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The Standardized Fish Bioassay Procedure for Detecting and Culturing Actively Toxic *Pfiesteria*, Used by Two Reference Laboratories for Atlantic and Gulf Coast States

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In the absence of purified standards of toxins from *Pfiesteria* species, appropriately conducted fish bioassays are the "gold standard" that must be used to detect toxic strains of *Pfiesteria* spp. from natural estuarine water or sediment samples and to culture actively toxic *Pfiesteria*. In this article, we describe the standardized steps of our fish bioassay as an abbreviated term for a procedure that includes two sets of trials with fish, following the Henle-Koch postulates modified for toxic rather than infectious agents. This procedure was developed in 1991, and has been refined over more than 12 years of experience in research with toxic *Pfiesteria*. The steps involve isolating toxic strains of *Pfiesteria* (or other potentially, as-yet-undetected, toxic *Pfiesteria* or *Pfiesteria*-like species) from fish-killing bioassays with natural samples; growing the clones with axenic algal prey; and retesting the isolates in a second set of fish bioassays. The specific environmental conditions used (e.g., temperature, salinity, light, other factors) must remain flexible, given the wide range of conditions from which natural estuarine samples are derived. We present a comparison of information provided for fish culture conditions, reported in international science journals in which such research is routinely published, and we provide information from more than 2,000 fish bioassays with toxic *Pfiesteria*, along with recommendations for suitable ranges and frequency of monitoring of environmental variables. We present data demonstrating that algal assays, unlike these standardized fish bioassays, should not be used to detect toxic strains of *Pfiesteria* spp. Finally, we recommend how quality control/assurance can be most rapidly advanced among laboratories engaged in studies that require research-quality isolates of toxic *Pfiesteria* spp. **Key words:** culture, dinoflagellates, functional type, standardized fish bioassay, toxic *Pfiesteria* complex. — *Environ Health Perspect* 109(suppl 5):745–756 (2001).

<http://ehpnet1.niehs.nih.gov/docs/2001/suppl-5/745-756burkholder/abstract.html>

The focus of this research is toxic strains of the two species, *Pfiesteria piscicida* Steidinger & Burkholder, and *P. shumwayae* Glasgow & Burkholder, that thus far have been confirmed as members of the toxic *Pfiesteria* complex (TPC) (1–4). Species within this complex (both known and those yet to be detected and formally described) not only resemble the type species, *P. piscicida*, under light microscopy but are strongly attracted to fish prey, can produce bioactive substances (biotoxins) (5–7) that are ichthyotoxic (cause erratic behavior, disease, and death in fish) and are stimulated to produce these substances in the presence of live fish or their fresh tissues, excreta, and secretions (separated from the live animal for <2 hr) (5,8). Other characteristics of secondary importance for membership in the TPC are a complex life cycle with an array of flagellated, amoeboid, and cyst stages, and the inability to photosynthesize unless kleptochloroplasts have been retained from algal prey that are often used as a food source when fish are not available (8–12). Like many other toxic algae, including cyanobacteria or blue-green algae, chrysophyte flagellates, diatoms, and dinoflagellates (13–17), the two formally described *Pfiesteria* spp. have both toxic and noninducible strains; the latter are apparently incapable of producing toxin or producing negligible/

nondetectable toxin in the presence or absence of live fish (3–5,10,18,19).

Species-specific molecular probes, first available for *Pfiesteria* in 1998, can detect the presence of the two known *Pfiesteria* spp. but cannot discern whether they are actively or potentially toxic (20,21). Both toxic and benign *Pfiesteria* strains are known, and there are three functional types (toxicity status) of *Pfiesteria*. Toxic strains may be either actively toxic (TOX-A functional type, exposed to live fish and in actively toxin-producing, fish-killing mode), or temporarily nontoxic (TOX-B functional type, separated from live fish and not engaged in toxin production. Benign or noninducible strains (NON-IND) represent the third functional type as mentioned. Efforts to diagnose whether actively toxic (TOX-A) *Pfiesteria* spp. were involved in an estuarine fish kill or fish epizootic also remain handicapped because sufficient purified toxin standard has not yet become available for development of detection assays. It should be noted that in August 2001, J.S. Ramsdell and P.D.R. Moeller of the National Oceanic & Atmospheric Administration (NOAA), National Ocean Service, Charleston, South Carolina, verified that a potent water-soluble neurotoxin has been isolated and purified from fish-killing, actively toxic *Pfiesteria* culture material in standardized fish bioassays

from our laboratory [patent process initiated by our research team (7)]. However, without additional purified toxin standards, assays cannot be developed for reliable toxin detection from natural samples (6). Therefore, properly conducted fish bioassays are the "gold standard," the only reliable technique presently available to test for the presence of actively toxic strains of *Pfiesteria* spp. and potentially toxic *Pfiesteria*-like dinoflagellates from natural water or sediment samples (4,8,19,22,23). See also the *Pfiesteria* Interagency Coordination Working Group (PICWG) (5) for a consensus document defining much of the correct terminology used in *Pfiesteria* research. This document was co-written by environmental and health officials from 10 states, the U.S. Environmental Protection Agency, the National Oceanic & Atmospheric Administration, the Centers for Disease Control and Prevention (CDC), and various academic specialists in research on toxic *Pfiesteria*. More recently, a panel of internationally renowned scientists was charged by the CDC to reevaluate all peer-reviewed publications with or about toxic *Pfiesteria*, and the panel endorsed the high caliber and scientific merit of our standardized fish bioassay (24).

Many heterotrophic dinoflagellates are difficult to grow in defined media because their nutritional requirements include unidentified organic substances (25). *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 (4,8). Appropriately conducted fish bioassays also must be used for

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the purpose of culturing toxic *Pfiesteria* and are required to maintain isolates capable of toxic activity in the laboratory for an extended duration (months). It should be noted that 95% of the 282 toxic *Pfiesteria* isolates [clones as defined by PICWG (5)] cultured from 1989 to 1999 by co-authors Burkholder and Glasgow have lost their ability to produce toxin after 6–8 months when grown with live fish, and within ≥ 6 weeks when grown in non-toxin-producing mode with other prey such as algae (9–11). The culturing of actively toxic *Pfiesteria* with live fish prey but with minimal bacteria, ciliate, or other microbial contaminants is difficult to accomplish, but with appropriate experience, fish disease and death with toxic *Pfiesteria* can be routinely and repeatedly reproduced (below).

We emphasize that this standardized fish bioassay procedure, including cross-corroboration of the data from each step by one or more independent specialists, was developed in 1991 and has been used and refined by the NCSU co-authors throughout the past decade (4); and that its existence was recognized and endorsed by the Samet et al. panel (25). We have served as the reference laboratories (J. Burkholder and H. Glasgow, for seven states and two federal agencies; H. Marshall, for Virginia) in diagnosing the presence of toxic *Pfiesteria* at fish kill and/or epizootic events. Our objectives here were to: a) Describe the steps of our fish bioassay. b) Provide the ranges of environmental conditions characterizing test samples. These data represent more than 2,000 fish bioassays. c) Make recommendations on quality control/assurance steps to improve interlaboratory consistency in characterizing test samples. We shall assess how quality control/assurance can be most rapidly advanced among laboratories engaged in studies that require research-quality isolates of toxic *Pfiesteria* spp.

Required Biohazard BSL-3 Facilities

All fish bioassays to detect and/or mass-culture actively toxic *Pfiesteria* should be conducted in biohazard Biosafety Level 3 (BSL-3) containment systems. Laboratory evidence indicates that people are at risk of serious health impacts if they sustain water contact or inhale aerosols in the immediate vicinity where fish are diseased or dying and actively toxic *Pfiesteria* populations are present (27). In early toxic *Pfiesteria* research (1993), 12 people in several laboratories had worked in standard laboratory BSL-1–2 facilities without protection from culture aerosols. In 10 of the 12 cases, the cultures were at typical field cell densities found at estuarine fish kills linked to *Pfiesteria* ($>3 \times 10^2$ to 1.3×10^4 actively toxic zoospores mL⁻¹) (7,27–31);

the others had worked with higher cell densities that were still within field range (9.0×10^4 actively toxic zoospores mL⁻¹ vs up to 1.09×10^5 actively toxic zoospores mL⁻¹ reported at field fish kills) (27). The subjects experienced burning skin and a tingling sensation during/following contact with the water from such cultures. More seriously, for several subjects, inhalation of the air over fish-killing cultures was related to blurred vision, burning skin and eyes, acute respiratory difficulty, muscle cramping, nausea, vomiting, severe headaches, and/or suppression of learning ability expressed as profound, Alzheimer's-like short-term memory dysfunction (27,31). The subjects recovered to within normal range of cognitive functioning within ≤ 3 months after cessation of exposure to the toxic cultures (27).

An outside review panel including federal and state safety specialists and specialists on toxic algae other than *Pfiesteria* was organized by North Carolina State University (NCSU) in Raleigh, North Carolina, and NOAA to evaluate our laboratory safety protocols after two people working with actively toxic *Pfiesteria* were seriously hurt in what was later determined to have been a BSL-2 rather than BSL-3 facility because of a defective airflow system. The panel supported our laboratory safety protocols and our previous decision, as well as that of NCSU, to require the use of biohazard BSL-3 facilities for research with actively toxic (TOX-A) *Pfiesteria* (32). In accordance with our formal agreement, NOAA required our laboratory to use such facilities before release of further funding on an in-progress grant (32) and advised other federal granting agencies such as the National Science Foundation to follow a similar course (33). Thus, as knowledge about *Pfiesteria* developed, we were required to use biohazard BSL-3 facilities in recognition of the association (27) between work with toxic *Pfiesteria* cultures and serious human illness. The improved biohazard BSL-3 facility prevented exposure to aerosols from toxic *Pfiesteria* cultures, and personnel working with toxic *Pfiesteria* cultures have presented no exposure symptoms since the establishment of the BSL-3 containment systems.

Considerations About Fish Kills, Fish Epizootic Kills, and Fish Disease

Pfiesteria has mostly been implicated as a primary or secondary causative agent in kills of juvenile Atlantic menhaden (*Brevoortia tyrannus* Latrobe) with ulcerative lesions (4,8,19), referred to as “kill/disease” or “epizootic kill” events. In formal reevaluation of the available published data by the previously mentioned national science panel, lethality of toxic *Pfiesteria* to estuarine as well as laboratory fish

was supported (24). Regarding ulcerative lesions and fish disease, Leatherland and Woo [(34), pp. 337–341] wrote,

... fish disorders can be used as biological indicators of environmental problems. This use [of fish] in the wild as the ‘miner’s canary’ of the quality of the environment has provided an invaluable first step toward the recognition and subsequent understanding of sometimes broad-based problems. . . . Epizootics of gross lesions . . . have been used . . . usually as indicators of the presence of [toxic] contaminants, [while recognizing that] few population indices are disease-, disorder-, or condition-specific.

We view “fish as sentinels” similarly regarding *Pfiesteria*. Our focus has been kills rather than epizootics without death, because uncertainties inherent in attempting to diagnose the initial cause of lesions, especially chronic lesions, are much greater than in assessment of kills, especially when an acute stress (e.g., anoxia) can be related to the kills (8,19). We also follow this conservative approach because a field-reliable assay to detect *Pfiesteria* toxin is not yet available as mentioned (6,7,19).

Use of dying or diseased fish as sentinels provides a first-cut visual indicator to rapidly screen for the potential presence of actively toxic *Pfiesteria* among other factors that we also consider (8,19). Thus, we regard menhaden lesions as valuable data in fish-kill assessments. We do not, however, use menhaden lesions as an absolute, reliable indicator of toxic *Pfiesteria*, which is in accordance with Samet et al. (24) and Boesch (35), and we have related many epizootic menhaden kills to factors other than *Pfiesteria* (4,8,19). Magnien et al. (36) similarly considered an observed association between menhaden lesions and *Pfiesteria*, within the required context of the set of conducive environmental conditions for toxic *Pfiesteria* activity (8,19), as evidence in support of the value of diseased and dying menhaden as one of several initial indicators of possible *Pfiesteria* activity. Our view is similar to that of Magnien et al. (36): Field evidence suggests a strong association between actively toxic *Pfiesteria* and menhaden lesions under conditions conducive for toxic *Pfiesteria* activity, but a field-reliable assay for *Pfiesteria* toxin will be needed to determine more about the nature of the association.

Standardized Fish Bioassay for Detecting and Culturing Toxic *Pfiesteria*

Fish bioassays, designed to be ecologically relevant [with small numbers of test fish and live *Pfiesteria* cells at densities commonly observed under field conditions (4,8,19), below], are conducted in *Pfiesteria*-related research for four reasons: a) in survey efforts,

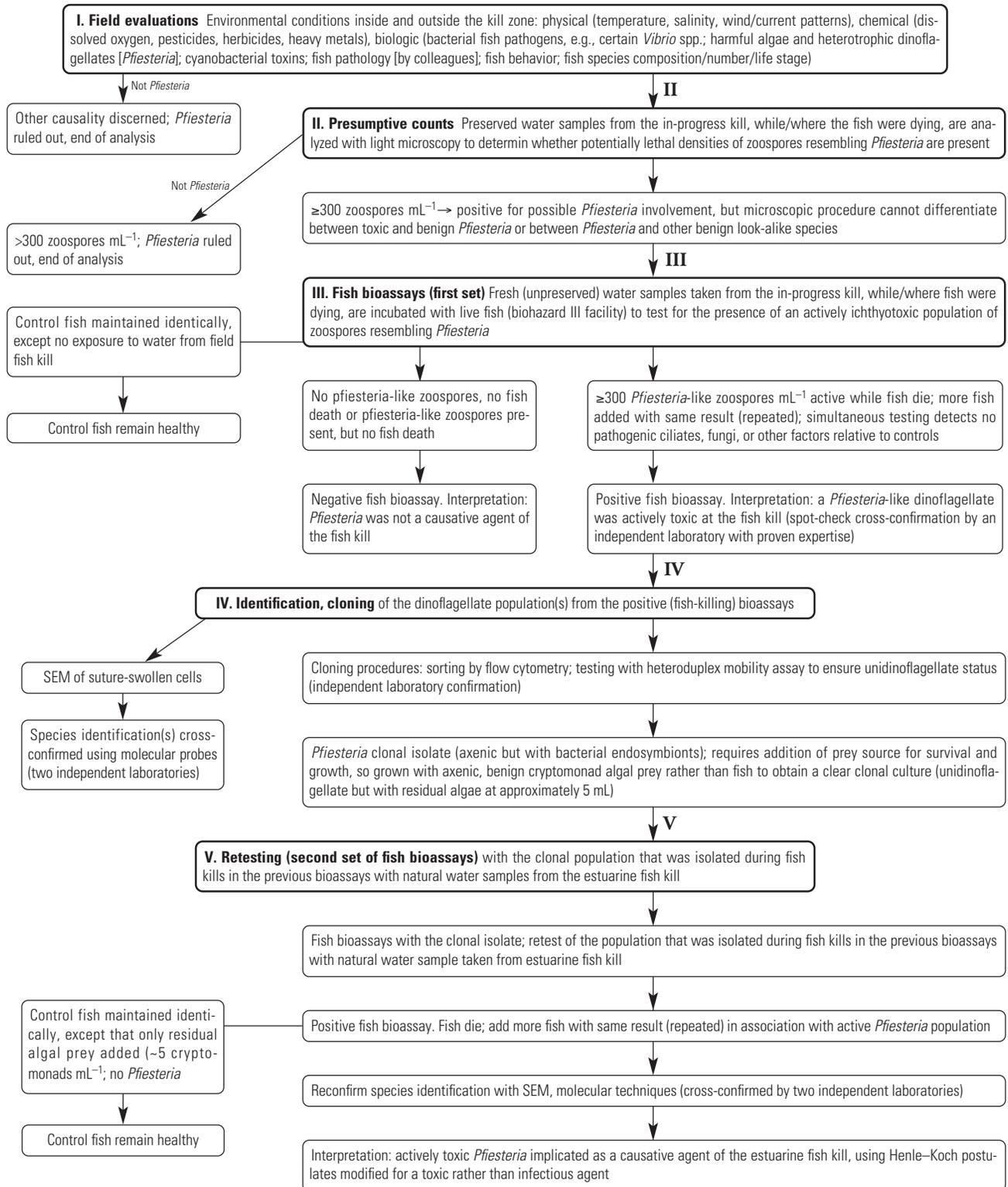


Figure 1. Schematic of the standardized steps in our fish bioassay procedure for evaluating the role of *Pfiesteria* in estuarine fish kills. The standardized fish bioassay includes two sets of trials with fish, following Henle-Koch postulates modified for toxic rather than infectious agents. The many details of this procedure are flexible to allow for modifications to accommodate highly variable estuarine conditions of sample origin [procedure cross-confirmed by A. Lewitus, University of South Carolina, Columbia, South Carolina; and by H. Marshall, Old Dominion University, Norfolk, Virginia; (4, 12, 22–24). Note that axenic clones (containing only *Pfiesteria* ± endosymbiont bacteria) were first available in late 1997. For isolates that were not possible to grow free of bacteria in the external medium, we eliminated most bacteria (see text). Then, to test fish bioassays, we added [*Pfiesteria* with endosymbiont bacteria + residual cryptomonads + residual bacteria]; to control fish cultures we added filtered medium (1- μ m porosity filters) with the low bacterial populations but no *Pfiesteria*. We also added similar quantity of cryptomonads from axenic culture grown without *Pfiesteria*. Figure modified from Burkholder et al. (4).

using water and/or sediment samples, to see whether potentially toxic strains of TPC species are present in an area; *b*) in response to a fish kill or fish epizootic, from water samples collected while/where fish are affected (i.e., behaving erratically, diseased, or dying), to assess whether actively toxic TPC species were present while fish were behaving erratically and/or were diseased and/or dying; *c*) to verify that a clonal culture of a *Pfiesteria* sp. is a toxic strain (= capable of ichthyotoxin production); and *d*) to mass-culture actively toxic *Pfiesteria* for research purposes (4,5,8). It should be noted that, in accord with PICWG (5) and given that it has not yet been possible to sustain significant cell production of *Pfiesteria* without a prey source, clonal culture is defined as an isolate of (unidentifiable) dinoflagellate, initiated from one isolated cell in the absence of other eukaryotes) *P. piscicida* or *P. shumwayae*, grown on axenic algal or other prey (4,10). In cloning procedures, toxic strains of *Pfiesteria* spp. grow well in temporarily nontoxic mode (TOX-B, without live fish) under bacteria-free conditions in the presence of algae from an axenic culture or other eukaryote prey (3,4).

In our fish bioassay procedure (Figure 1), samples are collected from an in-progress fish kill [Figure 1, step I; water, with collection techniques given in Burkholder et al. (4), Burkholder and Glasgow (8), and NOAA (26)], or from an estuary to be surveyed (water and/or surficial sediments), or from the aquaculture facility of focus (water and/or sediments, depending on the finfish or shellfish species being cultured). It should be noted that sampling must be conducted carefully to follow this caveat (19). 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 float at 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. Spatial as well as temporal mismatches between the fish kill and sampling must be avoided. 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 *Pfiesteria* is involved in a kill, samples taken in the immediate location of the dying/diseased fish have contained ≥ 300 zoospores/mL, but samples taken only about 70 m from the site have contained little or no *Pfiesteria*. The stipulation that water samples must be sampled while fish are dying/diseased 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 ~ 200 *Pfiesteria* zoospores mL⁻¹, representing a portion of the population that was actively toxic during the kill but that subsequently switched to other prey that were abundant in the area. Yet, by the above criteria, 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.

The samples should be transported to the laboratory in a shaded container at ambient temperature and should be maintained, loosely covered, in an isolated, quarantined biohazard BSL-3 facility at the same temperature and similar light regime at which they were collected (e.g., 50 μ mol photons m⁻² s⁻¹, 12-hr:12-hr light:dark cycle) prior to being tested for ichthyotoxic activity.

Fish bioassays must be conducted to detect and grow actively toxic (TOX-A) *Pfiesteria* strains (4,5,23). If test fish exposed to the samples show signs of disease, and/or if the test fish die (vs maintenance of healthy control fish without exposure to the sample), then water samples from the replicate culture vessels used for the test are examined for the presence of *Pfiesteria*-like zoospores. The presence of potentially harmful cell densities [≥ 100 zoospores mL⁻¹ for fish disease, ≥ 300 zoospores mL⁻¹ for fish death (4,5,8,28,29)] is evaluated as a positive fish bioassay, that is, with presumed actively toxic TPC species present. The dinoflagellates are identified to species using scanning electron microscopy (SEM) of suture-swollen cells and molecular identification using 18S rDNA polymerase chain reaction (PCR) probes (3,4,20,21).

The standardized fish bioassay procedure is a powerful tool in *Pfiesteria*-related fish kill assessment because it provides a reliable, although conservative, means to determine whether actively toxic *Pfiesteria* was present at the estuarine kill, while fish were dying (19). A positive fish bioassay can be used to implicate TPC species as a cause of estuarine fish death only when the following criteria are met (Figure 1):

Step I. Field evaluations. Extensive sampling is conducted for physical, chemical, and biological variables (19). To proceed beyond this step, the data should indicate no other evident cause of an in-progress fish kill that is occurring under appropriate environmental conditions for toxic *Pfiesteria* activity.

Step II. Presumptive counts. Light microscopy is used to assess whether potentially harmful *Pfiesteria*-like zoospore densities were present in field-preserved water samples that were collected when/where fish were dying, in estuarine or coastal waters with environmental conditions conducive for toxic *Pfiesteria* activity (4,5,26,29,30). If the

samples do not contain ≥ 300 *Pfiesteria*-like zoospores mL⁻¹, then *Pfiesteria* is ruled out as a cause of fish death. However, if such densities are present, standardized fish bioassays must be conducted to assess whether actively toxic *Pfiesteria* was present at the kill when/while fish were dying. It should be noted that our laboratories sometimes have been specially requested by state agencies to assess whether actively toxic *Pfiesteria* was involved in a fish disease event, absent fish death. In such cases, samples should contain ≥ 100 *Pfiesteria*-like zoospores mL⁻¹ to proceed to step III; otherwise, *Pfiesteria* is ruled out as a cause of fish disease.

Step III. First set of fish bioassays. Fresh (unpreserved) water samples collected at the same time/area as the above-described preserved samples are used for fish bioassays or for benthic species, water, and surficial sediments (4,5,29). The fish bioassays should yield *Pfiesteria*-like zoospores in cell densities that, if actively toxic, when collected from the kill (within <3 days, preferably <1–2 days, for appropriately treated samples), will cause fish disease or death within 21 days, usually 4–9 days (4,5,19) (below). The test fish become stressed, behave erratically, become ill, and die when such cell densities are present in the fish culture water. In contrast, control fish that were treated identically as the test fish, except for nonexposure to the natural water sample, remain healthy.

As an essential and integral component of the standardized fish bioassay, the cultures must be examined during both the first and second sets of fish bioassays (below) for other organisms [bacteria, fungi, other harmful algae, here including both auxotrophic and heterotrophic dinoflagellates (37–39)] and other toxins [e.g., microcystin from cyanobacteria, or heavy metals (34,38,39)]. The microflora/-fauna commonly associated with fish include various viruses, eubacteria, cyanobacteria, coccoid green algae, small chrysophytes, protozoan ciliates, amoebae, rotifers, parasitic copepods, and opportunistic fungi. If other fish pathogens are detected by repeated, rigorous examination throughout the fish bioassays, they are reported with the TPC species present as potentially multiple causative agents of the fish death (19). Where possible, separate tests are run to examine whether these agents could have been involved in fish death (e.g., isolation and separate testing for virulent *Vibrio* fish pathogens, if detected).

Step IV. Cloning the *Pfiesteria*-like organisms associated with fish death. The presumed-toxic *Pfiesteria*-like dinoflagellate species is/are allowed to increase zoospore production for several days of continued exchange of dead for live fish in the test bioassays, so that sufficient cells become available

for suture-swelling and SEM as well as PCR identification procedures (3,4). From this population(s), the *Pfiesteria*-like species is/are isolated (i.e., cloned in axenic culture with algae or other axenic prey) and again identified to species using SEM and molecular probe analyses (3–5,20,21).

Step V. Second set of fish bioassays. In this final, most critical step, the cloned toxic *Pfiesteria*-like dinoflagellate(s) species is/are retested separately for ichthyotoxic activity in an additional series of fish bioassays. If the cloned organism, at appropriate density, is associated with fish death; if there is no other apparent cause under rigorously controlled conditions; and if control fish (treated identically as the test fish, except for no exposure to the organism) remain healthy, then the organism is reisolated and recloned, and its identification is reassessed/verified with SEM and PCR.

Adherence to Henle-Koch Postulates, Modified for Toxic Agents

The above five standardized steps (I–V, Figure 1), when applied to assessment of a fish kill or fish epizootic event, follow Henle-Koch postulates (40,41) modified for toxic rather than infectious agents. The first Henle-Koch postulate is that the infectious organism must be present in the host. In the modification for a toxic rather than infectious agent, *Pfiesteria* must be present at a fish kill or fish disease event if it is implicated as a causative agent. The second Henle-Koch postulate (40,41) states that the infectious organism must be isolated from the host and grown in pure culture. In the modification, *Pfiesteria* must be isolated from a fish-killing sample [fish bioassay (4,5,8,28–30)] and grown in clonal culture (initiated from 1 isolated dinoflagellate cell); unidiflagellate and axenic, sometimes harboring endosymbiont bacteria, containing residual axenic algal or other benign prey such as ~5–10 cryptomonads mL⁻¹ (3–5). After selecting for toxic *Pfiesteria*-like zoospores using fish bioassays, the presumptive toxic *Pfiesteria* zoospores are cloned and grown on axenic algal prey prior to reinoculation into a second set of fish bioassays. Algal prey are used initially (first 3–4 weeks) in cloning efforts because they can be maintained (axenically) in clean cultures relative to fish. Clonal *Pfiesteria* cultures were achieved in 1991 using standard microscopic cell isolation techniques. The procedure was significantly advanced in our laboratory during 1999 with routine availability of a flow cytometer (below).

The third Henle-Koch postulate (40,41) states that the organism of interest must be injected from pure culture into a healthy host and infect the host. In our modification for

toxic rather than infectious agents, *Pfiesteria* from clonal culture (pure, except for residual benign algal prey) is added to healthy fish cultures, followed by fish death. In contrast, control fish are treated similarly, except that they are exposed to only residual axenic cryptomonad algae (without *Pfiesteria*), and the controls remain healthy. Prior to the second set of fish bioassays, the dinoflagellate clonal culture is allowed to consume most of the algal prey (to ~5–10 cryptomonads/mL). The presumed toxic clonal *Pfiesteria* or *Pfiesteria*-like species isolate and residual (remaining) axenic algal prey are then added to new, replicate bioassays with test fish. The fish have been grown in dinoflagellate-free conditions, confirmed by checking the outer mucus as well as the gut contents of representative fish and by repeated testing of culture water prior to inoculation (5,19). The test fish cultures are identical to control fish cultures, except that the control fish receive only algal prey (same concentration as added to test fish cultures) without *Pfiesteria*. That is, the only difference between the test fish and the control fish is the test fish were exposed to clonal

Pfiesteria grown on axenic algal prey. Thus, we emphasize that in the final set of fish bioassays (Figure 1, step V), the only difference between the test versus control fish is the addition of clonal *Pfiesteria* to the test fish. It should be noted that prior to 1997, without availability of axenic clones, we conducted test fish bioassays by adding *Pfiesteria* with endosymbionts + residual cryptomonads + residual external bacteria. Control fish cultures in these cases received filtered medium (1- μ m porosity filters) with the low bacterial populations but without *Pfiesteria*. We also added a similar quantity of cryptomonads from culture grown without *Pfiesteria*. The latter step, addition of residual axenic cryptomonads, continues to be standard procedure. It should also be noted that, given the expense of the required biohazard BSL-3 facilities and the necessity of having to feed each toxic *Pfiesteria* culture 15 live fish daily or more frequently, sterile fish have not been practicable.

The microbial consortium associated with the live fish [tilapia, *Oreochromis mossambicus* Peters, total length (t.l.) 5–7 cm; sheepshead minnow, *Cyprinodon variegatus* Lacepède, t.l.

Table 1. Recommended frequency for evaluation of water quality and microbial parameters in fish bioassays to detect or culture toxic strains of TPC species^a.

Variable	Sampling interval
DO (mg L ⁻¹)	Twice daily (near dawn, afternoon)
pH	Daily
Temperature (°C)	Daily
Salinity	Daily
Nitrogen series (μ g L ⁻¹) (NO ₃ ⁻ , NO ₂ ⁻ , NH ₄ ⁺ , urea, TKN, total AAs); phosphorus series (μ g L ⁻¹) (TP,SRP)	Initially; then at 3- to 4-day intervals in routine evaluation; daily or more frequently (depending on experimenter's purpose) when fish show abnormal behavior or disease. Finals when fish bioassays have presented positive ^b for fish mortality at least twice within a 24-hr period and molecular probes indicate the presence of TPC species (the molecular probe data should be cross-confirmed by an independent laboratory).
Microscopic evaluation (4,5,7) (presumptive TPC species)	Prior to inoculation with water sample suspected to contain TPC species; immediately after inoculation; twice weekly in routine evaluation during fish bioassays; and at least twice daily from when fish begin exhibit abnormal behavior through death.
Molecular evaluation (20,21) (probe confirmation of TPC species)	Prior to inoculation with water sample suspected to contain TPC species; immediately after inoculation; twice weekly in routine evaluation during bioassays; and at least twice daily from when fish exhibit abnormal behavior through death.
Other microbial evaluations (abundance; pathogenic bacteria, e.g., <i>Vibrio vulnificus</i> , <i>V. anguillarum</i> , phytoplankton abundance/dominant taxa; microfauna, e.g., ciliates, rotifers)	Prior to inoculation with water sample suspected to contain TPC species; immediately after inoculation; twice weekly in routine evaluation during bioassays; and daily from when fish exhibit abnormal behavior through death, except <i>Pfiesteria</i> -like zoospores twice daily or more frequently.
Reporter gene assay (43) (detects <i>Pfiesteria</i> toxin)	When a fish bioassay is positive ^b for fish mortality at least twice within a 24-hr period, and molecular probes (2 laboratories) indicate presence of TPC species.
SEM (2–5) (suture-swollen or membrane-stripped zoospores)	Needed for final confirmation of TPC species when a fish bioassay has presented positive ^b for fish mortality at least twice within a 24-hr period and molecular probes (2 different laboratories) indicate presence of TPC species.

Abbreviations: AAs, amino acids; DO, dissolved oxygen; NH₄⁺, ammonium; NO₂⁻, nitrite; NO₃⁻, nitrate; SRP, soluble reactive phosphate; TKN, total Kjeldahl nitrogen; TP, total phosphorus.

^aTwo known thus far as *P. piscicida*, *P. shumwayae*; Center for Applied Aquatic Ecology, NCSU protocols (4,5,8,27–29). ^bNo causality apparent other than an association with potentially lethal densities of presumed toxic zoospores of a TPC species [≥ 300 toxic zoospores mL⁻¹ (8,19)].

2–3 mm; juvenile hybrid striped bass, *Morone saxatilis* × *Morone chrysops* Rafinesque, t.l. 15–20 cm] could assist in the ichthyotoxic process by producing a cofactor(s) that reacts with substance(s) produced from the dinoflagellate population to create the toxic product. In addition, endosymbiont bacteria observed within *Pfiesteria* TOX-A zoospores (2,12) could be involved in toxic production [concept discussed for toxic dinoflagellates in Douchette et al. (42)]. However, the fact remains that with toxic *Pfiesteria*, fish die, often with lesions and other signs of disease. Without toxic *Pfiesteria*, fish remain healthy. Thus, we interpret such cases as positive fish bioassays for toxic *Pfiesteria*; the death of the test fish (while controls remain healthy) is attributed to

Pfiesteria, with the species identity reconfirmed using SEM and molecular probes.

The final Henle-Koch postulate (40,41) is that the infectious organism must be reisolated from the experimentally infected host, grown in pure culture, and reidentified to confirm that it is the same as the organism from the original culture (= first set of fish bioassays with the estuarine water sample). In the modification for a toxic rather than infectious agent, *Pfiesteria* must be reisolated from the second set of fish-killing bioassays (Figure 1) and its identification reconfirmed.

We consistently have followed the standardized steps (I–V) of the fish bioassay procedure in Figure 1 and the sampling frequencies shown in Table 1, described in

abbreviated form in our publications to follow journal policies (Table 2). The fish bioassay for detecting and growing actively toxic *Pfiesteria* spp. should not be considered a rigidly defined technique, because within the standardized steps there must be sufficient flexibility to accommodate samples from a wide variety of environments and circumstances (Tables 3–5). Specifics such as the quantity of estuarine water or sediment to add, the age/species/number of fish to use, the optimum size/type of culture vessel [e.g., with variations in salinity, organic load, temperature; a high surface area-to-volume ratio (SA/V) for samples collected from shallow sites, and a low SA/V for samples from deeper waters], the temperature/salinity/other

Table 2. Survey of 10 publications from issues selected randomly from each of three journals that publish finfish/shellfish experiments with fish, relative to data published from the Center for Applied Aquatic Ecology (NCSU).^{a,b}

Journal	Container	Medium	Environmental conditions ^a								Fish Age, size, sex	Vol., no. fish	Period ^c Equilibration, total	Food Type, date ^d	Total (of 20) ^e	
			Salinity	Temperature	Light	DO	pH	Nutrients	Metals	Bacteria						
Center for Applied Aquatic Ecology, NCSU ^f	+	+	+	+	+,+	(+)	+	NO ₃ ⁻ , NO ₂ ⁻	-	-	-	+, +	+, +	+, -	16	
Old Dominion University ^f	+	+	+	+	+, -	+	-	NH ₄ ⁺	-	+	-	+, +	+, +	-, -	13	
<i>Fish & Shellfish Immunology</i>																
Vol 10:1–20	-	-	-	+	+, -	-	-	-	-	-	-	+, -	+, NA	+, -	6	
Vol 10:21–30	+	+	-	+	+, -	-	-	-	-	-	-	+, +	+, +	+, -	10	
Vol 10:31–50	+	+	+	+	+, +	(+)	-	-	-	-	-	+, -	+, +	+, -	13	
Vol 10:47–59	+	+	+	+	+, -	-	-	-	-	-	-	+, +	+, +	+, -	12	
Vol 10:61–85	+	-	+	+	+, -	-	+	NH ₄ ⁺ , NO ₂ ⁻	-	-	-	+, +	-, NA	-, -	10	
Vol 10:229–242	-	-	+	+	-, -	-	-	-	-	-	-	+, -	+, +	-, -	5	
Vol 10:167–186	+	+	+	+	+, -	-	-	-	-	-	-	+, -	+, +	+, -	10	
Vol 10:243–260	-	+	+	+	-, -	-	-	-	-	-	-	-, -	-, -	+, +	7	
Vol 10:293–307	-	-	+	+	+, -	-	-	-	-	-	-	+, +	+, +	+, -	8	
Vol 10:187–202	+	-	-	-	-, -	-	-	-	-	-	-	+, -	+, NA	+, -	5	
Total (of 10)	5	4	6	8	7, 1	0	0	0	0	0	0	2, 8, 1	6, 3	7, 6	7, 1	X, 8.6
<i>Journal of Aquatic Animal Health</i>																
Vol 10:28–34	+	+	-	+	+, -	-	-	-	-	-	-	+, +	+, +	+, -	11	
Vol 10:69–74	-	+	-	+	-, -	+	+	NO ₂ ⁻ , NH ₄ ⁺	-	-	-	+, +	-, -	+, +	-, -	10
Vol 10:230–240	+	-	-	+	+, -	+	+	NH ₄ ⁺	-	-	-	+, -	+, +	+, -	11	
Vol 10:381–389	+	+	-	+	+, -	-	-	-	-	-	-	+, -	+, +	+, -	9	
Vol 10:397–404	+	+	+	+	+, -	+	+	+	+	-	-	+, +	+, +	+, -	17	
Vol 9:18–25	+	+	-	+	-, -	-	-	-	-	-	-	+, +	+, +	+, -	10	
Vol 9:49–57	+	-	+	-	-, -	-	-	-	-	-	-	+, -	+, +	+, -	6	
Vol 9:64–69 ^g	+	+	+	+	-, -	-	-	-	-	+	-	+, -	+, -	+, -	9	
Vol 9:127–131	-	+	+	+	-, -	-	-	-	-	-	-	+, -	-, -	+, -	6	
Vol 9:132–143	+	+	-	-	+, +	-	-	-	-	-	-	+, -	+, +	-, -	7	
Total (of 10)	8	8	4	8	5, 1	3	3	3	1	1	1	4, 10, 1	6, 3	8, 10	8, 0	X, 9.6
<i>Marine Biology</i>																
Vol 131:219–225	+	+	+	+	+, +	+	-	-	-	-	-	+, -	+, +	+, +	-, -	13
Vol 131:703–718	+	+	-	+	-, -	-	-	-	-	-	-	+, +	+, +	+, +	+, -	11
Vol 132:1–8	+	+	+	+	-, -	-	-	-	-	-	-	+, -	+, +	+, -	11	
Vol 136:175–184	-	+	+	+	-, -	+	+	+, +	-	-	-	+, -	+, +	+, -	13	
Vol 136:573–580	+	+	-	-	+, -	-	-	-	-	-	-	+, +, NA	+, +	NA, +	+, -	8
Vol 136:591–600	+	+	-	+	-, -	-	-	-	-	-	-	+, -	+, -	+, -	8	
Vol 136:615–626 ^h	+	+	+	+	-, -	+	+	+, +, +	NA	-	-	+, -	+, -	-, -	12	
Vol 135:561–572 ^h	+	+	+	+	-, -	+	+	+, +, +	-	-	-	+, +, NA	+, +	NA, +	+, -	15
Vol 135:341–345	+	+	+	+	+, +	+	-	-	-	-	-	+, -	+, +	+, -	11	
Vol 136:1087–1098	+	+	+	+	-, -	+	-	-	-	-	-	+, -	+, +	+, -	12	
Total (of 10)	9	10	7	9	3, 2	6	3	2, 3, 3	0	0	0	8, 9, (3)	9, 7	(8), 10	7, 0	X, 11.4

NA, not available.

^aParameters selected for comparison were taken from a draft workshop report (44) listing parameters recommended for measurement in fish bioassays. The workshop participants recommended these variables for research with cultured or experimental fish, then attempted to direct their attention more specifically to fish bioassays for detecting and growing toxic *Pfiesteria*. Positive (verified toxic *Pfiesteria* culture) and negative controls were maintained by the Center for Applied Aquatic Ecology. Light included two parameters as photoperiod and light intensity. ^bHere we excluded other parameters as fish species and negative controls (which all publications reported) and antibiotics/other additives (which one publication reported). ^cEquilibration or acclimation period and total experimental period. ^dDate indicated on the feed container, after which the feed should not be used. ^eX, the mean for the 10 publications of each journal. ^fWe described aerating the cultures (+) but did not provide the DO levels, which were maintained between 5 and 7 mg/L. That information was contained in Burkholder et al. (28,45) referenced in our publications. The information from Old Dominion University is from Marshall et al. (23). ^gMentioned use of a penicillin antibiotic mixture. ^hSex was indeterminable for the (larval and juvenile) shellfish species used.

environmental conditions, depend on the sample characteristics (for example, percent organic/nutrient content; origin from a fish kill vs from an area with no signs of fish disease or death, or from a toxic-laden urban watershed vs from an agricultural basin).

Isolating *Pfiesteria* Species

A Coulter Epics Altra flow cytometer with HyPerSort system (Coulter Corp., Miami, FL, USA), equipped with a water-cooled INNOVA Enterprise II Ion Laser (Coherent, Inc., Santa Clara, CA, USA), is used to sort and clone toxic strains of *Pfiesteria* spp. from positive fish bioassays. Excitation is provided by a 150-mW/488-nm argon laser line. Quality control calibrations are performed to optimize optical alignment and detector voltages, using 6- μ m diameter fluorescent latex microspheres (Molecular Probes, Inc., Eugene, OR, USA). Fish bioassay samples (~200 mL) are gravity filtered through 38- μ m Nitex mesh (Aquaculture Research/Environmental Associates, Inc., Homestead, FL, USA) immediately prior to flow cytometric analysis. Electronic sort gates are based on detection of optical parameters defined to select the detectable subpopulations of interest. Sort recovery and purity are checked with light microscopy and with PCR or the heteroduplex mobility assay (3,20,21). Isolation of highly purified cells via particle sorting yields ultra-clean cell preparations for further PCR and SEM analyses. From these subpopulations, a robotic Coulter AutoClone (Coulter Corp.) sorting system is used to establish multiple clonal isolates by directed deposition into multiwell microculture plates, where the populations are grown for 2 weeks with cryptomonad prey. They are then inoculated into fish bioassays and monitored daily or more frequently to confirm toxic activity and to grow toxic clones.

The AutoClone sorting system allows rapid cloning of single cells into multiwell microculture plates with 99.98% precision and purity. Once clonal sorting is complete, axenic (externally eubacteria- and cyanobacteria-free) prey are added to each well, and the dinoflagellates are allowed to grow and reproduce for several days under sterile conditions. To ensure that cultures are axenic and do not contain eukaryotes other than *Pfiesteria* and the added axenic prey, 10 μ L of each culture are plated onto agar growth medium (Triple Soy Agar; Difco Laboratories, Detroit, MI) following techniques described by Tanner (48). The plates consistently have been evaluated as free from bacterial, fungal, or other contaminants.

The Critical Importance of Delays Without Live Fish in the Time to Fish Death

Various microorganisms from natural water samples (e.g., fecal coliforms, *Vibrio* spp.) require incubation periods in the laboratory before their activity can be detected and/or quantified (48,49). The behavior we have repeatedly observed in *Pfiesteria* spp. indicates that they should be regarded analogously within a certain time interval (below). In the fish bioassay procedure, the time to first and second fish death is of critical importance in interpreting whether an actively toxic population of a TPC species was present at an in-progress fish kill or fish epizootic event. Transport-induced delays typically are encountered between sampling an in-progress estuarine fish kill and initiating fish bioassays to determine whether an actively toxic population of a TPC species was present at the kill. *Pfiesteria* spp. consistently have demonstrated a biochemical predisposition for recent stimulation by live fish (1,4,8), but they are sensitive to separation from fish.

We tested fish killing activity following separation from live fish (in standardized fish bioassays) for varying duration (0, 3, 7, 24, 48 hr; $n = 12$; one fish per replicate), using acute toxicity microassays with actively toxic *P. shumwayae* taken from mass-culture fish-killing bioassays and inoculated into multiwell culture plates [10-mL well volume; actively toxic clone 101235, Neuse Estuary in North Carolina; sheepshead minnow, *Cyprinodon variegates*, 25 days of age at assay initiation (Casper Environmental Services, Bohemia, NY, USA), acclimated to a salinity of 15 over a 3-day period; fish fed newly hatched *Artemia salina* L. (Aquatic Ecosystems, product no. BS01, Apopka, FL, USA), twice daily from 7 days of age to 25 days of age]. During the delay periods without live fish, the recently TOX-A subsample cultures (salinity 15) were maintained in loosely capped flasks at 21°C in darkness. Microassays were evaluated at 2-hr intervals throughout the 48-hr period using an Olympus SZX12 stereo microscope (10–60 \times). Mortality was assessed by gently contacting the fish with a disposable probe, and time of mortality was determined when there was no response. Negative controls (fish in water (salinity 15) from fish cultures maintained without *Pfiesteria*) were run for comparison with each treatment (duration without fish). Throughout these assays, environmental conditions were maintained as 20°C, salinity of 15, 50 μ mol $m^{-2} s^{-1}$, 7.8–8.0 pH, ≥ 6 –7 mg DO L^{-1} , and < 0.5 mg L^{-1} nitrite. When held for < 24 hr without live fish, the recently toxic populations killed fish in all 12 replicates within < 3 hr after being reexposed to live fish (Figure 2). In subcultures of *Pfiesteria* held without live fish for 48 hr, approximately 60% of the zoospores formed temporary cysts (50), and there was a 1-day delay before death occurred for in 6 of the 12 replicates. Fish death did not occur in the other 6 replicates for the remainder of the experiments. Actively toxic (TOX-A) subsamples that were held without live fish for more than 48 hr showed no fish-killing activity, with fish remaining apparently healthy in all 12 replicates.

It is important to note that acute toxicity (48-hr) microassays with larval finfish or shellfish (4,51) are only effective as an extension of the fish bioassay when testing for toxic *Pfiesteria* strains. They have not been reliable in initiation of growth or in continuous growth of actively toxic *Pfiesteria* cultures. Microassays can be used in short-term comparisons to reliably assess the fish-killing activity of clonal cultures of *Pfiesteria* spp./functional types that were physically active (= zoospores actively swimming) and taken from standardized fish bioassays. However, until a sufficient quantity of purified *Pfiesteria* toxin standard is available for

Table 3. Environmental conditions from which samples have been taken that were found to contain potentially toxic or actively toxic *P. piscicida* and *P. shumwayae* (1991–present; estuarine waters ranging from the Indian River Inland Bay, Delaware, to Galveston Bay, Texas; $n = 68$).

Variable	Median	Range
Temperature	26°C	15–33°C
Salinity	8	2–35 ^a
Light	40 μ mol photons $m^{-1} s^{-1}$	8–440 μ mol photons $m^{-1} s^{-1}$
pH	7.3	6.8–10.8
DO	5.6 mg L^{-1}	0.2–10.9 mg L^{-1}
Organic matter (sediment)	70%	30–80%
Finfish, shellfish species present	— ^b	Variable
TP ^c	145 μ g L^{-1}	60–1,220 μ g L^{-1}
SRP ^c	80 μ g L^{-1}	5–810 μ g L^{-1}
TKN ^c	930 μ g L^{-1}	620–2,000 μ g L^{-1}
NH ₄ ⁺ N ^c	15 μ g L^{-1}	0–240 μ g L^{-1}
NO ₃ ⁻ N ^c	40 μ g L^{-1}	0–480 μ g L^{-1}
Chlorophyll <i>a</i> (water column)	22 μ g L^{-1}	5–180 μ g L^{-1}
Fecal coliform bacteria	20 CFU 100 mL ⁻¹	0–3.6 $\times 10^6$ CFU 100 mL ⁻¹

Abbreviations: CFU, colony-forming units; SRP, soluble reactive phosphate; TP, total phosphorus.

^aAlso, salinity 0 in some aquaculture facilities (Ca²⁺ hardness > 20 mg L^{-1} ; but naturally occurring populations have not been found at salinities < 2 , except for occasional high-precipitation periods when salinity in normally brackish waters (e.g., mesohaline Neuse Estuary) can be depressed to a salinity of approximately 0 (30). ^bJuvenile Atlantic menhaden generally are abundant in surface waters (upper 1 m). ^cNutrient concentrations were rounded to the nearest 10 μ g L^{-1} .

development of reliable detection assays, the standardized fish bioassay, with the critically important cloning/retesting steps, is required to verify that a given population of *Pfiesteria* is toxic, and/or was actively toxic at a fish kill/disease event (24). In further clarification, it should be noted that neither *P. piscicida* nor *P. shumwayae* has been grown successfully using acute-toxicity microassays. After approximately 48 hr, cell production has declined significantly (>60%) and toxic (fish-killing) activity has not been observed. Clonal *Pfiesteria* zoospores or cysts inoculated into microassays with sheepshead minnows have not yielded cell production or excystment, respectively, in tests up to 1 week in duration; after 1 week, the larval fish have died from

starvation or, if fed, from accumulation of toxic waste products such as ammonia. Acute toxicity microassays have also yielded poor results with environmental samples; thus, they should not be used in place of standardized fish bioassays to test for toxic *Pfiesteria* involvement in fish kill/disease events. Nor have dockside microassays tested reliably in detecting actively toxic *Pfiesteria* populations. Under field conditions, physical disturbance from collection procedures, wave action, other boat movement, and sample transport can quickly cause *Pfiesteria* to cease toxic activity. The standardized fish bioassay procedure reliably tests environmental samples for the presence of *Pfiesteria* populations that may or may not have been actively toxic

when collected, but that had minimal physical activity or were not physically active when the assays were initiated.

The data from the acute toxicity microassays in the above-described experiment indicated that the duration of separation from live fish strongly influences the period required by toxic strains of TPC dinoflagellates to resume ichthyotoxic activity. In other experiments that were designed similarly, we used 48-hr microassays to compare the behavior of high densities (10^4 zoospores mL^{-1} , prey-replete) of TOX-A, TOX-B, and noninducible (NON-IND) *Pfiesteria* spp. (each species and functional type tested separately) and cryptoperidinioids (previously grown with cryptomonad prey because they

Table 4. Requirements for an appropriately conducted fish bioassay to detect or culture toxic *Pfiesteria*.

Variable	Recommendation
Culture vessel	Flexible. ^a Height-to-width ratio is not important; at least 300- to 500-mL volume is recommended. Attempts to use smaller volumes have not yielded useful information about toxic <i>Pfiesteria</i> ; we use small-volume tests only to conduct acute-toxicity, short-term final checks on larger-scale fish bioassays. A tightly closed culture vessel is needed unless suitably designed biohazard BSL-3 facilities are available. ^b
Fish species, age, size, number	Flexible. ^a \geq one 5-cm (total length) fish L^{-1} . <i>Pfiesteria</i> has been lethal to all finfish species tested to date. ^a If performing fish bioassays as a reference laboratory for a state agency, use of readily available fish without prior risk of <i>Pfiesteria</i> exposure, and with broad salinity/temperature tolerances is practical for comparative purposes.
Fish food	No specific brand recommended; special treatment not necessary; feeding schedule and amount per feeding should follow what is recommended for the fish species/age/size used. The fish should be fed food that does not contain dinoflagellate contamination. ^a
Medium	Flexible. ^a For most work, artificial salts such as Instant Ocean (Aquarium Systems, Inc., Carson, CA, USA) can be used; for toxin studies, an offshore, ultrafiltered (0.22- μm porosity) seawater source is recommended (4,47).
Filters	Flexible, but avoid use of carbon filters.
Initial inoculum of <i>Pfiesteria</i> or potentially toxic <i>Pfiesteria</i> / <i>Pfiesteria</i> -like isolate	Flexible; ^a depends on the characteristics of the sample (especially the nutrient enrichment status and the phytoplankton and/or <i>Pfiesteria</i> , suspected <i>Pfiesteria</i> cell densities) of the sample; can range from 10^1 to 10^3 cells mL^{-1} .
Temperature, salinity, light, pH	Flexible. ^a Should simulate the conditions from which the natural sample was collected, or from which the culture (obtained from another source) was previously maintained. If the natural sample is from the sediment, then should simulate conditions in the overlying water.
Dissolved oxygen	Should be $> 5 \text{ mg L}^{-1}$ and below supersaturation to maintain good fish health; can be gently aerated.
Nutrients	$\text{NO}_2\text{-N}$ should be $< 0.7 \text{ mg L}^{-1}$; $\text{NH}_4\text{-N}$ should be $< 1 \text{ mg L}^{-1}$; TKN, TP, SRP, other nutrients can be variable; no specific range recommended.
Duration	≤ 21 days to detect toxic <i>Pfiesteria</i> (or an as-yet-undetected toxic <i>Pfiesteria</i> -like species) from an in-progress fish kill or fish disease event in an estuary or aquaculture facility; ≤ 10 weeks to detect a toxic strain from a sample collected from areas without in-progress fish kills and/or fish epizootics.
Controls	Replicate fish bioassays set up and maintained identically as the replicate test or culture bioassays but without <i>Pfiesteria</i> or <i>Pfiesteria</i> -like cells, accomplished by ultrafiltering the natural sample or culture ^a and adding the filtrate (negative controls); and replicate fish bioassays set up and maintained identically to the replicate test or culture bioassays but with a tested, cross-confirmed ^d clonal culture (4,5,10) of toxic <i>P. piscicida</i> or <i>P. shumwayae</i> . ^g
Species identification	Scanning electron microscopy of suture-swollen (3,4,9) or membrane-stripped (2) zoospores is required for confirmation; supporting use of species-specific molecular probes (that have been tested and cross-confirmed as of dependable, research quality by at least one independent laboratory with demonstrated expertise in this area) is recommended. ^f
Clones [as defined in PICWG (5)]	Should be checked for unidinoflagellate status using the heteroduplex mobility assay (21).

^aDepends on the purpose of the fish bioassay and the specific conditions desired. We have grown toxic *Pfiesteria* in our fish bioassays using $< 1 \text{ L}$ up to 9,000 L. The quantity of culture we use depends, in part, on the culture volume needed. *Pfiesteria* has not been found in water samples at pH < 6.8 . We assume that the culturist has appropriate knowledge of how to culture healthy fish before undertaking efforts to culture toxic *Pfiesteria* with fish (see Popma and Masser (46); e.g., reagent grade-A calcium carbonate is used to adjust pH if initially necessary; salinity is adjusted with ultrafiltered (0.22- μm porosity) deionized water, or ultrafiltered seawater or, for some purposes, ultrafiltered synthetic ocean salts). The volume of water sample or mass of sediment sample added also depends on the initial cell densities and environmental conditions from which the sample was collected, as indicated. For example, we sometimes have used 50–100 mL of sediments in approximately 1-L volume—more sediment if the area is relatively oligotrophic, less if eutrophic. ^bLaboratory workers have sustained health impacts such as burning eyes and respiratory difficulty when exposed to small volumes (e.g., 1–2 L) of toxic, fish-killing *Pfiesteria* cultures (27,31). ^cMonetarily impractical to use only 1 sex of fish when mass-culturing toxic *Pfiesteria*. The choice of fish species, size, age, and number used must also depend on the environmental conditions associated with the sample to be tested. The fish should be checked to ensure that they are dinoflagellate-free so that they do not represent a potential source of contamination. To accomplish this, the fish should be maintained in separate facilities remote from where natural samples are to be tested or where *Pfiesteria* is grown; and fish should be examined periodically to ensure that no dinoflagellates can be detected in their outer mucus, gills, or gut tract. Note that use of antibiotics are not recommended; microflora/-fauna can be controlled to low levels with standard culture maintenance/cleaning procedures. ^dSee Burkholder et al. (4); also, sheepshead minnows, fathead minnows, and brine shrimp (*Artemia salina*) (4,6,49). ^eThe same number of cryptomonads can be added from cryptomonad culture maintained without *Pfiesteria*, if on step IV of the standardized fish bioassay procedure (Figure 1). Note that we do not recommend use of NON-IND in negative controls (despite the fact that we have repeatedly tested such isolates and found that they have caused no apparent harm to fish in standardized bioassays) because the toxin production by these strains may eventually be possible to stimulate via certain (as yet unknown) genetic controls that could inadvertently be triggered. Water and sediment samples from estuarine sites where *Pfiesteria* spp. have not previously been known cannot be used in negative controls, as potentially toxic populations may, in fact, be present. ^fCross-confirmation of toxicity and of the species identification by at least one independent laboratory with demonstrated cross-corroborated expertise in culturing toxic *Pfiesteria* and in plate tabulation determination on *Pfiesteria* [or, in the latter case, experienced in plate tabulation determination on weakly thecate dinoflagellates (i.e., species with thin cellulose deposits or plates)] is critical and should be a strict requirement for quality control/assurance before the data are reported in public forums, described in grant proposals, or published (see text). ^gNo other toxic organisms can serve as substitutes for toxic *Pfiesteria* as positive controls in this process. Toxic bacteria, toxic algae other than dinoflagellates, and toxic dinoflagellates other than TPC species behave very differently from TPC species under different optimal growth conditions, and many do not cause fish disease and death.

did not maintain cell production in repeated culturing attempts with live fish) in response to larval sheepshead minnows (4). All control fish (without dinoflagellates) remained healthy throughout the experimental trials. In contrast, all fish exposed to TOX-A *Pfiesteria* spp. ($n = 90$) died within 2.0–9.5 hr of exposure (mean \pm 1 SE, 5.8 ± 1.1 hr), and only 47% of fish exposed to TOX-B *Pfiesteria* spp. ($n = 82$) died, with mean time to death 16.8 ± 1.4 hr. Only 12% of the fish ($n = 90$) died in trials with NON-IND zoospores, with mean time to death 26.2 ± 12.2 hr. Death (low incidence) in the tests with NON-IND *Pfiesteria* appeared to result primarily from extended mechanical insult (>24-hr exposure) to fish tissues, rather than from a toxic effect as in TOX-A and TOX-B trials. Approximately 20% of the fish died in trials with the cryptoperidiniopsis isolates, also as an apparent physical effect, over an average time of 25.5 ± 3.3 hr. During a 48-hr period, 3 of 15 cryptoperidiniopsis clones caused >50% fish death, whereas there was no fish death in 8 other cryptoperidiniopsis clones. We observed visually in all treatments that high numbers of zoospores swarmed to the larval fish in attack and feeding mode, attaching by their peduncles and subsequently becoming engorged [see photos in Burkholder et al. (4)]. Although TOX-A zoospores sometimes attached to fish gill and cloacal areas, direct attachment to the fish body was more commonly observed for TOX-B and, especially, NON-IND zoospores. The lower incidence of attachment to the fish by TOX-A zoospores was visually obvious, and may have occurred because shortly after exposure to TOX-A zoospores, fish rapidly sloughed epithelial tissue, which did not occur in trials with the other two functional types. The TOX-A zoospores aggressively consumed the sloughing tissue. We hypothesize that toxin from *Pfiesteria* spp. can act as an escharotic substance that promotes the observed sloughing of epidermal tissue by fish exposed to TOX-A populations. Within minutes of exposure, cuticle damage was evident over the entire body. Other functional types and the cryptoperidiniopsis isolates, although displaying physical attachment and feeding behavior, did not cause the rapid mortality evident in TOX-A *Pfiesteria* trials. In tests of these clones, death in the TOX-B and NON-IND trials and in trials with the cryptoperidiniopsis appeared to be from physical attack and feeding rather than from toxins (4).

It should be clarified that, whereas some *Pfiesteria* isolates have been tested as capable of killing fish whether allowed direct contact with the prey or maintained within cellulose dialysis tubing (molecular weight cut-off 12,000–14,000 Da) to prevent direct contact (8,51), others have killed only when

allowed direct contact with the prey. The two species thus far have been shown to produce analytically comparable toxin (7), but considerable intraspecific differences among isolates apparently occur in toxin potency and in the extent to which toxin is released versus retained within the cells. A mechanism for *Pfiesteria* toxin impacts on fish and mammals has been described from experiments with clonal, toxic cultures (cross-corroborated by independent specialists), wherein the toxin mimics an ATP neurotransmitter that targets P2X₇ receptors (6). 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 (6,7).

Using the standardized fish bioassay procedure [with juveniles of various species; see Burkholder and Glasgow (8) and Burkholder et al. (4,10,28,29)], we have rigorously tested TOX-A cultures (ranging from weakly toxic with fish death at approximately 24-hr intervals to highly toxic with fish death at ≤ 1 -hr intervals) of both *P. piscicida* (clone B89B, Neuse Estuary) and *P. shumwayae* (clone 101125, Neuse Estuary) to assess resumption of fish-killing activity by a) populations that had been actively toxic but were held for 1–3 days without live fish prior to initiation of fish bioassays (recently TOX-A) versus b) TOX-B populations previously tested as capable of fish-killing activity, but grown on algal prey (*Cryptomonas* LB2423; required cloning as well as cleaning procedures because of prymnesiophyte contamination when received from the Culture Collection for Marine Phytoplankton in

Table 5. Environmental conditions in positive fish bioassays for the TOX-A functional type of *Pfiesteria* spp. (4,8); $n = 220$ unless noted].

Variable	Median	Range
Temperature	22°C	15–30°C
Salinity	15	0–35
Light	20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$	0–420 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$
pH	7.8	6.9–8.3
DO	6.2 mg L^{-1}	>5 mg L^{-1} to below supersaturation ($\leq 8.8 \text{ mg L}^{-1}$)
Finfish, shellfish species present	5	Variable
TP	470 $\mu\text{g L}^{-1}$	220–1,200 $\mu\text{g L}^{-1}$
SRP	340 $\mu\text{g L}^{-1}$	80–420 $\mu\text{g L}^{-1}$
TKN	1,800 $\mu\text{g L}^{-1}$	490–5,900 $\mu\text{g L}^{-1}$
NH ₄ ⁺ N	130 $\mu\text{g L}^{-1}$	70–810 $\mu\text{g L}^{-1}$
NO ₃ ⁻ N, NO ₂ ⁻ N ^a	180 $\mu\text{g L}^{-1}$	5–1,940 $\mu\text{g L}^{-1}$
Total dissolved AAs ($n = 42$)	2,550 FM	800–5,500 FM
Urea ($n = 42$)	30 $\mu\text{g L}^{-1}$	15–80 $\mu\text{g L}^{-1}$
Chlorophyll <i>a</i>	0 $\mu\text{g L}^{-1}$	0–1 $\mu\text{g L}^{-1}$
Phytoplankton (mostly cryptomonads, chrysophytes, cyanobacteria)	$2 \times 10^2 \text{ cells mL}^{-1}$	10^1 – $10^4 \text{ cells mL}^{-1}$
Total bacteria ($n = 42$)	$3 \times 10^5 \text{ cells mL}^{-1}$	10^3 – $10^7 \text{ cells mL}^{-1}$
Fecal coliform bacteria ($n = 54$) ^a	35 CFU 100 mL ⁻¹	0–104 CFU 100 mL ⁻¹
<i>Vibrio</i> spp. (<i>V. vulnificus</i> , <i>V. anguillarum</i> ; $n = 42$)	5 CFU mL ⁻¹	0–90 CFU mL ⁻¹
Ciliates	55 cells L ⁻¹	0–320 cells L ⁻¹
Rotifers	90 cells L ⁻¹	0–1,900 cells L ⁻¹

FM, femto mole.

^aNote that median NO₂⁻N in our fish bioassays was 35 $\mu\text{g L}^{-1}$ and has been maintained at <700 $\mu\text{g L}^{-1}$ (<0.7 mg L^{-1} as indicated in Table 4). Nutrient concentrations were rounded to the nearest 10 $\mu\text{g L}^{-1}$ (except urea, to the nearest 5 $\mu\text{g L}^{-1}$); total phytoplankton and total bacteria data were rounded as indicated; ciliate and rotifer data were rounded to the nearest 5 cells $\mu\text{g L}^{-1}$.

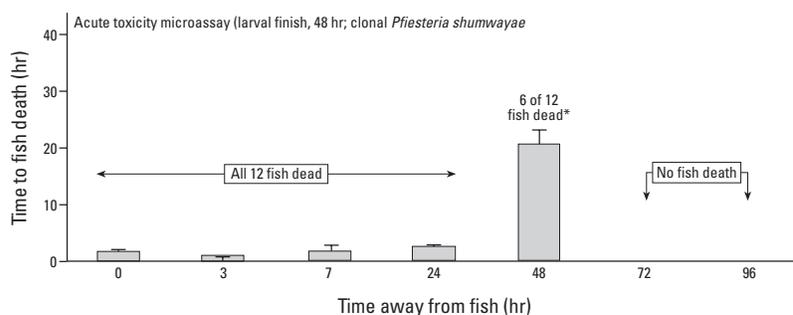


Figure 2. Acute toxicity microassays [48 hr, using clonal toxic strains of *P. shumwayae* previously maintained in (standardized) fish bioassays] on time of death of larval fish. Data are given as means \pm 1 SE; $n = 60$.

Bigelow, Maine) for 1–3 months prior to initiation of fish bioassays. These fish bioassays were conducted with *O. mossambicus* (juveniles, t.l. 5–7 cm, $n = 20$ with 10 fish per replicate in 10-L cultures) at a salinity of 15, 21°C, and 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 12 hr:12 hr light:dark cycle (see Tables 1, 4, and 5 for other environmental conditions). The TOX-A populations of varying potency required 4–9 days to show fish-killing activity (90% confidence interval; 95% confidence interval at ≤ 21 days). In contrast, the potentially toxic (TOX-B) populations that had previously not been in actively toxic, fish-killing mode required >40 days to show fish-killing activity (Figure 3). We have extended fish bioassays to test survey samples for toxic strains of TPC species (known or as yet undetected) for 14 weeks. However, among more than 400 such tests, only 2 have yielded toxic strains of TPC species after 10 weeks (≤ 12 weeks).

From these and other experiments with similar findings [e.g., Glasgow et al. (10) and Burkholder et al. (4)] (Figure 3), we interpret fish bioassays of samples collected from in-progress fish kill or epizootic events that yield dead fish within ≤ 21 days to indicate that an actively toxic (TOX-A) *Pfiesteria* or *Pfiesteria*-like population was present at the event. In the experimental series described above, condition *a* is considered analogous to tests of TPC populations that were actively toxic, in fish-killing mode, when collected prior to transport and other delays before testing in fish bioassays. Condition *b* simulated delays required before potentially toxic (TOX-B) rather than actively toxic populations can become lethal to fish and is analogous to samples from field surveys for potentially toxic *Pfiesteria* populations (4, 5, 8, 28, 29). With increasing time away from live fish, populations that were recently in actively toxic mode require progressively more time to resume lethal activity toward fish. Populations without recent active toxicity toward fish require a significantly longer duration before they show fish-killing capability. The time required would be expected to involve both excystment from temporary cysts that often form with sample disturbance during transport, and upregulation of biochemical pathways for initiating and increasing toxin production following separation from live fish. Such timing delays in the metabolic functioning of *Pfiesteria* in natural estuarine habitat might be exacerbated by the artificial conditions of laboratory culture. Heterotrophic dinoflagellates require various organic substrates, many of which have not been identified or are poorly understood (25). *Pfiesteria* clones commonly become significantly altered in toxicity, morphology, ploidy, and/or other traits over time in culture (4). Such changes suggest that the

culture conditions lack particular organic substrates (including, perhaps, one or more bacterial cofactors) that occur in the natural habitat, or some other vital factor(s) found in the natural habitat (4).

Algal Assays versus Fish Bioassays in Efforts to Detect *Pfiesteria*

Because toxin production in *Pfiesteria* spp. is triggered by the presence of live fish (3, 4, 8, 10, 22, 23), fish bioassays are the appropriate test to use in efforts to assess *Pfiesteria* involvement in estuarine fish kills [see consensus document by PCWG (5)]. However, lacking biohazard BSL-3 facilities, some laboratories have attempted to use algal assays to assess whether *Pfiesteria* spp. were present or involved in fish kill/disease events (52). Algal assays (4, 5) are conducted by adding cryptomonad or other algal prey, known to be commonly consumed by mixotrophic and heterotrophic dinoflagellates, to natural samples (5, 9, 25).

We hypothesized that algal assays would perform poorly in detection of toxic strains of *Pfiesteria* spp. by selecting for mixotrophic dinoflagellates that had not recently been targeting fish prey. To test this hypothesis, during June 1999 we collected 16 water-column + surficial sediment samples from the mesohaline Neuse Estuary (Minnesott Beach) and the Pamlico Estuary (Blount Bay) in North

Carolina [for locations see Glasgow and Burkholder (30)]. Each sample was gently mixed and subaliquoted into one subsample (100 mL each) for use in algal assays versus one subsample for fish bioassays. Algal assays were conducted by adding sufficient cryptomonads (*Cryptomonas* LB2423, acclimated to within a salinity of ± 2 of the samples) to effect an initial concentration of 10^4 cryptomonads mL^{-1} , compared to a total *pfiesteria*-like dinoflagellate density of approximately $2\text{--}6 \times 10^3$ zoospores mL^{-1} (environmental conditions 21°C and 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 14 hr:10 hr light:dark cycle). Fish bioassays were conducted with *O. mossambicus* as described above (100 mL natural sample added to 4-L volume). Both assays were run for 10 weeks and were subsampled for dinoflagellates minimally at 2-week intervals; in positive fish bioassays (with fish death, relative to no fish death in controls), samples were taken at 1- to 2-day intervals from the point when fish initially began to show signs of disease through death. Light microscopy was conducted on acidic Lugol's-preserved samples following the procedures in Burkholder and Glasgow (8) and Burkholder et al. (28), to evaluate the samples for *Pfiesteria* and *pfiesteria*-like dinoflagellates, as well as other potentially harmful microorganisms (including other dinoflagellates; as in Table 1). We also tested for the presence of *Pfiesteria* spp. using

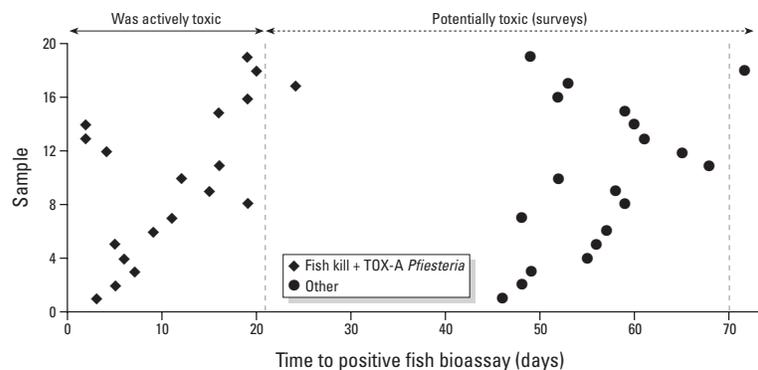


Figure 3. Comparison of the time interval required for positive fish bioassays (i.e., with fish-killing activity by TOX-A *Pfiesteria* spp.) for samples collected from estuarine fish kills in which TOX-A *Pfiesteria* spp. (4) were implicated as primary causative agents (closed diamonds; $n = 20$) versus samples that yielded toxic *Pfiesteria* in fish bioassays wherein the samples were taken during survey efforts in estuarine waters without diseased or dying fish (closed circles; $n = 20$). The latter estuarine areas were interpreted to contain TOX-B populations of TPC species. In all cases, sample transport and other delays extended for 1–2 days. Such handling is regarded as especially important for recently toxic populations in efforts to implicate versus rule out involvement of TOX-A *Pfiesteria* from in-progress estuarine fish kills. Note that 19 of the 20 samples from events that we diagnosed as having involved TOX-A *Pfiesteria* were positive for fish-killing activity within 20 days (longest lag period, 1 sample within 24 days). To err conservatively, we consider that samples appropriately handled (with <3-day lapse, including transport, following collection during an in-progress fish kill) should produce fish-killing activity within ≤ 21 days for actively toxic *Pfiesteria* to be implicated as a causative agent involved in the kill. Also note that samples which contained potentially toxic (TOX-B) *Pfiesteria* spp. populations that had not been recently in actively toxic mode toward fish did not show fish-killing activity until incubated with live fish for more than 6 weeks; 19 of the 20 samples from that set were ichthyotoxic by 10 weeks, with 1 sample requiring slightly longer. On the basis of well over 2,000 fish bioassays with estuarine samples, thus far we have not obtained toxic isolates of TPC species requiring more than 10.5 weeks to exhibit fish-killing activity, with two exceptions that required <12 weeks.

molecular probes [PCR, fluorescent *in situ* hybridization (FISH) (20,21)] together with SEM of suture-swollen cells [(3,4); at least 100 cells examined for complete plate structure in SEM for samples collected in association with fish death].

Of the 16 samples tested using algal assays, 12 were positive for cryptoperidiniopsisoid, scripsielloid, and/or gymnodinioid dinoflagellates (Table 6). Only 1 was positive for a *Pfiesteria* sp. (*P. piscicida*) and was subsequently tested as noninducible without ability to cause disease or death, in fish, bioassays. In marked contrast, 10 of the 16 samples tested using fish bioassays were positive for toxic strains of TPC species (*P. piscicida* and/or *P. shumwayae*). In 2 of the 16 fish bioassays, *Karoldinium micrum* (Leadbeater & Dodge) J. Larsen (53) (epifluorescence light microscopy, 600×) or gymnodinioid (54) dinoflagellates initially were detected, but these organisms rapidly declined to negligible or undetectable levels within 2–3 days, well before signs of fish distress, disease, and death (>40 days). PCR and FISH probe analyses on the original estuarine samples yielded results consistent with the fish bioassays and algal assays in combination. *Pfiesteria* spp. were detected from the same 10 of 16 samples (10 as in the 10 positive fish bioassays, detecting toxic strains; 1 of the 10 samples also tested

positive in the algal assay, a strain that was later confirmed as noninducible; thus, the latter sample had contained both toxic and noninducible strains). The data demonstrate that algal assays are not reliable in detecting actively or potentially toxic strains of TPC species from natural samples. Similar findings have been reported in other research (4,23).

Cross-Confirmation in Quality Control/Assurance

Fish bioassays, if conducted appropriately following this standardized procedure, are work-intensive undertakings that require practice and experience. The culturing of toxic *Pfiesteria* strains requires optimization of environmental conditions for growth of isolates from widely varying environmental conditions throughout the intensive, multi-step, lengthy procedure described in this writing. Dinoflagellate systematists strongly recommend cross-confirmation of dinoflagellate species identifications by independent laboratories with expertise in that specialized field [demonstrated through appropriate training and peer-reviewed international science publications (4,5)]. The standardized fish bioassay procedure requires not only expertise in dinoflagellate systematics, but expertise in the behavior and growth characteristics of *Pfiesteria* spp.,

which are animal-like dinoflagellates with complex behavior. Thus, cross-confirmation of the data by independent specialists is yet more important. Confirmation of results by proven specialists in the field is, historically, hallmark of good science and should be a standard step for quality control/assurance in scientifically sound harmful algal research [e.g., Burkholder et al. (4), Lewitus et al. (12), and Marshall et al. (23)]. There is a proven track record of quality control/assurance gained by laboratories that practice cross-confirmation with independent laboratories of specialists in toxic *Pfiesteria* research (4,5,23,24). The integrity of the findings is strengthened against reports of false positives (= reports of toxic *Pfiesteria* that are invalid) as well as false negatives (resulting from improperly conducted fish bioassays in environmental conditions that are not conducive to toxic *Pfiesteria* growth) and conveyance of related misinformation. Such corroboration should be regarded as an essential step in quality control/assurance. It should be routinely practiced by any laboratory engaged or attempting to engage in toxic *Pfiesteria* research.

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Table 6. Comparison of algal assays and fish bioassays in detecting *Pfiesteria* spp. among mixotrophic and heterotrophic dinoflagellates^a from natural estuarine samples.^b

Source, sample	Algal assays		Fish bioassays	
	LM/SEM	PCR (<i>Pfiesteria</i>)	LM/SEM	PCR (<i>Pfiesteria</i>) ^c
Neuse (Mesohaline, Minnesott Beach)				
1	Cryptoper, Gymno spp.	Negative	<i>P. shumwayae</i>	Positive
2	Cryptoper, Gymno spp.	Negative	<i>P. piscicida</i> , <i>P. shumwayae</i> ^d	Positive
3	Gymno spp.	Negative	Gymno spp. ^e	Negative
4	Cryptoper, Gymno spp.	Negative	<i>P. piscicida</i> , <i>P. shumwayae</i> ^d	Positive
5	Cryptoper, Gymno, Scrips spp.	Positive ^f	<i>P. piscicida</i> , <i>P. shumwayae</i> ^d	Positive
6	Gymno spp., <i>Karlo</i>	Negative	<i>K. micrum</i> , Gymno spp. ^e	Negative
7	Gymno, Scrips spp.	Negative	<i>P. shumwayae</i> , Gymno spp. ^g	Positive
8	Cryptoper, Scrips spp.	Negative	<i>P. piscicida</i>	Positive
9	Cryptoper, Gymno, Scrips spp.	Negative	<i>P. piscicida</i> , Gymno. spp. ^g	Positive
10	Cryptoper, Gymno spp.	Negative	Gymno spp. ^e	Negative
11	Gymno spp.	Negative	Gymno spp. ^e	Negative
12	Gymno spp.	Negative	Gymno spp. ^e	Negative
Pamlico (Mesohaline, Blount Bay)				
1	Gymno, Scrips spp.	Negative	<i>P. piscicida</i>	Positive
2	Crypto, Gymno spp.	Negative	Gymno spp. ^e	Negative
3	Crypto, Gymno spp.	Negative	<i>P. piscicida</i> , <i>P. shumwayae</i> ^d	Positive
4	Gymno spp.	Negative	<i>P. piscicida</i>	Positive

LM, light microscopy.

^aCryptoperidiniopsisoid spp. (Cryptoper), scripsielloid spp. (Scrips), gymnodinioid spp. (Gymno; would include both small *Gymnodinium* spp. and small *Gyrodinium* spp., and certain small *Katodinium* spp.). *K. micrum* = *Karoldinium micrum*, formerly *Gyrodinium galatheanum* (53). ^bIntegrated water-column sample + the uppermost 2 cm of surface sediments; total water column depth, 2.5–3.2 m; *n* = 100 cells per replicate in analyses with SEM of suture-swollen zoospores; note that LM was also periodically performed on acidic Lugol's preserved samples throughout the assay period to check for other mixotrophic and heterotrophic dinoflagellates. ^cPositive under fish bioassays; PCR indicates that the *Pfiesteria* species listed under SEM was/were also detected with PCR. ^dThe two *Pfiesteria* spp. alternated in dominance throughout the 10-week bioassay period. ^eIn fish bioassays of Neuse samples 3, 6, 10, 11, and 12, and of Pamlico sample 2, various other mixotrophic and heterotrophic dinoflagellates were detected, but their presence did not coincide with any signs of fish distress, disease or death. ^fIn algal assays of Neuse sample 5, *P. piscicida* was detected with PCR; however, when this isolate was cloned (using flow cytometric procedures) and tested separately in fish bioassays, it was evaluated as NON-IND (apparently incapable of causing fish distress, disease, and death). ^gThe *Pfiesteria* spp. and the gymnodinioid species from fish bioassays of Neuse samples 7 and 9 were each cloned and separately tested in additional sets of fish bioassays. In both cases, the gymnodinioid species did not promote fish distress, disease and death, whereas the clonal *Pfiesteria* spp. were ichthyotoxic and their presence led to fish disease and death.

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