

Field Ecology of Toxic *Pfiesteria* Complex Species and a Conservative Analysis of Their Role in Estuarine Fish Kills

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Within the past decade, toxic *Pfiesteria* outbreaks have been documented in poorly flushed, eutrophic areas of the largest and second largest estuaries on the U.S. mainland. Here we summarize a decadal field effort in fish kill assessment, encompassing kills related to *Pfiesteria* (49 major kills in North Carolina estuaries since 1991 and 4 in Maryland estuaries in 1997) and to other factors such as low oxygen stress (79 major fish kills in North Carolina estuaries). The laboratory and field data considered in developing our protocols are described, including toxic *Pfiesteria* behavior, environmental conditions conducive to toxic *Pfiesteria* activity, and impacts of toxic clonal *Pfiesteria* on fish health. We outline the steps of the standardized fish bioassay procedure that has been used since 1991 to diagnose whether actively toxic *Pfiesteria* was present during estuarine fish kills. Detailed data are given for a 1998 toxic *Pfiesteria* outbreak in the Neuse Estuary in North Carolina to illustrate of the full suite of diagnostic steps completed. We demonstrate that our conservative approach in implicating toxic *Pfiesteria* involvement in fish kills has biased in favor of causes other than *Pfiesteria*. Data are summarized from experiments that have shown stimulation of toxic *Pfiesteria* strains by nutrient (N, P) enrichment, supporting field observations of highest abundance of toxic strains in eutrophic estuaries. On the basis of a decade of research on toxic *Pfiesteria*, we present a conceptual model of the seasonal dynamics of toxic strains as affected by changing food resources and weather patterns. We also recommend protocols and research approaches that will strengthen the science of fish kill assessment related to *Pfiesteria* and/or other causative factors. **Key words:** dinoflagellates, dissolved oxygen, estuaries, fish kills, nutrients, toxic *Pfiesteria* complex. — *Environ Health Perspect* 109(suppl 5):715–730 (2001). <http://ehpnet1.niehs.nih.gov/docs/2001/suppl-5/715-730glasgow/abstract.html>

Dinoflagellates (*Pyrrophyta* or *Dinoflagellida*) include toxic species known to cause fish kills in estuaries and coastal marine waters (1–3). Within the past 15 years approximately 40 newly detected species of toxic dinoflagellates have been reported (3,4), including two species of ichthyotoxic *Pfiesteria* as *Pfiesteria piscicida* Steidinger & Burkholder and *P. shumwayae* Glasgow & Burkholder within the toxic *Pfiesteria* complex (TPC) (5–10). *Pfiesteria* spp. are considered unusual among toxic dinoflagellates in several characteristics: they have complex life cycles with multiple amoeboid stages and chrysophyte-like cysts (5,6,8); their prey range from bacteria to mammalian tissues (6,11,12); they express strong chemosensory attraction toward fish or their fresh excreta and tissues (8); and their toxic activity is triggered by live fish (5,8,13). However, they actually are similar to some benign (non-toxin-producing) freshwater and estuarine dinoflagellates in most of the above features (14,15). Toxic dinoflagellates produce among the most potent biotoxins known, including ichthyotoxins that act as neurotoxins in mammals (2,3,16). Ichthyotoxic activity of the two known *Pfiesteria* spp. has been reported from multiple laboratories in experimental trials (8,13,15,17–20). The two formally described *Pfiesteria* spp. produce bioactive substances with neurotoxic activity (21–23). These substances are called toxins, in accord with the *Pfiesteria* Interagency

Coordination Working Group (ICCWG) (24), acknowledging that these substances are only partially characterized (8,19,20,25), as is true for various other toxic dinoflagellates (2,3,16). Recently, a potent water-soluble *Pfiesteria* toxin was isolated and purified, and its chemical structure has been determined (26) (patenting process initiated).

In 1991 the toxic dinoflagellate eventually named as *P. piscicida* was first implicated as a causative agent of major fish kills in estuaries of North Carolina (5), especially the Albemarle–Pamlico Estuarine System, which is the second largest estuary on the U.S. mainland and among the most important fish nursery grounds on the U.S. Atlantic coast [(27,28); Figure 1]. This system is shallow, eutrophic, wind-mixed with little tidal effect, and poorly flushed, with a residence time in major tributaries of 50–100 days, on average, within an annual cycle (27,28,32). These features make the Albemarle–Pamlico especially sensitive to impacts from nutrient loading; winds easily resuspend nutrients deposited in the sediments, and poor flushing tends to retain nutrients in these waters (32–35). The Neuse and Pamlico Estuaries, major tributaries of the Albemarle–Pamlico, frequently have sustained phytoplankton blooms and bottom-water hypoxia/anoxia in violation of the state standards for water quality (36,37). Major fish kills and epizootics have occurred there during

warm seasons in most years since the early to mid-1980s (8,29,30,37,38). The mesohaline Neuse Estuary has been especially impacted by noxious algal blooms, oxygen deficits, toxic *Pfiesteria* outbreaks, and major fish kills and epizootics (29,32,35,37,38). Toxic strains of *Pfiesteria* spp. (6,9,24) thrive in estuarine waters affected by high nutrient loading from sewage, animal wastes, cropland runoff, and other sources (8,9,32,39,40) (Table 1).

It is important to assess whether actively toxic *Pfiesteria* is present at estuarine fish kills, because there is increasing clinical evidence that the toxin(s) produced by *Pfiesteria* can seriously impact mammalian as well as fish health (23,31,47). Toxic *Pfiesteria* spp. have been implicated in certain fish kills, called toxic *Pfiesteria* outbreaks (24), that have affected $>1 \times 10^9$ fish (8,9,29,30). Sometimes these kills have been referred to as fish kill/disease events, as nearly all have involve juvenile Atlantic menhaden (*Brevoortia tyrannus* Latrobe) with ulcerated lesions. These organisms have also been linked to the death of approximately 5×10^4 juvenile menhaden in several poorly flushed, shallow, nutrient-enriched tributaries of the largest estuary in area on the U.S. mainland, Chesapeake Bay in Maryland (8,9,41,42). Toxic strains of *Pfiesteria* spp. engage in toxin production when stimulated by substances from live fish, and under appropriate environmental conditions, dying and diseased fish have been used as sentinels for detecting potential toxic *Pfiesteria* activity (6,9,24,29).

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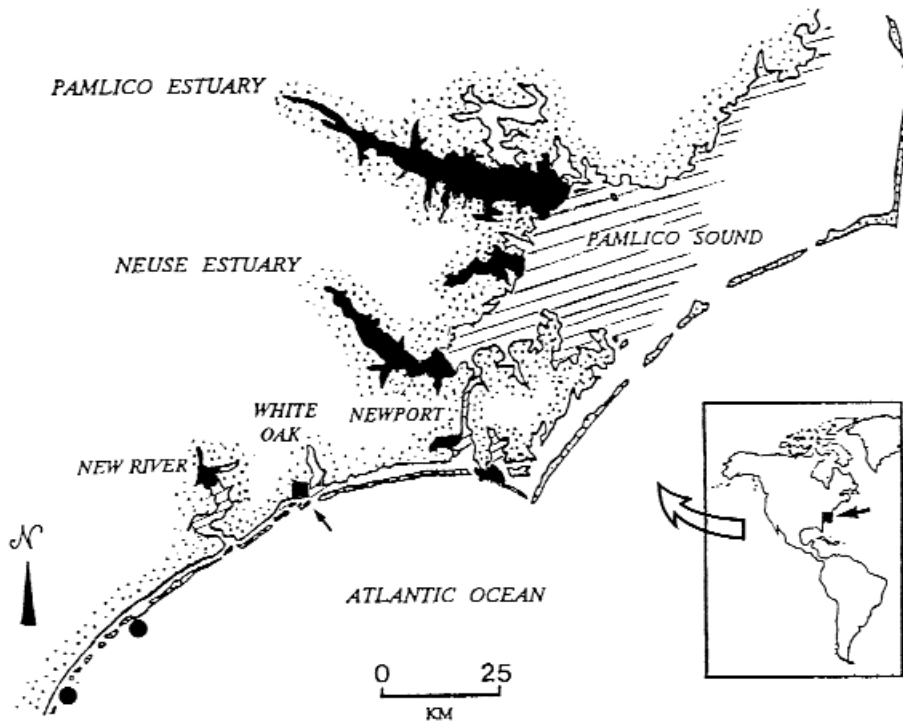


Figure 1. The Albemarle-Pamlico Estuarine System of North Carolina, second largest estuary in area on the U.S. mainland, and other locations affected by toxic *Pfiesteria* outbreaks in that state (27,28,34); blackened areas (5,8,9,29–32). Along the Atlantic coast these areas include (left to right) marine sites Wrightsville Beach and Topsail Beach (dots, lower left) and Taylors Creek, Beaufort; brackish sites include the Pamlico Estuary (Washington to Pamlico Sound), the Bay River (lower Neuse drainage), the oligohaline and mesohaline Neuse Estuary (New Bern to east of Oriental), and the Newport River. Also note the blackened square (arrow), indicating toxic *Pfiesteria* that contaminated a local aquaculture facility fed by Bear Creek, a brackish tributary of the White Oak River. Shaded areas (lines) indicate additional locations where we have documented potentially toxic *Pfiesteria* (TOX-B) populations in the presence of diseased fish.

Table 1. Ranges in some environmental conditions during toxic *Pfiesteria* outbreaks (i.e., in-progress fish kills linked to TOX-A *Pfiesteria*), including two North Carolina Estuarine Systems (Neuse and Pamlico within the Albemarle–Pamlico with means \pm 1 SE given for each, and the New River Estuary) and three Maryland estuaries within the Chesapeake Bay watershed for additional comparison [data given as means \pm 1 SE for the Pocomoke, and as ranges or medians [where available from the Maryland Department of Natural Resources (41) and Magnien (42)] for the Chicamaco and Kings Creek].^{a,b}

System	<i>n</i> ^c	Temp. (°C)	Salinity	DO ^c (mg L ⁻¹)	pH	Fish (no.)	Chl <i>a</i> ^c (µg L ⁻¹)	<i>Pfiesteria</i> -like cells mL ⁻¹	DOC (mg L ⁻¹)	TN (mg L ⁻¹)	DIN (mg L ⁻¹)	TP (mg L ⁻¹)	SRP (mg L ⁻¹)
Albe.-Pam.	44	18–29	2–16	4.8–10.4	6.8–8.4	>10 ⁹	12–112	3 × 10 ² –1.2 × 10 ⁶	4.5–19.0	0.5–1.7 ^d	0.01–0.85	0.07–0.70	0.03–0.65
Neuse	33	28 ± 1	10 ± 1	3.8–10.2	6.9–8.6	>10 ⁹	23 ± 0	970 ± 140	15.8 ± 3.2	1.01 ± 0.3 ^d	0.95 ± 0.15	0.21 ± 0.04	0.10 ± 0.01
Pamlico	11	29 ± 1	7 ± 1	4.2–10.4	6.6–8.3	>10 ⁶	19 ± 2	4,300 ± 2,510	8.3 ± 2.3	0.58 ± 0.0 ^d	0.11 ± 0.01	0.28 ± 0.11	0.29 ± 0.11
New River	2	31–33	9–13	6.8–8.0	7.7–8.4	1 × 10 ⁴	11 ± 1	1,200 ± 240	NA ^e	NA	0.06–0.08	0.16–0.30	NA
Chesapeake	4 ^f	18–28	1–18	4.9–8.0	6.1–8.0	5 × 10 ⁴	10–100	280–900	7.9 to >10	1.00 to >1.60	0.01–1.30	0.08–1.20	0.01–0.80

Abbreviations: Albe.-Pam. Estuarine System; Chl *a*, chlorophyll *a*; DIN, dissolved inorganic nitrogen; DOC, dissolved organic carbon; NA, not available; SRP, soluble reactive phosphate; TP, total phosphorus. ^aSee (9,29–32) for additional information on environmental conditions in North Carolina waters that have sustained toxic *Pfiesteria* outbreaks; 4–62 samples taken per event. In Chesapeake Bay, outbreaks occurred in the Pocomoke and Chicamaco Rivers and Kings Creek [Manokin drainage; see (41,42) for detailed information on environmental conditions in Maryland waters that have sustained toxic *Pfiesteria* outbreaks. *Pfiesteria*-like refers to dinoflagellate zoospores of similar appearance under light microscopy in water samples collected while/where fish were dying, later confirmed as toxic. ^bData modified from Burkholder et al. (9). ^cHere, we define a major fish kill as involving \geq 1,000 fish (8,9,43) and being sufficiently separated spatially and temporally to indicate that the events were distinct in occurrence. This approach to quantifying the kills is in accord with (8,9,29) and includes most of the fish kills described in [(30) and Table 1 therein]. In an alternate approach (32,34,41,42,44), the total number of fish kills was based on the number of field investigations in which dying fish were observed, irrespective of the number of fish counted during the investigation. Moreover, fish kills in close proximity (i.e., spatially as well as temporally) were considered separate events. Our approach here was to combine these events and consider them part of one overall kill with similar cause. For example, Glasgow and Burkholder (32) focused only on the Neuse Estuary from New Bern to Minnesott Beach/Cherry Point; thus, it did not include a kill that occurred in 1997 on the Bay River Estuary (lower Neuse drainage) and therefore listed 10 kills for the Neuse rather than 11 that year [(32) and Table 2 therein]. That study (32) reported 13 fish kills in the Neuse Estuary during 1995 from early July through late October; these events were collectively considered in Burkholder and Glasgow (8) for the Neuse within an early July period (encompassing 2 of the kills) and a late July–October period (encompassing 11 of the kills). Also note that Burkholder and Glasgow (8) reported information on other kills that was collected only by our laboratory. Here, we report information for major fish kills related to causes other than *Pfiesteria* in the state environmental agency database (37), which includes data from our laboratory and other sources. Overall, 49 major fish kills have been related to toxic *Pfiesteria* in North Carolina waters, but most environmental data were lacking for 3 of the kills (not included in this table) that occurred in marine waters (salinity of 35; locations Taylors Creek and the Atlantic Ocean off Topsail Beach and Wrightsville Beach). ^dUpper two-thirds of the water column considered for DO measurements in kills of surface-schooling, juvenile Atlantic menhaden, which accounted for >90% of the affected fish. In the Neuse Estuary, the value 3.8 mg DO L⁻¹ was recorded at 2–4 m depth in several of many *Pfiesteria*-affected sites nearly 2 weeks into an ~4-week kill in October 1995, but for the first ~13 days of the kill, DO had been >5 mg L⁻¹ throughout the water column. Chl *a* is given as an index of phytoplankton biomass (45). The state standard for acceptable water quality is <40 µg L⁻¹ in North Carolina waters and \leq 15 µg L⁻¹ in Maryland waters (36,46). ^eNitrogen values reported are for total Kjeldahl nitrogen rather than total nitrogen where indicated. Phosphorus values are reported as dissolved inorganic phosphorus rather than SRP where indicated. TP and DOC data are also included where available. Nutrient values (nitrogen, phosphorus, and carbon) are rounded to the nearest 10 µg L⁻¹; see (32) for methodologies. ^fThe Maryland Department of Natural Resources (41,42) considered toxic *Pfiesteria* activity separated by a 2-week interval in the same area of an estuary as two separate outbreaks (*n* = 9–13 samples per event in Maryland waters). Moreover, Maryland considers all fish kills involving \geq 50 fish (44). Here, we have considered major fish kills (\geq 1,000 fish affected) but if quantified following Maryland Department of Natural Resources protocols, Maryland waters have sustained 4 toxic *Pfiesteria* outbreaks (~50,000 fish affected), and North Carolina waters have sustained 88 toxic *Pfiesteria* outbreaks (>>1 billion fish affected).

When attempting to make the difficult step from correlation to implication of causality in a field setting, the available data for multiple causative factors should be considered [(8,43); and see Burkholder et al. (29) in formal correction of Paerl et al. (48)]. Here, we present an overview of our fish kill assessment protocols, which were designed on the basis of *a*) the known behavior of toxic *Pfiesteria* strains from laboratory studies compared with field observations; *b*) environmental factors that have been experimentally shown to be conducive to toxic *Pfiesteria* activity; *c*) the standardized fish bioassay procedure for detecting *Pfiesteria* populations that were actively toxic at estuarine fish kills; and *d*) known impacts of toxic *Pfiesteria* on fish health, from laboratory studies with clonal toxic *Pfiesteria* strains. We reemphasize the importance of the standardized fish bioassay procedure, developed in our laboratory from an early technique by Smith et al. (49), as the cornerstone technique used for our toxic *Pfiesteria* research, from laboratory experiments to estuarine fish kill assessment, during the period from 1991 to the present (5–10). After providing this information on the basis for our protocols, we summarize a decadal field effort in fish kill assessment, encompassing fish kills related to low oxygen stress, *Pfiesteria*, and miscellaneous causes such as pesticide spills. We demonstrate that use of our conservative approach consistently has biased in favor of causality other than *Pfiesteria*. A toxic *Pfiesteria* outbreak in July

1998 in the Neuse Estuary (37) is examined in detail to illustrate the full suite of diagnostic steps that were completed, including consideration of dissolved oxygen (DO) and other potential stressors. We also present comparative information on the nutritional ecology of *P. piscicida* and *P. shumwayae*. In addition, we provide an empirical model from a decade of research on the field ecology of *Pfiesteria*, emphasizing the seasonal dynamics of zoospores (the predominant planktonic stages) of toxic strains through changing weather patterns and nutrient dynamics from both correlative and experimental approaches. On the basis of our detailed, long-term data set on environmental conditions, fish kills, and toxic *Pfiesteria* activity, we also recommend protocols and research approaches that will strengthen both the science of fish kill assessment and insights about interactive environmental factors that influence both *Pfiesteria* and estuarine fish health.

Characteristics of Toxic *Pfiesteria* That Influence Fish Kill Assessment

Many heterotrophic dinoflagellates are difficult to grow in defined media because their nutritional requirements include as-yet-unidentified organic substances (50). *Pfiesteria* spp. have not been cultured successfully without a prey source, and thus far it has not been possible to induce toxin production unless live fish are added (8–10,13,19,20).

Like various other so-called toxic algae [in this article, including heterotrophic and mixotrophic dinoflagellates as defined in Burkholder (51); e.g., (52–57)], *Pfiesteria* spp. have both toxic and benign strains (non-inducible, NON-IND (9,24)). Benign strains apparently are incapable of toxic activity, or produce negligible toxin (9). Moreover, toxic strains exist as actively toxic (TOX-A) or temporarily nontoxic (TOX-B) functional types depending on environmental conditions, especially presence/absence of live fish (9,24,58) (below).

On the basis of >2,000 standardized fish bioassays (5,8,9,59) (below), toxic strains of *Pfiesteria* spp. (temporarily nontoxic, or the TOX-B functional type, having been without live fish) gradually become actively toxic (TOX-A functional type) when they detect live fish (8,9,24,58). Without live fish, *Pfiesteria* zoospores are uncommon in the water column of the assay vessel (<25 cells mL⁻¹, usually <10 cells mL⁻¹; $n = 60$), but they significantly increase in abundance after live fish are added. The rate of zoospore population increase depends on the frequency of fish additions. In trials with shorter time to availability of live fish, zoospores increased to sufficient densities to cause fish mortality (~300 cells mL⁻¹, $n > 2,000$) (5,8,9,59) more

rapidly than in trials with longer time intervals. Maximum zoospore densities (usually $>3 \times 10^2$ to 10^3 cells mL⁻¹, sometimes 10^4 cells mL⁻¹) consistently were observed around the time of fish death, followed by gradual to rapid declines (hours to days) in zoospores, depending on the *Pfiesteria* isolate (5,9). Zoospores encysted or formed palmelloid masses (5,6,8,58) and settled to the bottom of the assay vessel after fish death. Alternatively, if additional live fish were not added and dead fish were left in the cultures for >12 hr, zoospores of some toxic strains transformed to amoebae that attached to and fed upon the fish remains (5,7–10,58). If abundant alternate prey (e.g., certain algal species such as cryptomonads) were made available (6), zoospores sometimes remained in the water column as TOX-B forms.

TOX-A cells cease toxin production shortly after fish death (8). TOX-B cells gradually can be induced to become actively toxic again when they detect chemical stimuli from additional live fish (5,8). The biochemical pathways involved in *Pfiesteria* toxic production apparently require time for activation if the population has not recently been in toxic mode. Thus, a previously inactive (encysted or TOX-A or TOX-B) population can require days to weeks to become active in producing toxin (9). In contrast, a TOX-A population that killed fish recently (hours) can be lethal to fish within minutes to several hours, depending on the potency of the strain, its cell density, the number of fish per volume of medium, the health of the fish, and other factors (below) (5,8,9).

Laboratory trials, supported by field observations (Tables 1, 2), indicated that certain conditions are conducive to *Pfiesteria* toxicity. Toxic zoospore activity was documented at temperatures $>15^\circ\text{C}$ (North Carolina clones; it should be noted that apparent toxic activity by lobose amoeboid stages also was observed in laboratory fish cultures on two occasions at temperatures $<15^\circ\text{C}$) (5,8). Temperatures $\geq 25^\circ\text{C}$ were optimal for toxicity (8), with toxic activity and fish death occurring up to approximately 33°C (5,8–10). Field studies (together with standardized fish bioassays; below) indicated toxic *Pfiesteria* activity at temperatures $\geq 18^\circ\text{C}$ (rarely, -15°C), and at salinities ranging from approximately 2 to 16 (5,8,9,13,60). In laboratory trials the optimal salinity for toxic zoospore activity was approximately 15, with toxicity leading to fish death across a broad salinity range from 2 to 35 (5,8,10,60). Toxic activity has also occurred in dense fish cultures under freshwater conditions (salinity <1) (45) when calcium is ≥ 10 mg Ca⁺² hardness mL⁻¹, but *Pfiesteria* spp. grow slowly in fresh waters and likely survive poorly in natural freshwater environments (30).

Other conditions that influence toxic *Pfiesteria* activity in trials with laboratory fish are pH (≥ 6.8 required; $n > 400$, pH range of 6.8–8.4), dissolved oxygen [≥ 3.8 mg DO L⁻¹ needed in culture, although field populations have sometimes remained active at lower DO (8)], and turbulence. Like certain other dinoflagellates (64,65), toxic stages of *Pfiesteria* are sensitive to excessive turbulence. For example, in 1-hr laboratory trials, 29 ± 3% of toxic zoospore populations of *P. shumwayae* (clone 101238) formed temporary cysts under moderate turbulence (400 rpm; Fisher orbital shaker model 361, Fisher Scientific, Atlanta, GA, USA), versus negligible temporary cyst production in unmixed controls (10^3 zoospores mL⁻¹; $n = 12$). In estuaries during toxic *Pfiesteria*-related fish kills that were interrupted by moderate storm events of short duration (hours to <1 day), *Pfiesteria* zoospores encysted and/or sank from near the water surface (where fish were dying) down approximately 0.25 m above the sediments where the water had remained calm (8). The data suggest that TOX-A *Pfiesteria* tends to avoid high-wave-action, wind-mixed surface waters. Shortly after calm conditions reestablished (hours to 1–2 days), *Pfiesteria* zoospores moved back up to surface waters, and additional fish death occurred within hours to several days (8). Following major storms (e.g., Hurricanes Bertha and Fran in 1996), low *Pfiesteria* activity was documented for longer periods (weeks to months) (8,9,32). Additional laboratory trials with TOX-B zoospores (North Carolina and Maryland isolates) showed that relatively high nutrient levels (>100 µg NO₃⁻N or PO₄⁻³P L⁻¹) can stimulate TOX-B *Pfiesteria* growth, mediated through algal prey abundance and through direct inorganic nutrient uptake by kleptochloroplastidic cells (8,10,12,61,62). Moreover, TOX-B zoospores can utilize organic C, N, and nutrient forms (8,9,11,12,61,62). All points in this section were considered when designing scientifically sound protocols for evaluating *Pfiesteria* involvement in estuarine fish kills (below).

Standardized Fish Bioassays

We consistently have used a standardized fish bioassay procedure (59), which follows Henle-Koch postulates modified for toxic rather than infectious agents (66,67), to assess whether toxic *Pfiesteria* is involved in estuarine fish kills that occur under appropriate environmental conditions as indicated above (8,9,24,29,30) (Figure 2). In early research, our data for *Pfiesteria* fish-killing activity were cross-confirmed in parallel work by the independent laboratory of E. Noga (17,49). This standardized procedure has been cross-corroborated by Lewitus et al. (61,72) and Marshall et al. (15).

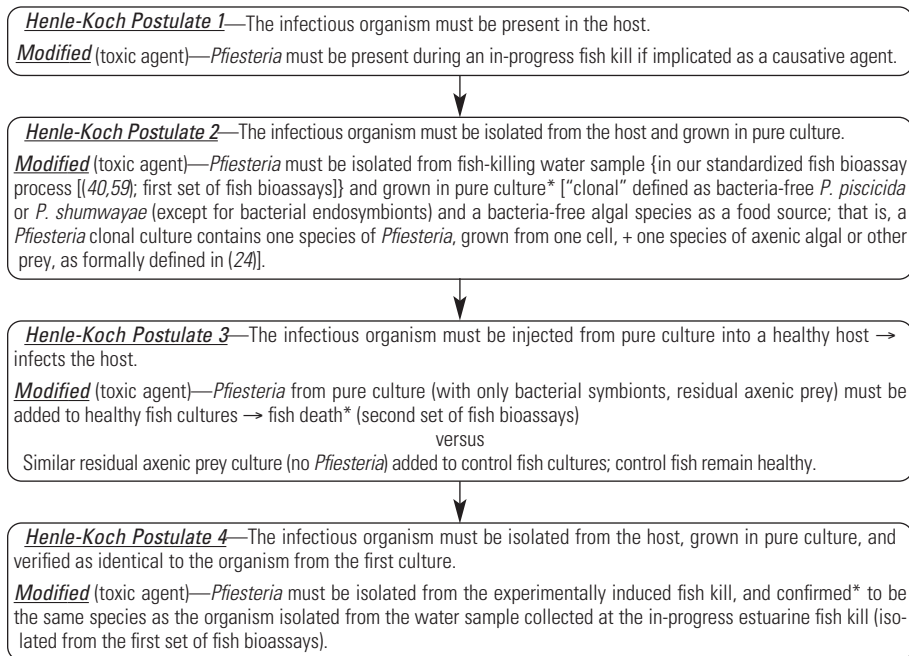


Figure 2. Schematic depicting how our standardized fish bioassay procedure to implicate TOX-A *Pfiesteria* in a fish kill follows the Henle-Koch postulates, modified for toxic rather than infectious agents. Asterisks (*) indicate the steps at which we obtain cross-corroboration by independent specialists with demonstrated expertise in *Pfiesteria* species identifications [for example, laboratories of P. Rublee (68–70) and D. Oldach (71), respectively] and toxicity [H. Marshall, Old Dominion University (15)].

Standardized fish bioassays must be used in fish kill evaluations for toxic *Pfiesteria* involvement for the following reasons. First, light microscopy (LM) cannot be used to distinguish *Pfiesteria* spp. from numerous benign estuarine look-alike species (so-called pfiesteria-like organisms that physically resemble *Pfiesteria*) (8,24). Second, species-specific molecular probes [first available in 1998 for *P. piscicida* (68) and in 1999 for *P. shumwayae* as *Pfiesteria* species B (71)] can detect the presence of *Pfiesteria* spp., but cannot discern whether they are in TOX-A (as opposed to nontoxic) mode (9,24,68–70). Third, efforts to diagnose whether TOX-A *Pfiesteria* spp. (or as-yet-undetected additional toxic *Pfiesteria*-like species) are involved in estuarine field fish kills or fish epizootics have remained handicapped because insufficient quantity of purified *Pfiesteria* toxin (26) has been available to develop field-reliable assays for toxin detection (19,20,25,59). Therefore, properly conducted fish bioassays are the “gold standard,” that is, the only reliable technique presently available, to test for the presence of TOX-A strains of *Pfiesteria* spp. (and of other, as-yet-unknown toxic *Pfiesteria*-like dinoflagellates) from natural water or sediment samples [(8,9,15,40,59); see *Pfiesteria* Interagency Working Group (PICWG) (24) for a consensus document defining much of the correct terminology used in *Pfiesteria* research]. The standardized fish bioassay procedure (59) is a powerful tool

in *Pfiesteria*-related fish kill assessment because it provides a reliable although conservative means to determine whether TOX-A *Pfiesteria* was present at the estuarine kill while fish were dying.

Standardized fish bioassays should be conducted in biohazard Biosafety Level 3 facilities to prevent human contact with toxic aerosols and water from fish-killing *Pfiesteria* cultures (59). Required quality control/assurance protocols should include in-house replication and, importantly, cross-corroboration of the results by independent laboratories with demonstrated expertise in culturing TOX-A *Pfiesteria* (59), replicated as recommended by the U.S. Environmental Protection Agency (U.S. EPA) (73). The procedure follows four basic steps:

- Step 1. Unpreserved water samples taken from the in-progress kill, while/where fish were dying (below), are incubated with live fish in the first set of test fish bioassays. Water quality conditions (temperature, salinity, light, pH, nutrients, etc.) (59) in the sampling site are simulated as closely as possible. Control bioassays are set up identically but without the estuarine sample, and control fish should remain healthy. If potentially lethal densities of *Pfiesteria*-like zoospores (≥ 300 cells mL⁻¹) (5,8,9,24) develop in association with fish death (repeated for two sets of fish), and if other cause(s) are not discerned among many monitored physical,

chemical, and biological variables (59), then the fish bioassay is positive for the presence of a toxic *Pfiesteria*-like organism. The organism is identified to species with scanning electron microscopy (SEM) of suture-swollen (6,8–10) or membrane-stripped cells (7,8). SEM of suitably prepared cells is considered the “gold standard” technique for identification (9,24) rather than molecular probes, which have tested thus far as species specific, but which might cross-react with as-yet-undescribed look-alike species.

- Step 2. From the first set of replicated positive fish bioassays, the *Pfiesteria*-like dinoflagellates are cloned using flow cytometric or LM procedures (9,10,58). Each clone initially consists of one axenic dinoflagellate cell, to which axenic algal prey is added as a food source. The clonal cultures (defined as in Burkholder et al. (9) and the PICWG (24) are grown to 10^2 – 10^3 zoospores mL⁻¹). The cultured dinoflagellates are allowed to graze the prey to low levels (~5–10 cryptomonad cells mL⁻¹).
- Step 3. The clonal populations (with residual algal prey) are added to a second set of test fish bioassays. Control fish bioassays are maintained identically, except that only residual algal prey are added, and control fish should remain healthy. If potentially lethal densities of *Pfiesteria*-like zoospores develop in association with fish death (repeated for two sets of fish), and if other causative factor(s) are not discerned among many monitored physical, chemical, and biological variables (59), then the second set of fish bioassays is positive for a toxic *Pfiesteria*-like organism. Culture toxicity is cross-corroborated by an independent laboratory with demonstrated expertise in conducting standardized fish bioassays (9).
- Step 4. The dinoflagellate population(s) associated with fish death in the second set of positive fish bioassays is re-cloned, grown with (initially axenic) algal prey, and identified to species (SEM as above; species identification cross-corroborated by an independent laboratory) (59).

The time to fish death is the key to interpreting the fish bioassays and is affected by sample handling (59). In assessment of a *Pfiesteria*-related fish kill, water (or sediment) sample collection would separate TOX-A *Pfiesteria* from dying fish. Additional, unavoidable stress on the population is imposed from jostling during sample transport, temperature changes (e.g., if samples are shipped by express mail or other means), and hypoxia (e.g., if samples are capped tightly for hours to days of transport). These factors promote encystment of *Pfiesteria* cells that were

actively toxic when sampled. In particular, TOX-A zoospores are highly sensitive to separation from live fish and often will encyst (usually within hours): We tested temporary encystment by a TOX-A clonal culture of *P. shumwayae* (#864T; isolated from the Neuse Estuary 6 months earlier and actively toxic in cultures with live fish for 2 months; 2×10^4 zoospores mL^{-1}) in response to separation from live fish for 1 or 3 days. Control subpopulations were not transported; treated subpopulations were transported by boat and automobile for 1, 3, or 7 hr ($n = 4$). After 1 day without live fish, $48 \pm 2\%$ (mean ± 1 SE) of control zoospores had formed temporary cysts, whereas 75% (1- to 3-hr transport) to 95% (7-hr transport) of transported cells had encysted. After 3 days without live fish, $\geq 85\%$ of zoospore subpopulations in controls and all treatments had encysted.

We also tested subpopulations of the same actively toxic *P. shumwayae* culture for ichthyotoxic activity after 1 or 3 days of separation from live fish, using standardized fish bioassays [tilapia, *Oreochromis mossambicus*, total length (t.l.) 5–7 cm, 4–5 fish per 7-L assay; initial *Pfiesteria* zoospores + temporary cysts, 2×10^3 cells mL^{-1} ; other conditions as in Burkholder et al. (59)]. Subpopulations were not transported (controls) or were transported as above for 1, 3, or 7 hr ($n = 4$). With increased duration of separation from live fish and/or increased duration of transport, the time required for *Pfiesteria* to resume lethal activity toward additional test fish increased. After 1 day without live fish, time to fish death was < 1.5 days for subpopulations that had been transported for 0–1 hr versus 4–6 days for subpopulations transported 3–7 hr. After 3 days without live fish, controls killed fish in 4 days, whereas transported subpopulations required 6 (1-hr transport) to 13 days (7-hr transport).

TOX-A *Pfiesteria* populations acclimated to culture conditions for 1–3 months have shown this relatively rapid ichthyotoxicity when reexposed to live fish. Recent field isolates generally have required longer (≥ 4 to ~ 21 days) to resume lethal activity toward fish following 3–7 hr of transport (via boat and automobile) and 1–3 days without live fish (59). Thus, following separation from fish for ≤ 3 days in combination with various modes of sample transport (via boat, automobile, and/or airplane), when recently TOX-A *Pfiesteria* cells in an estuarine water sample from an in-progress fish kill are added to standardized bioassays, they typically have encysted and resumed lethal activity toward newly added test fish within 4–9 days (90% confidence interval, $n = 20$ fish bioassays with 10 fish per replicate; 95% confidence interval at ≤ 21 days) (59). Populations that were not recently in TOX-A mode have required considerably

longer (6–8 weeks) to kill fish in bioassays (59). This lag time in response of recently toxic *Pfiesteria* to additional live fish or other prey, following separation from live fish along with disturbance during sample handling, is also an important consideration in designing mesocosm studies and other appropriate experiments in *Pfiesteria* research (Table 2).

Field diagnostics are not available for many factors harmful to aquatic life (e.g., various toxic substances, virulent strains of *Vibrio* spp. and other microbial pathogens) (74,75). Therefore, samples frequently must be incubated or otherwise treated so that inferences can be made about involvement of those factors in the death of wild fish (74,75). The standardized fish bioassay procedure somewhat analogously involves incubating estuarine water (or sediment) samples with live fish to obtain indication of whether TOX-A *Pfiesteria* was present at the fish kill. A national peer-review panel recently evaluated our fish kill assessment procedures in detail and concluded [(40), pp. 17, 23–25]:

That *Pfiesteria* can become ichthyotoxic upon exposure to live fish under appropriate laboratory conditions appears to be clearly established. . . . The preponderance of evidence from laboratory and field investigations [also] supports the proposition that *Pfiesteria* has caused fish kills in estuaries. . . . The behavior reported for *Pfiesteria* is consistent with the considerable global experience with other ichthyotoxic algal bloom species, their impacts, and established procedures applied by harmful algal bloom researchers. . . . Traditional methods of analysis typically cannot be applied to detecting and monitoring *Pfiesteria* involvement in fish kills for several reasons: a) its cryptic appearance, low abundance, and multiphasic life

cycle; b) its explosive, ephemeral bloom events; and c) the existence of morphologically similar, but ecophysiologicaly distinctive pfiesteria-like species. . . . A rigorously standardized fish bioassay process has been used to replicate and confirm key findings (8,30) concerning the ichthyotoxicity of *Pfiesteria*. [(15); also (18,60); fish bioassay procedure of the Center for Applied Aquatic Ecology (CAAE) (9,29–31)]

Impacts from Controlled Exposure of Fish to Toxic *Pfiesteria*

Standardized fish bioassays have been used to study impacts of toxic *Pfiesteria* on various species of cultured fish. Early experiments repeatedly documented death of test fish (76) in acute exposure to *P. piscicida* (5,17,30,49). Juvenile and/or adult stages of nine estuarine and seven exotic (nonnative) finfish species, as well as juveniles or adults of four shellfish species, were tested with toxic clonal *P. piscicida* in acute toxicity tests, with survival compared to that of control animals that had been similarly maintained but without toxic *Pfiesteria* exposure [(76) including scientific names]. Within minutes (bay scallops, some finfish species depending on the *P. piscicida* strain), hours (many finfish species), or days (blue crabs), all individuals ($n \geq 6$ –10 per species) of all species died when exposed to toxic *P. piscicida*, with the exception of adult eastern oysters, which showed depressed filtering rates but remained alive after > 3 weeks of exposure to toxic *P. piscicida* (5,8,30). The history of recent exposure to live fish influenced time to death of additional live fish. Over time, as live fish were added to toxic *P. piscicida* clonal cultures with dead fish

Table 2. Conducive conditions for *Pfiesteria* zoospore production, compiled from experimental laboratory and field data. Optima, where known, are indicated in parentheses, with ranges indicating data for isolates from different geographic regions.^a

Variable	Conducive to cell production	Negligible or slow cell production
Temperature	> 20 – 30°C ($> 26^\circ\text{C}$)	$< 20^\circ\text{C}$, $> 30^\circ\text{C}$
Salinity (60)	> 5 – 20 (10–15)	≤ 5 , ≥ 20
Light (quantity, photoperiod)	0–300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$	$> 300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$
Nutrient regime ^b	$\gg 100 \mu\text{g N}_i$, $\text{P}_i \text{ L}^{-1}$	$< 100 \mu\text{g N}_i$, $\text{P}_i \text{ L}^{-1}$
pH	6.6–8.6 (> 7.5)	< 6.6 , > 8.6
Water motion	Low turbulence	Mixed
Acclimation period (after transport, for cell production, toxic activity)	> 3 –7 days	< 3 –7 days
Algal prey (zoospores, amoebae)	Cryptomonads	Cyanobacteria, other picoplankton
Finfish prey (zoospores)	Many species	<i>Gambusia</i>

Abbreviations: N_i , inorganic nitrogen (nitrate, NO_3^- , and ammonium, NH_4^+), P_i , inorganic phosphorus (phosphate, PO_4^{3-}). ^aFrom laboratory experiments (5,6,8,11–13,61,62). ^bFrom laboratory experiments (5,8,12,13,58,62) together with field data from North Carolina and Maryland estuaries (31,32,41,42). Note that in contrast to these studies, a recent short-term mesocosm study by Pinckney et al. (63) found no apparent nutrient stimulation of pfiesteria-like organisms. From recent evaluation of that work, however, a national peer-review panel (40) concluded that “the study was compromised by serious experimental flaws (control populations usually died off) and taxonomic uncertainties, inadequate consideration of *Pfiesteria* life cycle stages and their trophic regulation, and a restricted data focus” [(40), p. 22]. The Pinckney et al. study (63) did not find *Pfiesteria* spp. among the look-alike organisms. In addition, the experiment duration was too short to enable *Pfiesteria*, had it been present, to increase cell production at the near-freshwater salinities that were imposed (30). The national peer-review panel (40) instead supported the research from our laboratory and others (8,12,61,62), indicating that under conditions conducive for growth, “*Pfiesteria* responds both via increased abundance and life cycle transformations, to enrichment with inorganic and organic nutrients [Burkholder and Glasgow (6,8), Burkholder et al. (5), Lewitus et al. (61)].” [(40), p. 22].

removed, time to fish death decreased from days to <30–60 min (5,9). Similar trials, with similar outcomes, have been conducted with toxic strains of *P. shumwayae* and tilapia (*O. mossambicus*), fathead minnows, sheepshead minnows, and adult guppies (13,25,26).

Toxic strains of *Pfiesteria* spp. zoospores (TOX-A, TOX-B functional types) commonly attack fish gills and skin and feed upon the tissues [via myzocytosis (50)—attachment and suctioning of tissue contents with the peduncle (8,9,58,77)]. The two *Pfiesteria* species thus far have produced analytically comparable toxin (26), but considerable intraspecific differences among isolates can occur in toxin potency and in the extent to which toxin is released (exotoxin) versus retained (endotoxin) within the cells. For some toxic strains, fish death has occurred whether *Pfiesteria* zoospores were allowed direct contact or were maintained within dialysis membrane (<0.22- μ m porosity) or cellulose dialysis tubing (molecular weight cutoff 12,000–14,000 Da) to prevent direct contact (8,77), indicating that exotoxin(s) from those

toxic *Pfiesteria* strains was involved. Other strains have killed only when allowed direct or nearly direct contact with the prey. 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 P2X₇ receptors (20). The cultures used for that research were tested as capable of killing fish when prevented from direct contact with prey. The mechanism of targeting P2X₇ receptors and the cascade of impacts (including extreme response to inflammation) that followed would be optimized with physical abrasion or damage (20). Physical attack by toxic *Pfiesteria* zoospores may help to promote toxin entry into 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.

In all of the above trials, $\geq 99\%$ of the control fish remained healthy with no signs of stress or disease. However, *Pfiesteria*-exposed fish showed neurological signs within minutes to hours, including depression, loss of equilibrium, episodic hyperexcitability, and decreased respiration (30,78,79). Densities of $\geq 1 \times 10^2$ (subacute exposure) to 5×10^3 toxic zoospores mL⁻¹ (clonal *P. piscicida* or *P. shumwayae* cultures, or *P. piscicida* + *P. shumwayae* mixed cultures) induced epithelial destruction and lesions. In repeat trials ($n = 12$), acute lesions formed within ≤ 12 hr (sometimes in ≤ 2 hr, typically in <8 hr), generally with hemorrhaging (sometimes within minutes) and often culminating in rupture of the peritoneal sac with exposure of the viscera (Figure 3). Diffuse superficial dermatological lesions involved intra- and extracellular edema and necrosis of epithelium (with pyknotic and eosinophilic cytoplasm), progressing to erosions that extended through the basement membrane (50–80% loss of epidermis). Epidermal and skeletal muscle tissues had mild to severe multifocal granulocytic and lymphocytic epidermatitis; moderate dermal edema; marked diffuse lymphocytic epidermatitis; and/or mild to marked necrotizing lymphocytic epidermatitis. Deep focal lesions often developed also, mostly on the ventral surface by the pectoral fins or the anus (Figure 3). Other impacts (78–80) ($n = 9–12$) have included severely increased osmolality with elevated serum levels of sodium, potassium, and chloride to similar levels as the surrounding medium (at a salinity of 15); depressed white blood cell count (to 40–60% of that in control fish); in gill, cytomegalic bacteria inclusions, moderate, diffuse edema of secondary lamellae epithelium (associated with moderate edema of primary lamellar epithelium) (Figure 4); in

hepatopancreas, mild multifocal lymphoplasmacytic, granulocytic (sometimes necrotizing) hepatopancreatitis; in kidney, mild multifocal tubular mineralization \pm granuloma formation; and in brain, occasional moderate subacute to chronic multifocal meningitis, mild to acute multifocal granulocytic optic neuritis, and encephalitis. In contrast, control fish, maintained similarly except without exposure to toxic *Pfiesteria*, remained healthy and did not show pathologies except for occasional mild epidermal granuloma formation.

When juvenile tilapia (*O. mossambicus*) or juvenile hybrid striped bass (76) that had developed lesions in acute, sublethal exposure to *Pfiesteria* were removed from toxic cultures and allowed to recover for 6 weeks, the lesions healed, but the fish were more susceptible to new infections from opportunistic bacteria and fungi. About 80% of the test fish developed lesions with moderate to severe, acute myonecrosis (78). Control fish, treated identically except for no prior exposure to toxic *Pfiesteria*, remained healthy without signs of disease. The observations from these controlled exposures of fish to toxic clonal and mixed *Pfiesteria* populations at field densities (8), collectively considered, were important in designing protocols to assess toxic *Pfiesteria* involvement in fish kills.

Estuarine Fish Kill Assessment

Our laboratory has monitored and assessed fish kills in North Carolina estuaries and coastal waters since 1991 (5,8,29–32). We have sampled the Neuse at least weekly throughout most years; on other occasions, kills were reported to us by the Neuse Riverkeeper, a citizen who was certified by the National Water Keepers Alliance and maintained a near-daily presence on the Neuse River and Estuary. Some kills were reported to us while they were in progress, particularly in recent years, by the North Carolina Department of Environment and Natural Resources (NCDENR) (37).

We have focused our assessments on fish kills rather than epizootics in the absence of dying fish (5,9,29–32). The uncertainties inherent in attempting to diagnose the initial causes of ulcerated lesions are greater than those confronted in fish kills, as kills often occur in response to an acute rather than chronic stressor. Within that context we have limited most of our fish kill assessments to major kills, defined as affecting $\geq 1,000$ fish (43), and to in-progress kills that can be sampled while fish are dying but not yet dead. The latter point is important because of the tendency for toxic *Pfiesteria* zoospores to transform to benthic stages (amoebae, palmelloid stages, cysts) and rapidly attach to fish remains or settle out of the water column after fish death (5,6,9,12,13,58).

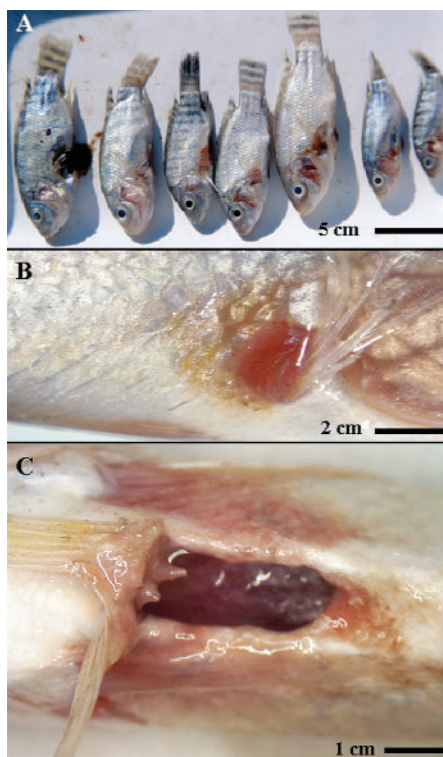


Figure 3. Focal lesion development resulting from exposure of tilapia (*O. mossambicus*, t.l. 5–7 cm) to TOX-A, clonal *P. piscicida* (Neuse isolate ND-PP990708) in controlled laboratory trials [fish bioassays (59)] including (A) tilapia after 8–12 hr exposure to $2.3–5.4 \times 10^3$ toxic zoospores mL⁻¹ (scale bar = 5 cm); (B) oblique lateral view, posterior to the pectoral fin, showing subepidermal hemorrhaging (scale bar = 2 cm); and (C) oblique lateral view showing a deep, bleeding, ulcerated focal lesion posterior to the pectoral fin (scale bar = 1 cm).

Sampling must be conducted carefully to follow this caveat of focusing only on in-progress fish kills. In practice, it is difficult to arrive at the scene of a fish kill while fish are still dying but not yet dead, because fish often float just below the water surface when they are moribund and come to the surface only after death. Nevertheless, to implicate toxic *Pfiesteria*, fish kills should not be sampled hours or longer after the fish are all dead. It is important to avoid spatial as well as temporal mismatches between the fish kill and sampling. By the time fish are sampled after capture, the boat may have drifted or the tide may have flushed out the water that was associated with the fish contained in, for example, a cast net held over the side. Commonly, when toxic *Pfiesteria* is involved in a kill, samples taken in the immediate location of the dying fish have contained ≥ 300 zoospores mL^{-1} , but samples taken only approximately 70 m from the site have contained little or no *Pfiesteria*. The stipulation that water samples must be sampled while fish are dying but not yet dead is highly conservative and probably underestimates toxic *Pfiesteria* activity. For example, water samples collected approximately 24 hr after fish death could contain approximately 200 *Pfiesteria* zoospores mL^{-1} , representing a portion of the population that was actively toxic during the kill but which subsequently switched to other prey that were abundant in the area. Yet, by our protocols requiring consideration of only samples from in-progress kills, the kill technically could not be related to toxic *Pfiesteria*. A field-reliable assay for *Pfiesteria* toxin, applicable for use in water samples as well as fish tissue, will enable appropriate consideration of events detected and sampled post-kill.

Causality of fish death by microbial pathogens or other factors can be inferred, but usually cannot be proven conclusively in a field setting (43,81). Other features of our protocols for assessing toxic *Pfiesteria* are also conservative in consideration of that fact. As a primary consideration, at least 300 *Pfiesteria*-like zoospores mL^{-1} must be present [presumptive count (24); LM, 400–600 \times ; basis: laboratory trials as stated (8,9,24)] (Figure 2). Then, to implicate toxic *Pfiesteria* as a factor in the kill, active toxicity of *Pfiesteria* cells collected at the in-progress fish kill must be confirmed by (standardized) fish bioassays as described (9,15,24,29,30,32,65). However, even if the fish bioassays are positive for toxic *Pfiesteria*, *Pfiesteria* is not implicated as the primary causative factor of the kill if other potentially lethal factor(s) are detected in the affected area (8,29,59).

For example, approximately 90% of the fish that have died in toxic *Pfiesteria*-related fish kills were juvenile Atlantic menhaden, which are schooling fish that typically reside

0.5–1.0 m below the water surface (82–84). In research comparing near-surface (0.5- to 1.0-m depth) versus total water-column fishing gear (cast nets and trawls, respectively), $\geq 90\%$ of the total juvenile menhaden were collected in cast nets (41). These data provide further indication that juvenile menhaden reside near the water surface where they are

captured by cast nets (82–84). Furthermore, although most estuarine fish (including juvenile menhaden) can withstand short periods (hours) of 2–3 mg DO L^{-1} , motile fish actively avoid waters with < 2 mg DO L^{-1} when adjacent refuge areas are available (85–87). Thus, menhaden would not be expected to move down from surface waters

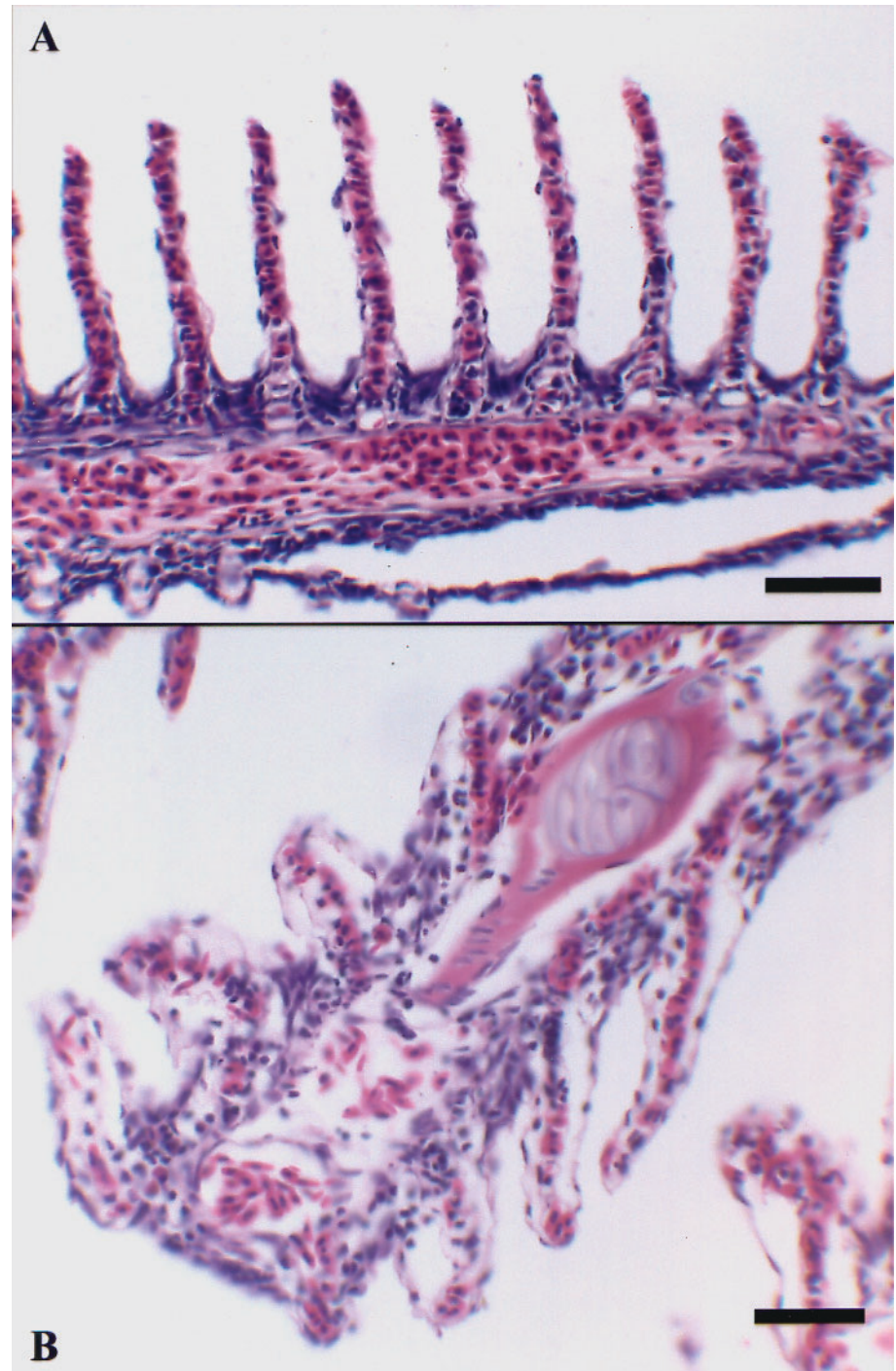


Figure 4. Gill tissue of (A) control tilapia (*O. mossambicus*, t.l. 5–7 cm), and of (B) test tilapia exposed for 8 hr to a culture of actively toxic (TOX-A) *P. piscicida* + *P. shumwayae* (isolated from the Pocomoke River, Maryland; 8×10^3 zoospores mL^{-1} at 8 hr; $n = 3$ fish examined, 2 of which were moribund at 8 hr). Scale bars = 0.1 mm. Photos courtesy of R. Smolowitz (80).

into a narrow band of hypoxic bottom water but would instead tend to move up toward the surface where refuge areas of oxygen-replete waters mostly occur. In a salinity-stratified water column with hypoxic bottom water, they would remain near the surface and would not encounter the low-oxygen conditions. Occasional encounters with low-oxygen bottom water by wind-forced upwelling would stress the fish, but would not kill them if DO was ≥ 2 mg L⁻¹.

In kills of these surface-schooling fish, we have implicated low-oxygen stress as the primary cause, even when actively toxic *Pfiesteria* was present based on fish bioassays (8,29); see Samet et al. (40) in correction of Stow (88). Low oxygen stress has been implicated as the primary cause if: a) anoxia—a condition of acutely low DO—approximately 0 mg L⁻¹ (29)—is present in more than the lower one-third of the water column of the affected area or immediately adjacent areas; or b) if hypoxia—a condition in which DO is < 4 mg L⁻¹ (29)—is present in more than the lower one-third of the water column over widespread areas within the kill zone. We have followed that practice in many cases wherein much of the upper water column was oxygen-replete [DO ≥ 5 mg L⁻¹ (29,36)] to provide refuge habitat (8,29,85–87). Considering these points, our protocol has been biased in favor of alternate causative factors such as low DO, rather

than *Pfiesteria*, as a primary cause of fish kills (Table 3, Figure 2). Accordingly, we have conservatively attributed 49 major fish kills to TOX-A *Pfiesteria* spp. as the primary causative factor(s) and 79 major fish kills to low DO stress or other causes (Table 4). Whereas 52 of 53 *Pfiesteria*-related fish kills (48 of 49 kills in North Carolina estuaries and 4 of 4 *Pfiesteria*-related kills in Maryland) have involved juvenile Atlantic menhaden with high incidence of ulcerative disease as mentioned, we have attributed many kills of diseased menhaden to other primary factors (Table 4).

The importance of environmental context in evaluation of the potential for involvement of TOX-A *Pfiesteria* in estuarine fish kill assessments cannot be overemphasized (Table 3). Thus, our range of focus has been limited to in-progress, major fish kills that do not occur following moderate to severe storms (e.g., hurricanes). *Pfiesteria*-related fish kills typically occur in quiet, warm, poorly flushed brackish waters, especially kills involving large schools of juvenile menhaden (8,29) (Table 3). Since nearly all *Pfiesteria*-related kills have involved a high percentage of menhaden with ulcerated lesions, we have also found the occurrence of ulcerated lesions to be helpful information. We have not used fish lesions as an absolute indicator of toxic *Pfiesteria* activity, as many stressors and microbial pathogens can be

involved in chronic lesion development (76,78). Our use of dying fish as sentinels, especially of dying menhaden with high incidence of disease, has been in accord with Leatherland et al. (81), while recognizing that few population indices, such as the existence of deep, bleeding lesions that are often chronic (78), are disease-, disorder-, or condition-specific (40).

Some fish kills that we have not related to toxic *Pfiesteria* occurred under environmental conditions conducive to toxic *Pfiesteria* activity (29–32) (Table 4). Several other non-*Pfiesteria* kills were tracked as a courtesy when requested by concerned citizens and were characterized by conditions in which TOX-A *Pfiesteria* had not occurred and was not expected, for example, kills following major storms such as hurricanes, kills along marine beaches (salinity of 30–35) (60) with high wave action, and kills in freshwater tidal rivers or other aquatic systems with salinity < 1 (45). Many kills that we have not related to toxic *Pfiesteria* involved juvenile menhaden with ulcerated lesions as mentioned and, among those events, several tested positive for the presence of *Pfiesteria* species. However, fish bioassays indicated that the *Pfiesteria* populations had not been actively toxic during the kills, and subsequent tests demonstrated that the populations had been noninducible or in nontoxic mode.

Table 3. Environmental conditions under which we evaluate a fish kill as potentially related to toxic *Pfiesteria* spp. [and/or to other, as-yet-undetected toxic *Pfiesteria*-like species; detailed field and laboratory protocols of the CAAE, North Carolina State University (NCSSU); followed in (8,9,29–32)]. These considerations are reflected in the official guidelines and protocols followed by various states [example included from (44)]. These guidelines are based on evolving scientific knowledge, with modifications anticipated as improved techniques and additional information [e.g., a reliable assay for the toxin(s) under field conditions (19,20,25)] become available.

Conditions for suspecting actively toxic (TOX-A) *Pfiesteria* in a fish kill (8,29–32)

Quiet, shallow waters (low turbulence, gentle current or wave action/wind-driven mixing).

Warm water temperatures ($\geq 15^\circ\text{C}$, usually $\geq 20^\circ\text{C}$).

Brackish (salinity ≥ 2 , generally > 5 and < 20).

No other cause of the fish kill is evident.

Fish behavior aberrant (acting erratically) and/or fish are obviously diseased with ulcers or other sores and/or fish appear to be dying but are not yet dead (i.e., event should be in progress, rather than after the fact with fish already dead).

Approximately 1,000 or more fish affected, i.e., only major kill and/or disease events [as defined by (43)] are considered in our research efforts on toxic *Pfiesteria*.

Other considerations—nutrient over-enriched waters offer optimal habitat for *Pfiesteria* (8,9,12,13,39–41,61,62).

Maryland protocol: evaluating a fish kill and/or fish epizootic as potentially *Pfiesteria*-related [quoted from (44)]

"The following guidelines generally describe the conditions considered for evaluating [toxic] *Pfiesteria* or [toxic] *Pfiesteria*-like [species] events. . . . Since many factors may cause lesions in fish, the guidelines do not reference lesions as a sole basis for closing rivers. . . . Factors to be considered include

- A significant fish kill is confirmed and there is no apparent explanation for the kill other than a toxic outbreak of *Pfiesteria* or *Pfiesteria*-like organisms.
- A significant number of fish are confirmed to be acting erratically and no other explanation for the behavior is apparent, such as low dissolved oxygen.
- 20% or more of one species (from a minimum of 50 fish of that species) are exhibiting fresh sores of a kind typically associated with [toxic] *Pfiesteria* outbreaks.
- There is evidence of increased [toxic] *Pfiesteria* or *Pfiesteria*-like activity, as reflected by an increase in the number of fish with sores typically associated with [toxic] *Pfiesteria* outbreaks.
- Environmental conditions (temperature, salinity, etc) are within ranges that may allow for a toxic outbreak. and
- An evaluation using the best available rapid technologies (such as light microscopy and molecular/toxin probes) reveals the presence of possible toxic *Pfiesteria* or toxic *Pfiesteria*-like cells."

Steps needed to implicate toxic *Pfiesteria*

Positive fish bioassays [standardized procedure of Burkholder et al. (8,29,30,59)] for a toxic *Pfiesteria* or *Pfiesteria*-like species [see Figure 1, Table 2, and text (5,8,9,24,29,30,42)].

Identification of the dinoflagellate(s) involved as a *Pfiesteria* species, and/or as a (newly recognized) toxic *Pfiesteria*-like species. Toxic strains of such newly detected species would have the three required characteristics for TPC species: they would be strongly attracted to fresh finfish secreted, excreta, and tissues; they would be capable of producing toxin(s) that cause erratic behavior, disease, and/or death in finfish and/or shellfish (24,29); and they would be stimulated to produce such substances by the presence of live fish (8,9,59).

Cloning the toxic *Pfiesteria*/*Pfiesteria*-like species involved (9,10,24,65) and retesting (separately, if more than one such species is present) in fish bioassays as per Figure 1; if positive, reconfirming species identification with SEM of suture-swollen (6,9,10,58) or membrane-stripped (7) zoospores.

Table 4. Summary of major fish kills^a by estuary or coastal area (Figure 1), by year, that met the criteria for analysis to determine whether actively toxic TOX-A *Pfiesteria* was present [compiled from (29–32)]. Overall information on kills that we did not relate to *Pfiesteria* is provided for comparison. “Ulcerated lesions” pertains to the percentage of the juvenile Atlantic menhaden population that was so affected ($n \geq 1,000$ individuals evaluated per event).

Event	Fish death	Ulcerated lesions (%)	<i>Pfiesteria</i> -like zoospores mL ^{-1b}	Fish bioassays ^c	<i>Pfiesteria</i> species identification ^d
1991 (9 toxic <i>Pfiesteria</i>-related major kills, 7 nonrelated)					
Neuse (Sept–Oct; $n = 2$)	Menhaden, catfish, mullet, blue crab; >1 billion	>65	$\leq 1,120$	+ toxicity confirmed ^f	SEM + <i>P. piscicida</i>
Pamlico (May–Aug; $n = 5$)	Menhaden, southern flounder, spot; >1 million	>50 ^e	$\leq 26,050$	+ toxicity confirmed	SEM + <i>P. piscicida</i>
Taylors Creek (Dec; $n = 1$)	Southern flounder, other; 2,000	n.a.	35,400	+ toxicity confirmed ^f	SEM + <i>P. piscicida</i>
Wrightsville (Dec; $n = 1$) ^g	Menhaden; >5,000	n.a.	50 ^g	+ toxicity confirmed	SEM + <i>P. piscicida</i>
Non- <i>Pfiesteria</i> -related (Pamlico, Neuse) ^h	Menhaden, southern flounder, croaker, spot; >10,000	<20	<100 (3 of 8 kills)	1 kill checked; no fish death, disease	–
1992 (6 toxic <i>Pfiesteria</i>-related major kills, 3 nonrelated)					
Neuse (July; $n = 1$)	Menhaden; <5,000	<50 ^e	≤ 630	+ toxicity confirmed	SEM + <i>P. piscicida</i>
Pamlico (July; $n = 4$)	Menhaden, croaker; >100,000	>50 ^e	$\leq 3,310$	+ toxicity confirmed ^f	SEM + <i>P. piscicida</i>
Topsail Beach (Dec; $n = 1$)	Menhaden; “thousands”	>50 ^e	$\leq 1,400$	+ toxicity confirmed	SEM + <i>P. piscicida</i>
Non- <i>Pfiesteria</i> -related (Pamlico, Neuse)	Menhaden, spot, croaker; >10,000	0–50	<80 (1 of 4 kills)	1 kill checked; no fish death, disease	–
1993 (4 toxic <i>Pfiesteria</i>-related major kills, 6 nonrelated)					
Neuse (July, Sept; $n = 3$)	Menhaden, spot, croaker; 290,000	>50 ^e	$\leq 1,100$; in foam, $\leq 10^6$	+ toxicity confirmed ^f	SEM + <i>P. piscicida</i>
Pamlico (July; $n = 1$)	Menhaden, croaker, spot, blue crab; 30,000	>50 ^e	≤ 270	+ toxicity confirmed	SEM + <i>P. piscicida</i>
Non- <i>Pfiesteria</i> -related (Pamlico, Neuse) ^h	Menhaden, croaker; >55,000	<50	<100 (4 of 6 kills)	1 kill checked; no fish death, disease	–
1995 (15 toxic <i>Pfiesteria</i>-related major kills, 13 nonrelated)					
Neuse (Sept–Oct; $n = 13$)	Menhaden, flounder, blue crab, croaker; >15,000,000	>50 ^c	$\leq 2,900$	+ toxicity confirmed ^f	SEM, + <i>P. piscicida</i>
New River Estuary (July; $n = 1$)	Menhaden; 10,000	<50 ^c	>300	+ toxicity confirmed	SEM, + <i>P. piscicida</i> , <i>P. shumwayae</i>
Pamlico (Aug–Sept; $n = 1$)	Menhaden; “100,000s”	>50 ^c	≥ 300	+ toxicity confirmed	SEM, + <i>P. piscicida</i>
Non- <i>Pfiesteria</i> -related (New, Pamlico, Neuse)	Menhaden, spot, croaker; “100,000s”	0–50	<80 (4 of 13 kills)	1 kill checked; no fish death, disease	–
1996 (3 <i>Pfiesteria</i>-related major kills, 6 nonrelated)					
Neuse (July; $n = 2$)	Menhaden; >1,000	>50 ^c	$\leq 1,200$	+ toxicity confirmed	SEM, + <i>P. piscicida</i>
New River Estuary (July; $n = 1$)	Menhaden, spot, croaker, mullet; >1,000,000	>50 ^c	≤ 400	+ toxicity confirmed	SEM, + <i>P. piscicida</i>
Non- <i>Pfiesteria</i> -related ^h (Neuse, Pamlico)	Many species; “millions”	<20	<80 (3 of 5 kills)	3 kills checked; no fish death, disease	–
1997 (11 <i>Pfiesteria</i>-related major kills, 6 nonrelated)					
Neuse (late June–mid-July; $n = 9$)	Menhaden, flounder, mullet, croaker; >500,000	>70	$\leq 1,400$	+ toxicity confirmed ^f	SEM, PCR + <i>P. piscicida</i> ; or SEM, PCR + both spp.
Pamlico (June; $n = 2$)	Menhaden; >640,000	>70	$\leq 1,400$	+ toxicity confirmed	SEM, PCR + <i>P. piscicida</i>
Non- <i>Pfiesteria</i> -related (Neuse, Pamlico)	Croaker, flounder, menhaden, perch; >50,000	None reported ^f	<50 (3 of 6 kills)	2 kills checked; no fish death, disease	PCR—3 kills checked; all negative
1998 (1 <i>Pfiesteria</i>-related major kill, 6 nonrelated)					
Neuse (late July–early Aug; $n = 1$)	Menhaden; 500,000 ^j	>75 ^c	$\leq 1,400$	+ toxicity confirmed ^f	SEM, PCR + <i>P. piscicida</i> , SEM, PCR + <i>P. shumwayae</i>
Non- <i>Pfiesteria</i> -related (Neuse, Pamlico)	Menhaden, croaker, carp, smallmouth bass, bluegill, perch; >10,000	0–50	<100 (6 of 6 kills)	3 kills checked; ^f no fish death, disease	PCR + <i>P. piscicida</i> , <i>P. shumwayae</i> ^h
1999 (0 <i>Pfiesteria</i>-related major kills, 16 nonrelated)					
Non- <i>Pfiesteria</i> -related (Neuse, Pamlico)	Menhaden, bluegill, largemouth bass, etc.; >10, 000	0–90	<100 (4 of 16 kills)	3 kills checked; ^f no fish death, disease	PCR + <i>P. piscicida</i> , <i>P. shumwayae</i>
2000 (0 <i>Pfiesteria</i>-related major kills, 16 nonrelated)					
Non- <i>Pfiesteria</i> -related (Neuse, Pamlico)	Menhaden, smallmouth bass, croaker, pinfish, flounder; >750,000	0–80	<85 (7 of 16 kills)	3 kills checked; ^f no fish death, disease	PCR + <i>P. piscicida</i> , <i>P. shumwayae</i>

^a“Fish kill/disease event” indicates that in all but one in-progress fish kill involving toxic *Pfiesteria* (Taylors Creek, 1991) >20% of the affected fish had lesions. Information on toxic *Pfiesteria* activity could not be obtained in 1994 because biohazard BSL-3 facilities were not available (31,65). ^b“Presumptive counts” (8,24,29,30) from acid Lugol’s-preserved water samples (45) collected where and while fish were dying (8,24,29,59) and completed by NCSU-CAAE. Extensive laboratory experiments [with similar results by (15,18,78)], have indicated that ≥ 100 toxic *Pfiesteria* zoospores mL⁻¹ can cause fish disease, and ≥ 300 cells mL⁻¹ can cause fish death (8). ^cWe consistently have followed the standardized fish bioassay procedure of (9,15,59), with $\geq 20\%$ of the fish bioassays done in duplicate in accord with U.S. Environmental Protection Agency (56) recommendations for replication of water sample analyses. ^dSEM, as well as PCR and FISH probe analyses of suture-swollen cells by NCSU-CAAE (8,24), were cross-corroborated by P. Rublee [PCR, FISH probes (68)] and D. Oldach [PCR (71)]. Species-specific molecular probes were not available for *P. piscicida* until 1998 (68,69,71) or for *P. shumwayae* until 1999 (70). Thus, molecular analyses prior to 1998 were retrospective on preserved/archived samples. The statistically significant detection limit for LM presumptive counts is ≥ 50 cells mL⁻¹, depending on the cell densities and the chamber area counted (45). In contrast, the detection limit for PCR probe analyses is ≥ 7 cells mL⁻¹ (68). ^eArchived records from the North Carolina Department of Environment, Health and Natural Resources [NC DEHNR] (37) and our laboratory through 1996 for percentages of fish with lesions generally were recorded qualitatively as “most” or “more than half.” ^fWe also conducted fish bioassays outside the kill zones for some *Pfiesteria*-related fish kills (31,37,42,59). These tests were all negative for TOX-A *Pfiesteria*. ^gThis kill is placed within brackets because it was evaluated as an exception to our standard protocols. NC DEHNR (Wilmington) requested our assessment; therefore, we examined this kill for potential toxic *Pfiesteria* involvement, despite having sampled it (though apparently fresh, with fish remains intact and little deterioration) after the fish were already dead, and despite low presumptive counts (<100 *Pfiesteria*-like zoospores mL⁻¹). The data were of interest because fish bioassays indicated that this (unusual) marine kill area (salinity of ~30) had a highly toxic population of *P. piscicida* that killed the test fish (30). The data also suggest that our standard protocols are conservative (see text), as this kill would not have been assessed for toxic *Pfiesteria* involvement had it not been for the agency’s request. Population stages (e.g., cysts, amoebae, zoospores) likely had attached to some of the juvenile menhaden before they moved from estuaries out to sea during their fall migration. ^hIn 1996 massive fish kills involving millions of fish were related by our laboratory to low DO stress (29) following level-3 Hurricane Fran. ⁱIn 1997 we were unable to monitor kills later in the growing season (past mid-July) because our laboratory was requested by Maryland State officials to assist intensively in assessment of fish kills there for toxic *Pfiesteria* activity (early Aug–late Sept). Thus, we report data from the NCDENR (formerly NC DEHNR) (37) on all North Carolina estuarine fish kills that were not *Pfiesteria* related. ^jWe estimated ~500,000 dead juvenile menhaden from intensive sampling each day over the 3-day kill period [transect procedure of (43)]. It should be noted that the state environmental agency sampled the kill for 1 day (day 3) and considered only that day in the agency’s estimate [250,000 dead fish (37)].

The 1998 Toxic *Pfiesteria* Outbreak in North Carolina

North Carolina estuaries have sustained approximately 98% of the known toxic *Pfiesteria* outbreaks, and the most affected has been the mesohaline Neuse Estuary of the Albemarle–Pamlico Estuarine System (8,9,29–32,34). As an example of our efforts to diagnose whether TOX-A *Pfiesteria* can be implicated as a primary causative agent of a given estuarine fish kill, here we describe a toxic *Pfiesteria* outbreak in the Neuse, which occurred during 28–30 July 1998, extended over an area of approximately 12-km², and affected approximately 500,000 juvenile Atlantic menhaden (Figure 5). The main kill zone occurred along the south shore of the Neuse (Flanners Beach), and the epicenter was relatively protected from wind disturbance (32). The toxic outbreak was terminated by a severe storm (by late morning of 30 July; maximum sustained northeast winds 32 km hr⁻¹) that apparently caused *Pfiesteria* to leave the water column [behavior similar to that described by Burkholder and Glasgow (8)].

The arrival of large schools of menhaden in the shallow, eutrophic, mesohaline Neuse Estuary about 3.5 weeks earlier coincided with moderate salinities of 6–8, warm water temperatures (28–30°C), and calm weather, conditions favoring toxic *Pfiesteria* outbreaks as mentioned (8,9,29) (Table 3). Other potential causative factors (e.g., microcystins, *Vibrio anguillarum*, *Vibrio vulnificus*) (74,75) were not detected. Prior to the kill we tracked what we have noted preceding other toxic *Pfiesteria*-related fish kills as a trend of increased incidence of ulcerated lesions and increased abundance of *Pfiesteria*-like zoospores [including *Pfiesteria* spp. as indicated by molecular probes in current or retrospective analysis (8,68–71)]. Three weeks before the kill, approximately 5% of the menhaden in the general area had ulcerated lesions, coinciding with 80–100 *Pfiesteria*-like zoospores mL⁻¹ (LM analysis); 2 weeks before the kill, 10–12% of the menhaden had ulcerated lesions, coinciding with approximately 130–160 *Pfiesteria*-like zoospores mL⁻¹; and 1 week before the kill, approximately 18% of the menhaden had ulcerated lesions, coinciding

with approximately 200–260 *Pfiesteria*-like zoospores mL⁻¹ ($n \geq 600$ menhaden sampled by cast net within ≤ 20 min on each date; $n = 3$ –4 samples per date for presumptive zoospore counts with LM).

During the 3-day kill, 75–80% of the affected menhaden developed ulcerated lesions, coinciding with 380 – $1,500 \pm 75$ zoospores mL⁻¹ of *Pfiesteria* spp. in the epicenter of the major kill zone (Flanners Beach area, depth 0.5–1.0 m from the surface where fish were dying) (Figure 5). *Pfiesteria* spp. were confirmed using species-specific, DNA-based fluorescent *in situ* hybridization (FISH) and polymerase chain reaction (PCR) probe analyses (68,69,71). A second region with high incidence of fish disease and low numbers of dying fish was observed around the Minnesott Beach (Figure 5) and coincided with elevated counts of *Pfiesteria* spp. zoospores (LM; approximately 100–210 cells mL⁻¹; *Pfiesteria* spp. confirmed with FISH and PCR analyses). Active toxicity of the *Pfiesteria* spp. populations present at the in-progress kill was confirmed from assays of water samples with test fish in standardized fish bioassays (8,9,29–32,65). Identification of toxic *Pfiesteria* spp. from the fish bioassays were verified with molecular probes (68,69,71) and with SEM of suture-swollen cells (9,10,58) (Table 3, Figure 2) [PCR confirmation by P. Rublee (University of North Carolina-Greensboro, Greensboro, North Carolina) (68,69,71); toxicity of isolates cross-confirmed by J. Ramsdell and P. Moeller (National Oceanic & Atmospheric Administration, National Ocean Service, Charleston, South Carolina) (26)]. Algal assays (6,24) were conducted separately and cryptoperidiniopsoids were detected, but these taxa did not cause fish death when cloned and retested separately in fish bioassays [e.g., data in Burkholder et al. (58)].

We had also tracked physical conditions during the previous 8 weeks (based on weekly spot sampling) and throughout the days and nights of the kill period (based on spot sampling at midnight, 0500 hr, 1200 hr, and 1800 hr). This effort was accomplished using a Hydrolab (model H2O, Hydrolab Corp., Austin, TX) that was recalibrated twice (early morning and afternoon) on each sampling date. Prior to and during the kill, in >25 sampling locations within the kill zones and surrounding areas, DO was >5 mg L⁻¹ [in compliance with the state standard for maintaining good fish health, which is ≥ 4 mg DO L⁻¹ (36)] throughout the 3.5- to 4.0-m water column, that is, in the upper two-thirds of the water column (hypoxic only in the bottom 0.5–1.0 m) (Figure 6). The surface-schooling menhaden were in the upper 0.5–1.0 m of the water column in the weeks prior to as well as during the kill, as

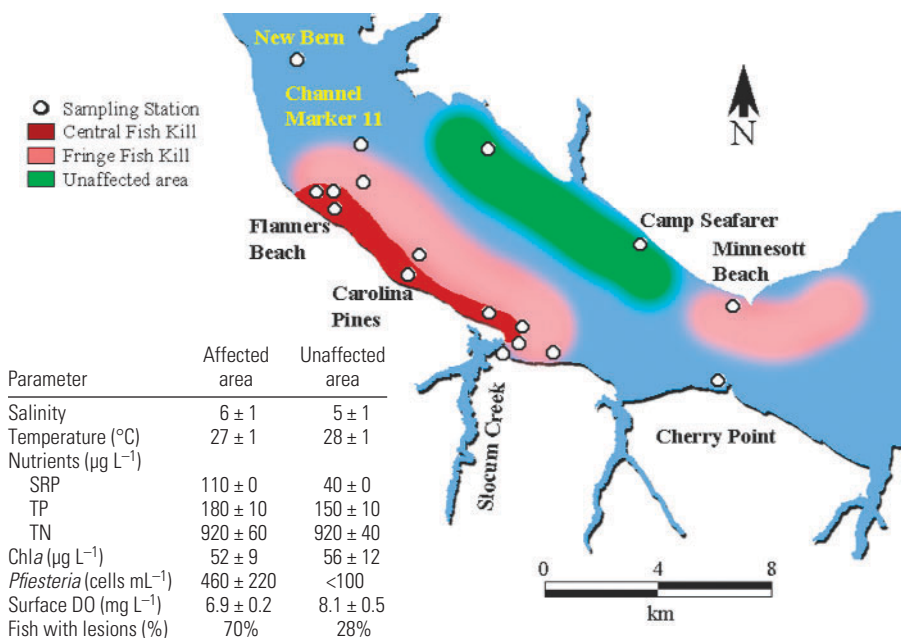


Figure 5. The main fish kill area (red, where 90% of the fish were dying) and fringe areas (pink, containing mostly diseased fish without erratic behavior, together with c. 10% of the dying/dead fish) with sampling locations during the 1998 toxic *Pfiesteria* outbreak in the mesohaline Neuse Estuary, which involved a kill of ~500,000 juvenile Atlantic menhaden. Also note sampling locations in unaffected areas (green). Environmental conditions are summarized in the accompanying table, with nutrient concentrations rounded to the nearest 10 µg L⁻¹ ($n = 14$ per parameter). For physical parameters (salinity, temperature), $n = 32$; for DO, $n = 32$; for chlorophyll *a*, $n = 14$; and for *Pfiesteria*, $n = 28$. PCR and FISH probe analyses [cross-corroborated by P. Rublee and co-workers (68–70)] confirmed the presence of *P. piscicida* in the water samples taken where there were fish with lesions prior to the kill, along with subdominant *P. shumwayae* in some samples. More than 100 water samples for presumptive counts, molecular probe evaluations, and water quality evaluations were collected during the 3 days of sampling the in-progress kill, including 28 samples collected outside the kill zones for comparison. Fish bioassays (8–11,15,29–32,59,61) conducted on samples inside the kill zone were positive for TOX-A *Pfiesteria* spp. ($n = 3$), compared with negative fish bioassays for water samples collected outside the kill zone ($n = 3$). The fish bioassays indicated that TOX-A *Pfiesteria* had been present in the fringe kill areas as well as the main kill zone while fish were dying, whereas TOX-A *Pfiesteria* was not detected outside the kill areas.

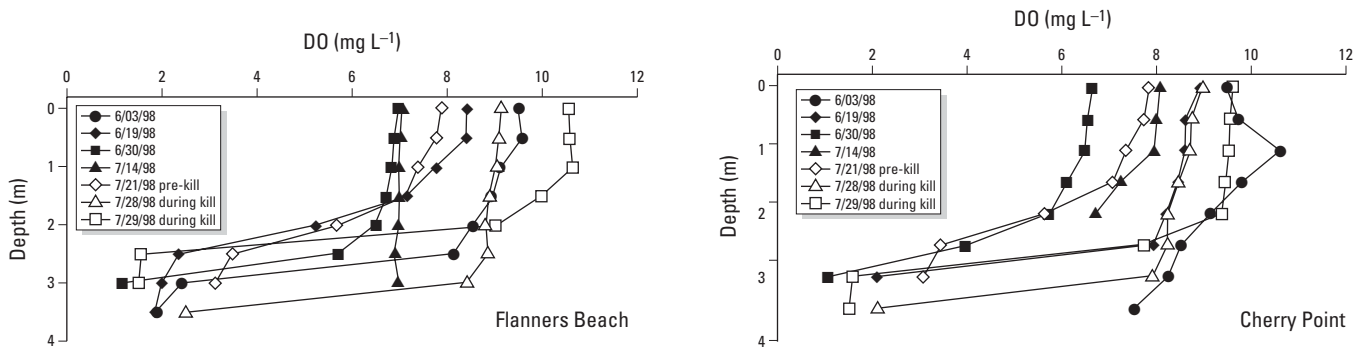


Figure 6. DO profiles in the Neuse Estuary at Flanners Beach, the location of the major kill zone in the 1998 toxic *Pfiesteria* outbreak, compared with DO profiles for an unaffected area, Cherry Point, prior to and during the menhaden kill (see Figures 1 and 5 for site locations). These data were recorded by a Hydrolab that was calibrated twice daily. Each plot prior to the kill (3 June to 21 July) represents site-specific DO profile recordings for that date. Each plot during the kill represents the mean of seven Hydrolab casts within the kill zones, and four casts outside the kill zones. Hydrolab casts during the fish kill were taken at midnight, 0500 hr, 1200 hr, and 1800 hr. It should be noted that within and around the kill zones during the three nights of the kill, DO was at 90–100% saturation throughout the water column or in at least the upper two-thirds (depth 0 m to 2.5–3.0 m) of the 3.5-m-deep water column. The juvenile menhaden populations were in the upper 0.5–1.0 m of the water column in the weeks prior to as well as during the kill [surface-schooling (82–84)], determined from routine cast net sampling and visual observations.

determined from routine cast net sampling and visual observations. A 72-hr evaluation (four times daily as above) within and around the two kill zones indicated no hypoxia throughout the water column, or hypoxia only in the lower third of the water column (with depths from 0 to 2.5 m having >5 mg DO L⁻¹). DO measurements during the three nights sampled (0100 and 0500 hr) indicated that DO was at 90–100% saturation and ≥ 5 mg L⁻¹, either throughout the water column or throughout the upper two-thirds of the water column. The nightly data indicated $\leq 3\%$ reduction in DO levels, relative to concentrations during the days, throughout the fish kill areas.

The U.S. Geological Survey (USGS) maintained an automated monitoring station at an open, windswept site in the mid-channel of the Neuse (at channel marker 11), which was positioned outside the main kill zone [depth 3.5 m, ~1 km from the kill zone epicenter (89)] (Figure 5). The station included two Hydrolabs (model H2O) to measure DO at 1.0–1.5 m from the surface (depending on the water level as altered by watershed inputs, winds, and wave action) and at 0.6 m from the bottom, respectively (89). The two USGS Hydrolabs were calibrated at 10- to 14-day intervals (below) and recorded average DO at 3- to 4-hr intervals (89). The USGS data indicated periods of <1 mg DO L⁻¹ in the bottom water for 3 days preceding the kill (89). Otherwise, DO levels generally were ≥ 4 mg L⁻¹ and consistently were >2 mg L⁻¹ [conditions wherein juvenile menhaden and other fish are stressed but not killed (85–87), as mentioned; Figure 7], even when strong winds from the northwest (250–325° from north) apparently mixed low-oxygen bottom water up to the 1.0–1.5 m depth. The *in situ* USGS Hydrolab in the upper water column recorded DO ≥ 4 mg L⁻¹ at depth 1.0–1.5 m for most of the kill, and

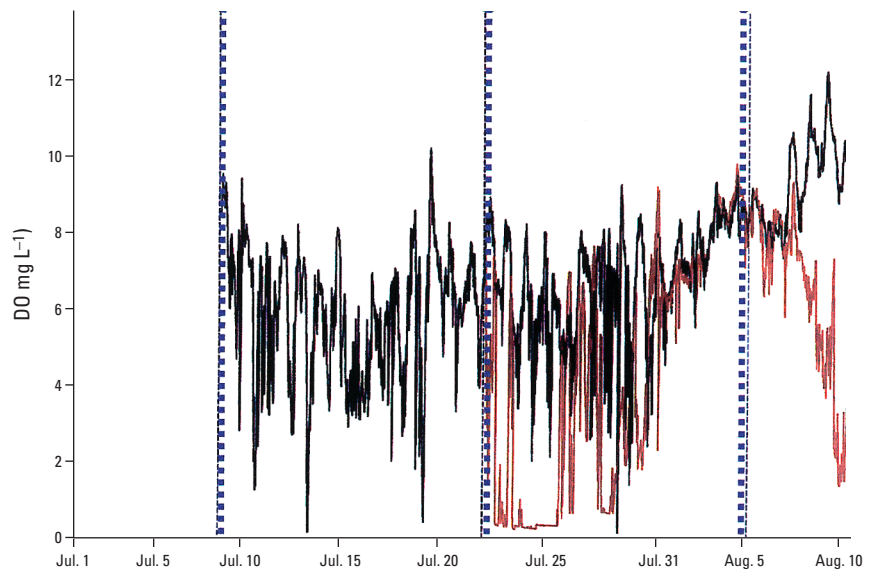


Figure 7. DO readings compiled from 15-min measurements (averaged hourly) taken by an automated USGS station located at channel marker #11 in the main channel of the Neuse Estuary in North Carolina, >1 km from the nearest area of the kill zones in the 1998 toxic *Pfiesteria* outbreak (89). This station used two Hydrolabs to monitor DO at two depths, ~2.45 m above the bottom (1–1.5 m from the surface; black line) and 0.6 m above the bottom (red line). The data were averaged, then uploaded via satellite at 3- to 4-hr intervals. USGS DO sensor recalibrations at 10- to 14-day intervals (89) are indicated by the dashed blue lines. Prior to 22 July (most recent recalibration prior to the fish kill on 28–30 July), bottom-water data were not available because the lower water column Hydrolab malfunctioned (89). Several days before the kill, the USGS data indicated several wind-driven upwellings of low-oxygen water into the surface layers. During these periods of upwelling followed by restratification, DO values remained above 4.0 mg L⁻¹ in the upper water column.

consistently recorded >2 mg DO L⁻¹ at that depth except for a brief excursion (one recorded data point) to levels approaching anoxia on 29 July near the end of the kill (Figure 7). However, it should be noted that an oxygen probe calibration problem with the USGS Hydrolabs had occurred by that date and the upper water-column Hydrolab significantly underreported DO levels (below). Thus, we found no evidence in support of lethal DO levels in the upper water column for a 6-day period preceding the kill or during the kill, although hypoxia likely contributed to fish stress (85–87).

Interpretations about the data from the USGS Hydrolabs versus our Hydrolab were strengthened from considering the flow patterns in and near the affected areas. We had documented well-oxygenated waters (5–7 mg L⁻¹) at 1.0–1.5 m depth on 22–23 July (prior to the kill) in what became the major kill site (Figures 5, 6). On the same date at the same depth, outside the kill site, the recently calibrated USGS Hydrolabs (calibrated on 22 July) showed a DO minimum of 3.5 mg L⁻¹ (Figure 7). The prevailing southwest/northwest winds (250–325° from north) and the flow patterns during 22–27 July supported

the formation of Langmuir circulation (90). The Langmuir cells were aligned parallel to the length (shores) of the estuary, with dead fish concentrated in areas of flow convergence (90). The dead fish exhibited the behavior of typical surface-drifting Lagrangian particles [e.g., drift cards (91,92)] and were transported away from the kill zones by the wind-driven downstream flow patterns. Thus, at the fish kill locations, DO was indicated to have remained >5 mg L⁻¹ in the upper water column prior to and during the kill. Moreover, from 28–30 July, DO was >5 mg L⁻¹ throughout most of the water column in the kill zones (except the bottom water, where the juvenile menhaden did not occur) and surrounding areas (Figures 5, 6).

Other evidence additionally indicated that infrequent probe calibration had adversely affected the quality of the USGS data. Extensive tests of CAAE automated sampling platforms in the Neuse have indicated that DO probes in this eutrophic, turbid estuary during summer require cleaning at ≤ 3 -day intervals for reliable function. Thereafter, DO sensor drift typically exceeds 2–3% of the 100% calibration standard, indicating membrane fouling by microflora and sediment particles. If left uncorrected, the fouling causes nonlinear fluctuations in DO readings, with drift values that can exceed 300%. Sensor drift is influenced by the type, thickness, and uniformity of the fouling, which in turn is influenced by changing characteristics of the sediment and plankton loads in the water. The nonlinear characteristic of the drift makes the data from infrequently calibrated probes unreliable, and not amenable to *a posteriori* correction [but see USGS (89)].

Therefore, the practice of attempting after-the-fact correction of DO data from infrequently calibrated probes is discouraged in rigorous quality control/assurance (93).

On 29–30 July 1998 we conducted a diel, *in situ* comparison of recordings from the USGS instruments (calibrated 7 days previously) versus those from our Hydrolab (calibrated twice daily). The two USGS Hydrolabs reported significantly different DO readings [Student's *t* test, $p < 0.01$; (94)] than the CAAE Hydrolab (Figure 8). The USGS data varied from the calibrated Hydrolab data by 13–36% and 33–207% (upper and lower water column, respectively). In the upper water column, the CAAE Hydrolab consistently yielded significantly higher DO readings than the infrequently calibrated USGS Hydrolab. These differences likely were the result of biofouling and microbial respiration for USGS probes in the upper water column, and chemical fouling (leading to reduced efficiency of the DO probe electrolyte solution) in the bottom-water readings (93). Overall, based on high concentrations of *Pfiesteria* at the in-progress kill, confirmation that TOX-A *P. piscicida* and *P. shumwayae* were present (based on fish bioassays), and lack of lethal DO levels or other lethal factors, we concluded that toxic *Pfiesteria* was the most likely primary causative agent of this fish kill, which involved fish that likely had been previously stressed by hypoxia.

Diagnosis of Toxic *Pfiesteria*-Related Fish Kills in Chesapeake Bay

Less than 2% of the known toxic *Pfiesteria* outbreaks have affected a relatively small area

of Maryland waters in Chesapeake Bay (41,42). We followed the above protocols in providing requested counsel to that state and evaluated whether TOX-A *Pfiesteria* had been involved in four major fish kills in Maryland estuaries during 1997 (41,42). We implicated toxic *Pfiesteria* as a primary causative agent of all four kill/disease events (each with $\geq 20\%$ lesioned fish), which collectively involved approximately 50,000 juvenile Atlantic menhaden (42). In all four events, DO was >5 mg L⁻¹ throughout the water column, based on day/night spot sampling (41). Water samples collected from the in-progress kills contained approximately 300–900 *Pfiesteria*-like zoospores mL⁻¹, and analysis of archived samples with FISH and PCR probes verified the presence of *P. piscicida* (all four kills) or *P. piscicida* with subdominant *P. shumwayae* (one of four kills) (68,70). All fish bioassays on samples taken where and while fish were dying during those events were positive for the presence of TOX-A *Pfiesteria* at the kills.

Fish bioassays (8,59) were conducted from two locations outside the kill zones at the time of the four *Pfiesteria*-related kills and were negative for the presence of actively toxic *Pfiesteria*, in contrast to positive fish bioassays for TOX-A *Pfiesteria* in the kill zones ($n = 8$). We also examined the water and the fish for, and did not find, other microorganisms (including other harmful algae, *Vibrio* spp., etc) that could potentially have been lethal to test fish in the positive fish bioassays. Algal assays were conducted (6,10,24) on sub-aliquots of the fresh samples from which fish bioassays were also completed, in attempts to detect other potentially toxic *Pfiesteria*-like species that subsequently could have been tested for toxicity with fish bioassays. Several cryptoperidiniopsoids ($n = 6$ clones) and *Karlodinium micrum* (Leadbeater & Dodge) J. Larsen [formerly *Gyrodinium galatheanum* (95); $n = 2$ clones] were isolated from the algal assays, although these organisms did not grow in fish bioassays from the natural water samples. The clones were grown on algal prey and then were retested in fish bioassays. None caused signs of stress or disease in fish, and fish remained healthy in the test bioassays as in the controls. The cryptoperidiniopsoid populations in fish bioassays declined to negligible zoospore densities after cryptomonad prey were depleted (9). The *K. micrum* clones grew in fish bioassays only when available light for photosynthesis of this obligate photosynthetic species was increased from 50 to approximately 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. To date there is no evidence that these and similar *Pfiesteria* look-alike organisms can cause fish death and disease as a toxin effect under ecologically relevant conditions, based on tests with natural live samples and with live clonal populations at typically encountered

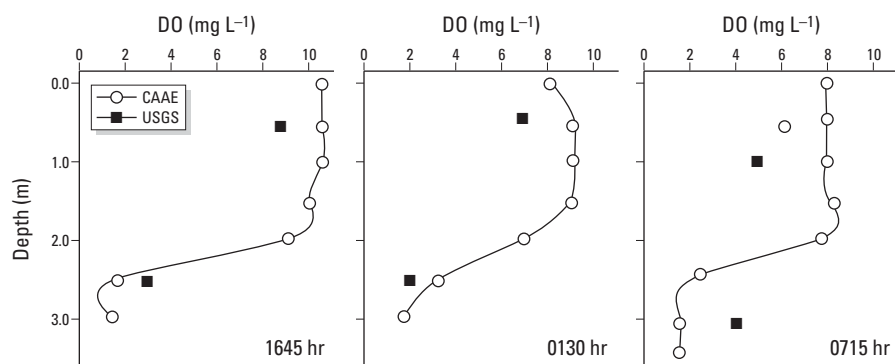


Figure 8. Side-by-side comparison of DO concentrations recorded from vertical-profile casts of a CAAE Hydrolab that was calibrated twice daily (morning and late afternoon) versus those from the USGS automated station (~1 km from the kill site) with two Hydrolabs (at depths 1.0–1.5 m from the surface, and 0.6 m from the bottom of the water column) that had been calibrated 7 days previously (89). The CAAE data were collected at 1645 hr, 0130 hr, and 0715 hr and compared to DO data measured simultaneously by the two USGS Hydrolabs. The USGS data varied from the calibrated CAAE Hydrolab data by 13–36% and 33–207% (upper and lower water column, respectively). In the upper water column where the kill of surface-schooling juvenile menhaden occurred, the calibrated CAAE Hydrolab consistently recorded significantly higher DO readings than the USGS Hydrolab. The lower water column USGS Hydrolab showed a greater range of variability from the calibrated CAAE data and gave both spuriously high and spuriously low readings (see text). Microbial fouling of USGS sensors was the most likely cause of the observed differences between the two data sets (93).

field densities (9). Similar findings from tests of live populations of cryptoperidiniopsoids and *K. micrum* have been reported by Marshall et al. (15).

Maryland officials additionally requested us to extend our approach to assist in assessment of seven fish epizootics (without fish death) that met the state's criteria for potential toxic *Pfiesteria* involvement (42). We assessed all seven events as not having involved toxic *Pfiesteria*, using our conservative approach. For example, an epizootic in the Middle River (1999) involved juvenile menhaden with lesions, and occurred under environmental conditions conducive for toxic *Pfiesteria* activity (42). About 53% of the menhaden had ulcerated lesions; *Pfiesteria*-like zoospores were $\leq 250 \text{ mL}^{-1}$, and 46 of 55 PCR analyses were positive for the presence of *P. piscicida*. However, replicate fish bioassays ($n = 3$) (8,59) were negative, indicating that a TOX-A *Pfiesteria* population had not been present (42).

***Pfiesteria*: Interactive Influences of Nutrient Enrichment, Algal Prey, and Fish**

The influence of estuarine nutrient dynamics on *Pfiesteria* is important both from an ecological and an economic standpoint. Many laboratory experiments (5,6,8,11,13) have shown that the two known *Pfiesteria* spp. are heterotrophic dinoflagellates with toxic strains, in particular, that exhibit ambush-predator behavior toward fish prey [(12); see Greene (96), Fulton (97), and Tjossem (98) for a description of this common term in aquatic biological literature]. When live fish are unavailable, certain algal species are rapidly consumed in myzocytotic feeding behavior by zoospores and phagocytosis by amoebae (6,8,58). Swarming behavior by zoospores occurs as prey become depleted (13). The nutritional ecology of *Pfiesteria* spp. is complex, and nutrient enrichment can stimulate these dinoflagellates through several general mechanisms (8). Both N and P have been shown experimentally, as organic and inorganic forms, to directly and indirectly stimulate toxic *Pfiesteria* strains (8,12,13,40,62). Organic nutrient forms (for example, glycerophosphate, amino acid mixtures, urea) can be taken up directly by TOX-A as well as TOX-B functional types of *Pfiesteria* zoospores, and amoebae (8,11,62). Inorganic nutrient forms (nitrate, phosphate) can be taken up directly by kleptochloroplastic *Pfiesteria* (61). Alternatively, inorganic nutrients can indirectly stimulate *Pfiesteria*, mediated through abundance of algal prey (8,9,40,61,62).

Other research has demonstrated that when certain flagellated algal prey are abundant, planktonic zoospores can predominate

among *Pfiesteria* stages, but if nonmotile prey such as the coccoid unicellular cyanobacterium *Cyanothece* or the diatom *Thalassiosira* are abundant, a higher proportion of the *Pfiesteria* population can consist of benthic lobose amoebae (58). Accumulating evidence indicates, as well, that *Pfiesteria* occurs in eutrophic or hypereutrophic environments rich in food resources (e.g., 10^4 – 10^5 algal prey mL^{-1} , at $\geq 15:1$ ratio of prey:*Pfiesteria* zoospores). Rather than competing for resources in the classical sense (99), it apparently switches from a planktonic to a benthic habit if preferred prey are not highly abundant in the water column (8,9,12,58).

Toxic strains of *Pfiesteria* species are widely distributed in eutrophic estuaries throughout the mid-Atlantic and Southeastern United States and elsewhere (e.g., Europe, New Zealand) (69), and nutrient-enriched waters appear to be a preferred habitat (40) (Table 2). Toward strengthening insights about environmental controls, our research team has amassed a decade of data on toxic *Pfiesteria* outbreaks and other field ecology of TPC species. This ongoing, long-term study has included emphasis on the mesohaline Neuse Estuary as the most active system for toxic *Pfiesteria* outbreaks. For the past 10 years, we have sampled 8 stations weekly and 16 biweekly (as well as 40 stations monthly in

1993–1998), with additional sampling during major storm events. This effort has yielded the most detailed, long-term data set available for the Neuse Estuary. The extended period has enabled us to construct a conceptual model of *Pfiesteria* seasonal dynamics in relation to various environmental factors (Figure 9), based on statistically significant interactions from trend analyses [e.g., (32)]. For example, based on archived sample analysis with recently available molecular probes (68,69,71), P decline with concomitant N increase has coincided with a shift in dominance from *P. piscicida* to *P. shumwayae* (9,13,32). These data support laboratory experiments that have shown comparatively higher P stimulation of *P. piscicida* zoospores, and higher N stimulation of *P. shumwayae* (13). This shift in dominance also occurred following several hurricanes (1996–1999), suggesting that *P. shumwayae* may have improved mechanisms for survival of flooding/scouring events relative to *P. piscicida*. The conceptual model is guiding collaborative research in progress to construct a quantitative, predictive model of *Pfiesteria* abundance and toxic activity.

Recommendations for Further Research

Most research on the field ecology of *Pfiesteria* spp. has emphasized planktonic stages

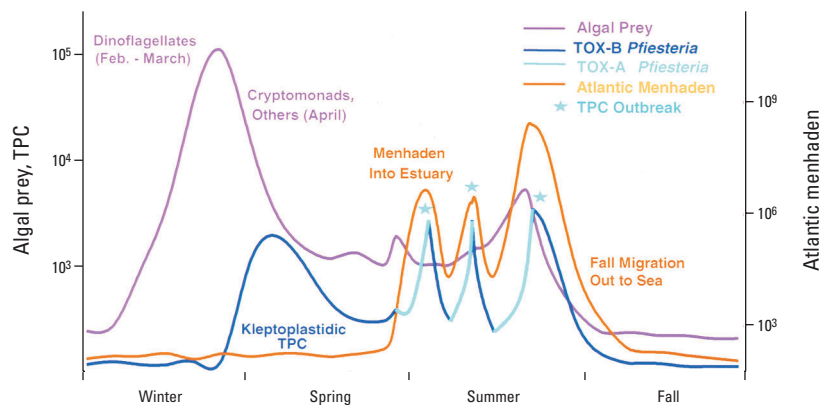


Figure 9. A conceptual model of the seasonal dynamics of toxic strains of *Pfiesteria* TPC species in the water column of the Neuse Estuary, on the basis of a decade of intensive field and laboratory data collection (5,6,8–13, 29–32,61,62,100). The model is extended from a conceptual model developed by Lewitus et al. [(61); basis, field and laboratory data (6,8,11,12,30,31)]. That model (61) had emphasized the role of algal prey and kleptochloroplasts in serving as major food resources for *Pfiesteria* spp. early in the growing season. Nutrient enrichment from major late winter/early spring storm events (32) can stimulate dense blooms of the dinoflagellates *Heterocapsa triquetra* Stein and *Prorocentrum minimum* Schiller ($\text{Chla} \leq 225 \mu\text{g L}^{-1}$) (35). These blooms typically are followed by an increase in other flagellates such as cryptomonads, which are a preferred algal prey source of *Pfiesteria* spp. (6,8,9,11–13,61). Certain strains of Neuse *P. minimum* also are readily consumed by *Pfiesteria* spp. as a food source, and an abundance of *P. minimum* during the bloom period has been positively correlated (with a 2-week lag) with *Pfiesteria*-like zoospores (100), including *Pfiesteria* spp. [from PCR and FISH probe analyses of archived samples (68,69,71)]. With exception of toxic *Pfiesteria* outbreaks during fish kills, *Pfiesteria* abundance can actually be highest in spring, including toxic strains as confirmed by fish bioassays [“survey” conditions of (59,101)]. Thus, the model depicts *Pfiesteria* cell abundance increasing during spring following a winter low-activity period, from utilization of late winter/early spring phytoplankton blooms, prior to the arrival of large schools of juvenile Atlantic menhaden in late spring/early summer (later in some years). Depending on the previous year’s toxic activity of *Pfiesteria* populations (8,9,32), fish abundance, and weather patterns, toxic outbreaks can occur. *Pfiesteria* zoospore populations decline with the onset of colder conditions (often coinciding with increased storm activity) and the fall migration of juvenile menhaden out to sea.

(8,9,13,29–32). The research has been strengthened by the development of species-specific molecular probes that have enabled focus on these species among various look-alike taxa which, thus far, have not exhibited toxicity to fish under ecologically relevant conditions (live cells at field densities) (9,15). Some success has been achieved in applying species-specific molecular probes to sediment samples (69,71). Such probes may finally enable more accurate tracking of *Pfiesteria* cyst deposits, as well as concerted focus on the ecology of active benthic (amoeboid and palmeloid) stages, which are still poorly understood.

Previous research has demonstrated that the three functional types of *Pfiesteria* spp. can show distinct differences in response to fish, algal prey, and nutrient enrichment (9,13,24,58,59). The importance of distinguishing among these functional types in field as well as laboratory *Pfiesteria* research cannot be overemphasized. Standardized fish bioassays have enabled distinction among the three functional types of *Pfiesteria*, but they are lengthy (days to weeks), complex (comprising multiple steps), and expensive (~\$1,500 U.S. including two sets of replicated fish bioassays, cloning, and SEM components) (59). Research on the field ecology of *Pfiesteria* spp. should continue to focus on toxic strains as the strains that are of interest in nutrient pollution, fish health, and human health issues (24). Research on toxic *Pfiesteria* strains will be greatly enhanced in the near future as field-reliable assays become available to detect *Pfiesteria* toxin (20,26). Such assays, together with molecular probes, will also enable insights from comparative studies on toxic versus benign (noninducible) strains.

We recommend caution in efforts to assess primary causality of estuarine fish kills, whether related to toxic *Pfiesteria* or other factors (8,29). The primary cause would be expected to depend, in part, on the behavior of the species involved. For bottom-dwelling finfish and shellfish, accumulation of toxic substances such as pesticides or heavy metals, low oxygen stress, or burial from a sudden major disturbance, or other factors such as toxic *Pfiesteria* could be lethal. For surface-schooling fish, ichthyotoxic *Pfiesteria* (and as-yet-undetected additional toxic *Pfiesteria*-like species), among other factors, may be suspected based on the presence of potentially lethal levels of zoospores in areas with suitable environmental conditions where fish are dying (Table 3) and assessed using appropriately conducted fish bioassays (Figure 2). TPC species should be implicated as primary causative agents of fish kills in the absence of other known lethal factors within the kill zone only after positive fish bioassays (59) indicate that a TOX-A strain of a TPC species (9) was present. Additional quantity

of purified *Pfiesteria* toxin is needed to enable development of assays for use with field samples and other improved toxin-based diagnostics can be developed (8,9,59).

Hypoxia/anoxia should be invoked on the basis of supporting data that demonstrate low oxygen conditions prior to (if possible) as well as during the period when, and where, fish are dying (29). Bottom-water hypoxia would be expected to be lethal to benthic finfish and shellfish, especially sessile forms that could not move to refuge areas with adequate oxygen. To interpret the importance of low oxygen stress in the upper water column to surface-schooling fish, the availability of adjacent oxygen-replete refuge areas should be assessed. The refuge areas should be large enough to support schools of fish such as juvenile menhaden during an upwelling event, when an excursion of low oxygen bottom water could rapidly depress DO concentrations in the surface waters. The practice of invoking low DO as a best guess when an area is examined after decomposing fish have been dead for hours to days in warm waters, or when an area some distance (e.g., kilometers) from the kill zone has low oxygen but the area where fish are dead/dying does not, should be avoided. Use of DO measurements from fixed-station buoys can be helpful, with the following caveats: *a*) the buoys should be within the kill zone; *b*) inversions of hypoxic bottom water to the surface should be documented in the area where fish were affected; and *c*) DO probes should be calibrated with sufficient frequency to prevent spurious data from fouling of probe membranes with fine sediments and microbial overgrowth (e.g., at ≤ 3 -day intervals in the eutrophic Neuse Estuary during summer to avoid spurious data from microbial fouling of probe membranes) (Figure 8) (29). Strengthened diagnosis of low oxygen stress will also be possible through development of experimental tests to support field data, such as certain enzyme assays that recently have become available to detect low oxygen stress in fish (102).

A continuing frustration in estuarine fish kill assessment has been the lack of information on conditions in the affected area immediately before, as well as during/after the kill. Toward that goal, we recently installed a series of eight automated platform stations in the mesohaline Neuse Estuary (www.pfiesteria.org), with maintenance/recalibration of the instruments at ≤ 3 -day intervals. These stations can measure physical, chemical, and biological conditions hourly throughout the water column. Near-real-time data on DO and other variables are transmitted to a freely accessible website. The stations have been strategically positioned in “hot spots” for major fish kills (related to *Pfiesteria*, low oxygen stress, and other factors) so that we can

strengthen acquisition of “before” and “during” data needed to improve diagnosis of the causative factors leading to fish kills. Such automated stations, with frequent calibration/maintenance to ensure data reliability, should be installed in other estuaries where major fish kills commonly occur.

Finally, we recommend, as we have in previous research, increased emphasis on documentation of factors that interact to promote fish kills (8,9,29). For example, although either variable alone, hypoxia or the TPC, can cause fish death, it is reasonable to expect that at sublethal/chronic levels, these as well as other stressors would interact to impair fish health, and that their roles as primary versus secondary factors could interchange depending upon the specific conditions. Such interactions between low oxygen stress and the TPC in impairing fish health, as well as interactions among these factors, other adverse environmental conditions, and other toxins and microbial pathogens, merit further examination. In addition to strengthening the science of fish kill assessment, this approach will help to foster greater appreciation of the multiple stresses confronted by estuarine fish populations.

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