Species of the Toxic *Pfiesteria* Complex, and the Importance of Functional Type in Data Interpretation

JoAnn M. Burkholder, Howard B. Glasgow, Nora J. Deamer-Melia, J. Springer, Matthew W. Parrow, Cheng Zhang, and Paul J. Cancellieri

Center for Applied Aquatic Ecology, North Carolina State University, Raleigh, North Carolina, USA

We describe the two species of the toxic *Pfiesteria* complex to date (*Pfiesteria piscicida* and *Pfiesteria shumwayae)*, their complex life cycles, and the characteristics required for inclusion within this complex. These species resemble *P. piscicida* Steidinger & Burkholder and also have *a*) strong attraction to fresh fish tissues and excreta, *b*) toxic activity stimulated by live fish, *and c*) production of toxin that can cause fish death and disease. Amoeboid stages were verified in 1992–1997 by our laboratory (various stages from toxic cultures) and that of K. Steidinger and co-workers (filose amoebae in nontoxic cultures), and in 2000 by H. Marshall and co-workers (various stages from toxic cultures), from clonal *Pfiesteria* spp. cultures, using species-specific polymerase chain reaction-based molecular probes with cross-confirmation by an independent specialist. Data were provided from tests of the hypothesis that *Pfiesteria* strains differ in response to fresh fish mucus and excreta, algal prey, and inorganic nutrient (N, P) enrichment, depending on functional type or toxicity status. There are three functional types: TOX-A, in actively toxic, fish-killing mode; TOX-B, temporarily nontoxic, without access to live fish for days to weeks, but capable of toxic activity if fish are added; and NON-IND, noninducible with negligible toxicity in the presence of live fish. NON-IND *Pfiesteria* attained highest zoospore production on algal prey without or without inorganic nitrogen or inorganic phosphorus enrichment. TOX-B *Pfiesteria* was intermediate and TOX-A was lowest in zoospore production on algal prey with or without nutrients. TOX-A *Pfiesteria* spp. showed strong behavioral attraction to fresh fish mucus and excreta in short-term trials, with intermediate attraction of TOX-B zoospores and relatively low attraction of NON-IND cultures when normalized for cell density. The data for these clones indicated a potentially common predatory behavioral response, although differing in intensity distinct from a toxicity effect, in attack of fish prey. The data also demonstrated that functional types of *Pfiesteria* spp. show distinct differences in response to fish, algal prey, and inorganic nutrient enrichment. Collectively, the experiments indicate that NON-IND strains should not be used in research to gain insights about environmental controls on toxic strains of *Pfiesteria* spp. *Key words*: amoebae, complex life cycle, culture, dinoflagellates, estuaries, fish, noninducible, nutrients, strains, toxic *Pfiesteria* complex. — *Environ Health Perspect* 109(suppl 5):667–679 (2001).
http://ehpnet1.niehs.nih.gov/docs/2001/suppl-5/667-679burkholder/abstract.html

Among the approximately 40 toxic dinoflagellate species reported within the past 15 years (*1,2*) are two species of ichthyotoxic *Pfiesteria, Pfiesteria piscicida* Steidinger & Burkholder and *Pfiesteria shumwayae* Glasgow & Burkholder (*3–5*). *Pfiesteria* spp. are considered to be unusual toxic dinoflagellates in their predominantly estuarine rather than marine coastal habitat (*1,5–7*) because of their direct attack behavior toward fish (*3,7*); their wide array of potential prey ranging from bacteria and algae, to finfish and shellfish, to mammalian tissues (*5,7,8*); their chrysophyte-like cysts not previously found in dinoflagellates (*3–5,7*); and their complex life cycles with multiple amoeboid as well as flagellated forms with maximum cell dimensions ranging from 5 to 120 µm (*5,7–9*). *Pfiesteria* produces bioactive substance(s) with neurotoxic activity. In the present article, we use the term "toxins" to describe this activity, in accord with the *Pfiesteria* Interagency Coordination Working Group (*10*). In so doing, we acknowledge that these substances are only partially characterized (*11,12*), which is true for various other toxic algae. In the present article, "algae" include heterotrophic dinoflagellates and cyanobacteria, as well as obligate photosynthetic eukaryotes (*13,14*). It should also be noted that in August 2001, J.S. Ramsdell and P.D.R. Moeller of the National Oceanic & Atmospheric Administration, National Ocean Service in Charleston, South Carolina, verified that a potent water-soluble neurotoxin has been isolated and purified from fish-killing, actively toxic *Pfiesteria* culture material in standardized fish bioassays from our laboratory [patent process initiated (*11*)].

Toxic dinoflagellates produce some of the most potent biotoxins known, including ichthyotoxins that can act as neurotoxins in mammals (*13,14*). *Pfiesteria* (type species, *P. piscicida* Steidinger & Burkholder) was unusual because it was the first toxic dinoflagellate found to be stimulated by the presence of live fish, whereas stimuli for toxin production in the other species are unknown (*15*). Exposure to toxic fish-killing cultures [(*16*); via water or aerosol contact] or toxic *Pfiesteria* outbreaks in estuaries (*7,17*) or exposure to toxic, fish-killing *Pfiesteria* culture medium [via subcutaneous injection in rats; filtered to remove the dinoflagellate population, or unfiltered (*18–20*)] have been linked to central nervous system impairment in mammals. Although *Pfiesteria* was unusual among toxic dinoflagellates, it actually is similar to some benign (nontoxin producing) dinoflagellates with complex life cycles, including freshwater and estuarine species with up to 38 stages [reviewed in (*4*); also see (*5,7–9,20*)]; wide range in sizes in freshwater (*21*) and estuarine species (*3–5,8*); ambush-predator behavior in estuarine species (*21*); and use of various prey as food in freshwater and estuarine species (*5,8,22,23*)].

Like many other toxic algal species (*1,24–30*), toxic *Pfiesteria* spp. have naturally occurring toxic as well as apparently benign strains. The latter are noninducible, that is, without toxic activity or capable of producing only negligible/undetectable toxin in response to live fish (*5,6,9,31*). Eventually as more is known about the effects of specific controls on toxin production, it may be possible to induce some or all of these strains to regain their toxicity, but at present the biochemical switches for toxin production apparently have been 'turned off' (*31*). More than 1,000 clones of *Pfiesteria* spp. have been isolated in our laboratory (1991–present). About 60% of the clones assayed with fish (*6,31–34*) have shown ichthyotoxic activity; the remainder have been noninducible. The proportion of toxic and noninducible isolates can vary substantially; occasionally for 1- to 2-year periods, most or all clones recently isolated from natural estuarine habitats have been toxic. Also like many other toxic algae (*25,29,30*), toxic strains of *Pfiesteria* spp. commonly lose

This article is based on a presentation at the CDC National Conference on *Pfiesteria*: From Biology to Public Health held 18–20 October 2000 in Stone Mountain, Georgia, USA.

Address correspondence to J.M. Burkholder, Center for Applied Aquatic Ecology, North Carolina State University, 620 Hutton St., Suite 104, Raleigh, NC 27606 USA. Telephone (919) 515-2726. Fax (919) 513-3194. E-mail: joann_burkholder@ncsu.edu. Center website: *http://www.pfiesteria.org*

Funding support was provided by the N.C. General Assembly, the U.S. EPA, NSF, ECOHAB, an anonymous foundation, the Z. Smith Reynolds Foundation, NOAA, ECOHAB, and the NCSU College of Agriculture & Life Sciences. P. Glibert, A. Lewitus, M. Mallin, R. Reed, and S. Shumway provided counsel on the manuscript.

Received 8 January 2001; accepted 6 August 2001.

ichthyotoxic activity in the presence of live fish when cultured for weeks to months (*31*). The environmental signals controlling toxin production have not yet been determined for any species where the phenomenon has been observed. However, loss of toxicity has been hypothesized to result from the lack of particular organic substrates, possibly including one or more bacterial cofactors, that occur in the natural habitat or from the lack of some other vital factor(s) in culture. If so, then strongly heterotrophic species or strains such as *Pfiesteria* spp. might be expected to be especially vulnerable to loss of toxin production in culture. Alternatively, loss of toxicity may arise from the loss of an essential gene over time in culture, as in certain toxic fungi (*35*). Other aberrations have been noted in cultured dinoflagellates, such as significant change in chromosome number over time (years) (*36*).

The objectives of this study were first to describe the species of the toxic *Pfiesteria* complex to date including their life cycles, with emphasis on stages found to date in both *P. piscicida* and *P. shumwayae* as well as further validation of amoeboid stages; and on the characteristics that must be manifested for inclusion within this complex. Following considerations used for other groups of toxic algae (Table 1) and the recommendations of a recent multifederal/multistate agency consensus document (*10*), we focused on the known toxic *Pfiesteria* species rather than considering all species of benign or unknown toxicity status and systematics that superficially resemble *P. piscicida* under light microscopy. All *Pfiesteria* look-alike species experimentally tested to date, for example, various strains of cryptoperidiniopsoid species, *Karlodinium micrum* [formerly *Gyrodinium galatheanum* (*42*)], and samples including an unnamed dinoflagellate informally referred to as "Shepherd's crook" (*44*) have shown no ability to grow, reproduce, or produce ichthyotoxins causing disease or mortality to fish under ecologically realistic conditions (tests of live cells in standardized fish bioassays) (*5,9,31,32,34*). Second, we tested the hypothesis that *Pfiesteria* differs significantly in response to environmental variables, depending on the functional type or toxicity status of the clone. We compared the response of the three functional types of *P. piscicida* and *P. shumwayae* to finfish materials, algal prey, and nutrient (N, P) enrichments (functional types: TOX-A, in actively toxic, fish-killing mode; TOX-B, temporarily nontoxic, without access to live fish but capable of toxic activity when live fish are added; and NON-IND, noninducible, with negligible/undetectable toxic activity in the presence of live fish) (*5,6,8,31*). We predicted that TOX-A *Pfiesteria* would be strongly attracted to fresh fish mucus/excreta, with low or negligible attraction shown by TOX-B and NON-IND *Pfiesteria*, respectively. We expected the opposite trend in response to inorganic nutrient enrichment and algal prey.

Materials and Methods

Culture and identification of* Pfiesteria *species. The zoospore cultures of *Pfiesteria piscicida* and *P. shumwayae* used in this research were isolated (cloned) from the mesohaline Neuse Estuary, North Carolina. For most experiments, the clonal populations had been isolated from the estuary ≤4 months before the experiments. The *Pfiesteria* amoeba clonal cultures used for certain experiments varied more in age, having been isolated from the Neuse and Pamlico Estuaries and from brackish waters of New Zealand (Table 2) months to years prior to the experiments. A Coulter Epics Altra flow cytometer with HyPerSort System (Coulter Corp., Miami, FL, USA) equipped with a water-cooled Innova Enterprise II ion laser (Coherent, Inc., Santa Clara, CA, USA) was used to sort and clone *Pfiesteria* spp. from positive fish bioassays. The procedure was conducted in biohazard Biosafety Level 3 facilities to obtain actively toxic *Pfiesteria* (*34*). Excitation was provided by a 150 mW/488 nm argon laser line. Quality control calibrations were performed to optimize

Table 1. Examples of the norm in science and management concerning toxic algae and animal/human health issues, i.e., consideration of only algal species known to have characterized or partially characterized toxins.[a]

Toxic algae	Co-occurring look-alike species (light microscopy, SEM)	Consideration (animal/ human health issues)
Cyanobacteria [e.g., certain *Microcystis, Anabaena* spp., *Nodularia* spp. (*25,26*)]	Many coccoid, colonial spp.; many filamentous spp. similar in appearance to toxic *Anabaena*, *Nodularia* spp.[b]	Toxic *Microcystis* spp., toxic *Anabaena* spp., toxic *Nodularia spumigena*[c]
Diatoms [*Pseudo-nitzschia, Nitzschia* (*29,38*)]	10^2–10^3 species[b]	Toxic *Pseudo-nitzschia* complex; recently, toxic *Nitzschia* complex
Dinoflagellates		
Alexandrium spp. (*1,39,40*)	Many species[c]	Toxic *Alexandrium* complex
Gyrodinium (*1,24*)	Many species[e]	Toxic *Gyrodinium* aureolum[c]
Gymnodinium (*1,24,42*)	Many species[e]	Toxic *Gymnodinium* spp. only [e.g., *G. catenatum* [(*39*)]
Pfiesteria (*1,3–7,10,31*)	Many small gymnodinioid, gyrodinioid spp. (*21*)	Toxic *Pfiesteria* complex (thus far, *P. piscicida, P. shumwayae*)[f]
Prorocentrum spp. (*1*)	Many spp.	Toxic *Prorocentrum* spp. only
Prymnesiophytes (*30,41*)	Many chrysophyte, prymnesiophyte spp.	Toxic *Chrysochromulina* (5 spp., mostly *C. polylepis*); toxic *Prymnesium* (3 spp., mostly *P. parvum*)[c]

[a]Thus, all coccoid cyanobacteria that resemble toxic *Microcystis* spp. under light microscopy are not considered in animal/human health issues; each diatom species that superficially resembles toxic *Pseudo-nitzschia* and toxic *Nitzschia* spp. is not considered in animal/human health management issues; all chrysophyte or prymnesiophyte species that superficially resemble the few toxic species known are not considered in animal/human health issues, etc. Among dinoflagellates, only the known toxic species among co-occurring look-alike species in light microscopy have been recommended for consideration at present in animal/human health management issues. *Pfiesteria* should be treated similarly; the known toxic species should be emphasized, as members of the toxic *Pfiesteria* complex, in animal and human health issues. [b]Systematics for many in flux (*25,37*). [c]Scientific names are given as *Nodularia spumigena* Mertens, *Gyrodinium aureolum* Hulburt, *Gymnodinium catenatum* Graham, *Chrysochromulina polylepis* Manton & Parke, and *Prymnesium parvum* Carter.[d]Including various peridinioids, scripsielloids (*41*). [e]Including some *Gymnodinium* and *Gyrodinium* spp. (*41*). Also note that the species previously known as *Gymnodinium breve* and *Gymnodinium mikimotoi* (*1,2,24*) recently were formally changed to *Karenia brevis* (Davis) G. Hansen & Moestrup and *Karenia mikimotoi*, respectively (*42*). [f]Alternate view from the Centers for Disease Control and Prevention (*43*): Any species that superficially resembles *Pfiesteria* must be considered as a "*Pfiesteria* complex organism" or "PCOs". This view was recommended against by a consensus document involving environmental agency officials from 10 states, officials from three federal agencies (U.S. Environmental Protection Agency, National Oceanic & Atmospheric Administration, Centers for Disease Control and Prevention), and academic scientists with expertise in toxic *Pfiesteria* research (*10*):

> PCOs (*Pfiesteria* complex organisms) is an incorrect acronym that should no longer be used. "Complex " has a specific meaning [that is improperly used in PCOs] and refers to a group of species within a genus or to an assemblage of species with certain behavioral characteristics (here, toxic activity, which is of critical importance when considering the *Pfiesteria* issue). Examples of the correct use of the term "complex " are the toxic *Alexandrium* complex, which refers to the species within the genus that produce saxitoxins and their derivatives; and the toxic *Pseudo-nitzschia* complex, which refers to the species within the genus *Pseudo-nitzschia* that produce the toxin, domoic acid. The TPC (toxic *Pfiesteria* complex) refers to dinoflagellate species with strains that resemble *Pfiesteria* when viewed under light microscope and produce toxins under appropriate experimental conditions (as demonstrated by a positive test with fish bioassays, see Glossary). To be included in the TPC, a species must have all of these characteristics: i) strong attraction to live fish prey, ii) ability to produce substances that are ichthyotoxic (i.e., cause erratic behavior, disease, or death in fish), and iii) toxic activity (production of toxic substances by some strains within the species) that is stimulated by the presence of secretions, excretions, or the presence of (usually large numbers of) live fish.

The consensus group also advised that dinoflagellates that are similar in appearance but not necessarily similar to *Pfiesteria* spp. in ability to produce toxin and other important behavioral characteristics should be called "pfiesteria-like organisms" (PLOs, which superficially resemble *Pfiesteria* under 400× in light microscopy), and that such organisms should (only) be of focus in management issues when completing presumptive counts in preliminary efforts to determine whether a toxic *Pfiesteria* or *Pfiesteria*-like species is actually present.

optical alignment and detector voltages, using fluorescent latex microspheres 6 µm in diameter (Molecular Probes, Inc., Eugene, OR, USA). Fish bioassay samples (200 mL) were gravity-filtered through 38-µm Nitex mesh (Aquaculture Research/Environmental Associates, Inc., Homestead, FL, USA) immediately prior to flow cytometric analysis. Electronic sort gates were based on detection of optical parameters defined to select the detectable subpopulations of interest. Sort recovery and purity were checked with light microscopy, and with the heteroduplex mobility assay (*47*). Isolation of highly purified cells via particle sorting yielded ultraclean cell preparations for polymerase chain reaction (PCR) and scanning electron microscope (SEM) analyses. From these subpopulations, a robotic Coulter AutoClone sorting system (Coulter, Miami, FL, USA) was used to establish multiple clonal isolates by directed deposition into multiwell microculture plates where the populations initially were grown for 2 weeks with cryptomonad prey. They were then inoculated into fish bioassays and monitored daily or more frequently to confirm toxic activity and to grow toxic clones.

The autoclone sorting system allowed rapid cloning of single cells into multiwell microculture plates with 99.98% precision and purity. Once clonal sorting was complete, axenic (externally eubacteria- and cyanobacteria-free) prey were added to each well, and the dinoflagellates are allowed to grow and re-produce for several days under sterile conditions. To ensure that cultures did not contain prokaryotes, or eukaryotes other than *Pfiesteria* and the added axenic prey, 10 µL of each culture was plated onto agar growth medium (triptic soy agar [TSA]; Difco Laboratories, Detroit, MI, USA) following techniques described by (*48*). The plates consistently were evaluated as free from bacterial, fungal, or other contaminants.

One clone of each species (#101161 and # 410T for *P. piscicida* and *P. shumwayae*, respectively) was confirmed as toxic to fish in the standardized fish bioassay process (*6,9,31,32,34*). It is important to clarify that this standardized fish bioassay procedure, including cross-corroboration of the data from each step by one or more independent specialists, was developed and has been used by our laboratory throughout the past decade (*31,34*). The existence of this standardized procedure was recognized and endorsed by a national science panel that was charged by the CDC to review all previously published *Pfiesteria* research (*49*). In the present experiments including fish bioassays, *Pfiesteria* toxicity was cross-corroborated by Marshall et al. (*9*). Unidinoflagellate clonal quality was determined by the heteroduplex mobility assay (D. Oldach, University of Maryland, Baltimore, Maryland, USA) (*47*). Species identifications were made from suture-swollen cells with SEM of ≥100 zoospores analyzed per clone following the methods of Burkholder and Glasgow (*8*) and Glasgow et al. (*5*) and were cross-confirmed with PCR] probes and the fluorescent *in situ* hybridization (FISH) probes of P. Rublee of the University of North Carolina at Greensboro (*46*) and D. Oldach (*47*), as well as cross-corroborated by those laboratories. A second isolate of each species (#B93B-N and #270A-2 for *P. piscicida* and *P. shumwayae*, respectively) tested as NON-IND in repeated fish bioassays (*6,9,31*). TOX-A *Pfiesteria* spp. were grown with live fish for 3–4 months prior to the experiments [tilapia, *Oreochromis mossambicus;* total length (t.l.) 5–7 cm; 3–6 fish day^{-1} in 5-L microcosms (*5,6,34*)]. TOX-B subcultures were taken from the TOX-A cultures after being grown with live fish for 2–3 months. The subcultures were switched for 3 weeks to a diet of cryptomonad prey containing *Cryptomonas* LB2423 cloned from commercial material provided by a culture collection at the University of Texas in Austin, Texas, USA. These phytoflagellates were grown in f/100 medium (*50*) at a salinity of 15 (*51*) and fed to *Pfiesteria* zoospores in a 1:15 zoospore:prey ratio at 3- to 4-day intervals (*5,6,31*). NON-IND cultures (of necessity, from different clones) were also grown with cryptomonad prey. Cultures and experiments were maintained at 23°C, 12 hr:12 hr light:dark (L:D)cycle, and 80 µmol photons m^{-2} s^{-1}. The medium was f/2-Si media (*50*) at a salinity of 15 made with sterile-filtered seawater [0.2 µm porosity, adjusted with sterile-filtered deionized water to a salinity of 15, which is optimum for TOX-A *Pfiesteria* spp. from North Carolina estuaries (*5,52*)] collected in the Atlantic Ocean, 2 km from Beaufort, North Carolina.

We estimated the chromosome number of *P. piscicida* using confocal laser scanning microscopy [(CLSM); Leica model TSC SP confocal laser scanning microscope equipped with a UV 351- and 363-nm laser, 63× water emersion optics, and a 1.2 numerical aperture; Leica, Solms, Germany] in combination with light microscopy (LM) and transmission electron microscopy procedures (*5*). CLSM was used to examine in more detail the morphology and localization of zoospore fluorescent cellular DNA structures (*53*). It provided high-resolution, three-dimensional reconstruction of chromosomal DNA by recording and stacking a series of two-dimensional images taken at 1-µm increment depths from the surface of each zoospore examined (n = 12). In addition, the relative DNA content of *P. piscicida* (14 clones, 12–50 months after isolation from fresh estuarine samples) and *P. shumwayae* zoospores (9 clones, 8–36 months after isolation) was evaluated using flow cytometry, as mean DNA fluorescence, (compared to chicken red blood cell DNA standard; ≥10^4 zoospores analyzed from each clone) under the G1 peak (*54*). Samples were preserved with 1% paraformaldehyde, stored for ≥24 hr in darkness at 4°C, treated with 1 µg RNAse A mL^{-1} for 1 hr at 20°C, and stained with 5 µM SYTOX Green [Molecular Probes, S-7020] for 12–16 hr in darkness at 4°C (*54–56*).

Transformations to filose and lobose amoebae from the clonal zoospore cultures were induced in the presence of live fish or their fresh tissues, secreta and excreta. Transformations from filose to lobose amoebae were observed after temperature and salinity shock and by manipulating the type and abundance of prey (*7,8*), for example, removal of fish and provision of bacteria [*Pseudomonas* isolate from the mesohaline Neuse Estuary, or the *Rhodomonas* CCMP757 cloned from commercial material obtained from the Culture Collection for Marine Phytoplankton (CCMP) from Bigelow Laboratory, Bigelow, ME, USA; the cloning procedure removed contaminant eukaryotic algae and bacteria]. Amoeboid cultures were grown at 21°C and a salinity of 15, on a 0 hr:24 hr L:D or 14 hr:10 hr L:D cycle at 80 µmol photons m^{-2} s^{-1}.

Table 2. Further confirmation of amoeboid stages in TPC species [in addition to (*3–5,7,8*)].[a–c]

Origin	Isolate	Species	Amoeboid stages	PCR[d]	FISH[d]
Neuse (North Carolina)	NRA5	*P. piscicida*	Lobose, filose	+	+
Neuse (North Carolina)	920A	*P. shumwayae*	Lobose, filose	+	+
Pamlico (North Carolina)	PRA-1A	*P. shumwayae*	Lobose, filose	+	+
Pocomoke (Maryland)	140A	*P. shumwayae*	Lobose, filose	+	+
New Zealand[e]	652T-A	*P. shumwayae*	Lobose, filose	+	+

[a]Clonal amoebae cultures were derived from clonal zoospore cultures of *P. piscicida* and *P. shumwayae*, confirmed as *Pfiesteria* species using species-specific molecular probes that had been developed for clonal zoospores of *P. piscicida* and *P. shumwayae*. PCR and FISH data were obtained in our laboratory and were cross-corroborated by the independent laboratory of P. Rublee. [b]Amoeboid stages have also been confirmed in *P. piscicida* by K. Steidinger and co-workers [filose stages from TOX-B cultures (*4*)] and H. Marshall [filose and lobose stages from TOX-A clones provided by our laboratory (*9*)]. [c]In more than 1,000 hr of observations on these clones, no zoospores have been observed. [d]The (+) symbol in each line under PCR and FISH (*45,46*) indicates that separate testing of lobose amoebae culture and of filose amoebae culture was positive for *Pfiesteria*. [e]TOX-A cultures of colleague L. Rhodes of the Cawthron Institute in Nelson, New Zealand (*45*).

The Complex Life Cycle and Behavior of *Pfiesteria*

Following the procedures of Glasgow et al. (*5,6*) and Burkholder and Glasgow (*7,8*) as indicated above, we used salinity shock (a sudden increase in salinity of 5–7, 1 hr, $n = 10$ populations of clonal zoospores, 50 cells population^{-1}), temperature shock (a sudden change from 22 to 8–10°C or to 32°C, 1–2 hr, $n = 12$ populations of clonal zoospores, 50 cells population^{-1}), the presence/absence of live fish (*O. mossambicus*), and the availability of different algal prey species to examine various stage transformations in these isolates (clones #B93BF1 and #416T of TOX-A and TOX-B *P. shumwayae*, respectively). We compared these observations from similar treatment of isolates of *P. piscicida* (*7,8*). As in Burkholder and Glasgow (*7*) for *P. piscicida* with finfish and Springer (*57*) for *P. piscicida* with shellfish, we documented attack, swarming, attachment to and feeding on live finfish by TOX-A zoospores of *P. shumwayae* [from mass-culture fish bioassays with tilapia, *O. mossambicus* (*31*)] using sheepshead minnows (*Cyprinodon variegatus*, 7–10 days of age) in acute toxicity microassay tests (*34*). Attack behavior of zoospores was monitored and recorded using an Olympus AX 70 light microscope (with water immersion lens, 200×) and an Olympus IX 70 inverted microscope (phase contrast, 40×; Olympus Corp., Melville, NY, USA). *Pfiesteria* zoospores were videotaped using a cooled-chip charged couple device video camera (Optronics Corporation, Goleta, CA, USA), S-VHS video recorder (Sony Electronics, Inc., Park Ridge, NJ, USA), and 53-cm video monitor (Sony).

Filose and lobose amoebae that had transformed from the clonal TOX-A zoospore cultures (*P. piscicida*, 1993–present; *P. shumwayae*, 1996–present) were maintained on a diet of *Cryptomonas* LB2423 or *Rhodomonas* CCMP757 (*8*) (after cloning the commercial-source algae in axenic culture; ≤8 cryptomonads were consumed hr^{-1} amoeba^{-1}), or bacteria (*Pseudomonas* isolate from the mesohaline Neuse Estuary). These amoebae initially ranged from 30 to 70 µm (major cell axis), with a nuclear diameter often approaching 20% of the total cell volume. Under prey-replete conditions, lobose amoebae averaged 17 ± 4 pseudopodia per cell (mean ± 1 standard deviation, $n = 100$ cells). Locomotion in these lobose amoebae typically occurred from steady cytoplasmic streaming, with slight anterior–lateral bulging. Clonal amoebae [clonal as defined in (*5,10,31*)] were grown with axenic cryptomonad algal prey at 21°C and a salinity of 15 (*51*), on a 0 hr:24 hr L:D or 14 hr:10 hr L:D cycle. They were monitored for changes in morphology and size over time in culture. We tested the influence of alterations in prey type/abundance in some subcultures (*5,7,8*). For example, change from the cyanobacterium *Cyanothece* [formerly Synechococcus (58); clone HP9001, diameter 1.5 µm, 10^6 cells mL^{-1}, 5000:1 ratio of prey:amoebae] to cryptomonad prey (e.g., 10^4 *Cryptomonas* LB2423 mL^{-1}, 40:1 ratio of prey:amoebae) has been shown to induce some amoeboid cells to produce zoospores (*7*). However, to retain pure cultures of amoebae without other life stages and thereby avoid the potential for uncertainty in interpretation, subcultures used for molecular probe analyses were maintained long-term (months to years) without altering the algal prey species used. Over these periods of observation, no zoospores were noted in the cultures.

FISH probes have been developed for *P. piscicida* and *P. shumwayae* zoospores (*45,46*). Specific Alexa Fluor fluorophores (Molecular Probes) were used to label an amine-modified oligonucleotide within the hypervariable region of the 18S ribosomal DNA gene (*59,60*) for the zoospores of each species (*P. piscicida*: Alexa Fluor 488, absorbance at 495 nm, emission at 519 nm, green fluorescence; *P. shumwayae*: Alexa Fluor 350, absorbance at 346 nm, emission at 442 nm, blue fluorescence). These probes were tested to assess for reactivity with amoeboid stages from our clonal cultures of *P. piscicida* and *P. shumwayae*. PCR testing was performed on amoebae isolates using 40 mL of sample from each clone (densities 800–1,200 cells mL^{-1}). DNA was extracted from cultured cells using a DNeasy plant mini-prep kit (Qiagen Corporation, Valencia, CA, USA). Amplification of target DNA was accomplished using a PCR-based procedure (*60*) with 18S rDNA-based primers that have tested as specific to *P. piscicida* and *P. shumwayae*. The amplified DNA was loaded into a 1.5% agarose gel containing ethidium bromide, and gel electrophoresis was mediated by a Tris-borate-EDTA buffer. Electrophoresed products were visualized and archived using a Gel Doc 2000 gel documentation system (BioRad Corporation, Hercules, CA, USA). Analyses were repeated and cross-confirmed by the independent laboratory of P. Rublee.

Algal Prey Species and the Amoeba:Zoospore Ratio

Assessment of the influence of algal prey species on the relative abundance of zoospores versus amoebae in clonal *P. piscicida* cultures were completed using TOX-B *Pfiesteria* zoospores as the initial test populations {batch cultures, 4 days; initial density, 150 + 30 zoospores mL^{-1} (means ± 1 SE; $n = 4$)]. These zoospores had been growing on cryptomonads (*Cryptomonas* LB2423) for 3 weeks. The algal prey were in exponential growth phase and included cyanobacterium *Cyanothece* sp. (clone HP9001, diameter 1.5 µm, 10^6 cells mL^{-1}), cryptomonads *Cryptomonas* sp. (LP 2423, 12 µm × 6 µm, 10^4 cells mL^{-1}) and *Rhodomonas* sp. (CCMP757, 12 µm × 6 µm, 104 cells mL^{-1}), and centric diatom *Thalassiosira weissflogii* (CCMP1335, diameter 8 µm, 10^4 cells mL^{-1}). Comparable total biovolume of each prey species was used.

Functional Types of *Pfiesteria*: Comparative Response to Algal Prey, Nutrients, and Fish

We examined zoospore production of the three functional types of each *Pfiesteria* species, using the above clones in three experiments. In the first experiment, we compared zoospore production of the three functional types of *Pfiesteria* (each *Pfiesteria* species tested separately) in response to cryptomonad prey: initially in batch culture mode for 6 days, 1:15 ratio of zoospores: prey, with initial zoospore densities of 150 ± 20 cells mL^{-1}; algal prey, *Cryptomonas* sp. LB 2423 [isolated in clonal axenic culture from the multi-species commercial culture source; grown in f/1000 media (*50*), $n = 3$; note that a NON-IND isolate was not available for *P. shumwayae* (*33*)]. Controls were assessed as zoospores alone (each *Pfiesteria* sp. separately, no algal prey) and prey alone (no *Pfiesteria*). In the second experiment, we compared zoospore production of the three functional types of each *Pfiesteria* sp. in response to N_i or P_i enrichment + cryptomonad prey (500 µg NO_3^-N or PO_4^{-3}P L^{-1}, 5 days, $n = 4$; otherwise, same initial conditions as above). Three types of controls were maintained in f/1000 media (*50*), including *a*) each *Pfiesteria* sp. without nutrient enrichment + cryptomonad prey; *b*) each *Pfiesteria* sp. + N_i or P_i enrichment but without cryptomonad prey; and *c*) each *Pfiesteria* sp. without nutrient enrich-ment and cryptomonad prey. Algal prey cell production without *Pfiesteria* ± N_i or P_i enrichment was also determined.

The third experiment tested *Pfiesteria* response to fresh fish mucus (minutes) using a microcapillary tube assay (*61*), wherein fresh mucus and excreta from several fish species were collected using consistent technique, sterile filtered (0.22-µm porosity), and added to replicate microcapillary tubes (aperture diameter ~30 µm; $n = 3$). The species included in the study were juvenile (all species) tilapia (*O. mossambicus*, t.l. 5–7 cm), Atlantic menhaden (*Brevoortia tyrannus* Latrobe, Neuse Estuary, t.l. 13–17 cm), juvenile hybrid striped bass *Morone saxatilis* × *Morone chrysops* Rafinesque; t.l. 15–20 cm), and bluegill (*Lepomis macrochiris*

Rafinesque, t.l. 8–11 cm). The cultured fish (all species except Atlantic menhaden) had been fed Tetra Marine fish food once daily prior to collecting excreta and mucus. The mucus and excreta were sterile-filtered immediately upon collection (including filtration of the menhaden materials in the field) and were separated from the live animals ≤3 hr prior to testing with *Pfiesteria* spp. The responses of the three functional types of *P. piscicida* were assessed as net entry of zoospores into the tubes over 10-min trials [10^3–10^5 cells mL^{-1}, normalized to 10^4 cells mL^{-1} for comparative purposes using a linear normalized term determined by least-squares regression and verified by ANOVA (62)], from analysis of videotapes [Olympus AX-70 research light microscope, 600×, water immersion objective (Olympus); and a cooled-chip CCD video camera (Optronics Corp.); S-VHS video recorder (model SVO-9500MD, Sony); and 53-cm video monitor (Sony)]. Control tubes contained sterile-filtered seawater at a salinity of 15. For each experiment, one-way ANOVA was used to test for differences between controls and treatments, and multifactor ANOVA was used to compare replicates and test for differences among functional types. All probability values (p) were considered significant at $p < 0.05$ (*62*).

Results

Toxic *Pfiesteria* Complex Species and Life Stages

The toxic *Pfiesteria* complex currently includes two species, *P. piscicida* and *P. shumwayae* (*4,5*). Both are heterotrophs (*6,7,8,31*), but zoospores are capable of photosynthesis when they retain kleptochloroplasts from algal prey (*5,63*). These species can be distinguished by the plate tabulation of the zoospore stages (Figure 1). The suture-swollen zoospores of the *P. piscicida* isolates (toxic and noninducible strains) in this study, as in previous research, yielded a plate tabulation of Po, cp, X, 4′, 1a, 5″, 6c, 4s, 5‴, 2‴′, with a three-sided anterior intercalary plate ["a"; as in (*4*)] (Figure 1). The plate tabulation for zoospores of the *P. shumwayae* isolates (toxic and noninducible strains) was Po, cp, X, 4′, 1a, 6″, 6c, 4s, 5‴, 2‴′, with a distinct four-sided "a" plate because of the additional precingular "c" plate (*5*). There are probably other toxic species of *Pfiesteria* not yet described (*7,31*). For example, a tropical species with a plate formula identical to that of *P. piscicida*, but with certain morphological differences has been reported from aquaria with fish kills (*64*). However, in that case, parasitic dinoflagellates were also present, which could have caused the fish death, and the culture was not cloned or formally tested for ichthyotoxicity.

Zoospores of *Pfiesteria* spp. varied considerably in size and shape (diameter usually 7–14 µm, but with a known range of ~3–24 µm, $n = 2125$), depending on the stage of origin and feeding activity (*5,7,23*). The epitheca and hypotheca were equal, subequal, or distinctly unequal in size, with the epitheca slightly to substantially larger. The flagella had a similar construction and insertion as has been described for other peridinoid species (*65*), including a helical transverse flagellum and a thick longitudinal flagellum (length ~20 µm, width ~0.1–0.2 µm, $n > 420$; mastigonemes were sometimes observed).

Other research has shown that the two *Pfiesteria* morphospecies are closely related. The isolates of *P. piscicida* and *P. shumwayae* examined in this study and in previous research differed by approximately 45 base pairs in their 18S rDNA sequence (*5,47*). The chromosome number of *P. piscicida* TOX-A and TOX-B zoospores (from clones isolated within ≤3 months from estuarine habitats) was 23 ± 2, as determined using light and epifluorescence microscopy, confocal microscopy, and transmission electron microscopy procedures (*31*). The small size and the thin but tough and highly impermeable theca have prevented use of stains and

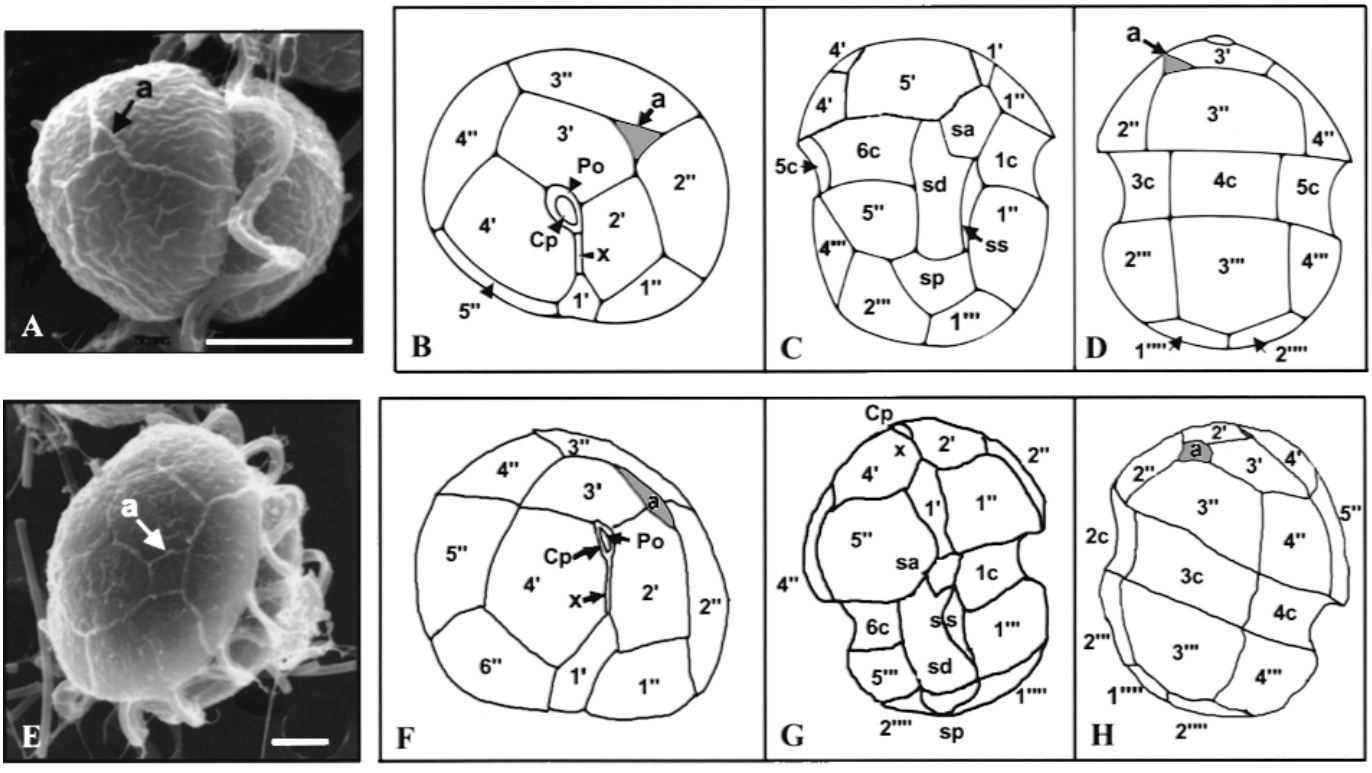

Figure 1. Scanning electron micrographs and plate structure of *Pfiesteria* spp. TOX-A zoospores as (*A*) *P. piscicida*, showing the 3-sided anterior intercalary plate "a" in this SEM of a suture-swollen cell (scale bar = 3 µm); and drawings of the plate structure of the apical view (*B*), the ventral view (*C*), and the dorsal view (*D*) of *P. piscicida* zoospores modified from Steidinger et al. (*4*); (*E*) *P. shumwayae*, showing the 4-sided plate "a" in this SEM of a suture-swollen cell (scale bar = 5 µm); the plate structure traced from zoospores, including the apical view (*F*), the ventral view (*G*), and the dorsal view (*H*) modified from Glasgow et al. (*5*). Reprinted from Burkholder et al. (*31*) with permission from *Phycologia*.

chromosome enumeration techniques employed, for example, by von Stosch (*66*), Holt and Pfiester (*36*), and Dodge (*67*). Flow cytometric procedures indicated that the DNA content of recent isolates of *P. shumwayae* TOX-A and TOX-B zoospores (clones isolated within ≤3 months from estuaries) was more than 2-fold higher than that of *P. piscicida* zoospores (23 clones tested; $>10^4$ zoospores per analysis) (*54*).

Thus, the chromosome number of *Pfiesteria* spp. is intermediate between the chromosome numbers of parasitic dinoflagellates (4–8 chromosomes) and some free-living species (≥20), on the one hand, and certain photosynthetic, free-living dinoflagellates with ≤270 chromosomes on the other (*68*). Over time in culture, some clonal zoospore cultures have become more variable in chromosome content. Although we have noted increasing ploidy over several months in TOX-A zoospores (*31*) [as observed in photosynthetic dinoflagellate species (*36*)], comparisons of DNA content of nine *P. piscicida* clones of varying age (1–4 years) have indicated that the DNA content can significantly decrease over longer periods in culture. This point is of interest, as toxin-producing capability of many toxic *Pfiesteria* clones has been lost over time in culture. We hypothesize that heterotrophic dinoflagellates, which likely require a more complex diet than obligate photosynthetic dinoflagellates, may more commonly lose than gain DNA when cultured for extended periods, as the artificial media probably is missing required substrates. It is not yet known whether the decrease in DNA content of cultures maintained for years reflects loss of genes involved in toxin production, as has been found for certain toxic fungi (*35*).

TOX-A zoospores are the most lethal stage in the life cycle of both *Pfiesteria* spp. (*5–7,31*) (Figure 2). In standardized fish bioassays (*34*), TOX-A zoospores have been lethal to fish in densities ≥300 cells mL^{-1}, with time to death of juvenile tilapia species (*O. mossambicus, O. aureus, Tilapia nolotica*, t.l. 5–7 cm) ranging from 20 min ($>3 \times 10^2$ to 10^3 toxic zoospores mL^{-1}) to 12 hr (10^3–10^4 toxic zoospores mL^{-1}), indicating highly to weakly toxic isolates, respectively (*6,7,33,52*). In most *Pfiesteria*-related estuarine fish kills, zoospore densities have been at $> 3 \times 10^2$ to 5×10^3 cells mL^{-1} (range up to 1.1×10^5 cells mL^{-1}) (*7,16,31*). Whereas some *Pfiesteria* isolates have been tested as capable of killing fish whether allowed direct contact with the prey (Figure 3) or maintained within dialysis membrane (molecular weight cut-off 12,000–14,000 Da) to prevent direct contact (*7,57*), others have killed only when allowed direct contact with the prey. The two species thus far have been shown to produce analytically comparable toxin (*12*), but considerable intraspecific differences among isolates apparently occur in toxin potency and in the extent to which toxin is released versus retained within the cells. A mechanism for *Pfiesteria* toxin impacts on fish and mammals has been described from experiments with clonal, toxic cultures (cross-corroborated by independent specialists) wherein the toxin mimics an ATP neurotransmitter that targets $P2X_7$ receptors (*11*). The cultures used for that research were tested as capable of killing fish when prevented from direct contact with prey (*7,57*). The mechanism of targeting $P2X_7$ receptors and the cascade of impacts (including extreme response to inflammation) that followed would be optimized with physical abrasion or damage (*11*). Thus, physical attack by toxic *Pfiesteria* zoospores may help to promote entry and damage by the toxin in fish tissues. Alternatively, for some *Pfiesteria* isolates, close proximity to fish may be required to stimulate toxin release, and/or external tissue damage or wounding may create areas where the toxin enters the fish.

TOX-A zoospores were observed to be produced by chrysophyte-like cysts (stage #11A), coccoid cysts (stage #6A), coccoid cells (stage #7A), benthic or suspended palmelloid masses (stage #5A, ranging from 6 to ≥32 coccoid cells), and planozygotes (stage #4) in the presence of live fish or their fresh materials (tissues, excreta, secreta) (Figure 2). They were produced following detection of materials from live fish by TOX-B zoospores. TOX-B zoospores were produced by stages to which they can directly [chrysophyte-like (chryso) cyst, temporary cyst, palmelloid mass—all haploid stages] or indirectly transform [from coccoid cells excysted from another cyst stage (both haploid) produced by TOX-B zoospores]; by large diploid lobose amoebae; and by diploid hypnozygotes. TOX-B zoospores were also formed from gametes through reversion of "–" anisogamous gametes (Figure 2) or of isogamous gametes (possible in both *Pfiesteria* spp.) when the signal from live fish that stimulates toxicity is no longer detected. In asexual reproduction TOX-A and TOX-B zoospores of *P. piscicida* either divide while swimming or form palmelloid masses where the cells undergo mitosis and cytokinesis. The latter pathway is more common in *P. shumwayae*. Most amoeboid stages in the complex life cycles of *Pfiesteria* spp. occur in actively toxic cultures, or in cultures that recently (hours to days) were in actively toxic mode. TOX-A zoospores can produce haploid filose, lobose, and rhizopodial amoebae, whereas thus far TOX-B zoospores have been verified to produce only filose and lobose amoebae (Figure 2).

TOX-A and TOX-B zoospores were identical in appearance to isogamous gametes (diameter 7–9 µm; n = 320), although their size varied four-fold depending on feeding activity. In strains with anisogamy, the smaller "+" anisogamous gametes were 4–5 µm in diameter (n = 280) and had a much longer longitudinal flagellum than the larger "–" anisogamous gametes (diameter 7–9 µm; n = 280). Gamete fusion in the presence of live fish or their fresh excreta, secreta, and/or tissues produced a triflagellate, diploid planozygote (diameter 14–60 µm, n = 125) with one transverse and two longitudinal flagella. The planozygote produced four zoospores or, alternatively, formed a hypnozygote (sexual resting cyst). Following a period of dormancy, four haploid zoospores emerged from the hypnozygote in *P. piscicida* or, alternatively in (*P. shumwayae*), two triflagellate cells (each with one transverse and two longitudinal flagella) emerged from the hypnozygote, and these each rapidly produced two biflagellate zoospores (each with one transverse and one longitudinal flagellum).

Amoebae in both *Pfiesteria* spp. were also produced by gametes, planozygotes, or cysts, resulting in considerable size variation (*5,7*) (Figure 2). The fine structure of the amoebae of *Pfiesteria* spp. is currently under examination and is less well known than that of the flagellated stages (which include the most toxic forms) or the cysts derived from them (*31*). *Pfiesteria* spp. amoebae had a normal eukaryote nucleus (diameter 3–11 µm, n = 75), with a double-membrane envelope. The chromosomes were not condensed during mitotic interphase (*4,5*), also reported for other dinoflagellate amoebae (*21,71–79*) (below). Depending on the clone, the food source, and environmental conditions, *Pfiesteria* amoebae included filose and/or lobose forms (both species) as well as rhizopodial forms [found thus far in *P. piscicida* (*7*)], with a smooth or rough outer covering (*4,5,7,8*). Transformations to amoeboid stages were common in some clones with live fish prey, but were rare in algal-fed clones. Amoebae were produced mostly during or following exposure to fresh fish materials, although filose amoebae were also sometimes produced by nontoxic cultures. The amoeboid stages generally ranged from 5 to 120 µm (n = 1,830) in maximum cell dimension, depending on the stage of origin (Figure 2), but like certain Sarcodinian amoebae (*80*), they sometimes grew much larger within ≥12 months [from ~40 µm initially to a maximum of ~750 µm in *P. piscicida* (n = 2 cultures) and from ~40 µm initially to a maximum of ~250 µm in *P. shumwayae* (n = 3 cultures); 70–90 cells measured initially in each culture and at ~6-month intervals]. By comparison, the largest *Pfiesteria* amoebae

that have been observed in fish-killing cultures 4–20 hr after fish death in this as well as previous research were approximately 120 µm on the major cell axis (*7,8*). The cultures also sometimes became multinucleate over time, probably as a cultural aberration; this phenomenon of uninucleate amoebae becoming multinucleate over long-term culture has also been reported for various Gymnamoebae (*80*). Filose amoebae generally were observed as ephemeral stages lasting minutes to hours. However, some clonal

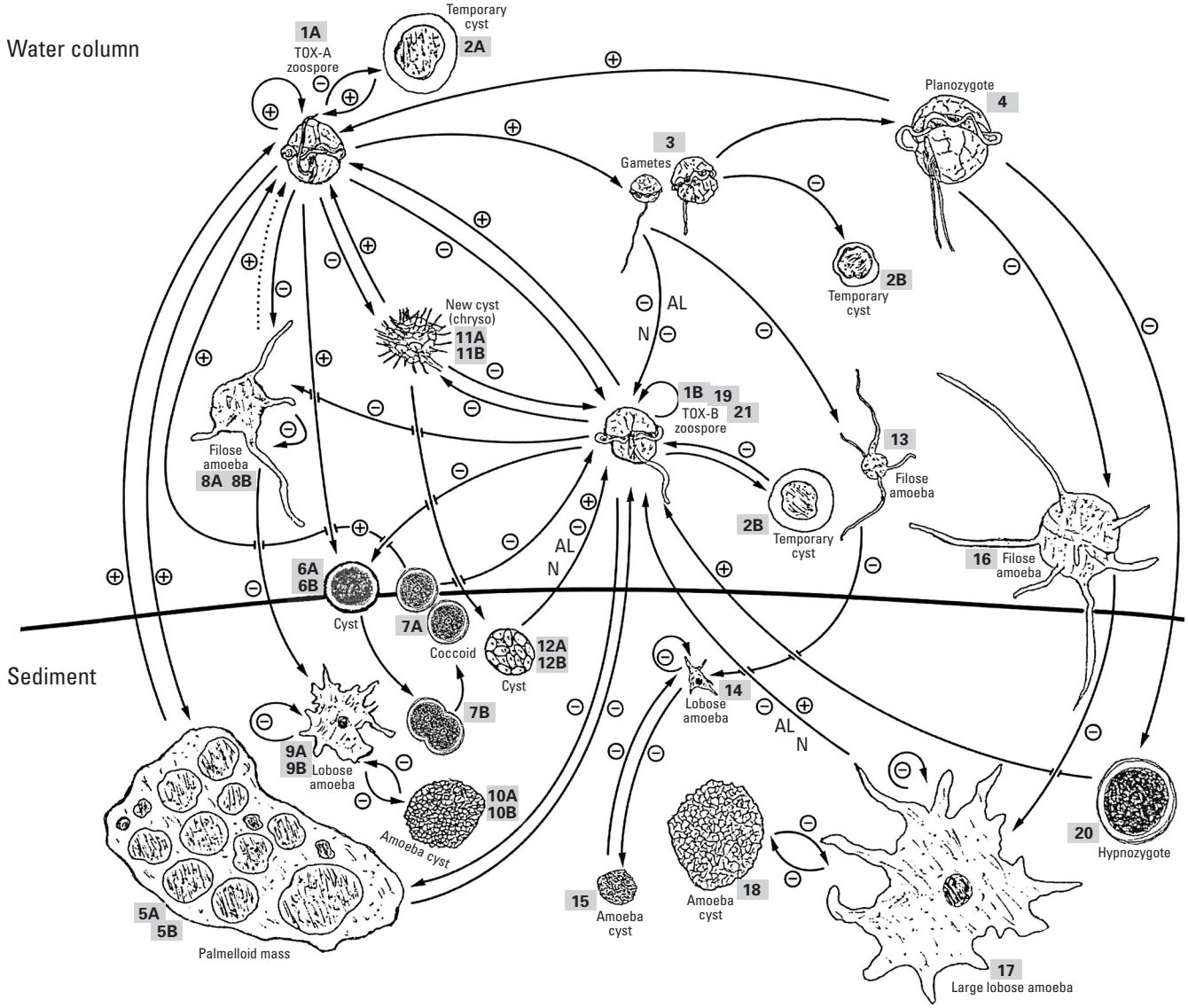

Figure 2. Schematic of the complex life cycle of *Pfiesteria shumwayae* as presently understood, showing stages and pathways that have been verified (solid lines) for toxic strains of *P. shumwayae,* and shared in common by *P. piscicida.* It should be noted that *P. piscicida* has been under study for a longer period, and several additional stages and pathways have been verified for that species (*7*). The pathways indicate the presence (+) versus the absence (–) of live finfish; AL = presence of cryptomonads and certain other algal prey; N = nutrient enrichment as organic and/or inorganic N and P; S = environmental stressor such as sudden shift in temperature or salinity, physical disturbance, or prey depletion). Dashed lines = hypothesized pathways. Stages have been conservatively numbered to facilitate description. Also note that varied approaches have been used for numbering stages. For example, complex life cycles of other dinoflagellates have sometimes numbered morphologically identical stages at each pathway or sequence of occurrence (*21*) which, if applied in this schematic, would result in at least nine additional stages. TOX-B zoospores [haploid; ploidy confirmed as in Burkholder (*31*)] are the temporarily nontoxic functional type in the absence of live fish prey [referred to as nontoxic zoospores in the life-cycle schematic previously published for toxic strains of *P. piscicida* (*7*)], although they can carry residual toxicity (*57*). TOX-B zoospores become TOX-A zoospores and produce toxin when sufficient live fish are added (*7,32*). As TOX-A and TOX-B zoospores are actually the same cells in the presence versus the absence of live fish, to stress that point we have designated morphologically identical stages as TOX-A versus TOX-B zoospores, or derived from those zoospores, with the same number followed by "A" or "B" (stages #1, 2, 5–12). TOX-B zoospores produced from diploid amoebae (stage #17) or hypnozygotes (stage #20) (rather than from stages directly derived from TOX-A or TOX-B zoospores) have been numbered as additional stages (stages #19, #21) in recognition of their distinct origin. TOX-A zoospores (stage #1A) can transform to filose (stage #8A) and lobose (stage #9A) amoebae (maximum cell dimension 15–60 µm). TOX-B zoospores (stage #1B) can transform to filose (stage #8B) and, less commonly, to lobose state #9B amoebae of similar size as those transformed from TOX-A zoospores. Planozygotes (stage #4) can transform to larger filose (stage #16) and lobose (stage #17) amoebae (maximum cell dimension 40–120 µm). Small filose (stage #13) and lobose (stage #14) amoebae (length 5–10 µm) can also be produced by gametes. Cysts include stages with *a)* roughened or reticulate covering (from amoeboid stages; haploid except when derived from diploid amoebae; stages #10A,B, #15, #18; diameter 4–30 µm); *b)* scaled covering ± bracts; from TOX-A and TOX-B zoospores; these chrysophycean-like cysts (stages #11A,B) are 4–25 µm (rarely 30 µm) in diameter and can lose their bracts (stages #12A,B) and scales over time so that they have a smooth covering. Also note that transitional forms to these cysts are not shown but occur in *P. shumwayae* as in *P. piscicida* (*7*); and *c)* hyaline covering [small cysts that can divide as in Spero and Morée (*69*)], with darkened contents; and hypnozygote (diploid cyst at lower right). Zoospores and gametes also form temporary cysts with thick mucus covering, which may settle out of the water column (arrows not shown).

filose amoeba cultures derived from clonal zoospore cultures were maintained over long periods (years). Lobose amoebae produced spherical to oval cysts with a reticulate outer covering (diameter ~4–25 µm; $n > 445$) (*4,5,7,31*).

Thus, overall, the stages observed so far in the complex life cycle of *P. shumwayae* were similar to those of *P. piscicida* (*5–9*) and include an array of flagellated (biflagellated zoospores and gametes, triflagellated planozygotes; diameter of flagellated stages ranging from 8 to 24 µm; $n = 4{,}050$ cells measured), amoeboid {lobose, filose, and less commonly observed rhizopodial stages [but see Marshall et al. (*9*)]; maximum length 7–120 µm observed thus far [$n = 1{,}200$], depending on the stage of origin}, and cyst forms (diameter 4–25 µm; $n = 620$) (Figure 2). Similarly, as shown for *P. piscicida* in previous research (*7,61*), *P. shumwayae* (at typical field densities) exhibited strong attraction to live fish (here, sheepshead minnow larvae) and their fresh tissues, followed by extension of the peduncle and feeding via myzocytosis (*70*) (Figure 3). *P. shumwayae* was also similar to *P. piscicida* (*7,8*) in that sudden salinity or temperature shock sometimes promoted transformations of TOX-A zoospores to cysts; sexual reproduction with planozygotes was observed in the presence of live fish; and removal of live fish in the presence of high abundance of algal or bacterial prey caused most TOX-A zoospores to transform to amoebae within 24 hr (*5*) (Figure 4). Alteration of algal prey type, especially a change from cryptomonad to cyanobacterial prey, induced some TOX-B zoospores from the clone tested (that had been in TOX-A fish-killing mode only 3 weeks previously) to transform to filose and lobose amoebae. When TOX-B zoospores were given different algal prey, the amoebae:zoospore ratio remained low with cryptomonads (~95% of the dinoflagellate population as zoospores), intermediate with *Thalassiosira* (70% zoospores), and lowest with *Cyanothece* (42% zoospores) (Figure 5).

The clonal amoebae derived from clonal *Pfiesteria* zoospores did react with the PCR and FISH molecular probes developed for *P. piscicida* and *P. shumwayae* zoospores [clonal amoebae cultures Neuse, Pamlico, 574A, 140A, 920A, 652TA (Table 2; Figures 6, 7); cross-confirmation of PCR and FISH probe reactivity completed by P. Rublee (*45,46*)]. Other estuarine Gymnamoebae [families Paramoebidae, Thecamoebidae according to Patterson (*80*); isolates 272A, 471A, 472A, 480A, 574A, 598A, 612A, 617A, 666A, and 872A from the Neuse and Pamlico Estuaries in North Carolina, and the Pocomoke Estuary in Maryland] were identical in appearance to certain *Pfiesteria* amoebae.

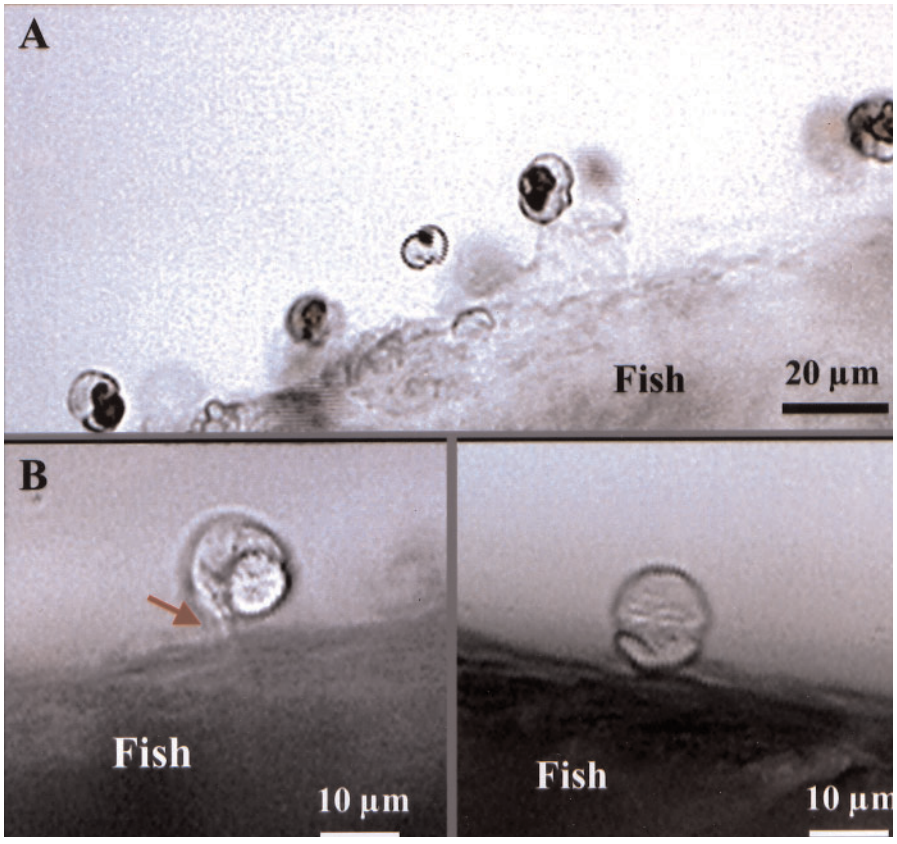

Figure 3. Color light micrograph of toxic zoospores of *P. shumwayae* attached to and actively feeding upon larval sheepshead minnows [fish; arrow in (*A*) indicating the extended peduncle of a toxic zoospore, attached to the fish and engaged in myzocytotic feeding activity (*70*)]. Note that some zoospores are pigmented in their food vacuoles from having previously consumed cryptomonad algal prey that were in the medium prior to the addition of fish; otherwise, zoospores are colorless (scale bar in *A* = 20 µm; scale bars in *B*, *C* = 10 µm).

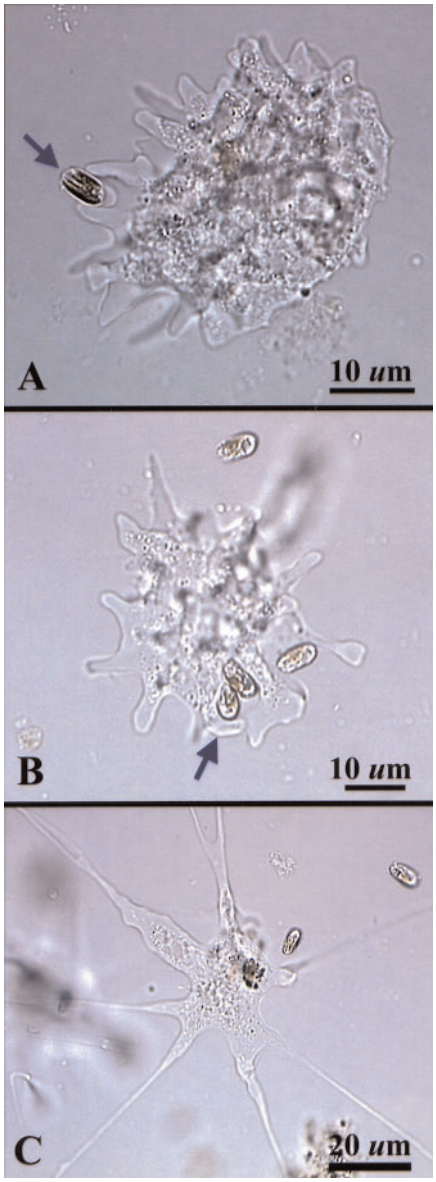

Figure 4. Color light micrographs of lobose and filose amoebae of *P. shumwayae* as (*A*) a lobose amoeba in the process of engulfing a cryptomonad (arrow; scale bar = 10 µm); (*B*) a lobose amoeba that recently engulfed two cryptomonads, contained within a food vacuole (arrow; scale bar = 10 µm); and (*C*) a filose amoeba (scale bar = 20 µm). These amoebae [confirmed in our laboratory with PCR and FISH probes specific to *P. shumwayae* and developed from zoospores; cross-corroborated with PCR and FISH testing by P. Rublee and co-workers (*45,46*)] were photographed from clonal cultures derived from clonal zoospores of *P. shumwayae*. They were maintained consistently on cryptomonad prey for 6 months, over which time no zoospores were observed.

These amoebae had been isolated and cloned from the Neuse Estuary (mesohaline segment near Minnesott Beach). They had not been derived from clonal *Pfiesteria* spp. zoospores isolated from the same area in the Neuse Estuary, and they did not react with the *Pfiesteria* species-specific PCR and FISH probes (Figure 7).

Response of Functional Types of *Pfiesteria* to Nutrients, Algal Prey, and Fish

In experiment 1 testing the response of functional types of *Pfiesteria* spp. to algal prey, controls of all functional types of both *Pfiesteria* spp. (without cryptomonad prey) showed negligible zoospore production, and most (~95%) of the zoospores encysted (33). With cryptomonad prey, zoospore production was highest in the NON-IND culture; Figure 8). TOX-B cultures of both *Pfiesteria* spp. were intermediate in zoospore production, and there was only a slight increase in zoospore abundance above that of controls in the initial TOX-A cultures (*P. piscicida* and *P. shumwayae*, tested separately; $p < 0.05$).

In experiment 2 with N_i or P_i, additions ± cryptomonad prey, after 5 days all three functional types of *P. piscicida* and *P. shumwayae* zoospores significantly increased in abundance within the (+ cryptomonads – nutrient) controls [from ~150 zoospores mL^{-1} initially to 1.5–2.2 × 10^3 zoospores mL^{-1} (recently TOX-A) to 1.1–4.2 × 10^4 zoospores mL^{-1} (TOX-B, NON-IND); $p < 0.05$]. In the (– cryptomonads + N_i or P_i) controls, zoospores of TOX-B and NON-IND functional types also increased (to 0.9–4.2 × 10^2 cells mL^{-1}; $p < 0.05$). However, in the (– cryptomonads – nutrients) controls, all functional types of both *Pfiesteria* spp. showed

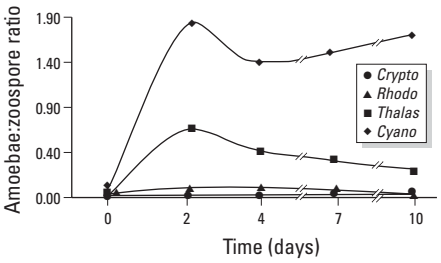

Figure 5. Influence of algal prey type on the ratio of amoebae to zoospores in clonal cultures of *P. piscicida* (TOX-B functional type, potentially toxic but without fish prey, previously grown on *Cryptomonas* LP2423 for 3 months; batch culture mode). Algal prey (single-species trials; normalized by total biovolume) included the cyanobacterium *Cyanothece* (*Cyano*, clone HP9001), cryptomonads *Cryptomonas* sp. (*Crypto*, cloned from commercial, multialgal species culture #LP2423) and *Rhodomonas* sp. (*Rhodo*, cloned from commercial, multialgal species culture CCMP757), and centric diatom *Thalassiosira weissflogii* Fryxell & Hasle (*Thalas*, cloned from commercial, multialgal species culture CCMP 1335). Note that when grown on cryptomonad prey, *P. piscicida* populations from this clone were dominated by zoospores, which comprised approximately 95% of the cells. In contrast, when given the cyanobacterium as prey, the proportion of the *Pfiesteria* populations as amoebae increased so that there were nearly twice as many amoebae as zoospores. An intermediate amoeba: zoospore ratio was obtained when *P. piscicida* was fed the centric diatom.

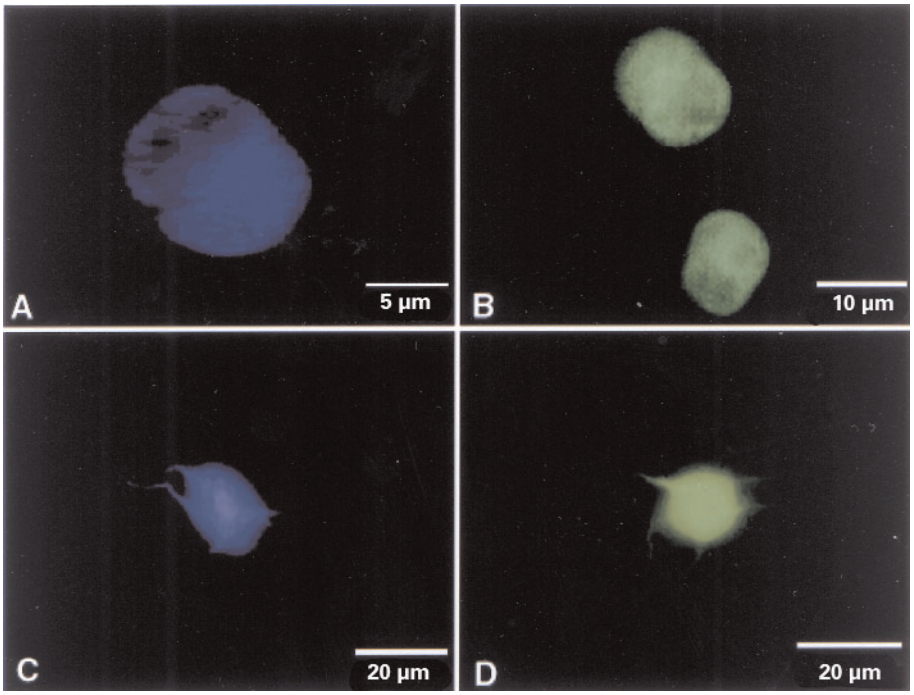

Figure 6. Whole-cell FISH molecular probes for species-specific identification of *P. piscicida* and *P. shumwayae* (*45*). Hyperfluorescence fluorophores were used to label an amine-modified oligonucleotide from the 18S rDNA of each species [cross-confirmed by P. Rublee and co-workers (*45, 51*)]. (*A*) Zoospore of *P. shumwayae* labeled with the Alexa-350 fluorophore. (*B*) Zoospore of *P. piscicida* labeled with the Alexa-488 fluorophore. (*C*) Filose amoeba of *P. shumwayae* labeled with the Alexa-350 fluorophore. Probe hybridization was to both the main cell (granuloplasm) and the pseudopodial extensions. (*D*) Filose amoeba of *P. piscicida* labeled with the Alexa-488 fluorophore, with visible labeled pseudopodia radiating from a central body. Application of these probes allows visual taxonomic identification and enumeration of these species [via epifluorescence microscopy (*81*)] in cultured and field samples. Images *A–C* are from our laboratory; image *D* was provided by P. Rublee and co-workers.

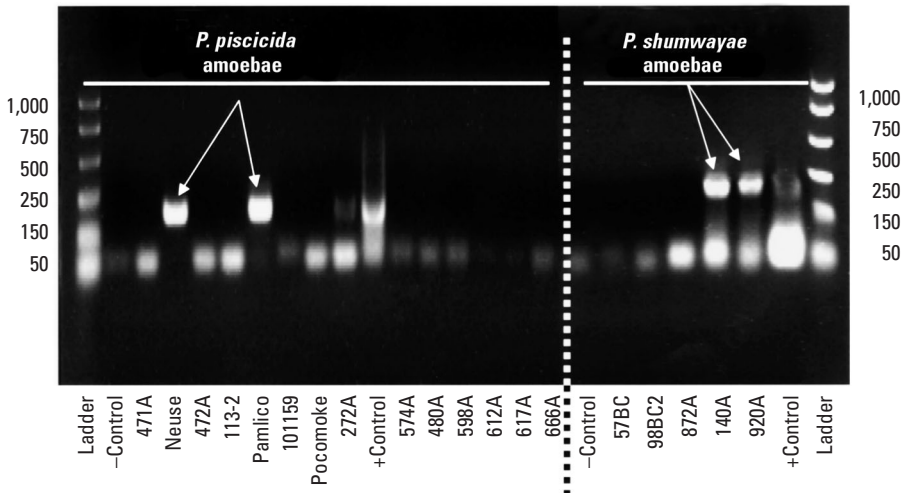

Figure 7. Gel electrophoresis of 19 lobose and filose amoebae isolates from the Neuse and Pamlico Estuaries in North Carolina (tributaries of the Albemarle–Pamlico Estuarine System) and the Pocomoke Estuary in Maryland (tributary of Chesapeake Bay). These clonal cultures were evaluated to assess whether they were *Pfiesteria* spp. life stages, using 18S rDNA molecular probes that were developed by P. Rublee and co-workers (*45,46*). The positive control was a lobose amoebae culture derived from clonal zoospores of *P. piscicida*. Of the 19 amoebae clones tested, 4 showed positive bands using PCR amplification (small white arrows). Analysis of all PCR-positive cultures was repeated in-house and was cross-confirmed by P. Rublee with identical results.

negligible zoospore production and most of the populations encysted. In treatments with cryptomonad prey + N_i or P_i enrichment, negligible increase in zoospore production over that of the (+ cryptomonad – nutrient) controls was detected for the initially TOX-A functional type in both *Pfiesteria* spp. (Figure 9). The greatest response to cryptomonad prey + nutrient enrichment was shown by the NON-IND functional type (both *Pfiesteria* spp.; $p < 0.01$), with intermediate zoospore production by TOX-B zoospores ($p < 0.05$) in response to N_i or P_i enrichment with cryptomonad prey.

In experiment 3 testing short term, response of TOX-A zoospores to fish (as net entry of into microcapillary tubes filled with fresh, sterile fish mucus and excreta; each *Pfiesteria* sp. tested separately) was significantly higher than that of either the TOX-B or NON-IND zoospores (data normalized for cell density; $p < 0.01$) (Figure 10). The data indicate significantly stronger chemosensory attraction of the TOX-A zoospores toward fish materials in these 10-min trials (*61*). The behavioral attraction response of each functional type to the sterile-filtered mucus and excreta was similar regardless of the fish species of origin.

Discussion

The *Pfiesteria* issue (*1,7,16,17,82,83*) has led to recent focus on various small, poorly described, cryptic estuarine gymnodinioid-appearing (actually including thinly armored peridinoid) dinoflagellates as pfiesteria-like because of superficial resemblance to *Pfiesteria* spp. Some of these look-alike species have been found to have complex life cycles similar to those of *Pfiesteria*, with amoeboid stages and ambush-predator behavior toward algal or ciliate prey (*22,76*).

In previous research with actively toxic cultures, we confirmed an array of amoeboid stages in the complex life cycles of *P. piscicida* (*7,8*) and *P. shumwayae* (*5*). We noted that flagellated stages (zoospores, gametes and, to a lesser extent, planozygotes) from TOX-A cultures were most active in transforming to filose, lobose, and rhizopodial stages. Filose, lobose, and rhizopodial stages of *P. piscicida*

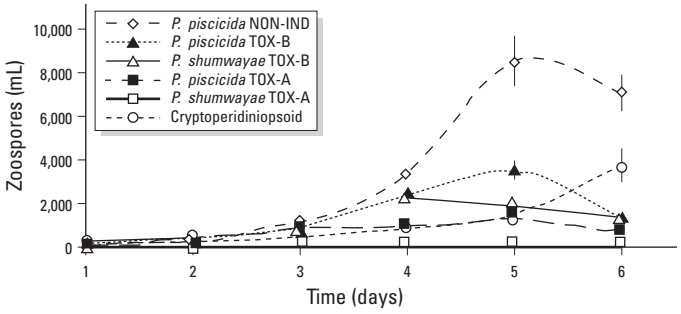

Figure 8. Response of different functional types of *P. piscicida* (TOX-A, TOX-B, NON-IND) and *P. shumwayae* zoospores (TOX-A, TOX-B; a NON-IND isolate was not available) to *Rhodomonas* prey in 6-day trials, additionally compared to the response of a cryptoperidiniopsoid species, which has not yielded ichthyotoxic activity in repeated fish bioassay tests as whole or sonicated cells (*5*). NON-IND *P. piscicida* and *cicida* the cryptoperidiniopsoid species attained highest zoospore production on algal prey, with less cell production by TOX-B and TOX-A *Pfiesteria* spp., respectively. Note that controls (each *Pfiesteria* species tested without algal prey) showed negligible zoospore production in the absence of an abundant prey source. Data are given as means ± 1 SE; $n = 3$ [modified from Parrow et al. (*33*)].

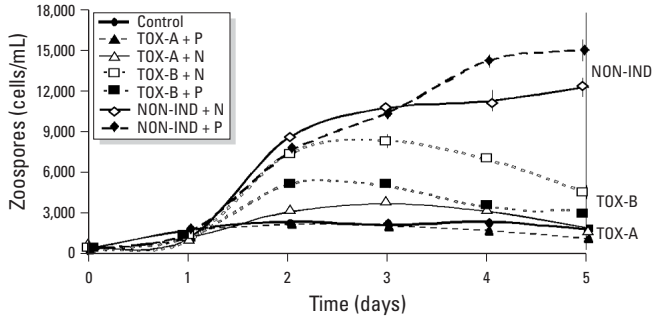

Figure 9. Zoospore production of the three functional types of *P. piscicida* and *P. shumwayae* (each species and each functional type tested separately) in response to N_i or P_i enrichment + N- and P-limited *Cryptomonas* prey (500 µg NO_3^-N or PO_4^{-3}P L^{-1}, 5-day trials in batch culture mode; 1:15 ratio of zoo-spores:prey, with approximately 150 zoospores mL^{-1} at T_0). In treatments with N_i or P_i enrichment + algal prey, TOX-A zoospores (both species) showed negligible increase in production over that of the [+ algal prey – nutrient] controls. Highest response to nutrient enrichment along with cryptomonad prey was shown by NON-IND *Pfiesteria*, with TOX-B zoospores intermediate in nutrient stimulation ($p < 0.01$). Data are given as means ± 1 SE ($n = 4$).

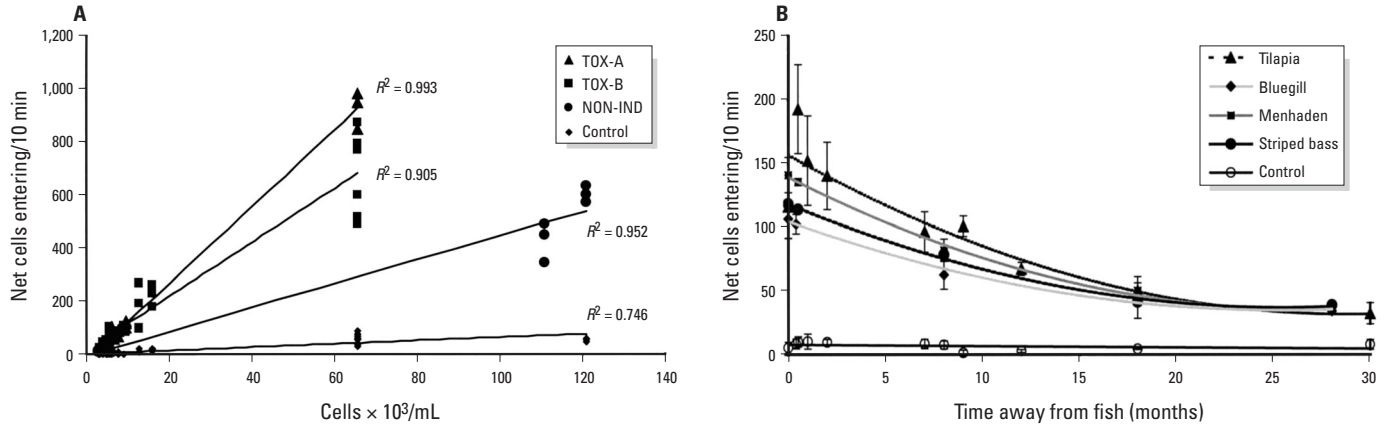

Figure 10. (*A*) Relationship between the rates of entry into micro-capillary tubes containing fish materials, and zoospore culture densities (10-min trials, salinity 15). The three functional types of each *Pfiesteria* species were tested separately in response to an extract of sterile-filtered mucus and excreta from tilapia (*Oreochromis mossambicus*) in microcapillary assays (see text; $n = 129$). TOX-A and TOX-B zoospores showed 3- to 5-fold higher attraction to the fish materials in comparison to their response to microcapillary tubes filled with sterile-filtered seawater (salinity 15). NON-IND zoospores of both *Pfiesteria* spp. were least responsive to the fish materials. The data indicate that the chemosensory attraction by zoospores toward fish materials is a biological phenomenon, independent of zoospore cell density within each functional type. (*B*) Attraction of zoospores of the three functional types of each *Pfiesteria* species (tested separately) to a mixture of sterile-filtered mucus and excreta from several finfish species (tested separately) in microcapillary assays [10-min trials, salinity 15; $n = 4$ per isolate tested; modified from Cancellieri (*61*)]. TOX-A and TOX-B zoospores consistently exhibited significant attraction to the fish materials relative to zoospore activity toward sterile-filtered seawater controls, whereas NON-IND populations showed much lower attraction to the fish excreta and mucus ($p < 0.01$). The chemosensory attraction by zoospores toward fish materials decreased with increasing duration of separation from live fish prey. Modified from Cancellieri et al. (*61*).

have been confirmed by Marshall et al. (*9*) in research with clonal cultures from our laboratory. Working with nontoxic (TOX-B and NON-IND) cultures from our laboratory, Steidinger et al. (*4*) also observed and verified transformations of *P. piscicida* zoospores to filose amoebae. PCR and FISH probes developed for *P. piscicida* and *P. shumwayae* zoospores reacted with amoeboid stages that had transformed from clonal zoospore cultures of both species. These findings were cross-confirmed by P. Rublee (Figure 6). Thus, various filose and lobose amoebae cultures have been developed from transformed clonal zoospore cultures of *Pfiesteria* spp. We have less frequently observed rhizopodial amoebae transformed from clonal *P. piscicida* zoospores, but they have been reported as common among toxic strains of cultured *P. piscicida* in H. Marshall's laboratory (*9*). Having commonly observed dinoflagellates with amoeboid stages in freshwater and marine coastal habitats, the late L. Pfiester and co-workers predicted that many, if not all, dinoflagellates would eventually be found with amoeboid stages [(*21*); also see (*9,71–79,84,85*)]. We hypothesize that in freshwater, estuarine, and marine coastal habitats, dinoflagellate life cycles have become increasingly complex across a gradient from obligate auxotrophic to mixotrophic, then to heterotrophic species, with the most complex life cycles found among free-living ectoparasites and predaceous dinoflagellates, especially those with certain stages dependent upon a specific type(s) of prey (here, for example, *Pfiesteria* spp. sexually reproduce in the presence of live fish). Amoeboid stages have been observed in various other dinoflagellates, mostly in ecto- and endoparasites but also in some mixotrophic predaceous species (*71–79,84,85*). For example, Buckland-Nicks et al. (*77–79*) observed formation of an amoeboid stage from a vegetative cyst of *Haidadinium ichthyophilum* Buckland-Nicks, Reimchen & Garbary [dinoflagellate ectoparasite of the freshwater fish known as the stickleback, *Gasterosteus* sp. (*77–79*)], wherein the cyst began to rotate; a small cytoplasmic extrusion was ejected; the cyst wall was shed; and an amoeboid protoplast emerged that moved across the culture dish, changing from spherical to oval in form. Rhizopodial amoebae of varying size were observed to form inside the common theca of a vegetative cyst from this dinoflagellate, and rhizopodial amoebae were released upon rupture of the theca. These amoebae were motile, consumed bacteria, and underwent asexual reproduction. Lobose amoebae of various sizes were also observed in this life cycle (*78,79*). Overall, Buckland-Nicks and co-workers (*77–79*) reported lobose, rhizopodial, and spheroid amoeboid stage, a vegetative dinokaryon, dinospores (zoospores) with condensed chromosomes, and amoeboid resting cyst.

The biochemical and ultrastructural mechanisms whereby the dinoflagellate zoospore mesokaryote nucleus with permanently condensed chromosomes can change [in *Pfiesteria*, sometimes within minutes (*7*)] to the eukaryote nucleus of amoeboid stages has not been examined in detail. Fensome and co-workers (*73*) noted that the condensed spiral structure of the chromatin varies during the life cycles of certain other dinoflagellate species. Chromosomal features in the heterotrophic dinoflagellates *Noctiluca* (*84*), *Blastodinium* (*84,85*), and *Oodinium fritillariae* (*74*), and in the mixotrophic predaceous dinoflagellate *Stylodinium sphaera* (*75*) have been observed to change from eukaryote to mesokaryote as swarmers are produced or as the reproductive cyst divides. For example, Timpano and Pfiester (*75*) described at least three distinct life stages in *S. sphaera,* including a phagocytic amoeba, an immobile reproductive cyst that could be produced by the amoeba, and gymnodinioid swarmers. Although the nuclear status of the amoeba stage was not ascertained, the nuclei of the multinucleate reproductive cyst from which the mesokaryotic gymnodinioid swarmers emerged were eukaryotic with dispersed chromatin material. In *H. ichthyophilum* ectoparasite of sticklebacks, Buckland-Nicks et al. (*77,79*) reported that the typical dinokaryon nucleus of the vegetative cyst transformed to a eukaryotic nucleus in the lobose amoebae. From research with the dinoflagellate, *O. fritillariae* (marine ectoparasite of Appendicularians), Cachon and Cachon (*74*) reported that the nucleus of young, attached ectoparasites had condensed, rodlike chromosomes similar to those of the free-living gymnodinioid stage. However, as growth of the trophont (attached parasitic) stage progressed, the nucleus became increasingly homogenous as in eukaryotes. When *Oodinium* left the host, nuclear reorganization processes occurred rapidly, corresponding to a peculiar prophase of the first sporogenic division. A conspicuous fusorial system appeared between two archoplasmic areas that were responsible for offspring chromosome segregation. The nuclear envelope remained intact, while the fusorial microtubules were attached to the nucleus at distinct, kinetochore-like structures. As the chromosomes became more condensed, the kinetochore-like formations disappeared. In ongoing research in our laboratory, we have begun to document in detail the ultrastructural changes in the nucleus of *Pfiesteria* spp. during zoospore–amoeba transformations.

We additionally hypothesize, as in previous work (*7,8*), that certain amoebae previously described as Sarcodinian or Gymnamoebae (*80*), without linkage to dinoflagellates, are actually dinoflagellate amoebae or are morphologically identical to dinoflagellate amoebae. As noted, we have maintained filose and lobose amoeboid cultures of both *Pfiesteria* species for months to years. Thus, although filose and lobose stages may sometimes function as transitional forms, they are not merely ephemeral but, rather, can be sustained stages of *Pfiesteria*. Moreover, throughout the extended periods in which we have maintained amoeboid cultures of *Pfiesteria* spp., we have not observed any zoospores in the cultures as mentioned. Zoospore production can be induced in some *Pfiesteria* amoebae by suddenly altering the available prey. For example, production of TOX-A zoospores has been induced in some *Pfiesteria* amoebae by adding live fish (*7*). As another example, when amoebae transformed from recently TOX-A zoospores are switched from *Cyanothece* prey to cryptomonads, we have observed large lobose amoebae (length ~50–70 μm or more) each produce four zoospores, followed by disintegration of the remainder of the amoeba cell. With increasing duration in culture, however, *Pfiesteria* amoebae appear to lose the ability to produce zoospores.

The array of amoeboid stages in the complex life cycles of *Pfiesteria* spp. would be identified within at least eight different genera of Sarcodinian amoebae using standard amoebae keys [e.g., Patterson (*80*)], indicating a need for reevaluation of the systematics of estuarine amoebae to include consideration of dinoflagellates. Molecular-based identification assays will be valuable in such efforts, as SEM often cannot discern among species of dinoflagellates or of amoebae (dinoflagellate or otherwise), within the same genus (*31,80*). Subtle differences in cell covering (e.g., scales) may be perceptible with SEM, but in attempts to differentiate among amoeboid species, nonmorphological characteristics (e.g., mode of locomotion, granuloplasmic inclusions) have been used in traditional amoebae keys [(*80*)]. A recent quote from amoebae specialists (*86*) succinctly framed the overall problem:

> The classification of the free-living amoebae is a most contentious area. . . . It is now evident that the free-living amoebae have evolved along many different lines. . . . There is striking evidence that various taxa regarded as species are in fact polyphyletic.

Ultimately, reevaluation of the systematics of estuarine amoebae to include dinoflagellates of similar appearance as various Gymnamoebae will likely depend in large measure on molecular and biochemical techniques.

Of approximately 3,000 dinoflagellate species, only about 55 are known or

suspected as toxic (*1,2,24*). Repeated tests (standardized fish bioassays) on estuarine samples from eight states (New York to Alabama) thus far have revealed only one additional toxic *Pfiesteria* / *Pfiesteria*-like species, *P. shumwayae* (*5–7,9,31,32,52*), indicating, as expected, that complex life cycles and attack behavior are more common than toxin-producing capability. We predict that additional toxic *Pfiesteria* / *Pfiesteria*-like species will be detected, although there probably will be few toxic forms among the many species of small, cryptic estuarine dinoflagellates that remain to be described.

This study has shown that functional types of *Pfiesteria* spp. are strikingly different in response to nutrient enrichment, algal prey, and fish prey. Within each of the two species known to date, the strains have shown high variability in physiological characteristics, and range from highly toxic to benign. Some strains produce potent ichthyotoxins that are lethal to fish [patenting process initiated on a water-soluble *Pfiesteria* toxin that has been isolated and purified (*12*)]. Some toxic strains can kill fish when prevented from direct contact with the prey, whereas others apparently require close contact. All three functional types can cause death of larval fish by physical attack as well, although this behavior is much more pronounced in toxic strains (*31*).

The data have important implications for policy considerations. Because of the high variability in toxicity among *Pfiesteria* strains [also characteristic, but unfortunately overlooked, for many other toxic algae (*25–30*)], federal and state agencies charged with managing natural resources and public health should require that reports of toxic *Pfiesteria* from any laboratory are both replicated (for internal confirmation) and, importantly, also cross-corroborated by an independent specialist with demonstrated expertise in culturing toxic *Pfiesteria* (*7,9,11,31,63*) as standard quality control/assurance procedure (*34*). This scientifically sound practice of cross-corroboration by another independent laboratory should also be followed for reports of other toxic algae. Agencies additionally should note that a national science panel (*49*) recently critically reevaluated the peer-reviewed literature on actively toxic *Pfiesteria* and supported as valid published findings (*7,9,11,63*) on the ichthyotoxicity and toxic, fish-killing activity of verified, cross-corroborated toxic *Pfiesteria* strains. As has been clarified for findings of toxic *Pfiesteria* after independent cross-corroboration (*7,9,11,31,63*), findings of "no toxic *Pfiesteria*" should be clarified as valid for [only] the strains tested, rather than being applied as a general statement regarding *Pfiesteria* spp. (*49*).

As additional policy considerations from these data and related studies (e.g, *7,22,87,88*), both *Pfiesteria* species thrive in eutrophic estuaries, and both can be stimulated by nutrient enrichment (*49*). TOX-A zoospores of these heterotrophic dinoflagellates, found where excreta and leached fish materials are abundant [with high dissolved organic N and P; (*7,22,23,31*)], would not be expected to be stimulated directly by inorganic nutrient enrichment (*31*). TOX-B zoospores retain kleptochloroplasts from algal prey and thus can adopt a plant-like nutritional mode including stimulation by inorganic nutrients (*6,87*). Among the three functional types, NON-IND zoospores may have highest reliance on kleptochloroplasts (*33*). Although nutrient stimulation effect is highest for NON-IND *Pfiesteria*, use of such cultures would significantly bias in favor of a higher nutrient stimulation effect, relative to the response of toxic strains. Therefore, TOX-B *Pfiesteria* should be used in tests to determine influences of anthropogenic nutrient sources on these dinoflagellates. Toxic *Pfiesteria* strains cannot be maintained in commercial culture clearinghouses (lacking biohazard BSL-3 facilities). The data from this study and from related research (*5,6,9,31*) indicate that NON-IND strains should be avoided in research to gain insights about environmental controls on toxic *Pfiesteria* and about impacts of (toxic) *Pfiesteria* on fish and mammalian health.

References and Notes

1. Burkholder JM. Implications of harmful marine microalgae and heterotrophic dinoflagellates in management of sustainable marine fisheries. Ecol Appl 8(suppl):S37–S62 (1998).
2. Hallegraeff HM, Blackburn S, Bolch C, Lewis R, eds. Proceedings of the Ninth International Conference on Harmful Algal Blooms, 7-11 February 2000, Hobart, Tasmania, Australia. Paris:Intergovernmental Oceanographic Commission of the United Nations Educational, Scientific and Cultural Organization (IOC UNESCO), 2000.
3. Burkholder JM, Noga EJ, Hobbs CW, Glasgow HB, Smith SA. New "phantom" dinoflagellate is the causative agent of major estuarine fish kills. Nature 358:407–410; 360:768 (1992).
4. Steidinger KA, Burkholder JM, Glasgow HB, Truby E, Garrett J, Noga EJ, Smith SA. *Pfiesteria piscicida* (Pfiesteriaceae, fam. nov.), a new toxic dinoflagellate with a complex life cycle and behavior. J Phycol 32:157–164 (1996).
5. Glasgow HB, Burkholder JM, Morton SL, Springer. A second species of ichthyotoxic *Pfiesteria* (Dinamoebales, Pyrrhophyta). Phycologia 40:234–245 (2001).
6. Glasgow HB, Burkholder JM, Morton SL, Springer J, Parrow MW. The fish-killing activity and nutrient stimulation of a second toxic *Pfiesteria* species. In: Proceedings of the Ninth International Conference on Harmful Algal Blooms, 7-11 February 2000, Hobart, Tasmania, Australia (Hallegraeff GM, Blackburn S, Bolch C, Lewis R, eds). Paris:IOC UNESCO, in press.
7. Burkholder JM, Glasgow HB. *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates: behavior, impacts, and environmental controls. Limnol Oceanogr 42:1052–1075 (1997).
8. Burkholder JM, Glasgow HB. Interactions of a toxic estuarine dinoflagellate with microbial predators and prey. Arch Protistenkd 145:177–188 (1995).
9. Marshall HM, Gordon AS, Seaborn DW, Dyer B, Dunstan WM, Seaborn M. Comparative culture and toxicity studies between the toxic dinoflagellate, *Pfiesteria piscicida* and a morphologically similar cryptoperidiniopsoid dinoflagellate. J Exp Mar Biol Ecol 225:51–74 (2000).
10. *Pfiesteria* Interagency Coordinative Working Group [*P*ICWG]. Glossary of *Pfiesteria*-Related Terms. *P*ICWG, chaired by J. Macknis. Baltimore, MD:U.S. Environmental Protection Agency. Available: *http://www.redtide.whoi.edu/pfiesteria/documents/glossary.html* [cited 2 November 2000].
11. Kimm-Brinson KL, Moeller PDR, Barbier M, Glasgow HB, Burkholder JM, Ramsdell JS. Identification of a $P2X_7$ receptor in GH4C1 rat pituitary cells: a target for a bioactive substance produced by *Pfiesteria piscicida*. Environ Health Perspect 109:457–462 (2001).
12. Ramsdell JS, Moeller PDR. Personal communication.
13. Falconer IR, ed. Algal Toxins in Seafood and Drinking Water. New York:Academic Press, 1993.
14. Hallegraeff GM, Anderson DM, Cembella AD, eds. Manual on Harmful Marine Microalgae. Intergovernmental Oceanographic Commission Manuals and Guides No. 33. Paris:UNESCO, 1995.
15. Burkholder JM, Springer JJ. Signaling in dinoflagellates. In: Microbial Signaling and Communication (England RR, Hobbs G, Bainton NJ, Roberts DMcL, eds). Fifty-Seventh Symposium of the Society for General Microbiology, 8-23 April 1999, Edinburgh, Scotland. New York:Cambridge University Press, 1999;220–238.
16. Glasgow HB, Burkholder JM, Schmechel DE, Fester PA, Rublee PA. Insidious effects of a toxic dinoflagellate on fish survival and human health. J Toxicol Environ Health 46:501–522 (1995).
17. Grattan LM, Oldach D, Perl TM, Lowitt MH, Matuszak DL, Dickson C, Parrott C, Shoemaker RC, Wasserman MP, Hebel JR, et al. Problems in learning and memory occur in persons with environmental exposure to waterways containing toxin-producing *Pfiesteria* or *Pfiesteria*-like dinoflagellates. Lancet 352:532–539 (1998).
18. Levin ED, Schmechel DE, Burkholder JM, Glasgow HB, Deamer-Melia N, Moser VC, Harry GJ. Persistent learning deficits in rats after exposure to *Pfiesteria piscicida*. Environ Health Perspect 105:1320–1325 (1997).
19. Levin ED, Simon BB, Schmechel DE, Glasgow HB, Deamer-Melia NJ, Burkholder JM, Moser VC, Jensen K, Harry GJ. *Pfiesteria* toxin and learning performance. Neurotoxicol Teratol 21:215–221 (1999).
20. Levin ED, Rezvani AH, Christopher NC, Glasgow HB, Deamer-Melia NJ, Burkholder JM, Moser VC, Jensen K. Rapid neurobehavioral analysis of *Pfiesteria piscicida* effects in juvenile and adult rats. Neurotoxicol Teratol 22:533–540 (2000).
21. Popovský J, Pfiester LA. Dinphyceae (Dinoflagellida). Stuttgart, Stuttgart:Gustav Fischer Verlag, 1990.
22. Burkholder JM, Glasgow HB, Lewitus AJ. Physiological ecology of *Pfiesteria piscicida* with general comments on "ambush-predator" dinoflagellates. In: Physiological Ecology of Harmful Algae (Anderson DM, Cembella A, Hallegraeff GM, eds). NATO ASI Series G: Ecological Sciences, Vol 41. Berlin:Springer-Verlag, 1998;175–191.
23. Glasgow HB, Lewitus AJ, Burkholder JM. Feeding behavior of the ichthyotoxic estuarine dinoflagellate, *Pfiesteria piscicida*, on amino acids, algal prey, and fish vs. mammalian erythrocytes. In: Harmful Microalgae (Reguera B, Blanco J, Fernandez J, Wyatt T, eds). Proceedings of the VIIIth International Conference on Harmful Algal Blooms, 25–29 June 1997, Vigo, Spain. Paris:Xunta de Galicia and IOC UNESCO 1998;394–397.
24. Steidinger KA. Some taxonomic and biologic aspects of toxic dinoflagellates. In: Algal Toxins in Seafood and Drinking Water (Falconer IR, ed). New York:Academic Press, 1993;1–28.
25. Skulberg OM, Carmichael WW, Codd GA, Skulberg R. Taxonomy of toxic Cyanophyceae (cyanobacteria). In: Algal Toxins in Seafood and Drinking Water (Falconer IR, ed). New York:Academic Press, 1993;1–28.
26. Chorus I, Bartram J, eds. Toxic Cyanobacteria in Water – a Guide to Their Public Health Consequences, Monitoring and Management. New York:World Health Organization, 1999.
27. Gentien P, Arzul G. Exotoxin production by *Gyrodinium* cf. *aureolum* (Dinophyceae). J Mar Biol Assoc (UK) 70:571–581 (1990).
28. Anderson DM. Toxin variability in *Alexandrium*. In: Toxic Marine Phytoplankton – Proceedings of the Fourth International Conference on Toxic Marine Phytoplankton (Granéli E, Sundstrom B, Edler L, Anderson DM, eds). New York:Elsevier, 1991;41–51.
29. Bates SS, Garrison DL, Horner RA. Bloom dynamics and physiology of domoic-acid-producing *Pseudo-nitzschia* species. In: Physiological Ecology of Harmful Algae (Anderson DM, Cembella A, Hallegraeff GM, eds). NATO ASI Series G: Ecological Sciences, Vol 41. Berlin:Springer-Verlag, 1998; 267–292.
30. Edvardsen B, Paasche E. Bloom dynamics and physiology of *Prymnesium* and *Chrysochromulina*. In: Physiological Ecology of Harmful Algae (Anderson DM, Cembella A, Hallegraeff GM, eds). NATO ASI Series G: Ecological Sciences, Vol 41. Berlin:Springer-Verlag, 1998;193–208.

31. Burkholder JM, Glasgow HB, Deamer-Melia NJ. Overview and present status of the toxic *Pfiesteria* complex. Phycologia 40:186–214 (2001).
32. Burkholder JM, Mallin MA, Glasgow HB. Fish kills, bottom-water hypoxia, and the toxic *Pfiesteria* complex in the Neuse River and Estuary. Mar Ecol Prog Ser 179:301–310 (1999).
33. Parrow MW, Glasgow HB, Burkholder JM, Zhang C. Comparative response to algal prey by *Pfiesteria piscicida*, *Pfiesteria shumwayae*, and an estuarine 'look-alike' species. In: Proceedings of the Ninth International Conference on Harmful Algal Blooms (Hallegraeff GM, Blackburn S, Bolch C, Lewis R, eds). Paris:IOC UNESCO, in press.
34. Burkholder JM, Marshall HG, Glasgow HB, Seaborn DW, Deamer-Melia NJ. The standardized fish bioassay procedure for detecting and culturing actively toxic *Pfiesteria*, used by two reference laboratories for Atlantic and Gulf coast states. Environ Health Perspect 109(suppl 5):745–756 (2001).
35. Cheng YQ, Ahn JH, Walton JD. A putative branched-chain-amino-acid transaminase gene required for biosynthesis and pathogenicity in *Cochliobolus carbonum*. Microbiology 145:3539–3546 (1999).
36. Holt JR, Pfiester LA. A technique for counting chromosomes of armored dinoflagellates, and chromosome numbers of six freshwater dinoflagellate species. Am J Bot 69:1165–1168 (1982).
37. Burkholder JM. Cyanobacteria. In: Encyclopedia of Environmental Microbiology (Bitton G, ed). New York:Wiley Publishers, in press.
38. Wood M, Shapiro LM, eds. Domoic Acid–Final Report of the Workshop. Oregon Institute of Marine Biology. Oregon Sea Grant Rep ORESU-W-92-003. Corvallis, OR:Oregon State University, 1992.
39. Scholin CA. Morphological, genetic and biogeographic relationships of toxic dinoflagellates *Alexandrium tamarense, A. catenella* and *A. fundyense*. In: Physiological Ecology of Harmful Algal Blooms (Anderson DM, Cembella AD, Hallegraeff GM, eds). NATO ASI Series G. Ecological Sciences, Vol 41. New York:Springer-Verlag, 1998;13–28.
40. Kao CY. Paralytic shellfish poisoning. In: Algal Toxins in Seafood and Drinking Water (Falconer IR, ed). New York: Academic Press, 1993;74–86.
41. Tomas C, ed. Identifying Marine Diatoms and Dinoflagellates. New York:Academic Press, 1995.
42. Daugbjerg N, Hansen G, Larsen J, Moestrup Ø. Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. Phycologia 39:302–317 (2000).
43. CDC. Possible Estuarine-Associated Syndrome. Atlanta, GA:Centers for Disease Control and Prevention, 1998.
44. Steidinger KA. Personal communication.
45. Allen IC Jr. Utilization of PCR and FISH to Determine Fine Scale and Global Distribution of *Pfiesteria* Species [MS Thesis]. Greensboro, NC:University of North Carolina-Greensboro, 2000.
46. Rublee PA, Kempton J, Schaefer E, Burkholder JM, Glasgow HB, Oldach D. PCR and FISH detection extends the range of *Pfiesteria piscicida* in estuarine waters. Va J Sci 50:325–326 (1999).
47. Oldach DW, Delwiche CF, Jakobsen KS, Tengs T, Brown EG, Kempton JW, Schaefer EF, Bowers H, Glasgow HB, Burkholder JM, et al. Heteroduplex mobility assay guided sequence discovery: elucidation of the small subunit (18S) rDNA sequence of *Pfiesteria piscicida* from complex algal culture and environmental sample DNA pools. Proc Natl Acad Sci U S A 97:4304–4308 (2000).
48. Tanner RS. Cultivation of bacteria and fungi. In: Manual of Environmental Microbiology (Hurst C, Knudsen G, McInerney M, Stetzenbach L, Walter M, eds). Washington, DC:American Society for Microbiology Press, 1997;52–60.
49. Samet J, Bignami GS, Feldman R, Hawkins W, Neff J, Smayda T. *Pfiesteria*: Review of the science and identification of research gaps. Report for the National Center for Environmental Health, Centers for Disease Control and Prevention. Environ Health Perspect 109(suppl 5):639–659 (2001).
50. Guillard RRL. Culture of phytoplankton for feeding marine invertebrates. In: Culture of Marine Invertebrate Animals (Smith WL, Chanley MH, eds). New York:Plenum Press, 1975;29–60.
51. UNESCO. The International System of Units in Oceanography. UNESCO Technical Paper no. 45. Paris:United Nations Educational, Scientific and Cultural Organization, 1985.
52. Burkholder JM, Glasgow HB, Hobbs CW. Distribution and environmental conditions for fish kills linked to a toxic ambush-predator dinoflagellate. Mar Ecol Prog Ser 124:43–61 (1995).
53. Salih A, Hoegh-Guldberg O, Cox G. Photoprotection of symbiotic dinoflagellates by fluorescent pigments in reef corals. In: Proceedings of the Australian Coral Reef Society 75th Anniversary Conference, October 1997, Heron Island, Australia (Greenwood JG, Hall NJ, eds). Brisbane, Australia:University of Queensland, 1998;217–230.
54. Parrow MW, Burkholder JM. Flow cytometric determinations of zoospore DNA content and population DNA distribution in cultured *Pfiesteria* spp. (Dinamoebales, Pyrrhophyta). J Exp Mar Biol Ecol (in press).
55. Veldhuis MJ, Cucci TL, Sieracki ME. Cellular DNA content of marine phytoplankton using two new fluorochromes: taxonomic and ecological applications. J Phycol 33:527–541 (1997).
56. Johnston JS, Bennett MD, Rayburn AL, Galbraith DW, Price HG. Reference standards for determination of DNA content of plant nuclei. Am J Bot 86:609–613 (1999).
57. Springer J. Interactions Between Two Commercially Important Species of Bivalve Molluscs and the Toxic Estuarine Dinoflagellate, *Pfiesteria piscicida* [Master's Thesis]. Raleigh, NC:North Carolina State University, 2000.
58. Zehr JB, Waterbury JB, Turner PJ, Montoya JP, Omoregie E, Steward GF, Hansen A, Karl DM. Unicellular cyanobacteria fix N2 in the subtropical North Pacific Ocean. Nature 412:635-638 (2001).
59. Sogin ML. Amplification of ribosomal RNA genes for molecular evolution studies. In: PCR Protocols: a Guide to Methods and Applications (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). San Diego:Academic Press, 1990;307–314.
60. Boni L, Rollo F. Molecular detection and characterization of marine dinoflagellates and their symbionts: potentials and pitfalls. In: Science and the Total Environment – Proceedings of an International Conference. Bologna, Italy, 1992.
61. Cancellieri P, Burkholder JM, Deamer-Melia NJ, Glasgow HB. Chemosensory attraction of zoospores of the estuarine dinoflagellates, *Pfiesteria piscicida* and *P. shumwayae*, to finfish mucus and excreta. J Exp Mar Biol Ecol 264:29–45 (2001).
62. SAS Institute, Inc. SAS/STAT Guide for Personal Computers, Version 7. Cary, NC:SAS Institute, Inc., 2001.
63 Lewitus AJ, Glasgow HB, Burkholder JM. Kleptoplastidy in the toxic dinoflagellate, *Pfiesteria piscicida*. J Phycol 35:303–312 (1999).
64. Landsberg JH, Steidinger KA, Blakesley BA. Fish-killing dinoflagellates in a tropical aquarium. In: Harmful Marine Algal Blooms (Lassus P, Arzul A, Erard le Denn E, Gentien P, Marcaillou-Le Bout C, eds). Paris:Lavoisier Intercept, 1995; 65–70.
65. Taylor FJR, ed. Dinoflagellate morphology. In: The Biology of Dinoflagellates (Taylor FJR, ed). Botanical Monographs, Vol 21. Boston:Blackwell, 1987;2–91.
66. von Stosch HA. Observations on vegetative and sexual life cycles in two freshwater dinoflagellates, *Gymnodinium pseudopalustre* Schiller and *Woloszynskia apiculata* sp. nov. Br Phycol J 8:105–134 (1973).
67. Dodge JD. The chromosomes of dinoflagellates. Int Rev Cytol 94:5–19 (1985).
68. Raikov IB. The diversity of forms of mitosis in protozoa: a comparative review. Eur J Protistol 30:253–269 (1994).
69. Spero HJ, Morée M. Phagotrophic feeding and its importance in the life cycle of the holozoic dinoflagellate, *Gymnodinium fungiforme*. J Phycol 17:43–51 (1981).
70. Schnepf E, Elbrächter M. Nutritional strategies in dinoflagellates—a review with emphasis on cell biological aspects. Eur J Protistol 28:3–24 (1992).
71. Bursa A. *Dinamoebidinium hyperboreum* spec. nov. in coastal plankton of Ellesmere Island, N.W.T., Canada. Arct Alp Res 1:152–154 (1970).
72. Bursa A. *Dinamoebidinium coloradense* spec. nov. and *Katodinium auratum* spec. nov. in Como Creek, Boulder County, Colorado. Arct Alp Res 2:145–151 (1970).
73. Fensome RA, Taylor FJR, Norris G, Sarjeant WAS, Wharton DI, Williams GL. A Classification of Living and Fossil Dinoflagellates. New York:Micropaleontology Press, 1993.
74. Cachon J, Cachon M. Observations on the mitosis and on the chromosome evolution during the life-cycle of *Oodinium*, a parasitic dinoflagellate. Chromosoma (Berl) 60:237–251 (1977).
75. Timpano P, Pfiester L. Observations on "*Vampyrella penula - Sylodinium sphaera*" and the ultrastructure of the reproductive cyst. Am J Bot 73:1341–1350 (1986).
76. Seaborn D, Seaborn A, Dunstan W, Marshall HG. Growth and feeding studies on the algal feeding stage of a pfiesteria-like dinoflagellate. Va J Sci 50:337–334 (1999).
77. Buckland-Nicks JA, Reimchen TE, Taylor FJR. A novel association between an endemic stickleback and a parasitic dinoflagellate 2: morphology and life cycle. J Phycol 26:539–548 (1990).
78. Buckland-Nicks JA, Reimchen TE. A novel association between an endemic stickleback and a parasitic dinoflagellate. 3. Details of the life cycle. Arch Protistenkd 145:165–175 (1995).
79. Buckland-Nicks J, Reimchen TE, Garbary DJ. *Haidadinium ichthyophilum* genov. et sp. nov. (Phytodiniales, n. Dinophyceae), a freshwater ectoparasite on stickleback (*Gasterosteus aculeatus*) from the Queen Charlotte Islands, Canada. Can J Bot 75:1936–1940 (1997).
80. Patterson DJ. The diversity of eukaryotes. Am Nat 154 (suppl):S96–S124 (1999).
81. Herman B. Fluorescence Microscopy, 2nd ed. Microscopy Handbooks 40, Royal Microscopical Society. Oxford, UK:BIOS Scientific Publishers, 1998.
82. State of Maryland. Water Quality Improvement Act of 1998. Annapolis, MD:General Assembly, 1998.
83. Burkholder JM, Glasgow HB. Toxic Pfiesteria in North Carolina estuaries from 1991 to the present. BioScience (in press).
84. Soyer M-O. Structure du noyau des *Blastodinium* (Dinoflagellés parasites). Division et condensation chromatique. Chromosoma (Berl) 33:70–114 (1971).
85. Soyer M-O. Les ultrastructures nucléaires de la Noctiluque (Dinoflagellé libre) au cours de la sporogenése. Chromosoma (Berl) 39:419–441 (1972).
86. Walochnik J, Aspöck H. Classification of the free-living amoebae: state of knowledge. In: Final Program and Book of Abstracts, Eleventh International Congress of Protozoology ICOP, 15–19 July 2001, Salzburg, Austria, 2001. Geneva:Kenes International, 2001.
87. Burkholder JM, Mallin MA, Glasgow HB, Larsen LM, McIver MR, Shank GC, Deamer-Melia N, Briley DS, Springer J, Touchette BW, Hannon EK, et al. Impacts to a coastal river and estuary from rupture of a large swine waste holding lagoon. J Environ Qual 26:1451–1466 (1997).
88. Lewitus AJ, Willis BM, Hayes KC, Burkholder JM, Glasgow HB, Glibert PM, Burke MK. Mixotrophy and nitrogen uptake by *Pfiesteria piscicida* (Dinophyceae). J Phycol 35:1430–1437 (1999).