History of Toxic *Pfiesteria* in North Carolina Estuaries from 1991 to the Present

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The fish kills for which *Pfiesteria* became well known began in North Carolina, when very little research had been conducted on this unusual dinoflagellate. North Carolina's Albemarle-Pamlico Estuarine System, the epicenter of toxic *Pfiesteria* outbreaks, is the second largest estuary in area on the US mainland and the most important fish nursery ground on the US Atlantic Coast (Burkholder and Glasgow 1997, Mallin et al. 2000). As a conservative estimate, the state had sustained 48 toxic *Pfiesteria* outbreaks by 1997, involving more than a billion fish in an area more than 100 km^2 (Burkholder et al. 2001a). These events had occurred nearly every summer beginning in 1991, when the organism was first recognized as an estuarine, fish-killing agent (Burkholder et al. 1992, 2001a, Burkholder and Glasgow 1997). In 1997, 50,000 fish died in a small area of the Chesapeake Bay from toxic *Pfiesteria* , and press coverage of the event was like an explosion. In the same summer, 1.2 million fish died during toxic *Pfiesteria* outbreaks in Albemarle-Pamlico estuaries, a 4-hour drive south of Washington, DC; those deaths went virtually unmentioned.

North Carolina was the first state to encounter toxic *Pfiesteria*, and the knowledge gained there, especially about toxic *Pfiesteria* outbreaks and health impacts on laboratory workers (Burkholder et al. 1992, 1995, Burkholder and Glasgow 1995, 1997, Glasgow et al. 1995), benefited Maryland officials, who were challenged to act quickly and decisively. They evaluated and rapidly verified the role of toxic *Pfiesteria* in the Chesapeake Bay outbreak (MDNR 1998). Maryland was the first state from which people who reported neurocognitive, respiratory, and other symptoms from environmental exposure were clinically evaluated within a short period (1–3 weeks) after being exposed (Grattan et al. 1998). Maryland was also the first state to address the *Pfiesteria* problem by making significant advances in legislation for protection of water quality (State of Maryland 1998). Congressional attention following the toxic *Pfiesteria* outbreaks in Maryland led to the appropriation of many millions of dollars to federal agencies to research and manage toxic *Pfiesteria* outbreaks, resulting in excellent progress in some areas and setbacks in others. Here we examine the history of toxic *Pfiesteria* outbreaks before the four in Chesapeake Bay and a few aspects of the aftermath, from the perspective and experience of our research in the Albemarle-Pamlico Estuarine System with high toxic *Pfiesteria* activity. An important part of the *Pfiesteria* story is how North Carolina—with 98% of the *Pfiesteria* problem—subsequently moved to strengthen water resource protection, environmental education, and support of *Pfiesteria* research, actions that would not have been possible without the events that unfolded in Chesapeake Bay.

MANY TOXIC *PFIESTERIA* OUTBREAKS HAVE PLAGUED THE ALBEMARLE-PAMLICO ESTUARINE SYSTEM, INCLUDING EVENTS BOTH BEFORE AND AFTER THE 1997 OUTBREAKS IN CHESAPEAKE BAY

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Beginnings

The *Pfiesteria* story began in 1988 with the death of some fish held in brackish aquaria at the College of Veterinary Medicine, North Carolina State University (NCSU). We were asked by fish pathologists to help characterize an unknown microbial contaminant from the fish cultures. The microbe resembled many other benign, estuarine dinoflagellates (Figure 1), but the timing of its appearance in the aquaria raised the suspicion that it had caused the death of the fish. The small dinoflagellates were abundant in water samples taken when fish became lethargic, developed open bleeding sores, and hemorrhaged. Shortly after the fish died, however, the dinoflagellates seemed to "disappear," by forming dormant cysts or other stages that left the water column and attached to fish or settled to the bottom of the aquaria. When more fish were added to the aquaria, the pattern was repeated, and higher dinoflagellate densities were attained (Burkholder et al. 1992).

From that accidental contamination, we first learned of the existence of a small, apparently toxic dinoflagellate exhibiting rapid, predatory behavior in attacking and killing fish prey (Burkholder et al. 2001b). We began to conduct many experiments that tested, then verified, its toxicity to fish (Burkholder et al. 1995, Burkholder and Glasgow 1997). But the pathologists had obtained fish from all over the world and did not know which fish, from which geographic source, had carried the toxic dinoflagellate. Therefore, basic questions remained unanswered: Where did this organism originate? Was it capable of killing fish in natural habitat?

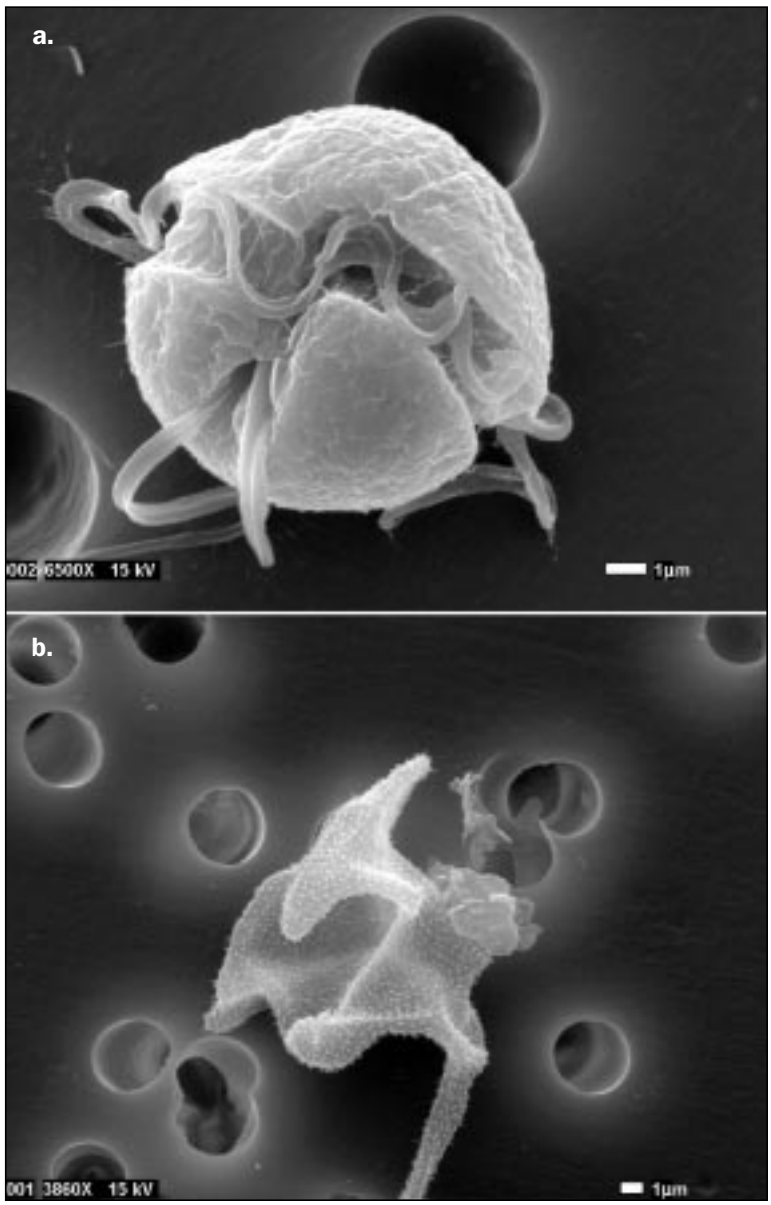

Figure 1. Scanning electron micrographs of two common stages of* Pfiesteria piscicida *(Steidinger and Burkholder). (a) A toxic zoospore (cell diameter ≈ 7–14 µm). Photos: NCSU Center for Applied Aquatic Ecology. (b) A lobose amoeba that transformed from a toxic zoospore (cell length ≈ 20–40 µm).

Detecting the organism at estuarine fish kills.

The Albemarle-Pamlico Estuarine System provides half the nursery ground area used by fish from Maine to Florida and, thus, is an extremely important estuary for sustaining fish populations on the Atlantic seaboard (Figure 2; Mallin et al. 2000). Isolated from the ocean by the Outer Banks except for several narrow inlets, this huge expanse of water is shallow (3–4 m deep, on average), poorly flushed under average flow conditions, and highly sensitive to pollutant loading. Like many estuaries, it has become increasingly eutrophic from nutrient pollution (Glasgow and Burkholder 2000, Mallin et al. 2000). In two of the major subestuaries, the Pamlico and Neuse, massive fish kills—many without known cause—had become so characteristic of summer and early fall from the mid-1980s through the mid-1990s that the local vernacular for that time of year was "fish-kill season" (Burkholder 1998).

Beginning with information gained from the accidental contamination of fish cultures, assistance from biologists at the state environmental agency, and funding from the US Environmental Protection Agency ($4,000 per year for the first 4 years of the research), we developed a field research component to examine whether the organism was present and actively toxic at estuarine fish kills. We obtained water samples from a kill of about 1 million Atlantic menhaden (*Brevoortia tyrannus* L.) in the Pamlico Estuary. The samples were taken while the kill was in progress, when most fish were moribund but not yet dead. Examination of water samples under a light microscope revealed abundant microbes that were similar in appearance to the culture contaminant from the NCSU College of Veterinary Medicine. Water samples taken less than 24 hours later, from a site where many dead fish floated at the surface, contained few dinoflagellates. When tested, the water samples

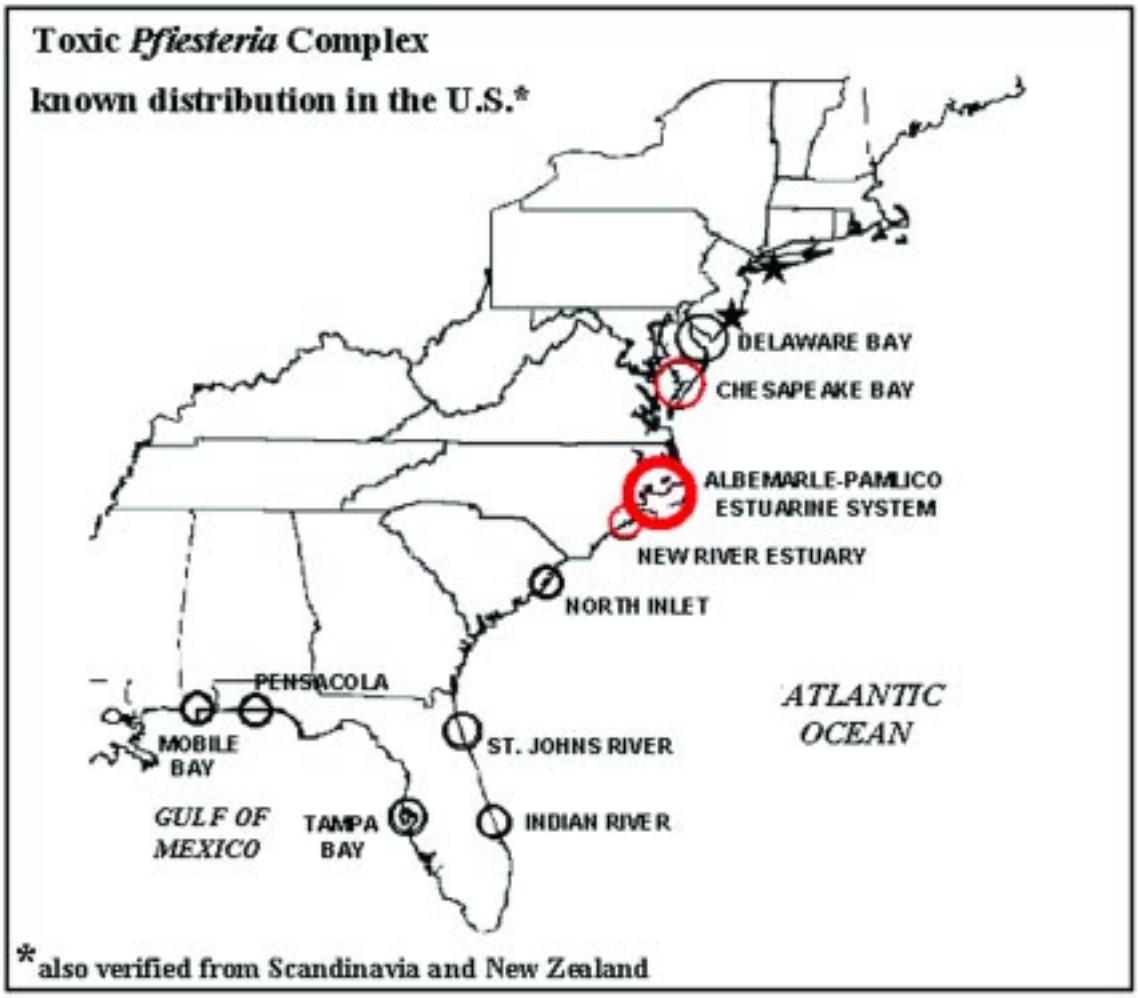

Figure 2. Known distribution of toxic strains of the toxic* Pfiesteria *complex (TPC) in US estuaries. Locations (black circles) where potentially toxic strains of* Pfiesteria *spp. (TOX-B functional type) have been documented through standardized fish bioassays are indicated. The Albemarle-Pamlico Estuarine System, Chesapeake Bay, and the New River Estuary locations (red circles) are where the two known actively toxic* Pfiesteria *spp. have been confirmed during major fish kills, using the same standardized procedure. Prior to the Chesapeake Bay outbreaks, our research team verified the presence of toxic strains of* P. piscicida *in the inland bays of Delaware (Indian River; Burkholder et al. 1995), Jenkins Creek (tributary of the Choptank River; Lewitus et al. 1995), Patuxent River (in the Chesapeake Bay system; Burkholder et al. 1995), many sites in North Carolina (Burkholder et al. 1995, Burkholder and Glasgow 1997), several sites in Florida (Burkholder and Glasgow 1997, Glasgow et al. 2001a), and Mobile Bay in Alabama (Burkholder et al. 1995). Stars indicate northern locations in the United States where* Pfiesteria *has been detected more recently using molecular probes (Rublee et al. 1999, Allen 2000), but these populations have not yet been rigorously tested for toxicity. Also since the Chesapeake Bay outbreaks, toxic strains of* Pfiesteria *spp. have been verified from Scandinavia (both species) and New Zealand (*P. shumwayae*; Allen 2000).

were shown to contain the same species of toxic organism as the culture contaminant (Burkholder et al. 1992). Thus, the microbe had behaved in the estuary as it had in the aquaria and appeared to be implicated in a major estuarine fish kill. Without completing detailed identification procedures (Popovský and Pfiester 1990) and obtaining corroboration of the results by other specialists (Steidinger et al. 1996), we could not determine whether the organism had been identified previously. Because a formal name would not be available until that process was completed, we nicknamed it the phantom dinoflagellate for its typically rapid appearance in the water in association with fish death, followed by its rapid disappearance (Burkholder et al. 1992).

Basic biology, ecology, and toxicity of Pfiesteria *as of 1997*

Species and optimal environmental conditions.

The newly known dinoflagellate previously had eluded detection as a fish-killing organism. Over the next 7 years, systematists helped to verify that it represented a new family, genus, and species of toxic dinoflagellate (Steidinger et al.

1996). The organism eventually was named *Pfiesteria piscicida* Steidinger & Burkholder, in honor of the late Lois Pfiester, whose elegant research on freshwater dinoflagellates revealed many fascinating characteristics about the complex life cycles of these organisms (Steidinger et al. 1996, Burkholder and Glasgow 1997). We developed proficiency in dinoflagellate species identifications in our laboratory (Glasgow et al. 2001a), but we continued to corroborate our identifications with other specialists, because we regard such action as an essential quality control step in science and thus in all toxic dinoflagellate research.

Our early laboratory tests established that *P. piscicida* can be active over a temperature range of 9° to 33°C, although generally at 18°C or higher (Burkholder et al. 1995). The organism is usually active in brackish waters of salinity 2 to 20 (freshwater is less than 1 and marine water is about 35 on the salinity scale), but it can kill fish in full-strength seawater and in the calcium-rich freshwater of aquaculture facilities near estuaries (Burkholder et al. 1995, 2001a, Burkholder and Glasgow 1997).

We initially suspected that the organism represented the first known member of a species complex with similar behavior (Burkholder et al. 1992, 1995). By 1994, our research team had verified the presence of toxic *Pfiesteria* strains from the inland bays of Delaware south and west to Mobile Bay, Alabama (Burkholder et al. 1995, Burkholder and Glasgow 1997), including two sites in Chesapeake Bay (Figure 2; Lewitus et al. 1995). In 1995 we first detected what appeared to be a second toxic *Pfiesteria*-like species in water samples collected from two North Carolina estuaries during fish kills (Burkholder and Glasgow 1997, Glasgow et al. 2001a), but subsequently the culture became noninducible (unable to engage in toxic activity; PICWG 2001). Such loss of toxicity in culture over time has been reported for toxic strains of various other harmful algae, including toxic dinoflagellates, and it is believed to be an artifact of the culture conditions (Burkholder et al. 2001a). By early 1997 we had cloned isolates of the second species, first referred to as *Pfiesteria* species B, that were attracted and toxic to live fish. After corroborating the morphologically unique features and toxicity with specialists in independent laboratories, we formally named the second species *Pfiesteria shumwayae* Glasgow & Burkholder (Glasgow et al. 2001a), in honor of the renowned scientist Sandra Shumway, who has significantly advanced understanding of toxic dinoflagellate impacts on shellfish (e.g., Shumway 1990).

Although there are many species that physically resemble *Pfiesteria*, the look-alikes would be of concern only if they had strains with toxin-producing capability, behavior, and impacts (Burkholder et al. 2001a, 2001b). Several other potentially toxic pfiesteria-like taxa have been reported among many look-alike species, but thus far only *P. piscicida* and *P. shumwayae* have been confirmed as toxic to fish under ecologically relevant conditions (standardized fish bioassays, below; Burkholder and Glasgow 1997, Marshall et al. 2000, PICWG 2001, Burkholder et al. 2001c). Therefore, at present, these two species form the toxic *Pfiesteria* complex (TPC) (Burkholder et al. 2001a, PICWG 2001).

A remarkable life cycle. Despite the common appearance of its small flagellated form, or zoospore, *P. piscicida* was a creature of firsts. It was the first dinoflagellate known to have cysts or dormant stages that resemble the cysts of an entirely different group of microorganisms called chrysophytes (Burkholder et al. 1992, Burkholder and Glasgow 1995, Steidinger et al. 1996). Moreover, whereas nearly all other known toxic, or "red tide," dinoflagellates are plant-like organisms with pigments that can discolor the water, this animal-like (heterotrophic) dinoflagellate is translucent or colorless unless it recently consumed pigmented prey (Burkholder and Glasgow 1995, 1997, Lewitus et al. 1999a). Nor does *Pfiesteria* act like a passive plant-like organism; it was the first toxic dinoflagellate shown to attack fish prey (Burkholder et al. 1992, Burkholder and Glasgow 1995, 1997). *Pfiesteria* is also difficult to track in estuaries because it can exist as more than 20 stages or forms, not counting transitional morphs between stages, that move between the benthic sediments and the water column (Burkholder and Glasgow 1997, Burkholder et al. 2001b, Glasgow et al. 2001a).

Its life cycle, with multiple amoeboid as well as flagellated stages, is among the most complex known for dinoflagellates (Figure 1; Popovsky and Pfiester 1990, Burkholder et al. 2001b), and the first such life cycle that was reported for a toxic species (Burkholder et al. 1992, Burkholder and Glasgow 1995, Steidinger et al. 1996). Amoebae produced by some strains have been identified as *Pfiesteria*, using species-specific molecular probes developed for zoospores of *P. piscicida* and *P. shumwayae* (Burkholder et al. 2001a, Glasgow et al. 2001a), and amoeboid stages of *Pfiesteria* have been corroborated by independent specialists (Steidinger et al. 1996, Marshall et al. 2000). *Pfiesteria* amoebae can be 5–120 μm in length; although they have grown to 750 μm, such increased size is believed to be an artifact of culture (as in other cultured amoebae; Burkholder et al. 2001b). Classical taxonomic keys lead to misidentification of *Pfiesteria* amoebae as belonging to at least eight different genera of unrelated sarcodinian amoebae (Burkholder et al. 2001b). Thus, the amoeboid stages of *Pfiesteria* probably have been misidentified as sarcodinian amoebae, suggesting a need to reevaluate the systematics of some amoeboid genera.

Benign and toxic strains. Like many other species of so-called toxic algae, *P. piscicida* populations isolated from estuarine waters and sediments show a range in toxicity—from highly toxic strains to noninducible strains that apparently are unable to engage in toxic activity in the presence of live fish (Burkholder et al. 2001a, 2001b, PICWG 2001). The toxic and noninducible, or benign, strains differ significantly in their behavior toward fish, suitability as food for predators, use of algal prey, and responses to nutrient enrichment and other environmental factors (Burkholder et al. 2001b). Our research focused only on toxic strains in the early years, because they are germane to questions about fish and human health (Burkholder and Glasgow 1997). To verify strain toxicity, we used a standardized, multistep fish bioassay (Burkholder et al.

1995, 1999, 2001c, Burkholder and Glasgow 1997, Marshall et al. 2000). The standardized steps follow Henle-Kochs' postulates, modified for toxic rather than infectious agents (Burkholder et al. 1995, 1999, Lewitus et al. 1995, Burkholder and Glasgow 1997, Marshall et al. 2000, Burkholder et al. 2001c). The toxic strains may be actively toxic (TOX-A functional type) or temporarily nontoxic, that is, capable of toxicity to fish but separated from live fish for days or longer, and, therefore, not engaged in toxin production (TOX-B functional type; Burkholder and Glasgow 1997, Burkholder et al. 2001a, *P*ICWG 2001).

The environmental signals that trigger toxin production in nearly all toxic algae are unknown. In contrast, *P. piscicida* was the first dinoflagellate shown to be stimulated to become toxic, producing toxin in the presence of live fish or their fresh secretions, excretions, and tissues (Burkholder et al. 1992). Filtrate from some toxic strains has killed fish, and those strains have also been lethal to fish when held in finely porous tubing to prevent direct contact with the fish prey (Burkholder and Glasgow 1997, Springer 2000). In the absence of live fish, toxic strains of *Pfiesteria* are only potentially toxic—that is, they are basically nontoxic consumers of other microbes, carrion, and mammalian tissues, as well as dissolved nutrients (Burkholder and Glasgow 1997). However, these nontoxic (TOX-B) cells can sometimes retain residual toxicity that can be lethal to fish at sensitive larval stages (Springer 2000). Cultures of toxic strains that are maintained on food sources other than live fish cannot kill fish for extended periods, indicating that the dinoflagellates were not actively toxic when the fish were introduced. Moreover, filtrate from TOX-B cultures, lacking the dinoflagellates, cannot kill fish (Burkholder and Glasgow 1997). In contrast, when maintained with live fish as prey, toxic strains of *P. piscicida* have been lethal to every individual and every species among more than 30 finfish and shellfish species that have been tested (Burkholder et al. 1995), with exception of subadult oysters (Springer 2000). Toxic strains of *P. shumwayae* have killed all finfish, but have not been lethal to shellfish, in the absence of finfish, in tests conducted thus far.

Most of our toxic isolates have been obtained from in-progress kills of juvenile Atlantic menhaden. Along mid-Atlantic shores, these fish move upriver from the ocean during late spring and early summer, and they linger to feed through early fall in poorly flushed, shallow estuaries with abundant plankton for food (Manooch 1988). During colder seasons, and even in warm seasons where menhaden are not abundant, toxic *Pfiesteria* strains act similarly to other members of the estuarine microfaunal community, consuming prey that range from bacteria to finfish and shellfish (Burkholder and Glasgow 1995). The Albemarle-Pamlico Estuarine System is believed to be ideal habitat for *Pfiesteria* (Figures 2, 3; Burkholder and Glasgow 1997). The shallow water minimizes the distance that must be traversed by benthic *Pfiesteria* populations to attack fish that occur near the water surface, such as juvenile menhaden (Manooch 1988). Menhaden are oily fish that travel in dense schools (Manooch 1988), concentrating the excretions and secretions detectable by *Pfiesteria*. In addition, these fish feed in poorly flushed, nutrient-rich areas with abundant algal prey, where the shallow habitat enhances the accumulation of both the fish materials and the toxin, with minimal dilution or washout. Such conditions, in the absence of fish, would provide a rich environment of potential food sources for temporarily nontoxic stages (Figure 3; Burkholder and Glasgow 1997).

Versatile nutrition. *Pfiesteria* cell production can be stimulated by nutrient pollution, as shown by research conducted in 1997 that became important to Maryland officials in assessing the Chesapeake Bay toxic *Pfiesteria* outbreaks and in developing water quality management strategies to discourage *Pfiesteria* growth (State of Maryland 1998). The diet of *Pfiesteria* potentially spans the estuarine food web from bacteria and algae to mammalian tissues, live and dead (Figure 4; Burkholder and Glasgow 1995, 1997). Such versatility in food sources had not been tested for or encountered previously in toxic dinoflagellates. *Pfiesteria piscicida* readily consumes dissolved organic nutrients, including certain substances found in poorly treated sewage and animal wastes (Burkholder and Glasgow 1997). Moreover, *Pfiesteria* can use photosynthesis as a borrowed nutritional mode by retaining the chloroplasts from algae that it consumes when fish are not readily available. *Pfiesteria* maintains these inclusions, called kleptochloroplasts, in a large food vacuole and allows them to function for hours to days in supplementing its nutrition (Lewitus et al. 1999a). *Pfiesteria* zoospores with kleptochloroplasts proliferate when directly stimulated by inorganic plant nutrients such as nitrate and phosphate, which are found in cropland or lawn fertilizers and other sources (Burkholder and Glasgow 1997, Lewitus et al. 1999b).

About 75% of the North Carolina toxic *Pfiesteria* outbreaks through 1997 occurred in estuarine waters for which available data show high nutrient concentrations from various anthropogenic sources, such as cropland fertilizer runoff, poorly treated human sewage, or animal wastes (Figure 5; Burkholder et al. 1995, 1997, Burkholder and Glasgow 1997). Nutrient data were not available for the other events, but they also occurred in a nutrient-enriched environment, namely, in aquaculture facilities where many fish (and their excretory materials) had been held in a relatively small, enclosed space (Burkholder et al. 1995, Burkholder and Glasgow 1997). The nutritional ecology of *Pfiesteria* is complex. More data are needed before it will be possible to quantify the relative importance of various nutrient sources in stimulating *Pfiesteria*. However, by 1997 the stimulatory effect of nutrient enrichment on toxic strains of *Pfiesteria* had been observed repeatedly in laboratory experiments to some extent (Burkholder et al. 1992, Glasgow et al. 1995, Burkholder and Glasgow 1997).

***Culturing and assaying for toxic* Pfiesteria.** Basic information about culturing toxic *Pfiesteria* also later figured prominently in the science and policy of the Chesapeake

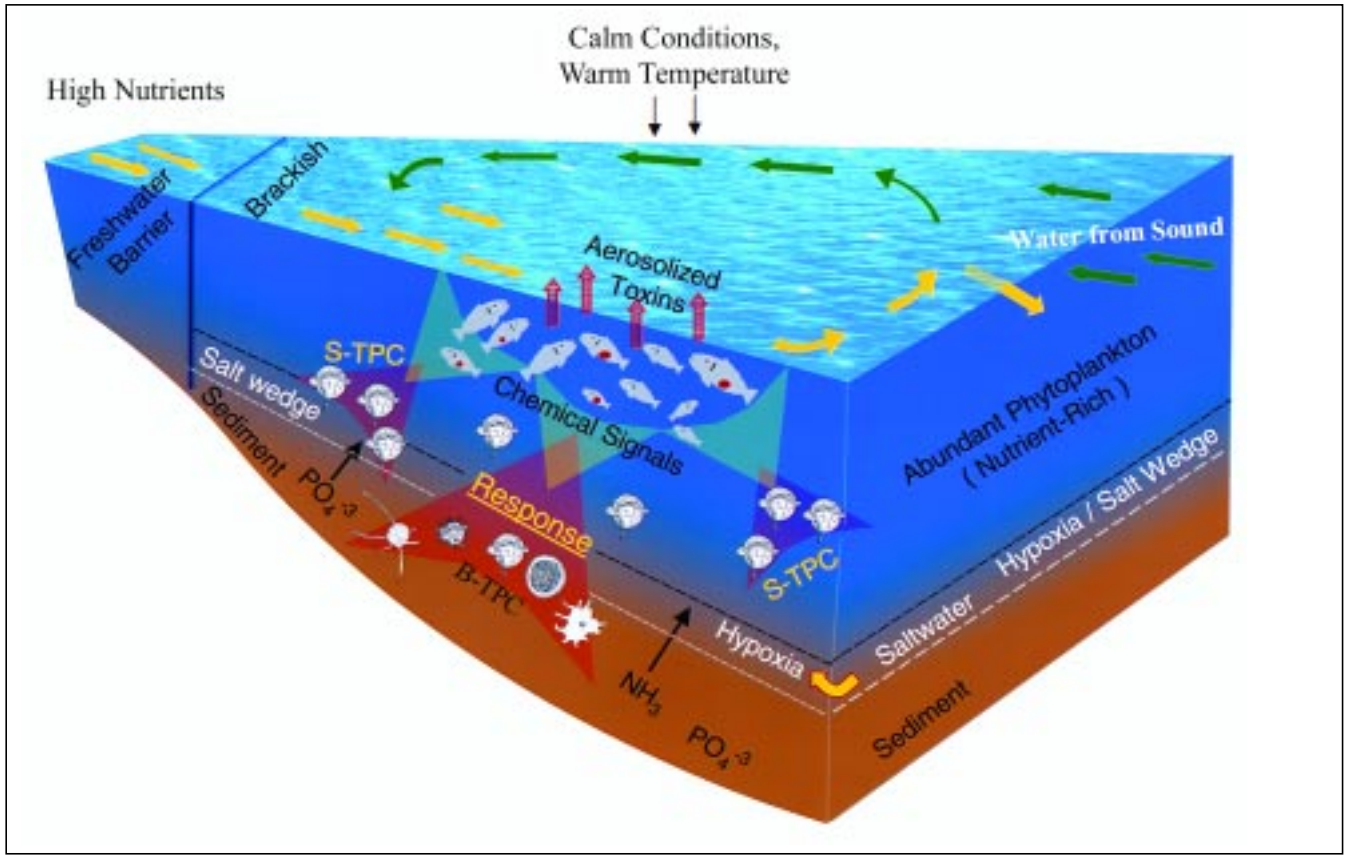

Figure 3. Schematic depicting toxic* Pfiesteria *response under calm conditions leading to fish kills in the mesohaline Neuse Estuary, showing the strong salt wedge and gyre that typically occur in late spring through summer, during periods of low precipitation and low flow. High-salinity water from Pamlico Sound flows up-estuary in the lower water column, whereas lower-salinity water (with freshwater river input) flows down-estuary in the upper water column. The resulting two water masses have distinct physical and chemical characteristics. The mid-to-upper water column, influenced mostly by water from up-river, is high in nutrients (e.g., nitrate) from the watershed, and is less dense from lower salt content in comparison to the bottom water. The mid-to-upper water column also is higher in available light and contains abundant phytoplankton and abundant dissolved oxygen from their photosynthesis. In contrast, the denser, more saline bottom water is often hypoxic (low in oxygen) and contains nutrients (e.g., ammonium and phosphate) released from decomposition of dead plant and animal remains that have settled out. A fish kill caused by toxic* Pfiesteria *results when large schools of fish such as juvenile Atlantic menhaden linger to feed. Their excreta and secreta act as chemical signals that are detected by both benthic (B-TPC) and suspended (S-TPC)* Pfiesteria *cells. These chemical signals stimulate* Pfiesteria *zoospores to produce toxin that causes fish disease and death. A waterborne toxin from* Pfiesteria *spp. can be aerosolized and has been linked with human illness, such as memory impairment, that can last for days to months.

outbreaks. Thus far, *Pfiesteria* spp. cultured in defined media have multiplied slowly or have grown well but for only a short duration (days); thus, significant, sustained growth has required other organisms as a food source (Burkholder and Glasgow 1995, 1997). Live fish stimulate both cell growth and toxin production (in toxic strains), as mentioned. Algae can serve as food, but if previously toxic cultures (fed live fish) are maintained on algal prey for more than a few weeks, many lose toxin-producing capability. *Pfiesteria* cultures maintained on algae remain cleaner (for example, bacteria free) than those reared on fish, but toxic *Pfiesteria* strains can also have endosymbiotic bacteria (Lewitus et al. 1999a), which are not removed in sterilization procedures. Thus, we refer to toxic *Pfiesteria* as TPC species (PICWG 2001) with associated endosymbiont bacteria. As for most toxic algae, the question of whether bacteria play a role in toxin-producing activity remains to be resolved (Doucette et al. 1998, Burkholder et al. 2001a).

Assays for toxic and noninducible strains of *Pfiesteria* involve adding fish or algae to natural water samples collected from an in-progress fish kill area (Burkholder and Glasgow 1997, Burkholder et al. 2001c). Most actively toxic *Pfiesteria* cells form dormant resting stages or cysts when they are separated from live fish during sampling at an estuarine fish kill, especially during transport to laboratories for analysis. Live fish must be added to stimulate these recently toxic cells to

excyst and move up into the water column of test containers, where they can then be evaluated to determine whether they were actively toxic at the estuarine fish kill (Burkholder et al. 2001c). Addition of prey other than live fish does not enable evaluation of estuarine samples to determine whether *Pfiesteria* was involved in the fish kill, because *Pfiesteria* becomes toxic only when given live fish.

For example, water samples taken from natural fish kills, with algal prey rather than live fish added as food, usually fail to produce detectable populations of toxic *Pfiesteria* although, occasionally, noninducible strains can be detected (Burkholder et al. 2001c, PICWG 2001). *Pfiesteria* strains that were very recently in actively toxic mode often require 2 weeks or more without fish to switch to an algal diet and increase their population density. During that time, benign heterotrophic dinoflagellates that co-occur in natural samples usually outcompete toxic strains of *Pfiesteria* for algal prey and attain high abundance. Thus, toxic *Pfiesteria* remains low to negligible in abundance, and generally is not detected (Table 1). When a natural mix of microorganisms is subjected to a given condition or stimulus in culture, competition among the species present is influenced by that condition, so that certain species proliferate while others decline (Fogg and Thake 1987). Here, addition of algal prey strongly stimulates certain dinoflagellate species to increase in number, whereas recently toxic *Pfiesteria* strains (that were consuming fish materials) respond poorly. In contrast, addition of live fish prey strongly stimulates populations of recently toxic *Pfiesteria*. These populations then excyst and, within a relatively short period (usually 4–9 days), become actively toxic again and increase their population density (Burkholder et al. 2001c). Our research team has shown repeatedly that toxic strains of *Pfiesteria* are reliably detected from natural samples by adding live fish in appropriately conducted, standardized fish bioassays (Burkholder et al. 1995, Lewitus et al. 1995, Burkholder and Glasgow 1997, Marshall et al. 2000, Burkholder et al. 2001c).

Impacts on fish. The most lethal strains of actively toxic *Pfiesteria* can narcotize juvenile and adult finfish within minutes, so that they become sluggish and swim erratically (Burkholder et al. 1992, 1995; Burkholder and Glasgow 1997). During acute exposure, fish commonly hemorrhage or develop skin lesions that are diffuse or nonfocal, as well as deep, localized or focal, bleeding sores or ulcerations. Focal lesions usually appear within 2 to 12 hours when fish are exposed to Pfiesteria densities (more than 3×10^2 to 10^3 cells per mL), comparable to those typical of estuarine fish kill or disease events (Figure 6; Burkholder et al. 1995, Burkholder et al.

Table 1. Comparison of the fish and algal assays for detecting the toxic and noninducible strains of* Pfiesteria. *Samples were collected during fish kills, and comparisons were initiated within 2 days following collection (n = 3). At least 100 zoospores with swollen sutures (technique in Glasgow et al. 2001a) were analyzed per replicate. This required examination of about 2,000 cells per replicate, because relatively few cells could be rotated to adequately discern the features in all four views (apical and posterior, and ventral and dorsal). See Burkholder et al. (2001b, 2001c) for the experimental details.

Culture	Fish bioassay[a]	Microalgal assay[a]
Pamlico (May 1996)	*P. piscicida* (100%)	*P. piscicida* (25%)[b] *Pfiesteria* look-alike sp.
Pamlico (July 1997)	*P. piscicida* (100%)	*P. piscicida* (30%)[b] *Pfiesteria* look-alike sp.
Neuse (July 1997)	*P. piscicida* (80%) *P. shumwayae* (20%)	*P. piscicida* (less than 10%)[b] 2 *Pfiesteria* look-alike spp.
Pocomoke (May 1997)	*P. piscicida* (100%)	*Pfiesteria* look-alike sp. *Gymnodinium* sp.
Pocomoke (Aug. 1997)	*P. piscicida* (100%)	*Pfiesteria* look-alike sp. *Gyrodinium* spp.
Pocomoke (Sept. 1997)	*P. piscicida* (70%) *P. shumwayae* (30%)	2 *Pfiesteria* look-alike spp. *Karlodinium micrum*[c] 2 *Gymnodinium* spp.
Chicamacomico (Sept. 1997)	*P. piscicida* (100%)	*P. piscicida* (40%)[b] *Pfiesteria* look-alike sp.

a. Percentage of cells that are toxic *Pfiesteria* complex (TPC) species is given; for microalgal assays, other species are listed but not quantified.

b. Subsequently tested and verified as a noninducible population, that is, incapable of causing fish distress, disease, or death (live cells at field densities). Note that *P. piscicida* from microalgal assays was tested also by H. Marshall at Old Dominion University (Norfolk, VA) and was noninducible.

c. *Karlodinium micrum* (Leadbeater and Dodge) J. Larsen; formerly *Gyrodinium galatheanum* (Daugbjerg et al. 2000).

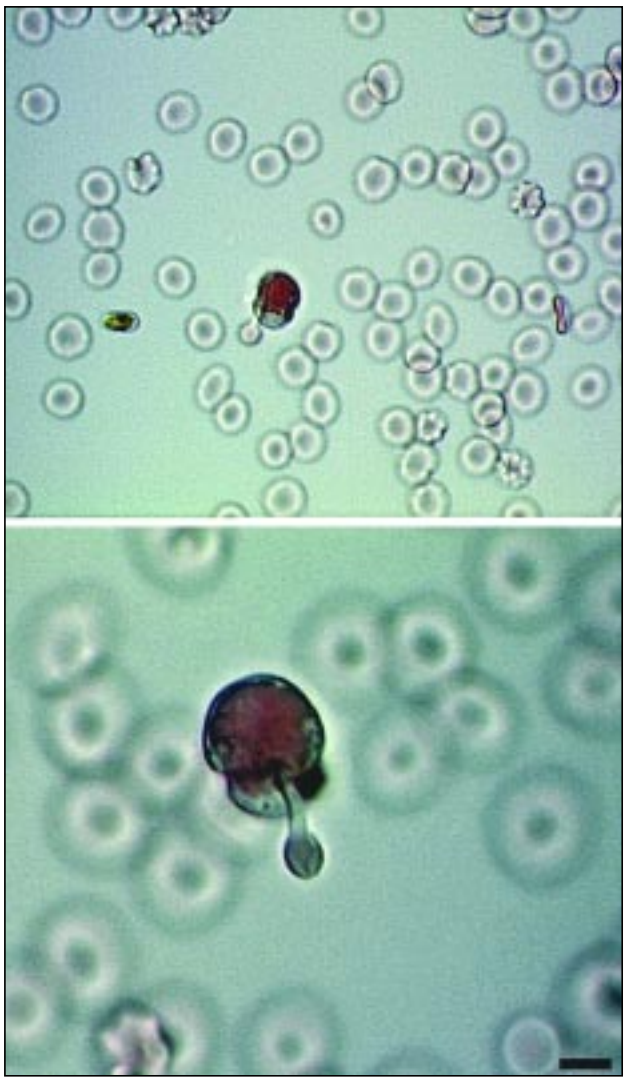

Figure 4. A zoospore of a toxic strain of* Pfiesteria shumwayae *(TOX-B functional type) consuming mammalian red corpuscles. Note that each zoospore feeds by extending its peduncle, attaching to a red blood cell, and suctioning the contents. Zoospores, typically translucent without pigmentation, display a reddish coloration within their food vacuole because of the pigmented (hemoglobin) RBCs that they consumed. Photos: NCSU Center for Applied Aquatic Ecology.

2001a, Glasgow et al. 2001b). For some toxic strains, lesions and hemorrhaging have developed in finfish, followed by death, whether *Pfiesteria* zoospores were in direct contact with fish (allowing both zoospores and toxin to attack the fish) or were separated from fish by a dialysis membrane (0.22-µm porosity) through which only toxins could pass (Burkholder and Glasgow 1997).

Pfiesteria can have serious chronic and sublethal impacts as well as acute impacts on finfish health. Acute exposure to toxic *Pfiesteria* followed by a *Pfiesteria*-free period, or sublethal exposure to toxic *Pfiesteria* over days to weeks, lowered disease resistance so that fish became more susceptible to attack by opportunistic pathogens, including those that can cause deep, focal lesions (Noga et al. 1996, Burkholder et al. 2001a). The data suggest that *Pfiesteria* toxin compromises the fish immune system, which is supported by the observed decrease in fish white blood cells to 40% to 60% of normal (Burkholder 1998). Fish also show prolonged damage of the osmoregulatory system, which is found in the epidermis and is destroyed by *Pfiesteria* and its toxin (Noga et al. 1996). Compromise of the osmoregulatory system would be a potentially serious problem affecting fish survival in the highly variable salinity characteristic of estuaries (Mallin et al. 2000). Eggs of commercially important fish such as striped bass (*Morone saxatilus* Walbaum) and killifish (*Fundulus heteroclitus* L.) have not hatched during or after exposure to toxic *Pfiesteria* populations, suggesting the possible reproductive impairment in exposed fish populations (Burkholder 1998, authors' unpublished data).

Pfiesteria piscicida also has been lethal to adult and larval shellfish, an effect that has been enhanced in the presence of finfish (Burkholder et al. 1995, Springer 2000). Adult scallops have died within minutes in the presence of finfish and actively toxic *Pfiesteria*, and adult blue crabs within days, of exposure to toxic *Pfiesteria* (Burkholder et al. 1995). Larval eastern oysters (*Crassostrea virginica* Gmelin) and both larval and adult bay scallops (*Argopecten irradians* Lamarck) have been killed within minutes of exposure to toxic *P. piscicida* when the dinoflagellates were contained within dialysis tubing (molecular weight cutoff 12,000–14,000 daltons) to prevent direct contact (Burkholder et al. 1995, Springer 2000). If allowed direct contact, some toxic *Pfiesteria* strains have attacked and devoured the shellfish pediveliger larvae that previously had discarded their vela (Figure 7; Springer 2000). Such attack is much less common for noninducible strains.

In estuaries, the basic factors conducive to a toxic *Pfiesteria* outbreak, other than a robust *Pfiesteria* population with toxic strains, include the following: the presence of high densities of fish prey, particularly large schools of oily fish; poorly flushed, shallow water that is overenriched in nutrients, warm, and brackish; and abundant phytoplankton or other prey that serve as an alternate food source for *Pfiesteria* when live fish are not detected (Burkholder and Glasgow 1997). During warmer seasons, the lower water column often contains a salt wedge, which consists of heavier saltwater from the ocean that underlies freshwater coming in from rivers (Figure 3; Glasgow and Burkholder 2000). Benthic *Pfiesteria* populations (active amoebae and cysts) in nutrient-rich, organic sediments produce zoospores that become toxic in response to unidentified chemical cues from abundant fish, for example, surface-schooling Atlantic menhaden (Figure 3). Toxins from the zoospores narcotize and then kill the fish. Note that in the natural environment, neither toxic *Pfiesteria* spp. nor other stressors likely act alone; instead, fish health may be stressed by *Pfiesteria*, co-occurring microbial pathogens, periodic or sudden exposure to water with low dissolved oxygen, a sudden salinity shift, and other factors. Although *Pfiesteria* can attack and kill fish in culture without other,

interacting stressors, in estuaries they may more readily attack fish that are weakened by other factors (Burkholder and Glasgow 1997, Burkholder et al. 1999).

Subtle but serious human health impacts. The pharmacological routes of human exposure to *Pfiesteria* toxins are distinct from most other dinoflagellate toxins, which have caused human illness and death primarily through consumption of toxin-contaminated seafood (Falconer 1993, Burkholder 1998). *Pfiesteria* did not appear to adversely affect people who ate seafood (against our advice) collected from areas where toxic outbreaks were in progress. However, the risk from consuming seafood from toxic *Pfiesteria* outbreaks cannot be ruled out until a reliable assay can be developed to test for *Pfiesteria* toxin in fish tissues (Wright 1998, Fairey et al. 1999). Moreover, when the water from a fish-killing culture is filtered to remove cells of some *Pfiesteria* strains, the remaining toxin typically kills fish for only 3–8 hours, indicating that the toxin(s) is labile and highly unstable in water (Burkholder and Glasgow 1997, Moeller et al. 2001). Therefore, shortly after a *Pfiesteria*-related fish kill ends in an estuary (within less than 24 hours), the area should be safe for human use. The data, although preliminary, were available in 1997 and were taken into consideration in forming Maryland's policy for reopening affected areas to fishing and recreation. As an extra precaution, areas were reopened 3 days after fish disease and death were no longer evident (Magnien et al. 2000, Glasgow et al. 2001b).

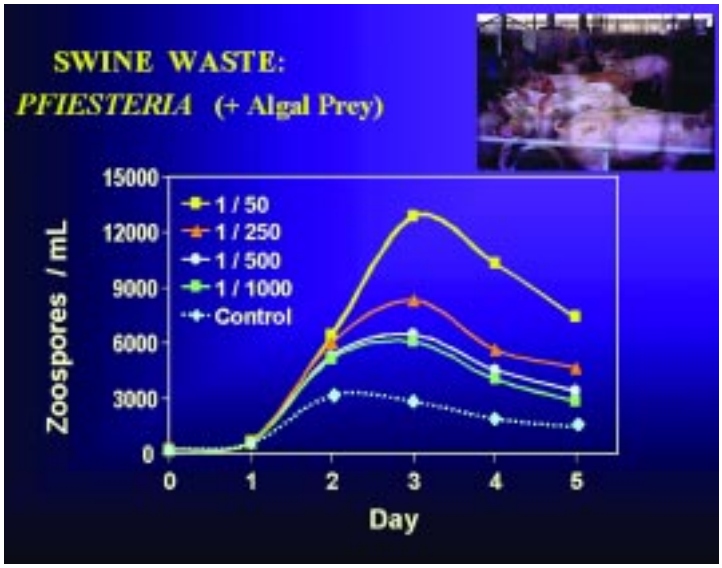

Figure 5. Response of a toxic strain of* Pfiesteria piscicida *(TOX-B functional type) to sterile-filtered swine effluent. The effluent was diluted in series (1:50, 1:250, 1:500, 1:1000) using sterile-filtered water (seawater diluted with sterile-filtered deionized water) at a salinity of 15.* Pfiesteria *zoospores in controls and all treatments were fed cryptomonad algae (5 x 10^3 cells per mL). Swine effluent clearly stimulated zoospore cell production, which significantly increased with increasing effluent concentration (p < 0.05; standard errors were less than 5% of the means in all cases).

Thus, based on sparse information, seafood may not be a major route for human exposure to *Pfiesteria* toxin (see more recent data in Springer 2000 suggesting a potential seafood route). Additional evidence, from laboratory staff exposures, indicated that people were at risk of serious health impacts if they sustained water contact or inhaled aerosols where fish were diseased or dying and actively toxic *Pfiesteria* populations were present (Glasgow et al. 1995; note that additional evidence for exposure through water contact and aerosol inhalation was later obtained by Grattan et al. 1998).

Toxins from a few other dinoflagellate species are known to be released into the air from cells broken by wave action along beaches, and they have caused respiratory problems when inhaled by humans and dogs (e.g., *Karenia brevis*, formerly *Gymnodinium breve*; Falconer 1993; Daugbjerg et al. 2000). Somewhat analogously, laboratory workers became ill after inhaling aerosols from fish-killing *Pfiesteria* cultures. Ten of 12 affected staff in several laboratories had worked with dilute concentrations, from 300 to 13,000 toxic zoospores per mL, of *Pfiesteria* in fish-killing mode (Burkholder et al. 1995, Glasgow et al. 1995). The others had worked with higher cell densities that were still within field range: 90,000 cells per mL, versus up to 109,000 cells per mL reported at estaurine fish kills (Glasgow et al. 1995). The subjects experienced burning skin and a tingling sensation during or following contact with the water from such cultures. More seriously, *Pfiesteria* was the first dinoflagellate linked to production of aerosolized substances that seriously affect the human nervous system. Inhalation of the air over fish-killing cultures was related to symptoms that included blurred vision, burning skin and eyes, acute respiratory difficulty, muscle cramping, nausea, vomiting, severe headaches, and profound memory dysfunction (Glasgow et al. 1995).

The memory dysfunction was an inability to create new memory, apparantly similar to an Alzheimer's-like suppression of short-term memory and learning ability. For days to weeks following exposure, several laboratory personnel could recognize words individually but could not form sentences, perform simple arithmetic, or remember more than the last words of a sentence directed to them. The most seriously affected person in our laboratory, who is a highly intelligent researcher, managed only a 7-year-old's reading level for 3 months after exposure and required reading lessons at first to help regain reading ability. This person recovered normal cognitive function about 3 months after exposure to the toxic cultures (Glasgow et al. 1995). For two others who were affected, long-term memory was compromised for several weeks, during which time they could not remember their names or where they lived. All the affected laboratory personnel were relatively young, mostly in their 20s or 30s, when they suffered these health impacts following repeated exposure to the dilute, fish-killing *Pfiesteria* cultures. Certain symptoms have recurred in some individuals, especially following strenuous exercise. More chronic problems, such as

Figure 6. Fish exposed to toxic* Pfiesteria.** ***A juvenile tilapia (*Oreochromis mossambicus *Peters; upper panel) following 8 hours of exposure to a clonal culture of actively toxic* Pfiesteria shumwayae *(2.5* x *10^3 cells per mL; Burkholder et al. 2001). A juvenile Atlantic menhaden (lower panel) with a fresh lesion from a toxic* Pfiesteria *outbreak on the Neuse Estuary, North Carolina. Although menhaden often develop chronic, deep focal lesions, they can also develop what appear to be fresh lesions, such as this, during toxic* Pfiesteria *outbreaks (Burkholder et al. 1995, Burkholder and Glasgow 1997). Photos: NCSU Center for Applied Aquatic Ecology.

autoimmune reactions, are suspected to be related and have been ongoing for up to 8 years in several cases (Schmechel and Koltai 2001).

The data from the laboratory cases were presented to the North Carolina state health director and his staff at a formal meeting in the spring of 1994, in the hope that the officials would evaluate the potential for health problems from *Pfiesteria* for people who use North Carolina's estuaries. We also conveyed information from anecdotal accounts of fishermen who unknowingly had been in toxic *Pfiesteria* outbreaks. These people experienced nausea, burning skin and eyes, vomiting, joint and muscle pain, and severe headaches; some also described losing consciousness and finding themselves lost in estuaries they had fished since childhood. Lingering complaints included chronic infections, respiratory problems, complete lapses in memory of events that had occurred minutes before, and other disorientation. Most of the fishermen who confided in us did so without making any link to *Pfiesteria*, instead attributing their health problems after engaging in estuarine water-related activities to "pollution." With our firsthand knowledge of health impacts from work with dilute cultures of toxic *Pfiesteria*, in discussions with the state health officials we stressed the need for management actions that erred on the side of protecting human health during toxic *Pfiesteria* outbreaks.

Biohazard–biosafety laboratory 3 facilities for toxic* Pfiesteria *research. When we had asked the National Oceanic and Atmospheric Administration (NOAA; Beaufort, NC, and Charleston, SC) and other federal agencies about recommended safety protocols for research with actively toxic *Pfiesteria*, we were informed that no federal regulations or guidelines were available for safety procedures in research with toxic algae. The officials advised us that standard laboratory practices (wearing a lab coat and gloves) were adequate and used by their personnel in research with other toxic algae. When we informed university safety personnel that a person had been hurt while working with toxic *Pfiesteria* cultures in our laboratory, a small biohazard biosafety laboratory 3 (BSL 3) facility was constructed for our use. However, the facility proved to have defective air flow, which led to a second person being seriously hurt (Glasgow et al. 1995). We then suspended all research with toxic *Pfiesteria* for about a year and a half, until an improved biohazard BSL 3 facility was completed (Burkholder et al. 2001c). Over that period, we learned of 10 others who had been affected, including people from three laboratories elsewhere, which strengthened the evidence that toxic *Pfiesteria* can cause human health impacts.

A site visit and detailed evaluation of our laboratory's safety protocols were mandated by NOAA which, in an unusual action, had suspended funding of a small grant to our laboratory because of uncertainties about the extent of the problem. The site visit panel included representatives from NOAA-Charleston and the Department of Defense, as well as various state personnel. The panel found our laboratory safety protocols satisfactory and supported NCSU's decision to require biohazard BSL 3 facilities for research with toxic *Pfiesteria*. NOAA required us to formally agree to use such facilities before further grant funding was released to our laboratory, and advised other granting agencies to follow a similar course. Thus, as our knowledge about *Pfiesteria* developed, we were required to use biohazard BSL 3 facilities in recognition of the connection (Glasgow et al. 1995) between work with toxic *Pfiesteria* cultures and serious human illness. Such caution proved to be prudent as well as necessary. The improved biohazard BSL 3 facility prevented exposure to aerosols from toxic *Pfiesteria* cultures, and no one else in our laboratory was hurt.

Attempts to characterize* Pfiesteria *toxin(s). The most critically needed information about *Pfiesteria* was, and remains, the identity of its toxin(s). The toxin must be identifiable and quantifiable in order to address all of these objectives: determining whether seafood is unsafe to eat because of toxin contamination; assessing the extent to which people have been exposed to toxic *Pfiesteria*; developing improved early warning systems to protect people from such exposure; and

understanding the modes of action by which the toxin(s) impact human health. This lack of information has seriously affected every facet of *Pfiesteria* science in both North Carolina and the Chesapeake Bay area, and it has been the greatest impediment to progress on the issue.

Although we sought specialists to analyze the toxin(s) from our fish-killing *Pfiesteria* cultures, the few who agreed to begin to examine it gave the research low priority, in part because only modest funding was available to support the research. Just before the Chesapeake Bay outbreaks, we began work with toxicologists at NOAA-Charleston (National Ocean Service [NOS]) and specialists from the intramural program of the National Institutes of Environment Health Sciences. Three days after receiving 1 L of culture, they isolated a heat-stable, water-soluble fraction from the culture with potent fish-killing activity. The data were presented in several scientific forums, and results were corroborated by an independent laboratory (Fairey et al. 1999). Toxin purification remained seriously impeded, however, because isolating enough of the highly unstable toxin for purification and identification proved more expensive than available funding could support.

Identification of dinoflagellate toxins typically requires many years. For example, brevetoxins are more amenable to analysis than *Pfiesteria* toxin because they are stable, lipid soluble, and produced in mass quantity by plant-like dinoflagellates that can be grown in simple media, without need of biohazard III facilities or live fish. Nevertheless, it took about 20 years to purify brevetoxins and fully identify their chemical structures (Falconer 1993, Hallegraeff et al. 1995). From the initial isolation work with toxic *Pfiesteria* culture, an assay was developed to detect the water-soluble *Pfiesteria* toxin in laboratory cultures. Production of water-soluble toxin material for analysis by NOS-Charleston became the central focus of our research team before the toxic *Pfiesteria* outbreaks in Maryland (Fairey et al. 1999). However, toxin assays cannot reliably detect *Pfiesteria* toxin from estuarine samples until sufficient quantity of purified toxin becomes available for use as standard in the assay. Thus, there was no reliable toxin assay available for use in diagnosing toxic *Pfiesteria* outbreaks in North Carolina, or Chesapeake Bay, in 1997.

Pfiesteria *research and socioeconomic concerns in North Carolina*

When *Pfiesteria* was first implicated in fish kills in North Carolina, it allayed some of the concern building since the mid-1980s about many fish kills of unknown cause. However, because many toxic dinoflagellates contaminate seafood with their toxins (Falconer 1993), the seafood industry viewed publicized information about *Pfiesteria* as an economic threat (Diaby 1996). Their concern was based partly on the fact that there often is public panic when people learn of a "red tide" or other toxic dinoflagellate outbreak. The panic leads to widespread losses to the seafood industry because of an "economic halo effect" (Shumway 1990), whereby all seafood is avoided, even when it comes from completely unaffected areas.

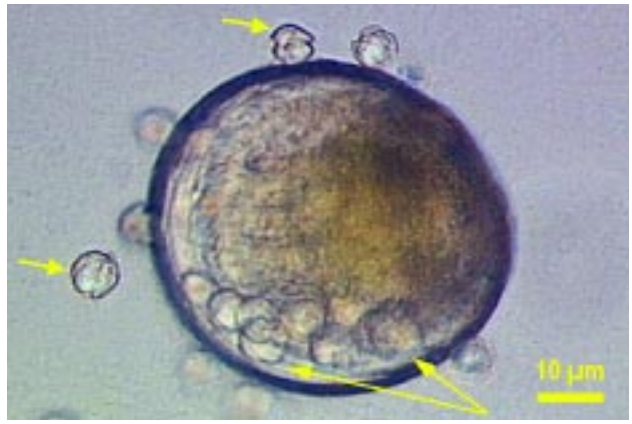

Figure 7. Actively toxic zoospores of* Pfiesteria piscicida *(TOX-A functional type), 2 hours after being taken from a fish-killing culture. Translucent zoospores (~10 µm in diameter) are shown swarming around (short arrows), penetrating, and consuming (long arrows) a live eastern oyster pediveliger larva that had discarded its velum. This photograph was taken 5 minutes after the* Pfiesteria *zoospores were added to the medium with the pediveliger. In this trial, within less than 30 minutes the zoospores had consumed most of the pediveliger's tissues except for the adductor muscle (Springer 2000). Photo: NCSU Center for Applied Aquatic Ecology.

Studies also showed that *Pfiesteria* can be stimulated by nutrient pollution (Burkholder et al. 1992, Glasgow et al. 1995, Lewitus et al. 1995, 1999a, 1999b, Burkholder and Glasgow 1997, Magnien et al. 2000, Samet et al. 2001). Nutrient overenrichment of the affected North Carolina estuaries comes from many sources, such as stormwater runoff, sewage treatment plants, row crop agriculture, and swine and poultry feed operations (Glasgow and Burkholder 2000, Mallin et al. 2000). The potential for adverse human health effects from toxic *Pfiesteria* exposure was also problematic for tourism and coastal development (Figure 8), as has occurred with outbreaks of other toxic algae (Burkholder 1998). Subsequent criticism of the validity of the published *Pfiesteria* research had long-term impacts on public opinion and influenced later treatment of the issue by state and federal authorities (Paolisso and Chambers 2001).

Complaints of disorientation, memory loss, burning eyes, burning skin, respiratory difficulty, nausea, and vomiting were conveyed to state and local health officials by people who had been exposed to fish kills that were confirmed to have involved toxic *Pfiesteria* (Burkholder and Glasgow 1997, Burkholder et al. 2001a). Such health problems were also described by 32 of 89 participants in an epidemiological questionnaire that was administered by state health officials (Morris 1996, McGeehin 1997). The participants had been in the toxic outbreak areas for minutes to hours. However, clinical evaluations were not performed to discern whether they manifested cognitive impairment, with the exception of three people who were examined 6–9 months after the exposures.

By that time, their symptoms could not be conclusively related to exposure to the toxic outbreaks (Morris 1996). The potential for health impacts from exposure to toxic *Pfiesteria* was also dismissed on the basis of a health questionnaire administered to fishermen long after fish kills had subsided (therefore, in the absence of actively toxic *Pfiesteria*; Griffith 1999, Lewitus et al. 1999c, Oldach 1999, Paolisso and Chambers 2001). Moreover, the health status of those exposed was based on reports from coastal physicians who had no diagnostic tool for relating symptoms to toxic *Pfiesteria* exposure (Smith and Music 1998). An evaluation by an independent, scientific panel, requested by the governor of North Carolina, thus concluded that insufficient data were available from 1991 to 1997 to evaluate the potential for human health effects from toxic *Pfiesteria* exposure in North Carolina estuaries (Wright 1998).

Pfiesteria *in Chesapeake Bay*

In 1993, potentially toxic strains of *P. piscicida* were first detected in Chesapeake Bay by scientists working on a tributary of the nutrient-enriched Choptank River on Maryland's Eastern Shore (Lewitus et al. 1995). Potentially toxic strains of this dinoflagellate had also been documented from the Patuxent Estuary on the western shore of the Chesapeake (Burkholder et al. 1995) and were later confirmed as toxic in fish bioassays (Burkholder and Glasgow 1997, Burkholder et al 2001c). In fall 1996 and early spring 1997, local fisherman suspected *Pfiesteria* of causing fish disease in the Pocomoke Estuary in Maryland, but the water samples were not tested with live fish in standardized fish bioassays; instead, they were tested using algal assays and, not surprisingly, *Pfiesteria* was not detected (MDNR 1998). In late spring 1997, we first examined samples from the Pocomoke Estuary that were sent by commercial fishermen, and we informed Maryland officials that the samples contained toxic *Pfiesteria* strains.

Fish bioassays and scanning electron micrographs (SEMs) of water samples collected later that spring and analyzed by our laboratory also indicated that toxic *P. piscicida* was present in the Pocomoke. (These samples were later confirmed as *P. piscicida* by other independent specialists, using molecular probes; Rublee et al. 1999, Oldach et al. 2000, Burkholder et al. 2001a, 2001c). We served on a technical committee that first met in early August 1997 to provide guidance for Maryland environmental agencies on fish disease in the Pocomoke. Because the Pocomoke receives pollution from various sources (MDNR 1998), we cautioned the officials that there very likely were many other factors in the estuary, such as pathogens in leachate from poultry wastes or other sources, that could promote fish disease. Less than a week later, the Pocomoke sustained a major fish kill with characteristics that suggested toxic *Pfiesteria* involvement, and within another week we confirmed the presence of actively toxic populations of *Pfiesteria*-like dinoflagellates. Clones of these organisms were toxic to fish in repeated, standardized fish bioassays,

Figure 8. Sample articles from North Carolina newspapers about the* Pfiesteria *issue in 1995, mostly stemming from two toxic* Pfiesteria *outbreaks, one in the New River Estuary following a major swine effluent spill (c. 10,000 juvenile Atlantic menhaden were killed), and one from a toxic outbreak in the Neuse Estuary (approximately 15 million fish were killed). Over a thousand related articles were published in local newspapers during that year alone, and many national and international articles and newscasts appeared about* Pfiesteria *and related water quality problems occurring in North Carolina from 1991 through1997, prior to the toxic* Pfiesteria *outbreaks in Maryland. Photo: NCSU Center for Applied Aquatic Ecology.

and SEMs and molecular probes verified that the organism involved in all four toxic outbreaks was *P. piscicida* (Magnien et al. 2000). In one event, toxic *Pfiesteria* species "B" (*P. shumwayae*) was subdominant. All other species that resembled *Pfiesteria* in the samples were found not to be toxic in fish bioassays.

After considering this information, the governor of Maryland chose to take extra precautions protecting human health. The efforts taken by the governor and his staff to assess the situation and the evidence, to build scientific consensus, to clinically determine whether serious human health impacts could be sustained from exposure to a toxic *Pfiesteria* outbreak under field conditions, and to act with long-range vision to improve water quality protection (e.g., State of Maryland 1998) are related in Burkholder and Glasgow (1999) and elsewhere in this issue of *BioScience*.

Positive aspects of the epilogue

Maryland officials later obtained additional evidence in support of their decision to follow our diagnosis on the presence of actively toxic *Pfiesteria* in the four toxic *Pfiesteria* outbreaks (Rublee et al. 1999, Magnien et al. 2000, Oldach et al. 2000). In other efforts to validate that research, a consensus group of officials and scientists from the Environmental Protection Agency, the Centers for Disease Control and Prevention (CDC), NOAA, 10 states, and several academic institutions formally recommended that research be focused on known toxic *Pfiesteria* species rather than on look-alike species that have not been toxic to fish under ecologically relevant conditions (*P*ICWG 2001). A national science panel later reevaluated *Pfiesteria* research for the CDC and also recommended a directed focus on known toxic *Pfiesteria* species rather than on look-alike taxa for which there is no evidence of similar toxicity (by live or sonicated cells at field densities) toward fish (Samet et al. 2001).

Funding constraints of laboratories with demonstrated expertise have continued to seriously impede progress in *Pfiesteria* toxin production and analysis. Therefore, assays to reliably detect *Pfiesteria* toxin from natural samples—the analytical tools that are critically needed by the CDC to conduct a meaningful, multistate epidemiological study, and by North Carolina and Maryland officials to design improved early warning systems to detect toxic *Pfiesteria* activity and thereby minimize human exposure—remain to be developed. During the 4 years since the Maryland toxic *Pfiesteria* outbreaks, NOS-Charleston has been the only research group to publish *Pfiesteria* toxin data that were obtained following appropriate quality control and assurance procedure (especially, use of toxic *Pfiesteria* cultures that were cross-corroborated by independent specialists as toxic strains of *Pfiesteria* spp.) (Fairey et al. 1999, Burkholder et al. 2001a, Kimm-Brinson et al. 2001). In mid-2001 the NOS-Charleston–based research team finished purification of a water-soluble neurotoxin from our *Pfiesteria* toxic culture material for chemical identification (J. Ramsdell and P. Moeller [NOS-Charleston, NC], personal communication, August 2001; patent process initiated).

Consensus by a panel of mid-Atlantic experts in support of the data for potential stimulation of *Pfiesteria* by nutrient pollution (Boesch 1997) provided the basis for Maryland's governor and legislature to pass the strongest regulations for nonpoint pollution control in the nation, including mandatory nutrient management practices for agricultural operations by 2002 (State of Maryland 1998, Magnien 2001). Linkages between *Pfiesteria* and nutrient pollution under some environmental conditions were also supported by a national panel of scientists who met at the request of North Carolina's governor (Wright 1998) and, later, by a national panel of scientists charged by the CDC to reevaluate the published literature on *Pfiesteria* (Samet et al. 2001). Samet et al. (2001) also endorsed the validity of our research on toxic *Pfiesteria* biology, detection and culture of toxic strains (including our standardized fish bioassays, with the required steps of cross-corroboration with other independent specialists), toxicity and lethality of *Pfiesteria* to fish in the laboratory, and lethality of *Pfiesteria* to fish in estuaries. With funding support from the US Department of Agriculture (USDA), EPA, and NOAA, many coastal states developed strengthened programs to improve nonpoint pollution control, monitor harmful algae in their regions, and assess chronic impacts of various stressors on fish health. The EPA and NOAA also have organized helpful workshops and provided publications for the citizenry (scientists and nonscientists) on *Pfiesteria* and other harmful algae. Environmental education has been expanded in many states, with greater overall national focus on water quality issues.

Spin-off initiatives have been many, and among the most encouraging were joint efforts by the USDA and the EPA to improve control of nonpoint pollution from concentrated animal operations, increased efforts by the poultry and swine industries to create value-added products from their wastes, and efforts to remove additional poultry wastes from the Delmarva peninsula (State of Maryland 1998). For example, the USDA (1999) directed $221 million to North Carolina farmers in the Albemarle-Pamlico watershed to help reduce nutrient pollution, and it cited fish kills, oxygen deficits, and toxic *Pfiesteria* outbreaks as the underlying basis for that action.

Several important steps were taken in North Carolina to alter state policy governing *Pfiesteria*. Information on all previous toxic *Pfiesteria* outbreaks was added to the official fish kill database (NCDENR 1998). The environmental and health agencies developed an early warning system to test for toxic *Pfiesteria* at fish kill or disease events, and a plan to clinically evaluate people exposed to in-progress fish kills shortly after they are exposed, when the clearest available diagnostic, cognitive impairment, is detectable (Glasgow et al. 1995, Grattan et al. 1998). Support for *Pfiesteria* research and for environmental education programs was also strengthened (Anderson and Stubbs 2000).

It remains important to consider *Pfiesteria* in its proper context. Many toxic dinoflagellates and other pathogenic microbes, including various species that have caused more serious health impacts than *Pfiesteria* (Falconer 1993, Burkholder

1998), inhabit estuaries. Like most pathogenic microbes, *Pfiesteria* spp. are cause for concern, but not for alarm, and proactive management can protect public health (Falconer 1993). Overall, the *Pfiesteria* issue has helped many people realize that water quality, fish health, and human health are strongly linked. Like many harmful algae (Falconer 1993), *Pfiesteria* outbreaks are sporadic (Burkholder and Glasgow 1997, Burkholder et al. 2001a). We hope that, beyond the short-term environmental and socioeconomic impacts of fish kills, beyond the progress realized in the two states that have had to confront toxic *Pfiesteria* outbreaks, and beyond the advances made in various other states as well as federal agencies to understand and protect fish and human health, this issue will eventually leave a more lasting legacy through strengthened efforts to manage and protect the quality of our nation's rivers, estuaries, and coastal waters.

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