Effects of the estuarine dinoflagellate *Pfiesteria shumwayae* (Dinophyceae) on survival and grazing activity of several shellfish species

Sandra E. Shumway [a,*], JoAnn M. Burkholder [b], Jeffrey Springer [b]

[a] *Department of Marine Science, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340, USA*
[b] *Center for Applied Aquatic Ecology, North Carolina State University, 620 Hutton Street, Suite 104, Raleigh, NC 27606, USA*

Abstract

A series of experiments was conducted to examine effects of four strains of the estuarine dinoflagellate, *Pfiesteria shumwayae*, on the behavior and survival of larval and adult shellfish (bay scallop, *Argopecten irradians*; eastern oyster, *Crassostrea virginica*; northern quahogs, *Mercenaria mercenaria*; green mussels, *Perna viridis* [adults only]). In separate trials with larvae of *A. irradians*, *C. virginica*, and *M. mercenaria*, an aggressive predatory response of three strains of algal- and fish-fed *P. shumwayae* was observed (exception, algal-fed strain 1024C). Larval mortality resulted primarily from damage inflicted by physical attack of the flagellated cells, and secondarily from *Pfiesteria* toxin, as demonstrated in larval *C. virginica* exposed to *P. shumwayae* with versus without direct physical contact. Survival of adult shellfish and grazing activity depended upon the species and the cell density, strain, and nutritional history of *P. shumwayae*. No mortality of the four shellfish species was noted after 24 h of exposure to algal- or fish-fed *P. shumwayae* (strains 1024C, 1048C, and CCMP2089) in separate trials at $\leq 5 \times 10^3$ cells ml^{-1}, whereas higher densities of fish-fed, but not algal-fed, populations (> 7–8×10^3 cells ml^{-1}) induced low ($\leq 15\%$) but significant mortality. Adults of all four shellfish species sustained $> 90\%$ mortality when exposed to fish-fed strain 270A1 (8×10^3 cells ml^{-1}). In contrast, adult *M. mercenaria* and *P. viridis* exposed to a similar density of fish-fed strain 2172C sustained $< 15\%$ mortality, and there was no mortality of *A. irradians* and *C. virginica* exposed to that strain. In mouse bioassays with tissue homogenates (adductor muscle, mantle, and whole animals) of *A. irradians* and *M. mercenaria* that had been exposed to *P. shumwayae* (three strains, separate trials), mice experienced several minutes of disorientation followed by recovery. Mice injected with tissue extracts from control animals fed cryptomonads showed no response. Grazing rates of adult shellfish on *P. shumwayae* (mean cell length ± 1 standard error [S.E.], 9 ± 1 μm) generally were significantly lower when fed fish-fed (toxic) populations than when fed populations that previously had been maintained on algal prey, and grazing rates were highest with the nontoxic cryptomonad, *Storeatula major* (cell length 7 ± 1 μm). Abundant cysts of *P. shumwayae* were found in fecal strands of all shellfish species tested, and $\leq 45\%$ of the feces produced viable flagellated cells when placed into favorable culture conditions. These findings were supported by a field study wherein fecal strands collected from field-collected adult shellfish (*C. virginica*, *M. mercenaria*, and ribbed mussels, *Geukensia demissa*) were confirmed to contain cysts of *P. shumwayae*, and these cysts produced fish-killing flagellated populations in standardized fish bioassays. Thus, predatory feeding by flagellated cells of *P. shumwayae* can adversely affect survival of larval bivalve molluscs, and grazing can be depressed when adult shellfish are fed *P. shumwayae*. The data suggest that *P. shumwayae* could affect recruitment of larval shellfish in estuaries and aquaculture facilities; shellfish can be

* Corresponding author. Tel.: +1 860 405 9282; fax: +1 860 405 9282.
E-mail address: sandra.shumway@uconn.edu (S.E. Shumway).

adversely affected via reduced filtration rates; and adult shellfish may be vectors of toxic *P. shumwayae* when shellfish are transported from one geographic location to another.

1. Introduction

Molluscan shellfish increasingly have been recognized as potential vectors of harmful algae and their toxins (Shumway et al., 1985, 1987; Shumway and Cucci, 1987; Gainey and Shumway, 1988a,b; Shumway, 1990, 1995; Matsuyama et al., 1999). Most previous studies of impacts of harmful algae have focused on photosynthetic species, especially species that attain high cell densities and frequently discolor the water (Shumway, 1995; Landsberg, 2002). With the exception of well-documented impacts on shellfish from certain parasitic dinoflagellates (e.g. crustacean epizootics and mortalities caused by *Hematodinium* and hematodinium-like species; Newman and Johnson, 1975; Taylor and Kahn, 1995; Stentiford et al., 2002), effects of harmful heterotrophic algae on shellfish behavior and survival are poorly understood (Burkholder, 1998).

Reports of toxic shellfish linked to free-living heterotrophic dinoflagellates mostly have been limited to *Dinophysis* sp. (e.g. Suzuki et al., 1996; Sidari et al., 1998; Miles et al., 2004a,b), *Protoperidinium* sp. (James et al., 2003, 2004), and *Pfiesteria piscicida* Steidinger et Burkholder (based on experimental data—Springer et al., 2002). Miles et al. (2004a) described a link between *Dinophysis* spp. and accumulation of pectenotoxin in shellfish, and *Protoperidinium* sp. also has been reported to produce a toxin that accumulates in shellfish (James et al., 2003). *P. piscicida* and a closely related species, *Pfiesteria shumwayae* Glasgow et Burkholder (Marshall et al., 2006), have toxic strains (Moeller et al., 2001; Gordon et al., 2002; Burkholder et al., 2004, 2005; Gordon and Dyer, 2005) that have been linked to fish mortalities and epizootics in estuaries of the mid-Atlantic and southeastern U.S. (Magnien et al., 2000; Burkholder et al., 2001a; Glasgow et al., 2001; Magnien, 2001). Adverse affects on fish have been associated with predation (Burkholder and Glasgow, 1997; Burkholder et al., 2001a,b; Lovko et al., 2003) as well as toxin production (Burkholder et al., 2001a,b, 2005; Melo et al., 2001; Moeller et al., 2001). Recent research also documented adverse impacts from *P. piscicida* on larval bay scallops (Argopecten irradians Lamarck, 1819) and eastern oysters (Crassostrea virginica Lamarck, 1819) (Springer et al., 2002). In that study, flagellated cells of *P.*

piscicida attacked, fed upon, and killed pediveliger larvae of both shellfish species; actively toxic cells held in dialysis membrane to prevent direct contact with *A. irradians* larvae caused high larval mortality as an apparent toxin effect; and actively toxic cells depressed grazing of adult *C. virginica*. Flagellated cells of *P. piscicida* also showed chemosensory attraction toward freshly dissected tissues of *A. irradians*, *C. virginica*, and northern quahogs (Mercenaria mercenaria Linneaus, 1758) (Springer, 2000). Moreover, *P. piscicida* survived passage through the digestive tract of adult *C. virginica* by forming temporary cysts, and ≥75% of the cysts produced viable flagellated cells when separated from the shellfish feces and placed into fresh culture media.

Impacts on shellfish from the second known toxigenic species of *Pfiesteria*, *P. shumwayae*, have not previously been assessed. The objectives of this study were to: (1) examine interactions between *P. shumwayae* and several shellfish species, based on a series of laboratory experiments and supporting field observations; (2) assess predation by *P. shumwayae* on shellfish pediveliger larvae and the potential for toxic effects on larvae, using different strains of *P. shumwayae*; (3) assess the grazing response of adult shellfish to different strains; and (4) determine the viability of *P. shumwayae* cells recovered from fecal strands.

2. Materials and methods

2.1. Field study

Eastern oysters (*C. virginica*) and ribbed mussels (Geukensia demissa Dillwyn, 1817) were sampled on 8 May, 18 August, and 30 August 2002 in the Bay River Estuary, a tributary of western Pamlico Sound (latitude $35°08'39''$N, longitude $76°46'15''$W, salinity ~15 ppt) where *P. piscicida* and *P. shumwayae* previously had been documented (Burkholder et al., 2004). The field sites included diverse sediment types ranging from fine-particulate sediments in the upper reaches of tidal creeks (sites 2, 3, 7–10, 13, and 14) to consolidated sands (sites 1, 4–6, 11, and 12). On the first sampling date, physical and chemical data were collected from eight sites (sites 1, 2, 5–7, 10, 11, and 14; Fig. 1). Water-
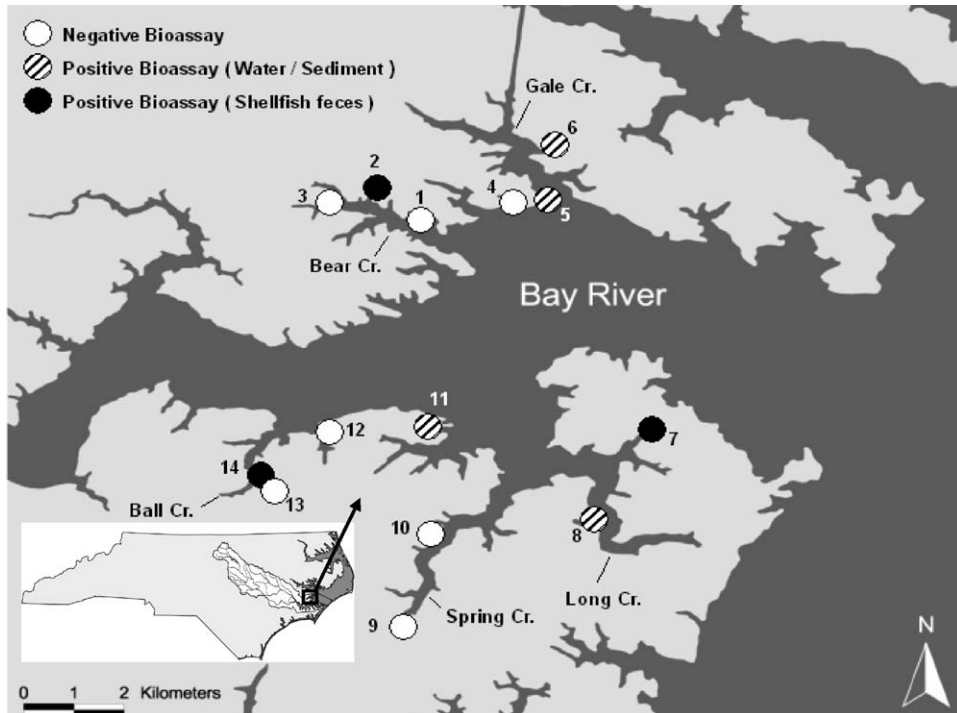
Fig. 1. Map of sites sampled in the Bay River Estuary (latitude, longitude): site 1, 35°15′53″, −76°48′42″; site 2, 35°16′22″, −76°49′21″; site 3, 35°16′10″, −76°47′12″; site 4, 35°16′12″, −76°46′54″; site 5, 35°17′1″, −76°46′47″; site 6, 35°9′38″, 76°48′58″; site 7, 35°12′41″, −76°48′36″; site 8, 35°11′43″, −76°50′45″). White circles indicate sites where *Pfiesteria shumwayae* was not detected by PCR or standardized fish bioassays (inoculated with shellfish fecal strand material); hatched circles show locations where water-column or sediment samples were positive for *P. shumwayae* with PCR; and black circles indicate sites that were positive for *P. shumwayae* with standardized fish bioassays inoculated with shellfish fecal material.

column and surficial sediment samples were collected at those sites and six additional sites for polymerase chain reaction (PCR) analyses (Fig. 1). On the second and third dates, samples were collected from eight sites (sites 1, 2, 5–7, 10, 11, and 14) for PCR analysis. A datasonde (Hydrolab Inc., Loveland, CO) was used to characterize background environmental conditions (water temperature, salinity, dissolved oxygen, and pH). An integrated water-column sampler (after Cuker et al., 1990) was used to collect samples of the upper and lower water columns for nutrient analyses (ammonia [NH_4^+], nitrate + nitrite [$NO_3^- + NO_2^-$], total phosphorus [TP], and soluble reactive phosphorus [SRP]), using procedures of Burkholder et al. (2006). Water-column and surficial sediment samples collected for PCR were analyzed using an 18S rDNA molecular probe specific for *P. shumwayae* (Bowers et al., 2000; Rublee et al., 2001).

Shellfish were collected by hand or using a rake, and were transported immediately to the laboratory (≤5 °C above ambient water temperatures), gently cleaned to remove epibiontic contaminants, and rinsed with filtered seawater (0.2 μm pore size; 34-ppt salinity natural seawater source water collected ~2.5 km

offshore from Wilmington, NC). Individual shellfish (*C. virginica* from sites 1, 2, 4, 5, and 8, *n* = 3 animals per site; *G. demissa* from site 5, *n* = 3; and *M. mercenaria* from sites 2 and 14, *n* = 3) were each placed into separate 2-l glass containers with gentle aeration. After 24 h, structurally intact fecal strands were collected, and the material was inoculated into standardized fish bioassays [SFBs] as in Burkholder et al. (2001c) (*n* = 1 SFB per site, 7 l, salinity 15 ppt, two tilapia [*Oreochromis mossambicus* Peters, length 5–8 cm] per SFB). Control SFBs were maintained identically except that shellfish fecal strands were not added. Bioassays with negative results (no fish mortalities) were monitored for 12 weeks; bioassays with fish death were monitored for 40 weeks. Bioassays were considered positive for the presence of toxic *Pfiesteria* if: (a) successive fish kills were recorded over a 48-h period; (b) microscopic analyses confirmed the presence of pfiesteria-like cells at densities ≥400 cells ml^{-1} (sufficient to cause fish death if *Pfiesteria* spp.; Burkholder et al., 2001c); (c) PCR molecular probe analysis confirmed the presence of *P. shumwayae*; (d) further testing confirmed the presence of *Pfiesteria* toxin (Burkholder et al., 2005).

Table 1
Origin of the clonal *Pfiesteria shumwayae* strains used in this study (strains from the NCSU CAAE unless otherwise noted)

Clonal strain	Site	Collection[a]	Date collected	Notes
1024C (CCMP2359, February 2004)	Chincoteague Bay, MD	NCSU CAAE	August 2000	Bloom of 2.5×10^3 flagellated cells ml^{-1}. Lethal to fish in standardized fish bioassays (SFBs); verified to produce toxin (NOS-Charleston[a])
1048C (CCMP2358, February 2004)	Neuse Estuary, NC	NCSU CAAE	May 2000	Menhaden with lesions observed at time of collection. Lethal to fish in SFBs; verified to produce toxin (NOS-Charleston[a])
CCMP2089 (1930A/VIMS1049, August 2001)	Pamlico Estuary, NC	VIMS	January 2000	Lethal to fish in SFBs; verified to produce toxin (NOS-Charleston[a]; Burkholder et al., 2004, 2005; Gordon and Dyer, 2005) when cultured and tested appropriately
270A1/270A2	Neuse Estuary, NC	NCSU CAAE	July 1998	Collected during a toxic *Pfiesteria*-related fish kill. Lethal to fish in SFBs; verified to produce toxin (NOS-Charleston[a])
2172C	Kiawah Island Pond, SC	SC DNR	April 2003	Pfiesteria-like cells abundant (1.7×10^4 cells ml^{-1}); no diseased or dying fish noted. Lethal to fish in SFBs; verified to produce toxin (NOS-Charleston[a])

[a] NCSU CAAE, North Carolina State University Center for Applied Aquatic Ecology; CCMP, Center for Culture of Marine Phytoplankton, Boothbay Harbor, ME and date deposited at the commercial Culture Collection for Marine Phytoplankton (CCMP), Boothbay Harbor, ME; VIMS, Virginia Institute of Marine Science; SC DNR, South Carolina Department of Natural Resources; NOS, National Ocean Service of the National Oceanic and Atmospheric Administration.

2.2. Algal culturing

In previous research, the grazing response of *C. virginica* to *P. piscicida* varied significantly depending upon whether the dinoflagellates had been maintained on live fish prey in actively toxic mode, or on algal prey (Springer et al., 2002). Therefore, tests of shellfish with sub-cultures of *P. shumwayae* fed fish versus algal prey were included in this study. Five clonal isolates of *P. shumwayae* were used in this study (Table 1), with three strains compared in most experiments. A sub-clone of each strain was initiated 1 month prior to experiments in SFBs following Burkholder et al. (2001c). A second sub-clone of each was maintained for 1 month prior to experiments using cryptophytes as a food source (*Storeatula major* = *Cryptomonas* sp., HP9101 isolate). All cultures were held at 15-ppt salinity (for trials with larval *C. virginica*, and for grazing studies using field-collected shellfish; filtered seawater diluted with deionized water) or 30-ppt salinity (trials with larvae of other shellfish species, and other grazing studies), at 19–22 °C under low illumination (20–50 µmol photons m^{-2} s^{-1}) and a 14-h light:10-h dark ratio. The salinity optimum for *Pfiesteria* spp. has been reported to be ~15 ppt (Burkholder et al., 2001a; Sullivan and Andersen, 2001), but *Pfiesteria* spp. have been associated with fish kills in waters of higher salinity (Burkholder et al., 2001a), and have been successfully cultured in the laboratory at salinities of 25–30 ppt (0.2–0.5 divisions day^{-1}; e.g. Springer et al., 2002). The five species of shellfish involved in this study are common

inhabitants of intertidal estuarine areas or nearshore embayments (Castagna and Chanley, 1973; Brand, 1991; Seed and Suchanek, 1992; Shumway, 1996; de Bravo et al., 1998; Grizzle et al., 2001), and were hatchery-reared or abundant in natural habitats at the salinities maintained during this study.

Cultures were cloned from field samples collected from estuaries along the mid-Atlantic Coast that had tested positive for *P. shumwayae* using a species-specific PCR probe (18S-rDNA; Rublee et al., 2001). Clones were established following the flow cytometric sorting procedures of Parrow et al. (2002). Prior to experiments, the species identification of each strain was re-confirmed using PCR (Bowers et al., 2000) and scanning electron microscopy following Burkholder et al. (2001a).

Fish-fed, actively toxic isolates of *P. shumwayae* were maintained in 7-l aquaria following Burkholder et al. (2001c). Salinities were established at 15 or 30 ppt as above. Fish-fed cultures were held in a biohazard III facility (Burkholder et al., 2001c). Flagellated cells (zoospores, gametes, and planozygotes, not distinguished under light microscopy in this study; diameter 8–12 µm, mean ± 1 standard error [S.E.], 9 ± 1 µm) attained densities up to $\leq 2 \times 10^4$ cells ml^{-1}. Algal-fed cultures of *P. shumwayae* were given cryptomonads (*S. major* HP9101 from A. Lewitus, University of South Carolina, Charleston, SC; maximum cell dimension 7 ± 1 µm, $n = 100$ cells measured; grown using f/2-Si; Guillard, 1975) at ~10 prey cells per *P. shumwayae* cell, supplied at 3–4-day intervals. These cultures

Table 2
Experimental crosses (shellfish species × algal- and fish-fed *Pfiesteria shumwayae* strains)

Experiment	Field study	Larval bioassays	Grazing studies and fecal analyses
Argopecten irradians (bay scallop)	– (not found in study area)	1024C, 1048C, CCMP2089, 2172C[a]	1024C, 1048C, CCMP2089
Crassostrea virginica (eastern oyster)	Feces collection, grazing study	1024C, 1048C, CCMP2089, 2172C[a]	1024C, 1048C, CCMP2089, 270A1[a]
Geukensia demissa (ribbed mussel)	Feces collection, grazing study	– (few larvae collected)	1024C, 1048C, CCMP2089, 270A1[a]
Mercenaria mercenaria (northern quahog)	Feces collection	1024C, 1048C, CCMP2089, 2172C[a]	1024C, 1048C, CCMP2089
Perna viridis (Asian green mussel)	– (not found in study area)	– (few larvae collected)	1024C, 1048C, CCMP2089

[a] Strains 270A1 and 2172 strains were used during some preliminary experiments, but demonstrated variable activity and flagellated cells sporadically encysted. Strains 1024C, 1048C, and CCMP2089, with more consistent activity, were used throughout most of the study.

attained densities of $\geq 8 \times 10^3$ to 1.3×10^4 flagellated cells ml^{-1}.

2.3. Shellfish in laboratory experiments

Five shellfish species (bay scallops, *A. irradians*; eastern oyster, *C. virginica*; ribbed mussel, *G. demissa*; northern quahog, *M. mercenaria*; and green mussel, *Perna viridis*) were used for various experiments in this study (Table 2), based on their commercial and ecological importance or their presence at field sites. Shellfish were acquired from academic sources or commercial hatcheries (*A. irradians*, Martha's Vineyard Shellfish Group, MA; *C. virginica* and *M. mercenaria*, Middle Island Aquaculture, Foster VA; *P. viridis*, Mote Marine Laboratory, FL) or field sites (Bay River Estuary, NC (Fig. 1): *C. virginica*, sites 1, 2, 6, and 8; *G. demissa*, site 5; *M. mercenaria*, sites 2 and 8). They were acclimated to experimental conditions (wild animals collected at 15–18-ppt salinity, 48 h; hatchery animals grown at 30-ppt salinity, 1 week). With the exception of the wild animals and green mussels, shellfish were acquired from areas where toxic *Pfiesteria* has not been known to be active (Burkholder et al., 2001a) or from hatcheries without known *Pfiesteria* contamination. Green mussels were collected from field sites in Florida where *Pfiesteria* was previously documented (Burkholder and Glasgow, 1997).

2.3.1. Larvae

Prior to experiments, hatchery-spawned larvae of *A. irradians*, *C. virginica*, and *M. mercenaria* were fed an algal diet consisting of a 50:50 mixture of the haptophyte, *Isochrysis galbana* Parke (isolate LB2307, University of Texas [UTEX] Culture Collection of Algae, Austin, TX) and the prasinophyte, *Tetraselmis sueicica* Kylin et Butcher (isolate CCMP904). Larvae were maintained prior to experiments in hatchery conditions (30-l holding tank

equipped with gentle aeration and biological filtration as a fluidized bed filter—Rainbow Lifeguard FB900, Pentair Aquatics, El Monte, CA).

2.3.2. Adults

Shellfish were held in either a 570-l recirculating seawater system maintained at 15-ppt salinity, or a 1130-l recirculating rack system maintained at 30-ppt salinity. Both systems were equipped with biological filters (15-ppt salinity, bubble-washed bead filter; Aquatic Ecosystems Inc., Apopka, FL) or a 1.2-m biofilter tank filled with BioBall Media (30-ppt salinity, Part T9; Model Aquatic Ecosystems Inc.). Both seawater systems were equipped with an ultraviolet sterilizer (Model QL-40; Pentair Aquatics, Inc.) to reduce microbial contaminants. During the acclimation period, all shellfish were maintained on a semi-continuous drip of a commercially available shellfish diet (Shell 1800; Reed Mariculture, Inc., Campbell, CA). Before experiments, shellfish were gently brushed to remove epibionts, and were transferred to a 400-l tray and held without food for 24–48 h to clear previously consumed algal prey from their digestive systems.

2.4. Behavioral interactions between P. shumwayae and shellfish larvae

In separate tests, 100 larvae of *A. irradians*, *C. virginica*, or *M. mercenaria* were pipetted into glass Petri dishes (diameter 25 mm; Bioptechs Inc., Butler, PA). Larvae were acclimated for 15 min to conditions on the microscope stage, where light was set as low as possible while allowing for adequate imaging; the change in temperature was $\leq +2\ °C$ over the observation period. Treatments ($n = 6$ replicates) consisted of shellfish exposed to filtered seawater (pore size 0.2 μm), or to three cell densities (1×10^2, 1×10^3, or 3×10^3 cells ml^{-1}) of the following: algal-fed *P. shumwayae* (strains 1024C, 1048C, CCMP2089 =

1930A, tested separately), fish-fed *P. shumwayae* (strains 1024C, 1048C, CCMP2089, tested separately), or the cryptomonad *Storeatula major* (benign micro-algal control, isolate HP9101). Each Petri dish was examined on an Olympus AX70 microscope equipped with water-immersion lenses (Uplan Fluorite objective 20×). Observations were videotaped (analog, S-VHS; digital camera head in time lapse mode at 1280×1024 resolution, effective 1.3 mega-pixel resolution) using a Sony SVO-9500MD video recorder or an Olympus digital camera head (model DP-70). General behavior and swimming motion of larvae and *P. shumwayae* cells were also noted.

2.5. Survival and behavior of larval C. virginica with P. shumwayae

The ability of *P. shumwayae* to induce mortality of *C. virginica* larvae, and to impact their behavior, was tested with direct versus no direct contact, as in previous work with *P. piscicida* (Springer et al., 2002). The larvae were exposed to $\sim 3 \times 10^2$ or 3×10^3 flagellated cells ml^{-1} of algal-fed *P. shumwayae* (strains 1024C, 1048C, and CCMP2089) or fish-fed *P. shumwayae* (strains 1024C, 1048C, and CCMP2089), or to similar densities of the cryptomonad *S. major*, with or without a semi-permeable plate barrier (0.4-μm pore size; Corning Inc., Corning, NY). For each treatment (*n* = 6 replicates with 100 larvae in each), larvae were gently pipetted into wells (6-well plates, 6-ml volume; Fisher Scientific International, Hampton, NH) containing 2 ml of filtered seawater (0.2-μm pore size). Plates were gently inserted into some wells (randomly selected) to prevent direct contact of *P. shumwayae* cells with shellfish larvae. Treatment cell densities were then added, considered as time zero (T_o). Shellfish behavior and viability were observed at 15-min intervals using a stereo dissecting microscope (Olympus SZX-12, 10×). Mortality was defined as absence of ciliary movement for >1 min, as in Springer et al. (2002).

2.6. Adult shellfish exposed to P. shumwayae

2.6.1. Survival
Adult shellfish (*A. irradians*, *C. virginica*, *M. mercenaria*, and *P. viridis*) were examined at 4–6-h intervals during 24 h of exposure to algal- and fish-fed *P. shumwayae* (strains 1024C, 1048C, CCMP2089, 2172C, and 270A1, tested separately) at a density of $\sim 5 \times 10^3$ or $\geq 7–8 \times 10^3$ flagellated cells ml^{-1}, to determine the LT$_{50}$ value for each species (time when half of the animals had died, *n* = 10). Mortality was defined as the loss of valve closure ability, a condition

that typically precedes death (Ray and Aldrich, 1967). A needle was also used to prod the mantle cavity and check for any movement. Control animals were treated identically except that they were maintained in filtered seawater media without algal food, or with similar densities of cryptomonad algae.

2.6.2. Toxicity of shellfish tissues
Whole *A. irradians* and dissected tissues (adductor muscle and mantle), and whole *M. mercenaria* that had been exposed to $\sim 5 \times 10^3$ flagellated cells ml^{-1} of *P. shumwayae* (fish-fed strains 1024C, 1048C, and 2172C) for 24 h as above were tested by the Maine Department of Marine Resources (J. Hurst and associates) in mouse bioassays (*n* = 3; Hallegraeff et al., 2003). Tissue homogenates from animals exposed to *P. shumwayae* were administered by intraperitoneal injection. The results were compared to data from mouse bioassays with tissue homogenates from control animals that had not been exposed to *P. shumwayae* (*n* = 3).

2.6.3. Grazing activity
A series of experiments was used to examine differences among shellfish species, and among adult individuals of the same species, in grazing three strains of algal- and fish-fed *P. shumwayae* (1024C, 1048C, and CCMP2089). The grazing rate was defined as the number of algal cells removed from solution ml^{-1} h^{-1} (Shumway et al., 1985), here, the number of cells ml^{-1} initially present minus the number of cells ml^{-1} remaining in the media after the 1-h assays. All experiments were conducted in gently aerated (1 bubble s^{-1}) glass beakers at 20 °C. Grazing experiments (21 °C, 30 ppt, *n* = 10) included animals of the following sizes (shell length, grand mean ± 1S.E.): *A. irradians* (38.3 ± 0.9 mm), *C. virginica* (44.9 ± 1.0 mm), *M. mercenaria* (29.2 ± 0.8 mm), and *P. viridis* (31.3 ± 0.6 mm). The shellfish (*n* = 6–10 replicate animals per experiment) were maintained in 1 l glass beakers containing filtered seawater (0.2-μm pore size, 15- or 30-ppt salinity), with the container size selected to prevent complete clearance of cells from solution and minimize accumulation of metabolites during the experiments.

All shellfish were allowed to clear their digestive tracts for 48 h prior to the grazing experiments. They were then placed into containers with filtered seawater media (0.2 μm pore size) and exposed to cryptomonads (algal controls, 3×10^3 cells ml^{-1}) or *P. shumwayae* for 1-h trials to assess grazing rates. To simulate bloom conditions, moderate phytoplankton densities (crypto-monads or *P. shumwayae*) initially were imposed at 2.5–8.0×10^3 cells ml^{-1}, mostly at 5×10^3 flagellated

cells ml^{-1}. Bloom concentrations of *P. shumwayae* ($5–8 \times 10^3$ cells ml^{-1}) were cleared from solution by all five species of shellfish tested. At $7–8 \times 10^3$ algal- or fish-fed flagellated cells ml^{-1}, most shellfish produced copious pseudofeces containing high abundance of *P. shumwayae* temporary cysts with a thick mucus covering. About 65% of these cysts from pseudofeces ($n = 3$ replicates per treatment) produced viable flagellated cells over a 2-week period when placed into culture media conducive to growth. Based on these observations, experimental cell densities were maintained at $<5 \times 10^3$ cells ml^{-1} during the rest of the grazing studies to minimize pseudofeces production.

Addition of *P. shumwayae* to the shellfish containers was considered as T_o. Water samples (1.5 ml) were collected at 15-min intervals and preserved with acidic Lugol's solution (Vollenweider, 1974) for phytoplankton analysis. This sampling interval allowed ample time for the shellfish to graze the prey (as in Springer et al., 2002). Phytoplankton cells were quantified at $400\times$ using Palmer-Maloney chambers (Wetzel and Likens, 2001) with an Olympus AX70 light microscope (Olympus Corporation, Melville, NY). Samples from each treatment were counted with 20% replication (U.S. EPA, 1998; variation $<5\%$ among replicates). Pseudofeces production (i.e., rejected algal cells bound in mucus and expelled into the surrounding medium rather than passing through the digestive tract) was closely monitored and was minimal; any pseudofeces were collected and examined under light microscopy for the presence of *pfiesteria*-like cells or temporary cysts.

At the end of each grazing experiment, the shellfish were each rinsed with filtered seawater (0.2 μm pore size), placed into identically sized containers with a similar volume of filtered seawater, and allowed to clear their digestive tracts for 24 h. Water samples and fecal strands were then collected for microscopic analysis (as above) and for cyst survival experiments (below). During these experiments, care was taken to minimize disturbance, which would have stressed shellfish and promoted encystment of *P. shumwayae* flagellated cells. Shellfish were observed for behavioral indicators of stress including irregular valve movement and mantle retraction (*A. irridians* and *P. viridis*), as reported for other dinoflagellate–shellfish interactions (e.g. Shumway and Cucci, 1987; Shumway, 1990; Lassus et al., 1999; Springer et al., 2002). In addition, during collection of feces, care was taken to preserve the integrity of the delicate fecal strands. Excess fluid was gently blotted onto autoclaved glass fiber filters (1.2-μm pore size, Millipore GF/C, Billerica, MA) to maintain no more than 1 ml in the pipette chamber. This step was taken to minimize fluid carryover that potentially could have contained free-swimming cells. Microscopic analyses were used to confirm the absence of free-swimming cells associated with fecal strands that had been placed onto slides or inside glass-bottomed Petri dishes. If free-swimming cells were noted, the fluid medium was gently withdrawn and replaced with fresh medium until free-swimming cells were not observed.

2.7. Survival of P. shumwayae following passage through shellfish digestive tracts

Fresh (unpreserved) fecal strands and the media from the grazing experiments with adult shellfish (above) were examined under light microscopy immediately after collection for flagellated *P. shumwayae* cells and cysts. Fecal strands containing >200 *P. shumwayae* cysts were viewed at $400\times$ with an inverted microscope (Olympus IX70), and were disrupted by successive aspiration of a micropipette tip. A micropipettor was then used to collect 100 cysts, which were transferred to a second glass-bottomed Petri dish and rinsed three times with 30-ppt salinity, sterile-filtered (0.2-μm pore size) f/2 media (Guillard, 1975) to remove associated organic material. Sterile-filtered media (4 ml) were added, and the covered Petri dishes were placed in an environmental chamber (21 °C, 14-h light:10-h dark illumination) and observed daily for 2 weeks. Preserved material was analyzed for algal and cysts within 48 h of collection. Phytoplankton were quantified using Palmer-Maloney chambers with an Olympus AX70 microscope ($400\times$). Each treatment was analyzed with 20% replication (two Palmer chambers counted per sample; U.S. EPA, 1998; variation $<5\%$ among replicates).

Fecal strands from six individual shellfish (1 ml wet-packed volume from each animal) were inoculated into Falcon flasks containing 200 ml of f/2 media (30-ppt salinity) and placed in a culture incubator (20 °C, 14-h light:10-h dark cycle, ~ 80 μmol photons cm^{-2} s^{-1} illumination) for 2 weeks. A sub-sample from each flask was examined daily under light microscopy for the presence of flagellated cells, and a second sub-sample was preserved for quantification of cell densities.

2.8. Statistical analyses

For each experiment, a repeated-measures analysis of variance [ANOVA] model was used to assess main and interactive effects of treatment and time on the response variable (SAS Institute Inc., 1999). The

repeated-measures factor was time; treatments consisted of shellfish exposed to *P. shumwayae* (algal- or fish-fed) versus controls as shellfish exposed to the benign cryptomonad, *S. major* HP9101. Response variables were shellfish larval survival or juvenile/adult grazing rates.

3. Results

3.1. Field study

Environmental conditions in the eutrophic Bay River Estuary (means ± 1 S.E., $n = 42$; 26.3 ± 0.5 °C, pH 7.6 ± 0, salinity 18.1 ± 0.3 ppt, $\sim 755 \pm 40$ µg TP l^{-1}, 45 ± 5 µg SRP l^{-1}, 5.3 ± 1.9 mg TN l^{-1}, 10 ± 0 µg NH$_4^+$ l^{-1}, 765 ± 30 µg NO$_3^-$ + NO$_2^-$ l^{-1}, 14 ± 3 µg chlorophyll a l^{-1}) were conducive for *Pfiesteria* spp. growth, based on previous field research (Glasgow et al., 2001). Other environmental conditions assessed included dissolved oxygen (7.1 ± 0.3 mg DO l^{-1} at depth 0.5 m), suspended solids (26 ± 7 mg l^{-1}), and turbidity (40 ± 8 NTU). Dominant shellfish were *G. demissa* (sites 7 and 8) and *C. virginica* (sites 1–5, 8, and 14). *M. mercenaria* was also found in deeper waters (depth 2–3 m) near sites 2 and 14. The remaining six sites, with chronic anoxia in the bottom water, were mostly devoid of bivalve molluscs.

Water column, sediment, or feces from shellfish collected in May were positive for *P. shumwayae* at 7 of 14 field sites, based on molecular probe analysis. Microscopic analyses confirmed the presence of *pfiesteria*-like cells in water-column samples (sites 1 and 2, $\sim 1 \times 10^2$ cells ml^{-1}; site 3, $\sim 7 \times 10^2$ cells ml^{-1}; remaining four sites detectable but with <100 cells ml^{-1}). Shellfish fecal strands contained mostly diatom frustules and low densities of partially digested dinoflagellate cells or cysts.

Three of six SFBs that were inoculated with fecal strands from *C. virginica* (sites 2 and 14) or *G. demissa* (site 7) sustained repeated fish mortalities after 48 days (site 2, 38 days; site 7, 40 days; site 14, 48 days). PCR analyses of water samples collected from these SFBs were positive for the presence of *P. shumwayae*, and analysis with light microscopy indicated *pfiesteria*-like densities $> 1 \times 10^3$ cells ml^{-1}. By comparison, fish mortality was low in control SFBs (one of two fish dead in two controls on two dates, or 20% fish death, versus 100% fish death repeatedly in test SFBs), and was caused by aggressive behavior and cannibalism (as in Oliveira and Almada, 1996a,b). PCR and microscopic analyses of samples collected from the controls did not detect *Pfiesteria*.

P. shumwayae was not detected by PCR molecular probe analysis from water or sediment samples collected on 18 August, and shellfish fecal strands from animals allowed to clear their digestive tracts for 24 h (*C. virginica*, site 14; *M. mercenaria*, sites 2 and 8) did not yield positive SFBs for the presence of *P. shumwayae* after 100 days. PCR analysis did detect *P. shumwayae* from sediment samples collected on 30 August at sites 8 and 14. SFBs were conducted for 98 days with fecal strands collected on 30 August from *C. virginica* (sites 1, 2, 5, 8, and 14), *G. demissa* (sites 7 and 8), and *M. mercenaria* (site 2). Only the fecal strands from *G. demissa* (site 8) yielded a positive SFB for fish death and *P. shumwayae* (after 76 days, with *pfiesteria*-like cell densities $\geq 5 \times 10^2$ flagellated cells ml^{-1}; *P. shumwayae* presence confirmed using PCR). No fish mortalities occurred in control SFBs.

Shellfish collected from the Bay River Estuary on the May sampling date readily consumed *P. shumwayae* during the short-term (1 h) grazing experiments. The clearance rate for adult *C. virginica* fed *P. shumwayae* (strain 270A1, initial density, $\sim 5.2 \times 10^3$ cells ml^{-1}) was 4.3×10^2 fish-fed cells ml^{-1} h^{-1} and 4.7×10^3 algal-fed cells ml^{-1} h^{-1}. When given *P. shumwayae* strain 1048C (initial density, $\sim 5.0 \times 10^3$ cells ml^{-1}), the clearance rate for *C. virginica* was 3.9×10^3 fish-fed cells ml^{-1} h^{-1}, and *C. virginica* cleared algal-fed cells completely from solution (grazing rate 5.0×10^3 cells ml^{-1} h^{-1}). Algal controls (cryptomonad *S. major*) were cleared completely from solution by *C. virginica*.

Ribbed mussels, *G. demissa*, were also effective at clearing *P. shumwayae* from solution. Adult animals ($n = 6$) grazed fish- and algal-fed flagellated cells of *P. shumwayae* strain 270A1 (initially $\sim 2.5 \times 10^3$ cells ml^{-1}) at comparable rates ($1.9 \pm 0.1 \times 10^3$ fish-fed cells ml^{-1} h^{-1}; $2.1 \pm 0.1 \times 10^3$ algal-fed cells ml^{-1} h^{-1}). When given *P. shumwayae* (strain 1048C), *G. demissa* cleared both algal- and fish-fed cells completely from solution after 1 h. Algal controls (*S. major*) were removed from solution by *G. demissa* at a rate of $4.62 \pm 0.30 \times 10^3$ cells ml^{-1} h^{-1}.

Within the 24-h period following these grazing experiments, field-collected shellfish (*C. virginica* and *G. demissa*) fed *P. shumwayae* produced fecal strands that contained high densities of *P. shumwayae* cysts (~ 35–50 cysts per 500-µm length of fecal strand). The cysts were uniformly distributed throughout the fecal strand lengths of *G. demissa*, whereas *C. virginica* fecal strands were less consolidated and contained small aggregations of cysts (~ 10–25 cysts per aggregate).

3.2. Interactions between P. shumwayae and shellfish larvae

3.2.1. Behavioral interactions

With the exception of algal-fed strain 1024C, all fish- and algal-fed *P. shumwayae* cultures showed direct attack behavior toward larvae of the three tested shellfish species (*A. irradians*, *C. virginica*, and *M. mercenaria*). Feeding behavior of *P. shumwayae* appeared to be more aggressive at 15 ppt (e.g. 15–30 flagellated cells attacked individual *C. virginica* larvae) than at 30 ppt (5–8 flagellated cells attacked individual *C. virginica* larvae). During the first 15 min of exposure, flagellated cells aggregated within 400–650 μm of a given larva while maintaining swimming velocities of ∼40–60 μm s^{-1}. Occasionally, a cell rapidly moved toward the valve margin and made repeated contact with the shell margin, which in turn appeared to attract more flagellated cells (e.g. *M. mercenaria*; Fig. 2A). After 15–25 min, *M. mercenaria* larvae developed a noticeable valve gape that the *P. shumwayae* cells breached. Once inside the shells, the flagellated cells consumed the soft tissues via myzocytosis (Schnepf and Elbrächter, 1992), wherein

a feeding organelle (peduncle) was extended from each flagellated cell to pierce and suction the contents from tissue cells. This process continued until the food vacuoles of the flagellated cells were filled, corresponding to an increase of 2–3 μm in cell diameter. After 1 h of exposure, 60–70% of *P. shumwayae* cells inside the excavated larvae had encysted (Fig. 2B). Most larvae that had not been attacked settled out to the bottom of the containers.

Larvae of the three tested shellfish species sustained high mortality (70–85%) after 45–70 min of direct contact with *P. shumwayae* flagellated cells (toxic strains 1024C and 1048C; 15 ppt, 2.5×10^3 cells ml^{-1}). Video-taped footage revealed that ciliary activity of remaining live larvae was severely depressed, especially for larvae exposed to fish-fed *P. shumwayae* strains 1024C and CCMP2089. For example, the ciliary activity of *C. virginica* larvae directly exposed to fish-fed *P. shumwayae* strains 1024C and 1048C was 321 ± 6 and 303 ± 7 beats min^{-1}, respectively ($n = 10$ larvae per treatment). Ciliary activity of *C. virginica* larvae exposed to the same *P. shumwayae* strains fed algae was significantly higher (367 ± 8 and 366 ± 7 beats min^{-1}, respectively; $p < 0.05$, Student's *t*-test, SAS Institute
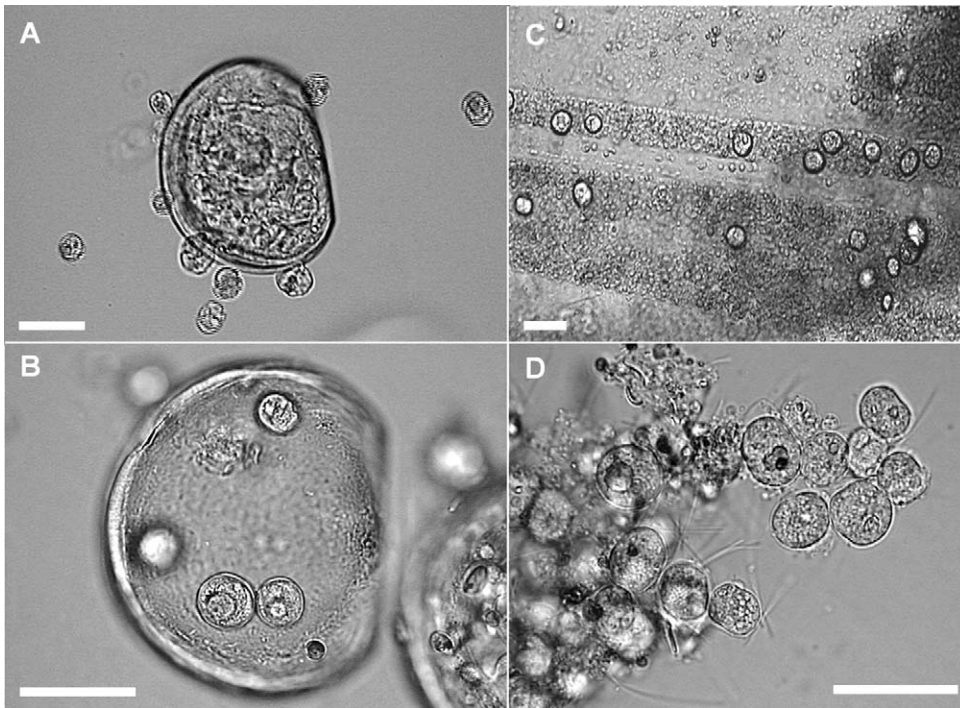

Fig. 2. Light micrographs of: (A) myzocytotic feeding behavior of fish-fed *Pfiesteria shumwayae* flagellated cells (strain 1024C) on a larval northern quahog (*Mercenaria mercenaria*) at 30 ppt; (B) *P. shumwayae* flagellated cells (strain 1024C) encysted after feeding on the shellfish soft tissue for 24 h (salinity 30 ppt, magnification 400×); (C) *P. shumwayae* cysts (strain 2172C) constrained within a fecal strand from a green mussel (*Perna viridis*), 24 h after completion of a grazing experiment; and (D) *P. shumwayae* cysts (strain 1048C) in pseudofeces of a ribbed mussel (*Geukensia demissa*) 36 h after completion of a grazing experiment. Scale bars = 20 μm.

Inc., 1999), and was also significantly higher for larvae fed cryptomonad controls (*S. major*; 380 ± 9 beats min^{-1}; $p < 0.05$).

3.2.2. Survival of larval oysters exposed to P. shumwayae with versus without direct contact

In the 24-h experiments testing impacts of *P. shumwayae* on *C. virginica* larvae with versus without direct contact (for the latter condition, separated from larvae by 0.4-μm well plate inserts), mortality was highest for larvae in direct contact with *P. shumwayae* at either density used (three strains tested: low,

$\sim 3 \times 10^2$ flagellated cells ml^{-1}; moderate, $\sim 3 \times 10^3$ flagellated cells ml^{-1}) (Fig. 3). At the low density of *P. shumwayae*, larvae directly exposed to algal- or fish-fed populations (all three strains) sustained significantly higher mortality than controls (means $\pm$ 1S.E.: algal-fed strains, $19 \pm 5\%$ mortality; fish-fed strains, $29 \pm 11\%$ mortality; controls, $2 \pm 0\%$ mortality; $p < 0.001$) (Fig. 3). The minor mortality that occurred in controls was attributed to larval injury that may have been sustained during transfer into the well plates and/or damage from interactions with the semi-permeable barrier. Strain CCMP2089 induced significantly less
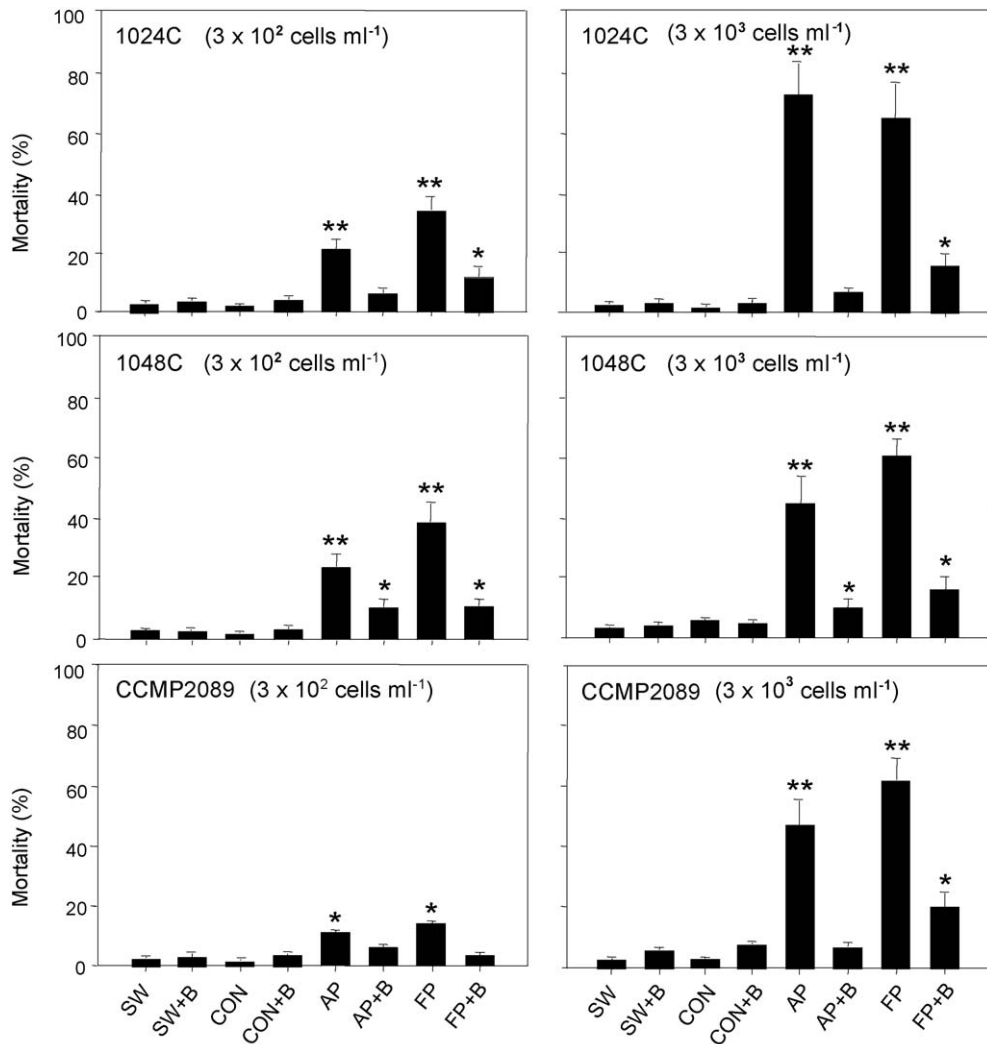

Fig. 3. Mortality of larval shellfish (*Crassostrea virginica*) during exposure to *Pfiesteria shumwayae* (strains 1024C and 1048C; $\sim 3 \times 10^2$ or $\sim 3 \times 10^3$ flagellated cells ml^{-1}). Treatments include SW (filtered seawater, 0.2 μm pore size); SW + B (filtered seawater + barrier); control (cryptomonad *Storeatula major* as the prey source); control + B (*S. major* + barrier); AP (algal-fed *P. shumwayae* flagellated cells); AP + B (algal-fed *P. shumwayae* + barrier); FP (fish-fed *P. shumwayae* flagellated cells); and FP + B (fish-fed *P. shumwayae* + barrier). Data are given as the percentage (%) of 100 larvae killed (means $\pm$ 1S.E., $n = 6$); *significantly different from controls at $p < 0.05$; **significantly different from controls at $p < 0.01$ (Student's t-test; SAS Institute Inc., 1999).

mortality than strains 1024C and 1048C ($p < 0.025$), and larval mortality without direct contact of strain CCMP2089 was not significantly different from that of controls (note that strain CCMP2089 has been evaluated as a weakly toxic strain—Burkholder et al., 2005; Gordon and Dyer, 2005; its low toxic activity has sometimes been missed, e.g. Berry et al., 2002; Vogelbein et al., 2002).

Among algal-fed cultures of *P. shumwayae*, only one strain (1048C, both densities) caused significantly higher larval mortality without direct contact than mortality in controls, suggesting the presence of sufficient *Pfiesteria* toxin in that algal-fed strain to cause some larval oyster death. Considering fish-fed cultures, at the low density, two of three strains (1024C and 1048C) caused significantly higher larval mortality without direct contact than mortality in controls ($p < 0.05$). The highest mortality observed in these experiments occurred with fish-fed cultures, at the higher density, in direct contact with oyster larvae (mean for all three fish-fed strains, $63 \pm 2\%$ mortality) (Fig. 3). Fish-fed strains additionally caused significantly higher mortality without direct contact than mortality of control larvae (mean for the three fish-fed strains, $17 \pm 3\%$, versus controls at $4 \pm 1\%$ mortality; $p \leq 0.05$), suggesting that physical attack acted as a primary mechanism for the death of oyster larvae exposed to these *P. shumwayae* strains, with toxin as a secondary, interactive factor.

3.3. Adult shellfish and P. shumwayae

3.3.1. Survival

No mortality of four shellfish species tested in these experiments (*A. irradians*, *C. virginica*, *M. mercenaria*, and *P. viridis*) was observed with any of three algal- or fish-fed *P. shumwayae* strains (1024C, 1048C, and CCMP2089) at 5×10^3 flagellated cells ml^{-1} for 24 h. At higher densities (≥ 7–8×10^3 flagellated cells ml^{-1}), shellfish mortalities were not observed with algal-fed strains, whereas mortalities of shellfish exposed to fish-fed strains were low ($\leq 15\%$), but significantly higher than in controls ($p < 0.05$). Shellfish response varied with two other strains of *P. shumwayae*. There was $>90\%$ mortality of *C. virginica*, *M. mercenaria* (LT$_{50}$, 14 h), and *A. irradians* (LT$_{50}$, 12.5 h), but no mortality of *P. viridis*, after 24 h exposure to fish-fed strain 270A1 (8×10^3 flagellated cells ml^{-1}). In contrast, *M. mercenaria* and *P. viridis* exposed to a similar density of fish-fed strain 2172C sustained $<15\%$ mortality over 24 h. No mortality occurred for control adult shellfish in filtered seawater media alone or with similar densities of cryptomonads.

3.3.2. Toxicity of shellfish tissues

After tissue homogenates from *A. irradians* and *M. mercenaria* that had been exposed to *P. shumwayae* ($\sim 5 \times 10^3$ flagellated cells ml^{-1} of fish-fed strains 1024C, 1048C, and 2172C) were separately administered to mice via intraperitoneal injection, mice experienced several minutes of disorientation followed by recovery in <5 min. Mice injected with tissues from nontoxic control shellfish that had been fed cryptomonads did not show temporary disorientation.

3.3.3. Grazing activity

Grazing rates of adult shellfish varied significantly depending upon the species, the strain of *P. shumwayae*, and strain feeding history. Although all shellfish species tested were able to remove *P. shumwayae* from solution at bloom densities, grazing rates were significantly higher (in 21 of 24 trials; $p < 0.05$) on the cryptomonad, *S. major* than on algal- or fish-fed *P. shumwayae*, except for green mussels, *P. viridis*, fed two strains of algal-fed and one strain of fish-fed *P. shumwayae* (Fig. 4). In addition, grazing rates were significantly higher on algal-fed than on fish-fed *P. shumwayae* ($p < 0.001$ to <0.05) except for *M. mercenaria* fed strain CCMP2089, and *A. irradians* which had low grazing rates on both algal- and fish-fed populations (all three strains) and the lowest grazing rates of the four shellfish species on *P. shumwayae*. Highest grazing rates on *P. shumwayae* were attained by *C. virginica* (Fig. 4). Grazing rates of *A. irradians* and *P. viridis* were significantly higher on *P. shumwayae* strain 1024C than the other two strains tested ($p < 0.04$). Overall, considering the three strains of *P. shumwayae* (both algal- and fish-fed) tested in the grazing trials, grazing rates ranked from the average of all trials as follows: *C. virginica* $>$ *P. viridis* $>$ *G. demissa* $>$ *M. mercenaria* and *A. irradians* (Fig. 4).

3.4. Analyses of fecal strands

P. shumwayae produced temporary cysts during passage through the gut tracts of all five tested species of bivalve molluscs (Table 2; Fig. 2C and D). These cysts were readily discernible under high magnification ($400 \times$). Epifluorescence microscopy revealed a high level of autofluorescence associated with intact cysts.

In all but 2 of 51 experimental trials, *P. shumwayae* cysts were found in fecal strands and, thus, had passed through the shellfish digestive tract. Exceptions included one trial with *A. irradians* and algal-fed *P. shumwayae* strain 1048C, and one trial with *M. mercenaria* and algal-fed strain CCMP2089. In 8 of 49 (16%) experimental trials, *P. shumwayae* cysts ($n = 100$ per shellfish species)
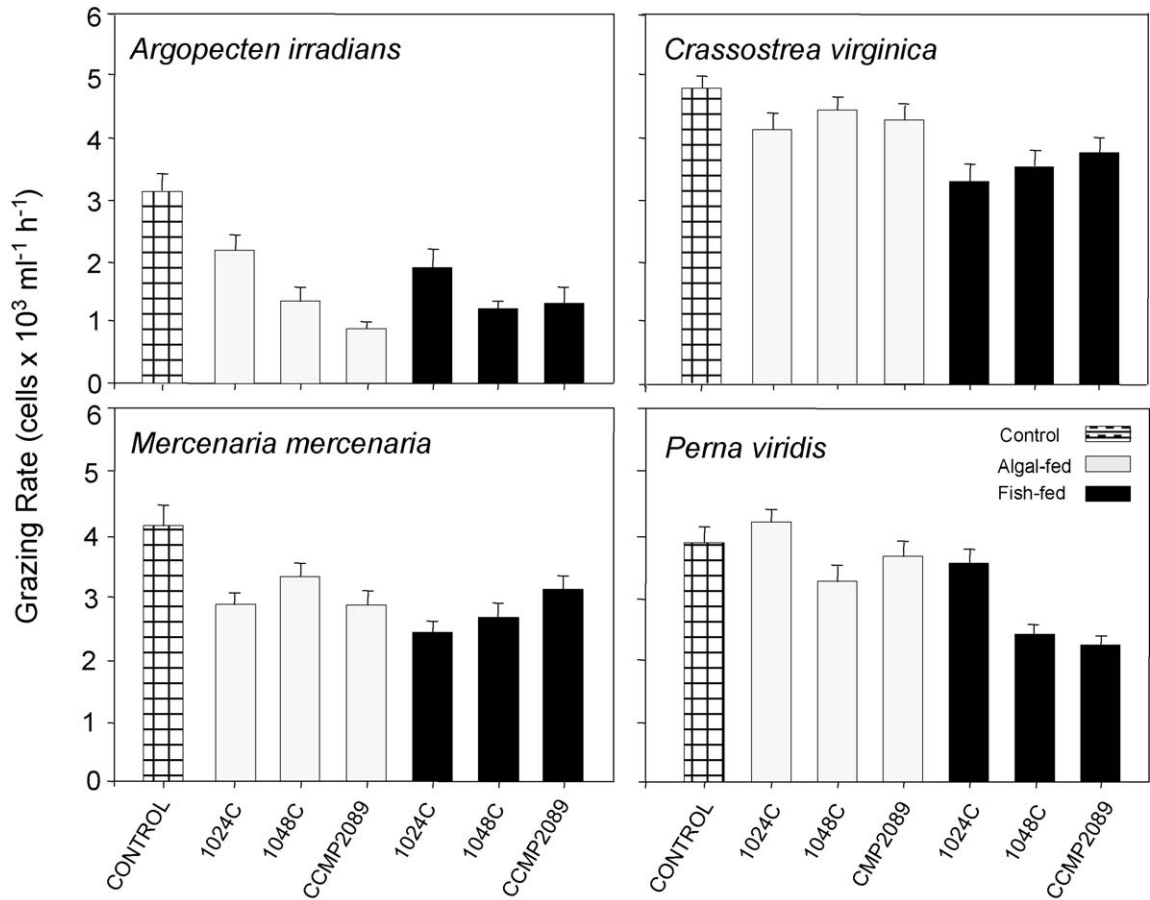
Fig. 4. Grazing rates (defined as the number of cells ml^{-1} initially present minus the number of cells ml^{-1} remaining in the media after 1 h of grazing activity) of four shellfish species on three strains of algal- and fish-fed *Pfiesteria shumwayae* (1024A, 1048A, and CCMP2089), and an algal control (cryptomonad *Storeatula major* HP9101).

isolated from fecal strands produced viable populations of flagellated cells (0.10–1.20 × 10^3 cells ml^{-1}) when the isolated cysts were placed into fresh f/2 media for 2 weeks. A higher percentage of experimental trials (23 of 49, or 47%) produced viable flagellated cells when the fecal strands with cysts were placed directly into fresh f/2 media. In SFBs, *C. virginica* feces yielded the most viable *P. shumwayae* populations (10 of 23 SFBs), whereas *P. viridis* feces yielded only 1 viable population from 12 SFBs.

4. Discussion

This study is the first to document adverse impacts of the toxigenic estuarine dinoflagellate, *P. shumwayae*, on larval shellfish (*A. irradians*, *C. virginica*, *M. mercenaria*, and *P. viridis*) and adults (these species, and *P. viridis*). Flagellated cells of the tested strains aggressively began to consume larvae of *A. irradians*, *C.*

virginica, and *M. mercenaria* within seconds of introduction, as seen in previous studies with the related species, *P. piscicida* (Springer et al., 2002), and similarly encysted within the larval shells after feeding. The shellfish larvae appeared to elicit a chemosensory swimming response in *P. shumwayae*, as shown for *P. piscicida* with shellfish larvae (Springer et al., 2002) and for *Pfiesteria* spp. with fish (Burkholder et al., 2001b; Cancellieri et al., 2001). The data suggest that *P. shumwayae* could adversely affect shellfish recruitment at the population level, given that even relatively small changes in larval mortality can have a major influence on recruitment (Olafsson et al., 1994; Eckman, 1996; Young et al., 1998).

In addition to the larval mortality resulting as a physical effect of feeding activity by *P. shumwayae* flagellated cells, the data from this study suggest that (toxin see Moeller et al., 2001; Burkholder et al., 2005) produced by cultures of *P. shumwayae* flagellated cells

also caused some larval mortality. Higher mortality was expected when *Pfiesteria* was allowed direct contact with prey, since physical attack by *Pfiesteria* likely facilitates toxin entry into tissues while also generally weakening the prey (Burkholder et al., 2001b). Here, when flagellated cells were prevented from making direct contact with shellfish larvae, significantly less larval mortality occurred than when direct physical contact was allowed, but mortality was still significantly higher than that of control larvae (ANOVA, $p < 0.05$). Thus, physical attack apparently acted as a primary mechanism for the death of oyster larvae exposed to these *P. shumwayae* strains, with toxin acting as a secondary, interactive factor (as in Burkholder et al., 2001b, 2005; Gordon and Dyer, 2005). It should be noted that some *Pfiesteria* spp. toxic strains have been lethal to fish when prevented from direct contact (Burkholder and Glasgow, 1997; Gordon et al., 2002; Gordon and Dyer, 2005), as in this study with shellfish and in Springer et al. (2002; shellfish with *P. piscicida*), whereas other strains have required direct contact for lethal effects (Burkholder et al., 2001b). Such variability may be due to differences in toxin production among strains (Burkholder et al., 2001b, 2005), as has been found for other toxigenic dinoflagellates (reviewed in Burkholder et al., 2001b; Hallegraeff et al., 2003).

Adults of all shellfish species tested were able to remove *P. shumwayae* from solution at bloom densities, although grazing generally was lower on *P. shumwayae* than on the cryptomonad *S. major*. Other researchers also have shown that clearance rates of *M. mercenaria* and several other species of bivalve molluscs were significantly depressed when the animals were fed toxigenic dinoflagellates such as *Alexandrium fundyense* Balech, in comparison to clearance rates of nontoxic algae. For example, Bricelj et al. (1991) reported that *M. mercenaria* adults ceased feeding and closed their valves when exposed to the toxigenic dinoflagellate, *A. fundyense*, whereas the animals resumed feeding when fed a mixture of *A. fundyense* and the benign diatom, *Thalassiosira weissflogii* (Grunow) G. Fryxell et Hasle. The underlying mechanism responsible for feeding rate inhibition (e.g. chemosensory detection of toxic cells and avoidance, or depressed metabolism following toxin incorporation) remains to be determined.

This study also documented changes in grazing activity of shellfish that were fed different *P. shumwayae* strains. Influences of algal-produced substances on shellfish grazing have been observed in previous research. For example, Ward et al. (1992) found that the sea scallop, *Placopecten magellanicus* (Gmelin,

1791), can detect intra- and extracellular organic compounds from phytoplankton (the diatom *Chaetoceros muelleri* Lemmerman, $\sim 5.0 \times 10^3$ cells ml^{-1}), and these substances stimulated filtration rates and particle ingestion rates. Gainey and Shumway (1991) showed that direct contact of several bivalve mollusc species with cells of the ochrophyte (brown tide organism), *Aureococcus anophagefferens* Hargraves et Sieburth, or with amylase digests of these cells, caused inhibition of ciliary beat of excised gill tissues. They concluded that the grazing inhibition likely resulted from shellfish detection of a bioactive substance produced by *A. anophagefferens*. Springer et al. (2002) reported that juvenile *C. virginica* cleared significantly less actively toxic populations of *P. piscicida* than populations with low or negligible toxicity.

Fecal strands from wild shellfish collected from the Bay River, NC, yielded fish-killing populations of *P. shumwayae* in SFBs. Cell viability after ingestion and gut passage has been reported for other dinoflagellates (e.g. the Pacific oyster, *Crassostrea gigas* [Thunberg, 1793] fed four species of thecate dinoflagellates—Laabir and Gentien, 1999; *C. gigas* fed *Alexandrium minutum* Halim—Lassus et al., 1999; *A. irradians* fed *Prorocentrum lima* [Ehrenberg] Dodge—Bauder and Cembella, 2000; *P. piscicida*—Springer et al., 2002), and the cysts of these dinoflagellates in shellfish feces have produced active toxic populations in aquaculture facilities (Harper et al., 2002). A proportion of the temporary cysts taken from fecal strands of shellfish in this study also yielded viable motile populations of these *P. shumwayae* strains. Temporary cysts are produced by dinoflagellates in response to sudden environmental stress, and are an important short-term survival mechanism (Taylor, 1987; Burkholder, 1992). Other algae also have been reported to remain viable following passage through shellfish digestive tracts (Fielding and Robinson, 1987). For example, persistent blooms of *Prorocentrum* spp. have been linked to reduced growth of *M. mercenaria* in Long Island Sound, and many intact, undigested *P. minimum* cells were found in the shellfish feces (Wikfors and Smolowitz, 1995). Laabir and Gentien (1999) found intact and viable cells of three species of thecate dinoflagellates (*A. minutum*, *A. tamarense*, and *Scrippsiella trochoidea* [Stein] Loeblich III) in fecal strands from *C. gigas*. These cells formed up to 50% of the fecal strands and regained motility within 24 h of removal to favorable culture conditions.

The data from this study clearly demonstrate the potential for shellfish to concentrate toxic *P. shumwayae* cells through filter-feeding, and also indicate the potential for transfer of viable *P. shumwayae* toxic

strains as cysts from one geographic region to another, via movement of brood stock and relaying (and see Shumway, 1990; Hallegraeff, 1993). Based on the field component of this study, toxic populations of *P. shumwayae* co-occur with adult *C. virginica*, *M. mercenaria*, and *G. demissa* in estuaries of the southeastern U.S. The field and laboratory data were also considered within the context of the potential for overlap of toxic *P. shumwayae* strains with the habitat of the other two shellfish species tested, and with recruitment periods of the five shellfish species. The major growing season for *Pfiesteria* spp. ranges from March to October, and they have been active in salinities ranging from ∼2 to 35 ppt (Burkholder et al., 2001a). Salinity ranges for the known distribution of the shellfish species used in this study overlap with that of *P. shumwayae*: *A. irradians* generally is found in salinities ranging from 20 to 30 ppt (Shumway and Parsons, 2005); *C. virginica* has a broad salinity range of ∼3–31 ppt (Carriker, 1951; Kennedy et al., 1996); *M. mercenaria* generally occurs in salinities of 15–32 ppt (Grizzle et al., 2001); and *G. demissa* and *P. viridis* are euryhaline, found in salinities of 5–35 ppt (Seed and Suchanek, 1992).

The spawning activities of all five shellfish species also overlap warmer seasons (May–October) when high abundance of *P. shumwayae* has occurred (Burkholder et al., 2001a). *A. irradians* generally spawns during the warmest months of the year, but can spawn from May to October depending on the latitude (Rhodes, 1990), whereas *C. virginica* has significant spawning activity in the spring and fall, with minor spawning during the summer depending on the geographic location (Thompson et al., 1996). Peak spawning of *M. mercenaria* occurs in May–June in the mid-Atlantic (Virginia, NC), but can vary by geographic location (e.g. March–April in Florida; June–July in New York; Eversole, 2001). *G. demissa* and *P. viridis* can spawn from spring to fall, but maximal spawning activity typically occurs in early spring and late autumn (Seed and Suchanek, 1992). *P. viridis*, in particular, can spawn throughout the year in warmer climates (Walter, 1982). Thus, at least a portion of the spawning cycle of all species tested in this study overlaps periods of toxic *Pfiesteria* activity, and *P. shumwayae* potentially could adversely affect all of these shellfish species in their natural habitat.

In summary, this study demonstrates the potential for larval shellfish (bay scallops, eastern oysters, and northern quahogs) to be adversely affected by an aggressive feeding response of *P. shumwayae* flagellated cells on soft tissues, and also by *Pfiesteria* toxin(s) (Moeller et al., 2001; Gordon et al., 2002; Burkholder et al., 2005; Gordon and Dyer, 2005). Grazing rates of adult shellfish (bay scallops, eastern oysters, green mussels, northern quahogs, and ribbed mussels) generally were depressed when animals were fed toxic strains of *P. shumwayae*, in comparison to grazing rates on benign algae. Fish-fed *P. shumwayae* strains generally were grazed less than algal-fed cultures of the same strains. In supporting research, reduced shellfish grazing rates were linked to the presence of toxin(s) from *P. piscicida* (Springer et al., 2002). In addition, *P. shumwayae* cells were shown to be capable of surviving passage through the gut tract of sub-adult *C. virginica* as temporary cysts. Thus, the possibility exists that shellfish transfer may inadvertently contribute to the distribution of viable *P. shumwayae* cysts between geographic locations.

Algal blooms represent a major economic threat to nearshore fisheries and aquaculture operations (Shumway, 1990; Sorokin et al., 1996; Matsuyama, 1999; Heil et al., 2001; Matsuyama et al., 2001), and the nutrient-enriched conditions associated with aquaculture operations may stimulate some toxic dinoflagellate populations (Sorokin et al., 1996; Harper et al., 2002). *Pfiesteria* spp. are most abundant in nutrient over-enriched estuarine waters (Burkholder et al., 2001a). The data from this study and previous work (Springer et al., 2002) suggest that high cell densities associated with *Pfiesteria* spp. blooms (e.g. $\geq 10^3$ flagellated cells ml^{-1}) could adversely affect recruitment of larval shellfish and food resources available to wild and farmed shellfish.

Acknowledgments

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