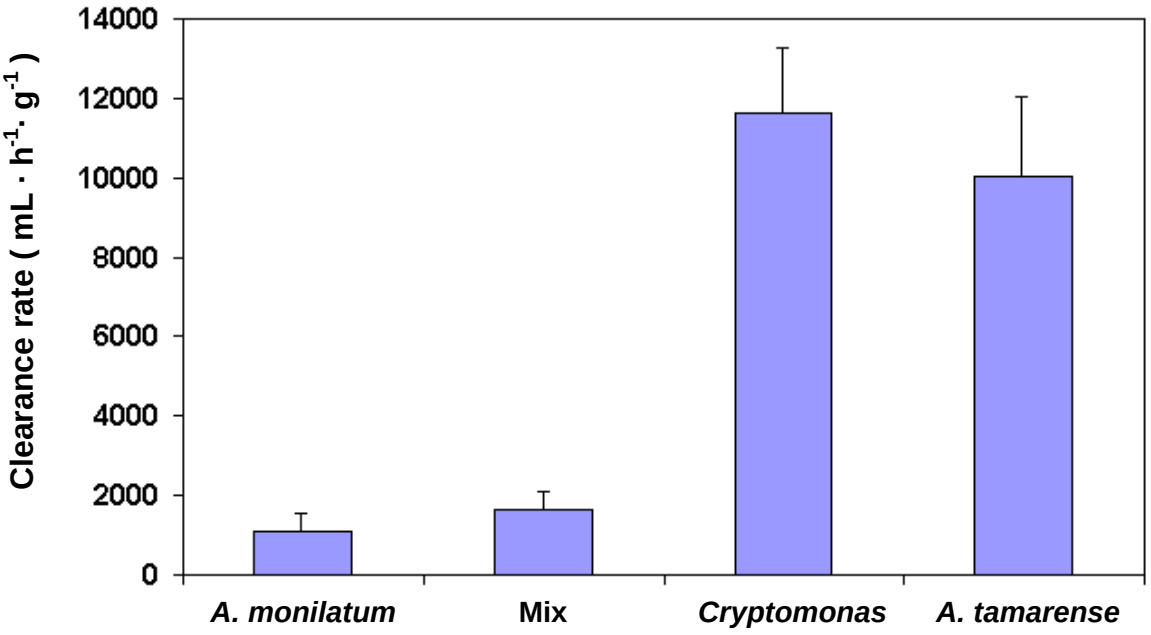

Figure 6. Mean weight-specific clearance rates (± 1 SE) of juvenile *Perna viridis* exposed to toxic *Alexandrium monilatum* (n = 7), *A. monilatum*/*Pavlova* mix (n = 7), *Cryptomonas* sp. (n = 10) and nontoxic *Alexandrium tamarense* (n = 5).

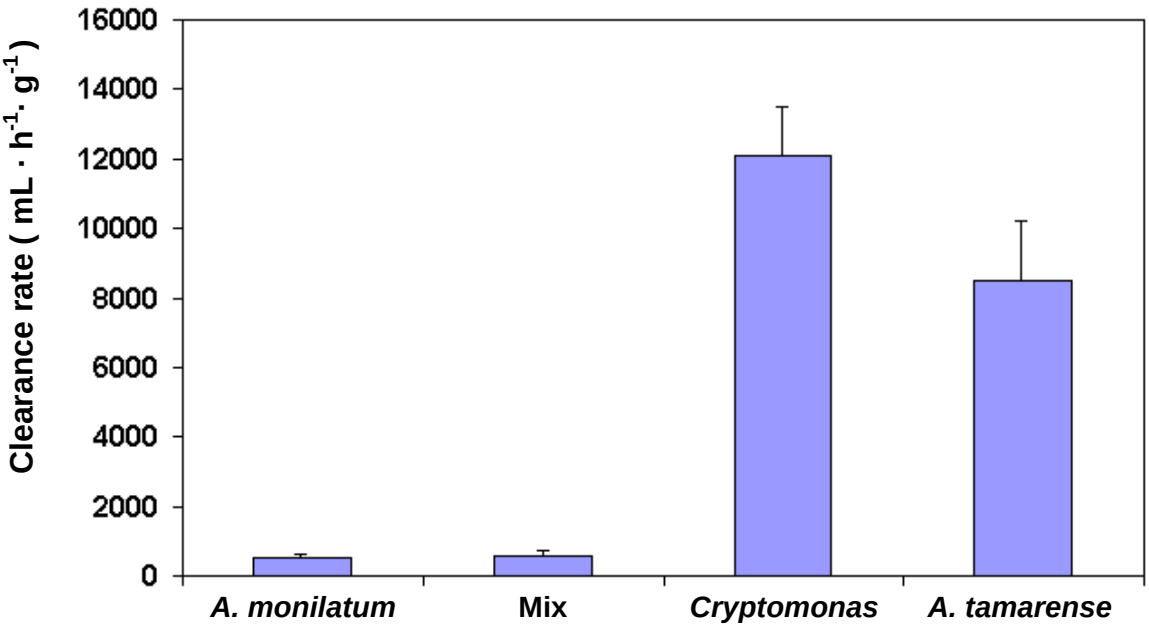

Figure 7. Mean weight-specific clearance rates (± 1 SE) of juvenile *Mercenaria mercenaria* exposed to toxic *Alexandrium monilatum* (n = 7), *A. monilatum*/*Pavlova* mix (n = 6), *Cryptomonas* sp. (n = 9) and nontoxic *Alexandrium tamarense* (n = 8).

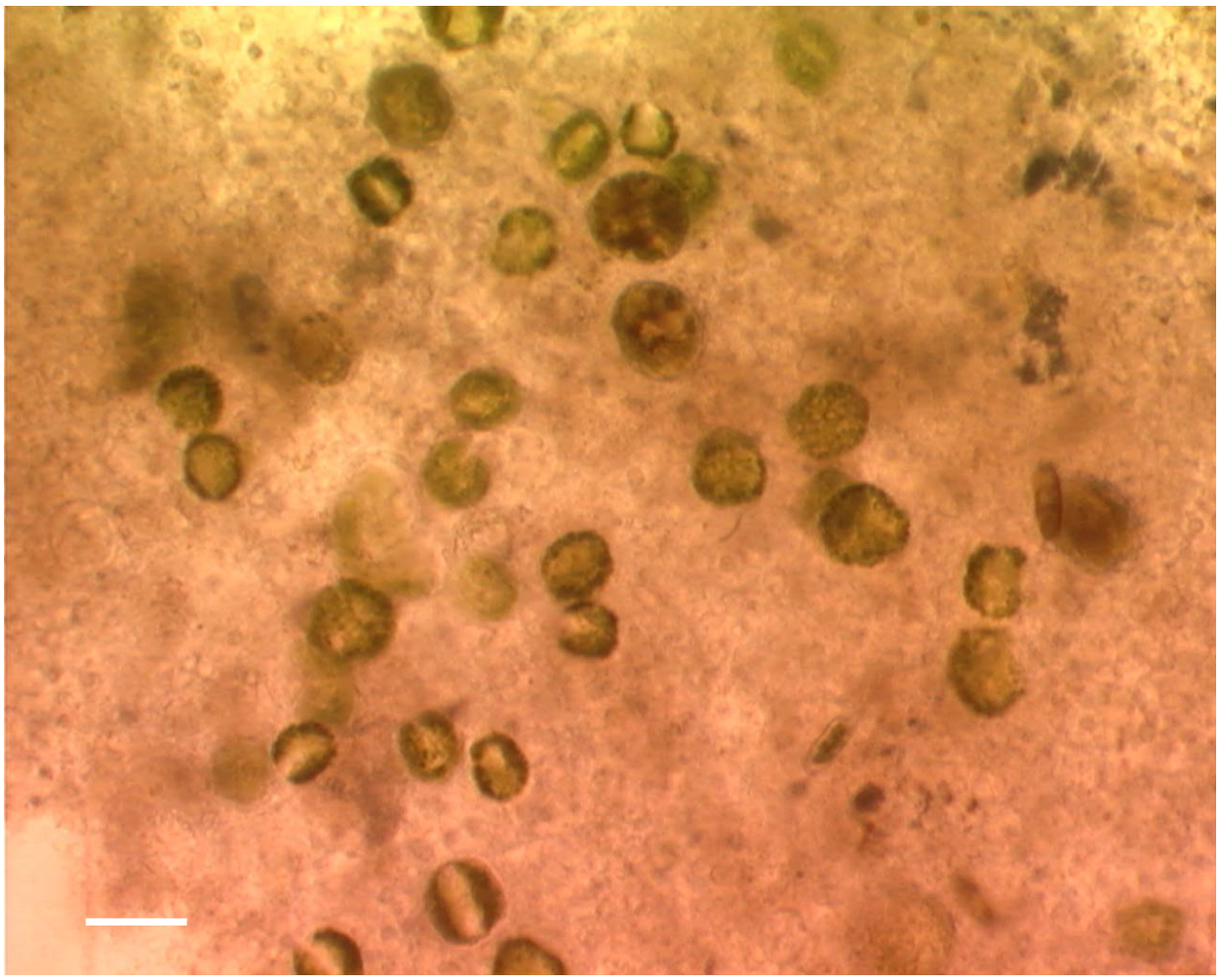

Figure 8. Adult *Crassostrea virginica* feces collected after 48 h of depuration, showing intact *Alexandrium monilatum* cells (scale bar = 50 μm, 100x).

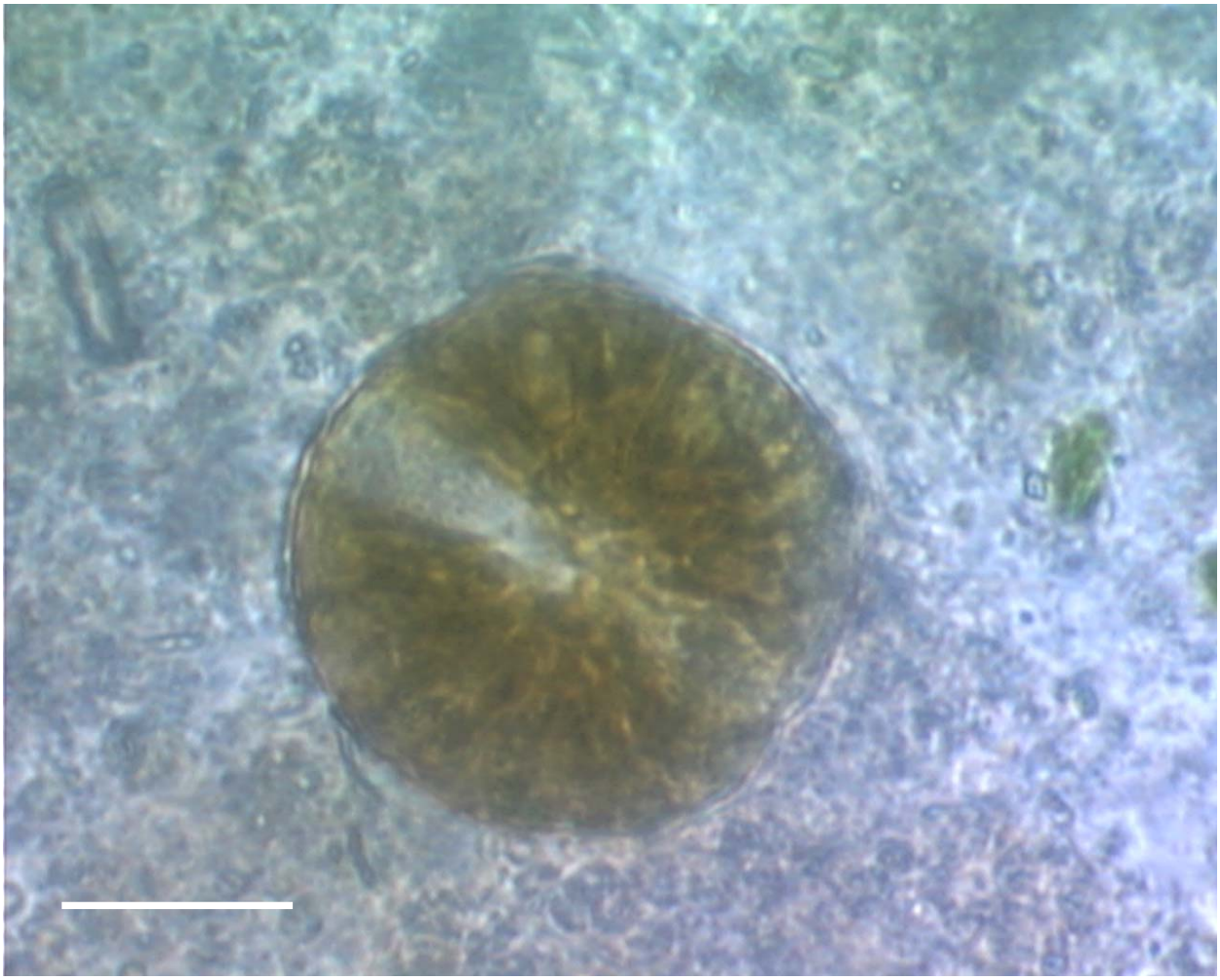

Figure 9. Intact *Alexandrium monilatum* cell in adult *Perna viridis* feces collected after 24 h of depuration (scale bar = 20 μm, 600x).

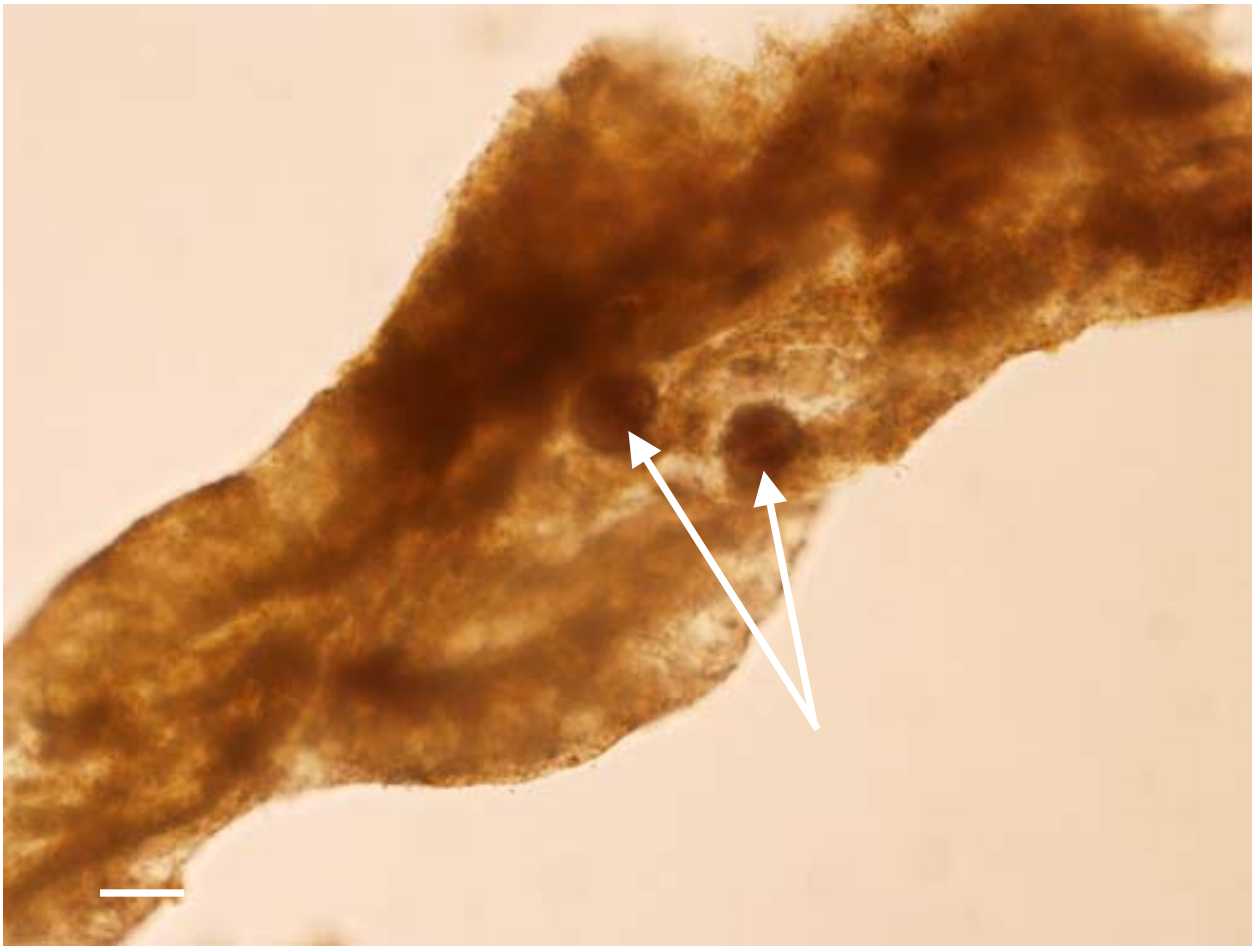

Figure 10. Intact *Alexandrium monilatum* (arrows) in adult *Mercenaria mercenaria* feces collected after 24 h of depuration (scale bar = 50 μm, 40x).

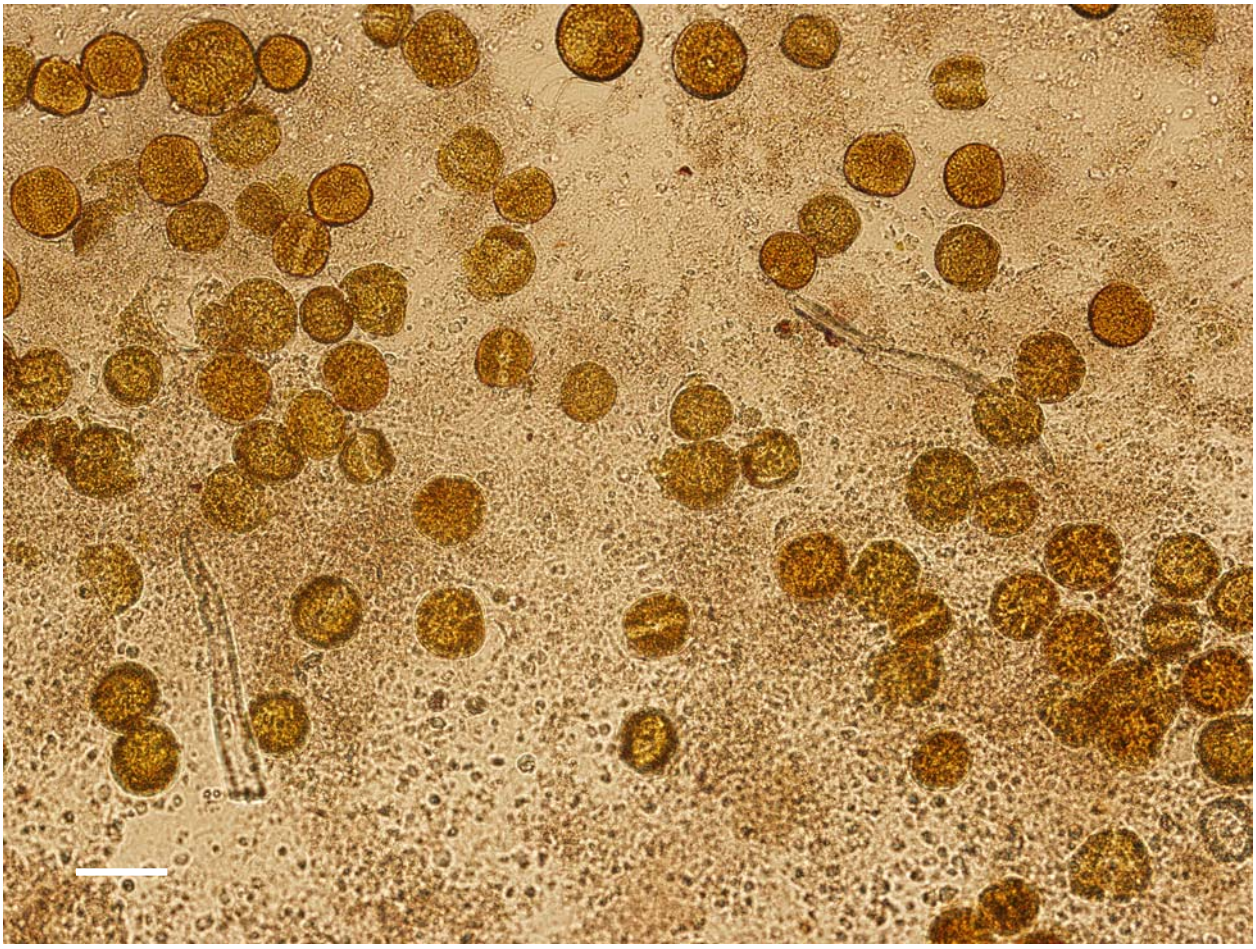

Figure 11. Adult *Perna viridis* pseuduofeces after 8 h of exposure containing *Alexandrium monilatum* cells (scale bar = 50 μm, 100x).

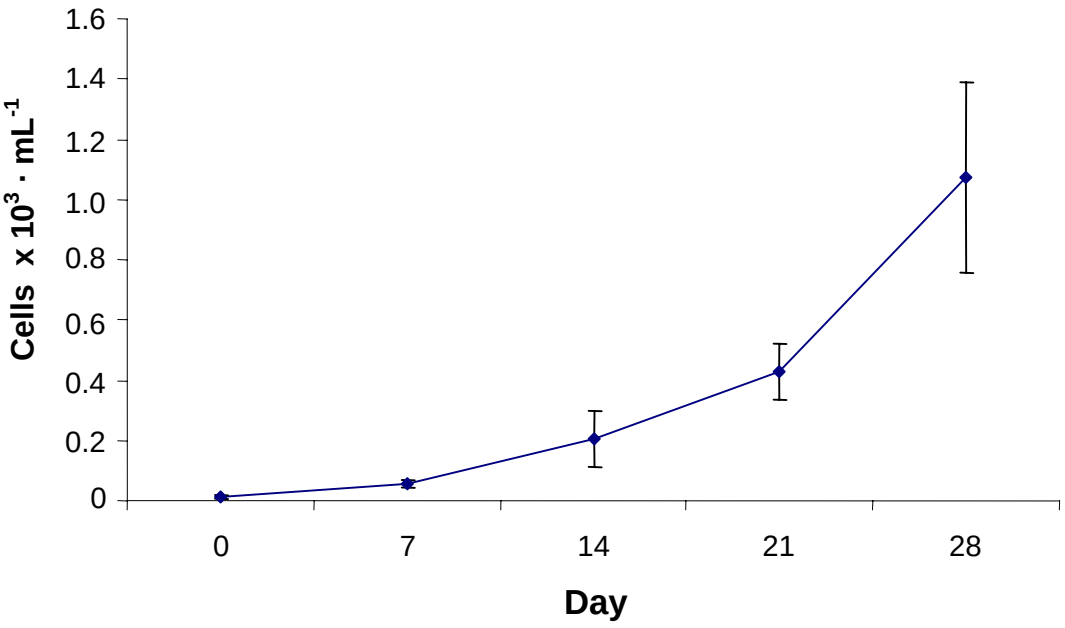

Figure 12. Cell production by *Alexandrium monilatum* (k = 0.19 d^{-1}) in L1 medium (means ± 1 SE, n = 10).

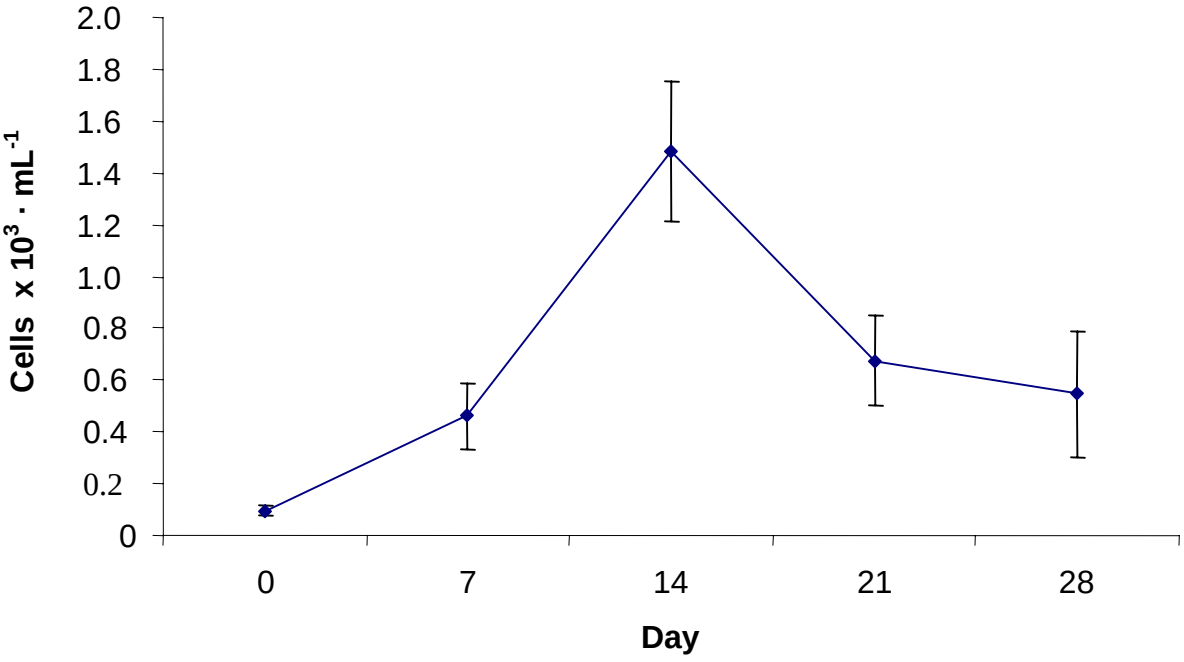

Figure 13. Cell production by *Alexandrium monilatum* (k = 0.24 d^{-1}) in natural seawater (means ± 1 SE, n = 10).

3.4. Valve gape

There was a significant effect of algal treatment on the mean valve gape of adult shellfish, but there was no difference in valve gape among shellfish species, nor a 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). Exposure to toxic *Alexandrium monilatum* caused a significant decrease in the mean valve gape of adult *Crassostrea virginica* and *Perna viridis* (Figs. 14, 15) relative to animals given nontoxic *Pavlova* (*C. virginica*: p = 0.0021; *P. viridis*: p = 0.0013). The mean valve gape of *Mercenaria mercenaria* exposed to *A. monilatum* was also significantly lower than animals given nontoxic *Pavlova*, but at a 1% statistical level (*M. mercenaria*: p = 0.1024) (Fig. 16).

3.5. Larval bioassays

There was no difference in percent survival of *Crassostrea virginica* or *Mercenaria mercenaria* larvae exposed to intact and restricted *Alexandrium monilatum* and *Alexandrium tamarense* (Fig. 17, 18). In contrast, sonicated *A. monilatum* caused a 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 38% of the *M. mercenaria* larvae survived.

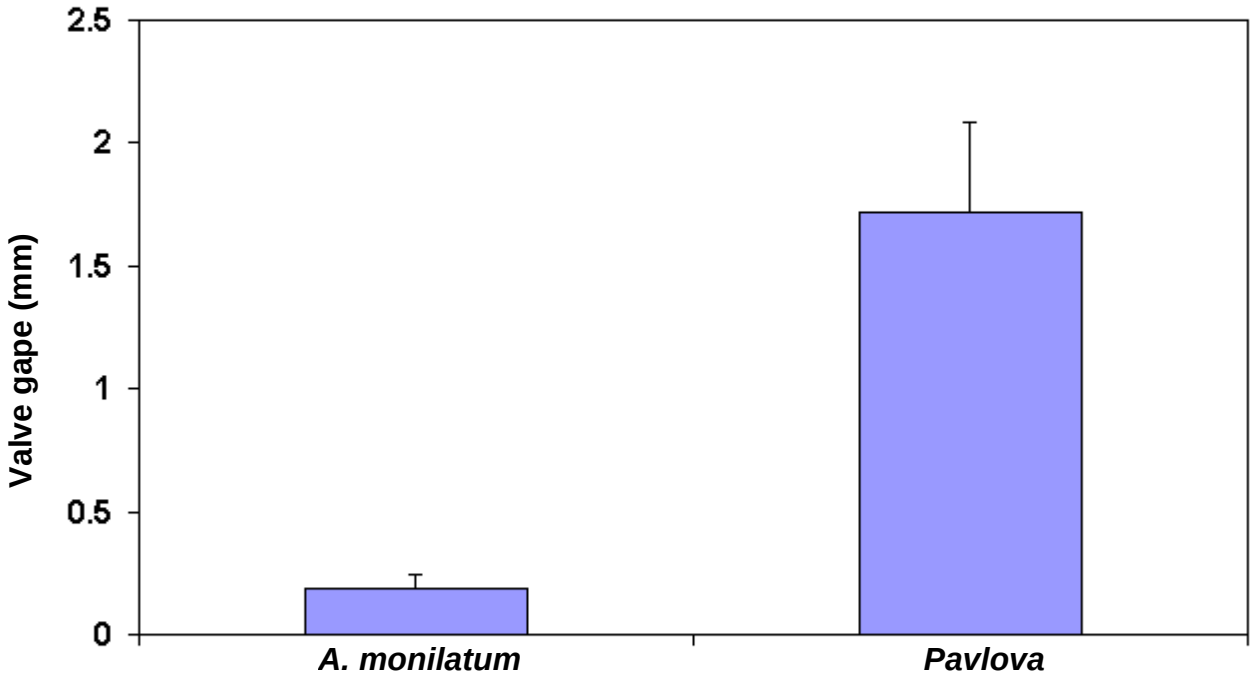

Figure 14. Mean valve gape (± 1 SE) of adult *Crassostrea virginica* exposed to toxic *Alexandrium monilatum* versus benign algal prey (*Pavlova*) for 2 h (n = 3) (significantly different; Tukey-Kramer multiple comparisons test; p = 0.0021).

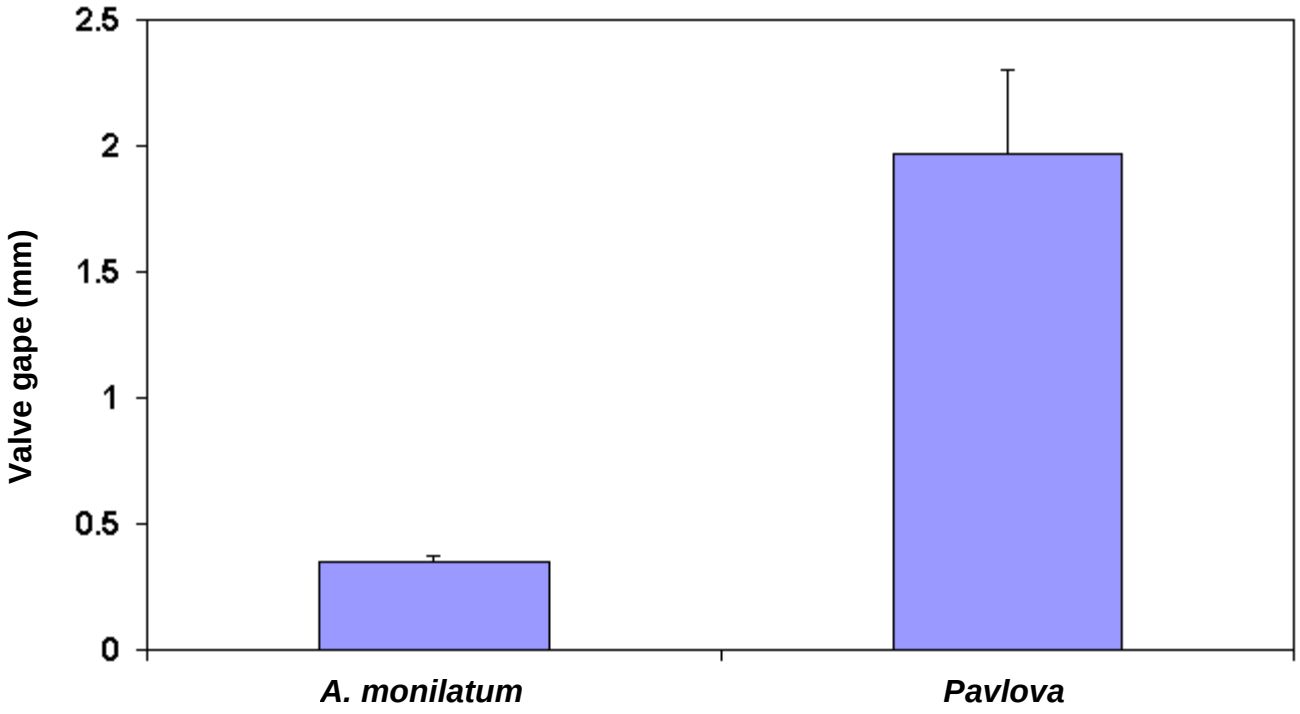

Figure 15. Mean valve gape (± 1 SE) of adult *Perna viridis* exposed to toxic *Alexandrium monilatum* versus benign algal prey (*Pavlova*) for 2 h (n = 3) (significantly different; Tukey-Kramer multiple comparisons test; p = 0.0013).

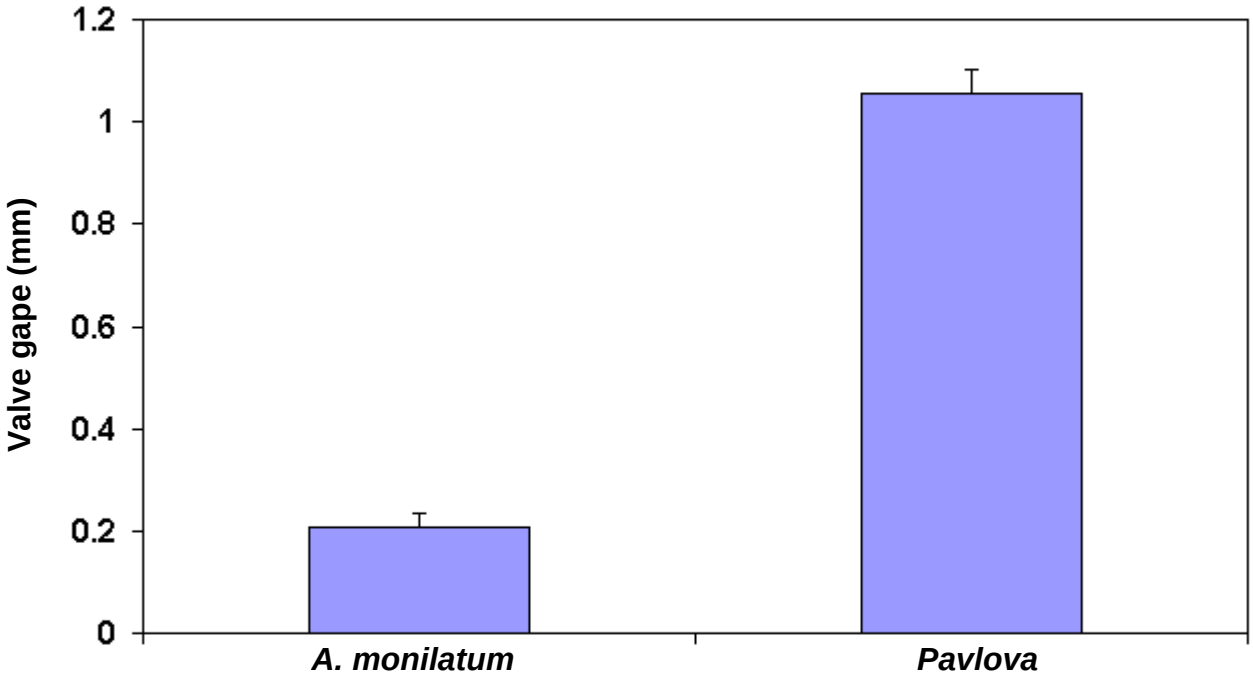

Figure 16. Mean valve gape (± 1 SE) of adult *Mercenaria mercenaria* exposed to toxic *Alexandrium monilatum* versus benign algal prey (*Pavlova*) for 2 h (n = 3) (significantly different; Tukey-Kramer multiple comparisons test; p = 0.1024).

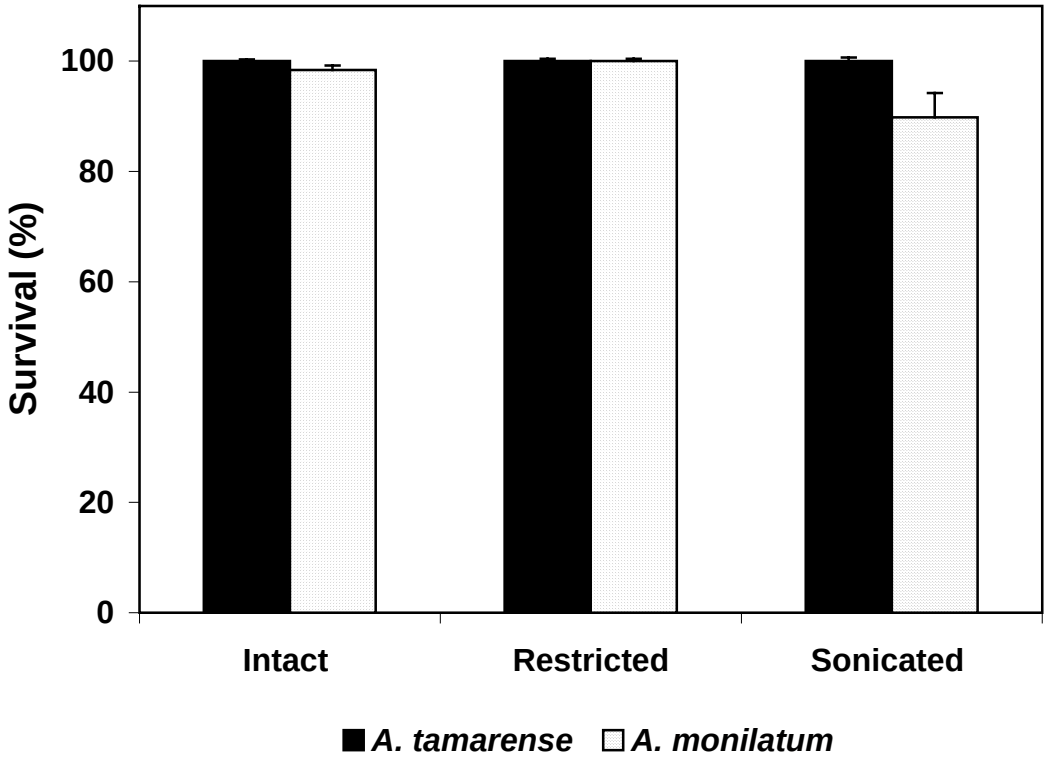

Figure17. Survival of larval *Crassostrea virginica* exposed to intact, restricted and sonicated toxic *Alexandrium monilatum* or nontoxic *Alexandrium tamarense* for 2 h (means ± 1 SE; n = 10).

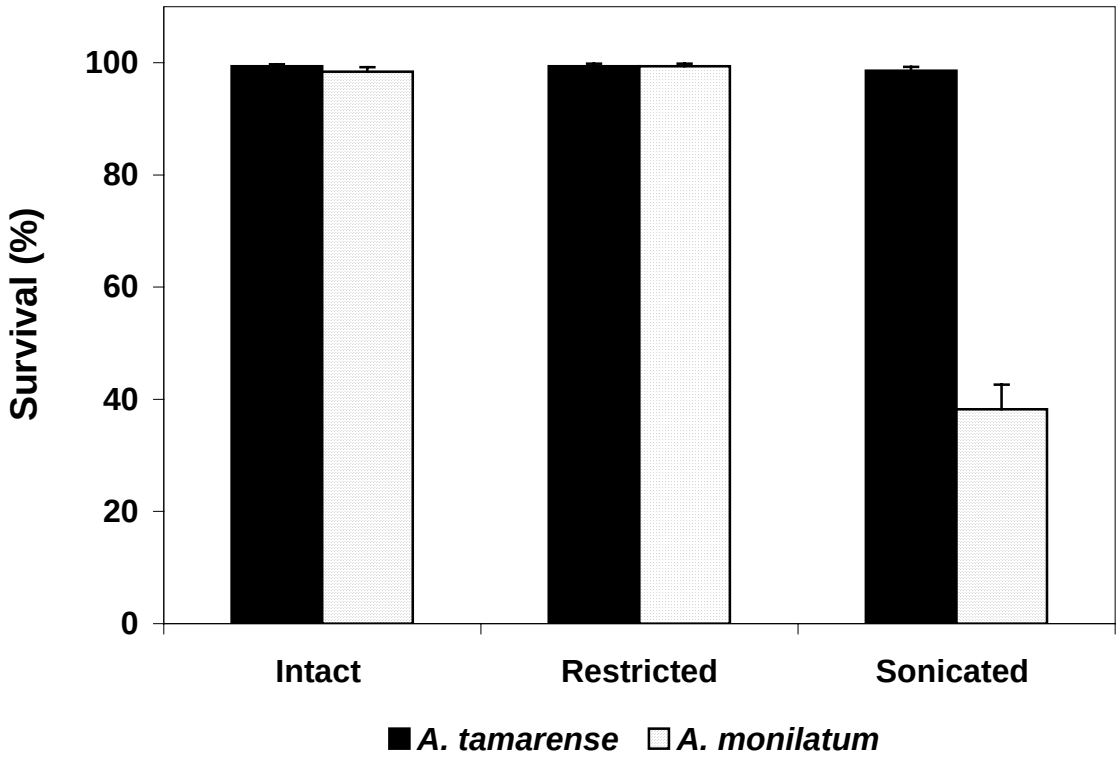

Figure 18. Survival of larval *Mercenaria mercenaria* exposed to intact, restricted and sonicated *Alexandrium monilatum* (n = 10) or *Alexandrium tamarense* (n = 10) for 2 h (means ± 1 SE; n = 10).

4. DISCUSSION

This is the first systematic study of the impacts of *Alexandrium monilatum* on shellfish. Previous research has demonstrated that bivalve responses to toxic dinoflagellates are variable, ranging from avoidance behavior (e.g. valve closure, clearance rate reduction) to normal feeding activity, and species specific (see review in Gainey and Shumway 1988b). In this study, the responses of *Mercenaria mercenaria, Crassostrea virginica,* and *Perna viridis* experimentally exposed to a toxic strain of *A. monilatum* were species-specific. Shellfish could be exposed to this dinoflagellate at any stage of their life history, and it was shown that *A. monilatum* blooms could potentially affect the survival of these shellfish species by reducing clearance rate and valve gape, affecting their food intake, and inducing mortality in some species.

4.1. Effect of *Alexandrium monilatum* on clearance rates of juvenile and adult shellfish

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), the amount of toxin accumulated in the bivalves (Bricelj et al 1991) and history of exposure to harmful algal blooms (Shumway and Cucci 1987). Bricelj et al. (2005) found that clams (*Mya arenaria* Linnaeus) inhabiting areas with frequent harmful algal blooms were more resistant to paralytic shellfish poisoning (PSP) toxins and accumulated toxins at a high rate. The authors concluded that this resistance is due to a natural sodium channel mutation, which causes a 1,000-fold decrease in affinity at the saxitoxin-binding site.

Oysters and clams generally exhibit lower levels of toxicity than other species tested concurrently after exposure to harmful algal blooms, with mussels accumulating toxins much more rapidly (Lassus et al. 1989, Bricelj et al. 1990, Shumway 1990, see review Shumway et

al. 1990). Previous research has shown that *Mercenaria mercenaria* and *Crassostrea virginica* show avoidance behavior in response to toxic dinoflagellates usually resulting in valve closure or reduced filtration (Ray and Aldrich 1967, Shumway and Cucci 1987b, Shumway 1990, Bricelj et al. 1990, Shumway 1990, Bricelj et al. 1991). For example, *M. mercenaria* closed their shell valves when exposed to toxic *Protogonyaulax tamarensis* (Lebour) Taylor and did not re-open until placed into fresh seawater (Shumway and Cucci 1987). Shumway and Cucci (1987) also found that the oyster, *Ostrea edulis* Linnaeus, significantly increased their clearance rate when fed toxic *P. tamarensis*, but the filtration rate of *C. virginica* was significantly reduced. Avoidance behavior isolates the animal from the external environment and can affect toxin accumulation (Gainey and Shumway 1988b).

It has been reported that *Mercenaria mercenaria* is not as sensitive to saxitoxins as some shellfish species, whereas *Crassostrea virginica* is the most sensitive (Twarog and Yamaguchi 1974). Bricelj et al. (1991) reported evidence of a toxin recognition mechanism in *M. mercenaria*: in their study, adult animals ingested and consumed weakly toxic strains of *Alexandrium tamarense*, whereas very little of the more toxic *Alexandrium fundyense* strain was ingested, and only in the presence of a nontoxic diatom supplement. This species has also 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 their normal distribution at ~15 cm (Shumway 1990). Nerve fibers of *M. mercenaria* apparently are resistant to PSP toxins, but whole animals are capable of avoidance behavior, which may be mediated by a toxin recognition mechanism (Bricelj et al. 1991, Shumway 1990). The mechanism for recognition of harmful algal toxins by shellfish is poorly understood. Li et al. (2001) exposed *Perna 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 PSP toxin content. It was hypothesized that their feeding rate was not affected either due to the low toxicity of the dinoflagellate clone that was used in the study or toxic algae does not affect their clearance rates (Li et al. 2001).

Ray and Aldrich (1967) reported that *Crassostrea virginica* rarely opened or filtered when exposed to *Alexandrium monilatum.* In this study, all adult and juvenile shellfish filtered *A. monilatum*, but at a significantly lower rate than shellfish fed benign *Cryptomonas* sp. Also, clearance rates of adult and juvenile shellfish fed *A. monilatum* were significantly lower than clearance rates of shellfish exposed to the nontoxic *Alexandrium tamarense*, except for adult *Mercenaria mercenaria.* There was a decrease, although not statistically significant, in the clearance rate of adult *M. mercenaria* fed *A. monilatum* compared to quahogs given nontoxic *A. tamarense*. Based on previous research with *M. mercenaria* and toxic dinoflagellates (Bricelj et al. 1990, Shumway 1990, Bricelj et al. 1991) the lack of statistical significance in clearance rates of adult *M. mercenaria* fed toxic *A. monilatum* versus nontoxic *A. tamarense* may have occurred due to low numbers of replicates. Clearance rates of shellfish fed *Cryptomonas* sp. versus *A. tamarense* were not significantly different, except that the clearance rate of adult green mussels was depressed by ~20% when fed the nontoxic strain of *A. tamarense*. Thus, the nontoxic *A. tamarense* strain generally did not adversely affect clearance rates of adult and juvenile shellfish, indicating that the reduction in the clearance rate of shellfish fed *A. monilatum* was not due to particle size. Clearance rates of shellfish fed a mixture of toxic *A. monilatum* and nontoxic Instant Algae® *Pavlova* were not significantly different from clearance rates of animals fed toxic *A.*

monilatum; thus, the addition of nontoxic algal prey to toxic *A. monilatum* did not result in higher consumption of *A. monilatum* by these shellfish species.

The depressed clearance rates observed for adult and juvenile *Crassostrea virginica*, *Perna viridis*, and *Mercenaria mercenaria* fed toxic *Alexandrium monilatum* likely occurred in response to toxin(s) produced. The dinoflagellate sub-cultures used during the short-term grazing experiments in this study were in stationary growth phase, in order to maximize the potential for toxin exposure. Aldrich et al. (1967) reported that *A. monilatum* produces an endotoxin that is released during cell autolysis. In that study, peak toxicity to fish occurred when *A. monilatum* populations had been declining for a month.

4.2. Adult shellfish behavior (valve gape) in response to *Alexandrium monilatum*

Clearance rates of bivalve molluscs have been positively correlated with valve gape (Jørgensen et al. 1988, Jørgensen and Rissgård 1988, Shumway and Gainey 1992). Reduction in valve gape is usually accompanied by retraction of the mantle edges and exhalent siphon, affecting pumping rates (Jørgensen et al. 1988, Jørgensen 1990). Exposure to toxic *Alexandrium monilatum* not only depressed clearance rates of adult *Crassostrea virginica*, *Mercenaria mercenaria* and *Perna viridis* (above), but also significantly reduced mean valve gape in comparison to that of control animals exposed to nontoxic *Pavlova*.

4.3. Impact of *Alexandrium monilatum* on shellfish survival

Sievers (1969) reported that *Crassostrea virginica* sustained increased mortality when exposed to toxic *Alexandrium monilatum* for 48 h, and naturally occurring *A. monilatum* blooms have also been associated with mortality of various fauna (Table 1). Ray and Aldrich (1967) found that *C. virginica* lost valve closure ability, a condition that

usually precedes death, when exposed to *A. monilatum*. In this study, of the three shellfish species tested, only *Perna viridis* were moribund after 24 h of exposure to toxic *A. monilatum*. Adult *P. viridis* had an LT_{50} of 16 h and juveniles of 10 h. This species, which naturally occurs in the Indo-Pacific (Siddall 1980), could be more sensitive to *A. monilatum* because it was recently introduced to coastal U.S. waters (Bensen et al. 2001). History of exposure to harmful algal blooms can influence the response of shellfish to harmful algae (Shumway and Cucci 1987).

Larval shellfish are especially sensitive to toxic dinoflagellates (Wikfors and Smolowitz 1995, Yan et al. 2001, Springer et al. 2002). In this research, larval *Crassostrea virginica* and *Mercenaria mercenaria* were more sensitive to the toxic *Alexandrium monilatum* than the adult and juvenile stages, and *M. mercenaria* larvae were more sensitive to *A. monilatum* toxicity than *C. virginica* larvae (~38% and ~90% survival, respectively). Interestingly, there was no major decrease in survival of larval *C. virginica* or *M. mercenaria* exposed to intact and restricted *A. monilatum* versus a nontoxic strain of *Alexandrium tamarense*, whereas sonicated *A. monilatum* 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 these experiments were in log growth phase to reduce the amount of toxin potentially present from cell autolysis (as suggested by Aldrich et al. 1967). Thus, as expected, the larvae were not affected by *A. monilatum* until the cells were lysed through sonication.

Endotoxins may be released minimally into the surrounding water under optimal conditions, but under stressful conditions or during senescence or collapse of a bloom more

toxins can be released (Landsberg 2002). Cellular retention of toxins may confer protective benefit to *A. monilatum* by minimizing both grazing pressure, as indicated in this study, and competition with other algae for available resources. Growth rates and biomass yields of natural phytoplankton assemblages have been suppressed in laboratory experiments when *A. monilatum* culture filtrates and cell lysates were added (Juhl 2003). In this study, *Cryptomonas* sp. lysed when mixed with *A.monilatum*. This effect on phytoplankton may enhance the nutrient availability for *A. monilatum* in its natural habitat.

4.4. Survival of *Alexandrium monilatum* after ingestion by adult and juvenile shellfish

In this study, *Alexandrium monilatum* cells were rejected in the pseudofeces of adult *Perna 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 adult and juvenile shellfish species tested, indicating that the shellfish did not digest at least a portion of the ingested cells. Various other toxigenic dinoflagellate species have been documented to pass intact through the digestive tract of bivalve molluscs (e.g. Fox and Coe 1943, Dupuy and Sparks 1968, Shumway et al. 1985b, Shumway and Cucci 1987, Bardouil et al. 1993, Bricelj et al. 1993, Laabir and Gentien 1999, Bauder and Cembella 2000, Springer et al. 2002). For example, based upon inspection of fecal ribbons, Shumway et al. (1985b) reported that *Ostrea edulis* preferentially ingested the dinoflagellate *Prorocentrum minimum* (Pavillard) Schiller cells relative to other microalgal species. Although, high incidence of *P. minimum* fragments and intact cells in the fecal ribbons indicated that the cells were rejected from the oyster gut.

Successfully invading species are difficult or impossible to eradicate (see review in Clout and Veitch 2002); therefore, potential vectors of such species should be carefully

considered in efforts to minimize dispersal of harmful algal species. Introduction of harmful algal bloom species via cargo ship ballast water has received considerable attention, whereas other potential modes of transfer mostly have been ignored (Shumway et al. 1985a, Hallegraeff and Bolch 1992, and see review Hallegraeff 1993). 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). For example, a strain of the dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge exhibited cell division following passage through the gut tract of bay scallops (Bauder and Cembella 2000). Egested cells of a strain of *Alexandrium fundyense* were capable of doubling times comparable to those of ungrazed control populations (Bricelj et al. 1993). Strains of *Pfiesteria piscicida* Steidinger et Burkholder formed temporary cysts when ingested by adult 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 *Alexandrium minutum* Halim and *Alexandrium tamarense* were able to pass intact and viable through the digestive tract of the Pacific oyster, but the unarmored dinoflagellate *Karenia mikimotoi* Miyake et Kominami was not. The toxic dinoflagellates, *Alexandrium fundyense* and *Prorocentrum minimum* were also able to re-establish growing populations after passage through the digestive tract of several shellfish species (Hégaret et al. in prep).

In this study, intact *Alexandrium monilatum* cells were found within the feces of all shellfish species tested. Moreover, culture media inoculated with feces from adult *Crassostrea virginica* and *Perna viridis* contained swimming *A. monilatum* cells within the first week, but subsequent cell production was not observed. Control populations of *A.*

monilatum were able to grow under the same culture conditions, suggesting that the egested *A. monilatum* cells, at least this strain tested, were incapable of cell division. The inoculated feces were checked for 4 weeks for cell production. In other work, it took 4-6 weeks for *Prorocentrum minimum* and 6-8 weeks for *Alexandrium fundyense* populations to recover after passage through the digestive tract of shellfish (Hégaret et al. in prep). Therefore, a longer incubation period for the inoculated feces may be necessary for *A. monilatum* populations to re-establish.

4.5. Shellfish as potential vectors of toxic *Alexandrium monilatum*

The toxic dinoflagellate *Alexandrium monilatum* can form resting cysts in stationary cultures and in nitrogen-deficient medium (Walker and Steidinger 1979). 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 *A. monilatum* blooms. In the present study, population growth did not occur for *A. monilatum* cells that passed intact through the digestive tract of shellfish. Thus, shellfish feces may not be a vector for the introduction of *A. monilatum* to new areas. However, since some shellfish species are capable of surviving 24 hr of exposure to *A. monilatum*, the movement of these shellfish to new areas may be a potential vector for *A. monilatum* dispersal.

4.6. Conclusions

This study documents species-specific responses of *Mercenaria mercenaria*, *Crassostrea virginica* and *Perna viridis* experimentally exposed to a toxic strain of the dinoflagellate, *Alexandrium monilatum*. All three species showed avoidance behavior by reducing their clearance rate and valve gape when fed toxic *A. monilatum*. The most sensitive species to toxic *A. monilatum* was *P. viridis* based on survival experiments; they were moribund after 24 h of exposure. Larvae of both species tested (*M. mercenaria*, *C. virginica*) were more sensitive to toxic *A. monilatum* than juvenile and adults, and larval *M. mercenaria* were more sensitive than larval *C. virginica* (38% and 90% survival, respectively).

Intact cells of *Alexandrium monilatum* were noted in the feces of all three shellfish, but these *A. monilatum* cells appeared to be incapable of further cell division after passage through the gut. Shellfish feces may not be a vector for the introduction of *A. monilatum* to new areas, but further tests should be conducted to verify that intact cells within shellfish feces can not re-establish populations after a longer incubation period. Transfer of shellfish from one area to another has been suggested as a potential vector for the transport of harmful algae into new environments (Bricelj et al. 1993, Scarratt et al. 1993, Vila et al. 2001, Lilly et al. 2002, Penna et al. 2005). Shellfish could be a vector for the dispersal of *A. monilatum*, since some species of shellfish can survive at least 24 h of exposure to *A. monilatum* blooms.

Reported *Alexandrium monilatum* blooms and associated fish and shellfish kills in the Gulf of Mexico and along the Atlantic coast of Florida were frequent 20 to 50 years ago (Howell 1953, Gates and Wilson 1960, Williams and Ingle 1972, Wardle et al. 1975, Perry et al. 1979, Norris 1983). Since that time, blooms have been sporadic in these areas, except for recurrent *A. monilatum* blooms in the Mississippi Sound that have been reported since 1998

(Moncreiff et al. 2002). Nevertheless, it should be noted that areas where *A. monilatum* blooms historically have been reported coincide with areas increasingly used for shellfish aquaculture and, thus, this harmful alga represents a potential threat to commercially important shellfish culture. For example, the Indian River Lagoon of Florida supports an important commercial *Mercenaria mercenaria* fishery (Norris 1983, Vaughan 1988). This area also has the most productive natural *M. mercenaria* beds and the highest concentration of active quahog aquaculture leases (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 (Arnold et al. 2000).

Overall, the data from this study suggest that *Alexandrium monilatum* blooms could potentially affect the recruitment and survival of some shellfish species by reducing clearance rate and valve gape, affecting food intake, and inducing larval mortality. Moreover, 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 population growth (Tuner and Tester 1997; Johnson et al. 2003).

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