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Pfiesteria: Review of the Science and Identification of Research Gaps. Report for the National Center for Environmental Health, Centers for Disease Control and Prevention

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In connection with the CDC National Conference on *Pfiesteria*, a multidisciplinary panel evaluated *Pfiesteria*-related research. The panel set out what was known and what was not known about adverse effects of the organism on estuarine ecology, fish, and human health; assessed the methods used in *Pfiesteria* research; and offered suggestions to address data gaps. The panel's expertise covered dinoflagellate ecology; fish pathology and toxicology; laboratory measurement of toxins, epidemiology, and neurology. The panel evaluated peer-reviewed and non–peer-reviewed literature available through June 2000 in a systematic conceptual framework that moved from the source of exposure, through exposure research and dose, to human health effects. Substantial uncertainties remain throughout the conceptual framework the panel used to guide its evaluation. Firm evidence demonstrates that *Pfiesteria* is toxic to fish, but the specific toxin has not been isolated or characterized. Laboratory and field evidence indicate that the organism has a complex life cycle. The consequences of human exposure to *Pfiesteria* toxin and the magnitude of the human health problem remain obscure. The patchwork of approaches used in clinical evaluation and surrogate measures of exposure to the toxin are major limitations of this work. To protect public health, the panel suggests that priority be given research that will provide better insight into the effects of *Pfiesteria* on human health. Key gaps include the identity and mechanism of action of the toxin(s), the incomplete description of effects of exposure in invertebrates, fish, and humans, and the nature and extent of exposures that place people at risk. *Key words*: dinoflagellate organism, ecophysiology of *Pfiesteria*, epidemiology, fish kills, fish pathology and toxicology, Henle-Koch postulates, ichthyotoxins, literature review, neurological effects, state of the science. — *Environ Health Perspect* 109(suppl 5):639–659 (2001).
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Overview*

The *Pfiesteria* organism was first identified and named in the early 1990s and its ability to kill fish was documented at the time. Research on the effects of exposure to its toxin on humans is more recent, sparked largely by widely publicized fish kills in the late 1990s and reports of adversely affected persons present at the scenes of the kills. Consequently, the peer-reviewed literature is still limited and substantial research is in progress at institutions in the Atlantic coast states of Maryland, North Carolina, and Virginia, and elsewhere. Dramatic reports of fish kills and illness among persons exposed to water at the time of the kills sparked public concern and reviews by a variety of agencies in the late 1990s. The primary data sources, secondary reviews, and expert panel reports were evaluated by this panel.

In approaching its charge, the panel turned to a toxicologically based framework for conducting its review, characterizing the state of the science, and identifying research gaps (Figure 1). Prevention of adverse effects of *Pfiesteria* will need to be based in an understanding of each element in this framework: the ecological factors that drive concentrations of the organism and its state; the stimuli that lead to toxin production and the nature of the toxin; the mechanism of the toxin's effect on fish; the circumstances and routes of human exposure; and the mechanisms by which human health is adversely affected by the toxin. Sufficient certainty is needed on each of these points to evolve policies that will assure protection of public health and the fish populations. In addition, we need enhanced understanding of the still poorly characterized effects of *Pfiesteria* on human health.

Other reviewers have commented on many elements of this framework; their reviews make clear that there are gaps in scientific understanding, with attendant uncertainty for decision makers, for each of these elements. With substantial research now under way, the panel views the timing of this review as an opportunity to examine the gaps and the extent to which new research findings will set aside uncertainties.

Pfiesteria has proved to be a challenging problem. Researchers have referred to the organism as "ephemeral" and "phantom-like." It has multiple forms, transforming itself into a toxin-producing dinoflagellate when it somehow senses the presence of fish. Its toxin apparently destroys the integrity of the skin of fish, allowing the organism to feed; after feeding, the organism transforms into a dormant form. (Throughout this report, we refer to "*Pfiesteria* toxin," recognizing that the organism may well produce more than one toxin. We use the plural "toxins" in describing the basis for supposing that more than one toxin exists.) There is debate as to whether a pathognomonic lesion occurs in fish. Humans seem to be at risk when their activities lead to contact with toxin-containing water at the time of fish kills. Whether the toxin reaches target tissues by dermal contact or inhalation is unclear. A diffuse syndrome has been described in individuals likely to have been exposed to toxin; its components include dermal irritation, respiratory and gastrointestinal symptoms, systemic symptoms such as fatigue and malaise, and impaired neuropsychological functioning. The Centers for Disease Control and Prevention (CDC) has offered criteria for possible estuary-associated syndrome (PEAS) for the purpose of surveillance (*1–3*).

While the scientific evidence on *Pfiesteria* is only now being accumulated, driven by funding made available over the last several years, there are sufficient credible human case

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

†These authors make up the Peer Review Panel on the state of of the science concerning *Pfiesteria*.

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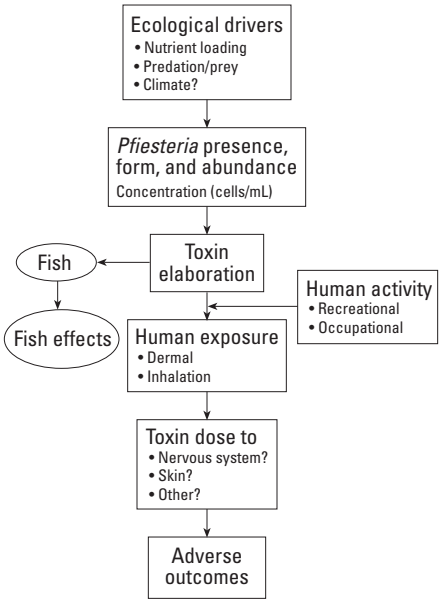

Figure 1. A framework for addressing *Pfiesteria* and public health.

reports to suggest adverse effects of *Pfiesteria* toxin on people. The panel viewed this clinical evidence as sufficiently compelling to motivate a research agenda that would comprehensively address the many gaps in our understanding of *Pfiesteria* and human health.

Principles for Interpreting Evidence

In addressing its charge, the panel reviewed evidence associating *Pfiesteria* with fish kills, toxin elaboration, and adverse effects on human health; it also considered ecological data on the presence of *Pfiesteria* and changes in nitrogen and phosphorus concentrations driven by man's activities. The various lines of data were derived from observation and also from the laboratory setting. No single framework can be applied across disciplines for evaluating such diverse evidence for its strength in establishing linkages of hypothesized sets of causes with observed outcomes, e.g., *Pfiesteria* with fish kills, waste effluents into rivers with the presence of *Pfiesteria*, or *Pfiesteria* toxin with human health effects.

To identify causes of human disease, criteria for determining the causal nature of associations have been proposed, beginning with Koch's postulates, offered at the end of the 19th century for judging if an infectious disease were caused by a particular microbe as described by Evans (*4*). Koch's postulates, sometimes referred to as the Henle-Koch postulates to acknowledge the contribution of Henle, Koch's professor, included *a*) an organism needs to be present in every case of the disease and "under circumstances which can account for the pathological changes and clinical course of the disease"; *b*) the organism is not found in other diseases as a fortuitous and nonpathogenic bystander; and *c*) after isolation from affected people and growth in culture, the organism can induce the disease (*4*). Koch applied these criteria in describing the causal link of the tubercle bacillus with human tuberculosis.

Recently, Fredricks and Relman (*5*) described an extension of these criteria to sequence-based identification of microbial pathogens. They specifically addressed sequence-based identification of microbial pathogens, now possible with modern assay techniques. Marshall and colleagues (*6*) modified the Koch postulates and applied them to findings of fish bioassays for the toxicity of *Pfiesteria*. In analogy to Koch's first postulate, they proposed that *Pfiesteria* must be present at a fish kill or disease event. For the second postulate, they suggested that *Pfiesteria* must be isolated from a fish-killing sample and then grown in clonal culture. For the third, addition of the clonal *Pfiesteria* to healthy fish cultures results in death. Finally, *Pfiesteria* must be isolated from the experimentally induced fish kill and recloned. Their report describes experiments intended to meet these criteria.

Criteria for causality in relation to human disease have evolved substantially since Koch's writings over 100 years ago. In the mid-20th century, substantial epidemiologic research was initiated on the emerging epidemics of the "chronic diseases"—cancer, coronary heart disease and stroke, and chronic lung disease. There was soon recognition that the Henle-Koch postulates were not appropriate for interpreting the evidence on these multifactorial, complex diseases. A set of guidelines evolved (*4,7,8*) that was applied, for example, in interpreting the evidence on smoking and lung cancer in the 1964 Surgeon General's Report (*9*). The guidelines consist of the following elements: consistency of association, strength of association, specificity of association, temporal relationship of association, and coherence of association. These elements are intended to be criteria for judgment and not a rigid checklist. They call for consistency of findings with replication and coherence with other relevant observations, including understanding of pathogenesis. The argument for causality is strengthened by the finding of a stronger level of association, less likely to be attributable to chance or bias, and the presence of a dose–response relationship. Specificity is generally not relevant to multicaused human diseases.

The scientific evidence on *Pfiesteria* is still limited, with only a small number of research groups involved and insufficient time for replication and validation of key findings. Thus, regardless of the criteria applied for causal interpretation, the paucity of the data limits the strength of inferences that can be drawn. Consequently, in carrying out its review, the panel first attempted to characterize the extent of the evidence available, enumerating to the extent possible the number of independent observations, e.g., cases of human disease reported and the numbers of samples studied in the laboratory setting. The panel judged that some reports included overlapping data and attempted in its review to document the evidence available.

Beyond this detailed enumeration of the data available, the panel considered the Henle-Koch postulates as appropriate for linking *Pfiesteria* to either fish kills or human disease. As a minimum, there should be documented presence of the organism at the time of fish kills or, in the instance of human disease, the presence of a fish kill when persons who become ill were in contact with water. Effects on fish can be addressed in the laboratory, thus fulfilling the last postulate, but human effects cannot be investigated experimentally. There is anecdotal evidence in the form of experience of persons exposed in the laboratory who have become ill. However, establishing causal linkages between exposure to *Pfiesteria* and human disease is complicated by the nonspecificity of the clinical picture as presently characterized.

The evidence on ecological determinants of the organism's presence and concentration is also observational. The panel's approach for interpretation paralleled that used for the human health data.

This report follows the panel's framework offered in Figure 1. We begin with a review of the ecophysiology of *Pfiesteria* and then shift to its effect on marine organisms. The subsequent section covers approaches to measurement and detection of the organism and its toxin. After that, we address human health effects, including clinical and epidemiological reports. Finally, we offer conclusions and suggest directions for further investigation.

Ecophysiology of *Pfiesteria piscicida**

P. piscicida Relative to Other Dinoflagellates and Harmful Algal Blooms

Pfiesteria piscicida Steidinger & Burkholder represents a novel group of dinoflagellates first discovered in the 1980s, and collectively termed "ambush predators." (Throughout this report, we use "*Pfiesteria*" to encompass "*Pfiesteria piscicida* Steidinger & Burkholder," "*Pfiesteria shumwayae* Glasgow & Burkholder," and other as yet unidentified species of the genus. We use the standard shortened forms "*P. piscicida*," "*P. shumwayae*" whenever we can do so without causing confusion.) *P. piscicida* appears to be unique among this

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group and among dinoflagellates generally in its life cycle, nutritional behavior, and toxicity, although much remains to be learned (*10,11*). The ichthyotoxic blooms of *P. piscicida* were first described in 1992 (*10*) and the organism was classified taxonomically in 1996 (*12*). Knowledge of the life cycle and feeding behavior of the organism is based primarily on research conducted in the laboratory and on less extensive field research.

Current understanding of the salient features of the life cycle and of the behavior of *P. piscicida* has been described in several peer-reviewed publications (*6,10,11,13,14*). The organism's polymorphic life cycle (Figure 2) consists of three distinct life-form stages—flagellated, amoeboid, and encysted—that live in bottom sediments or as free-swimming organisms in the water column. These stages involve at least 24 size, shape and morphotypic variants, ranging from 5 to 450 μm in size. The stages include rhizopodial, filose [i.e., the star amoebae in Figure 2; (*15*)] and lobose amoebae; toxic and nontoxic zoospores (asexual flagellated spore); cysts of various structure; and gametes (mature sexual reproductive cells having a single set of unpaired chromosomes) (*14*).

Under laboratory conditions in the presence of live fish, its sediment-dwelling amoeboid and resting stages transform rapidly into free-swimming flagellate stages in response to unknown chemical cues secreted or excreted by fish (*10*). The induced (excysted) flagellate stages swarm into the water column and become toxic during their continued exposure to the fish-derived (sometimes shellfish-derived) chemical stimulants. The toxic zoospores gather together, alter their random swimming pattern into directed movement, doubling their swimming speed in the process (to 670 μm sec^{-1}; median), and commence predatory behavior directed toward targeted fish.

The toxic zoospores produce a neurotoxin of unknown structure, soluble in water, and which may be liberated as an aerosol under some conditions. Fish are first narcotized by the toxin, die suddenly, and slough off tissue, which the attacking zoospores consume by sucking out the cell contents through the attached peduncle. The zoospores sometimes ingest other microscopic plant and animal prey at the same time. During this killing period, the zoospores reproduce both asexually (mitotic division) and by producing gametes that fuse to produce toxic planozygotes (actively swimming offspring formed by sexual reproduction, i.e., the union of two gametes) (Figure 2). The presence of live fish is required both for completion of the sexual cycle and for toxin induction.

Upon fish death or their retreat, the toxic zoospores and planozygotes transform into (mostly) nontoxic amoeboid stages that gather onto the floating fish carcasses on which they feed for extended periods, and follow the sinking fish remains to the bottom sediments. Not all toxic zoospores and planozygotes transform into amoebae. Some encyst and sink into bottom sediments; a lesser number revert to nontoxic zoospores that remain in the water column. The proposed 24 stages of the complex life cycle are based on laboratory observation. Although several stage transformations have been documented photographically, photographic or videotape documentation for other proposed phases has not been published.

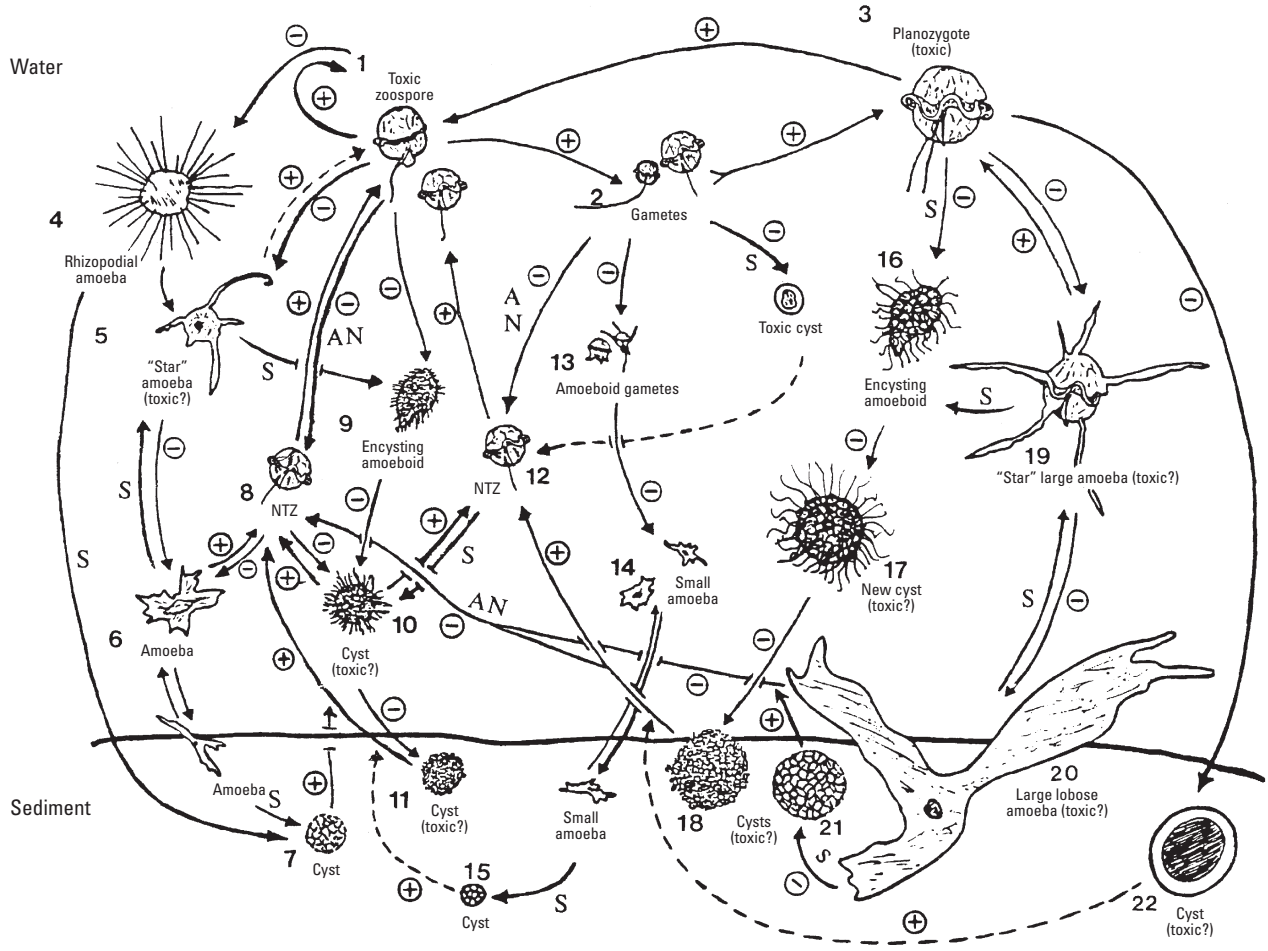

Figure 2. Schematic of the complex life cycle of *P. piscicida* with (+) and without (–) live finfish. Other environmental controls are indicated by: A, presence of flagellated algal prey; N, nutrient enrichment (e.g., organic or inorganic N and P); S, environmental stress such as shift in salinity, temperature, or physical disturbance. NTZ, nontoxic zoospore. Adapted from Burkholder and Glasgow (*11*) with permission from the American Society of Limnology and Oceanography.

Neither is molecular confirmation available to demonstrate that each of the proposed stages has the same genome.

Other dinoflagellate species have multiple life stages that transition from bottom sediments to the water column, feed on multiple trophic levels, are toxic to fish, "bloom" explosively under certain environmental conditions, and are toxic to other marine organisms and to humans. *Pfiesteria*'s distinctive characteristics are its extraordinarily complex life cycle, nutritional diversity, trophic control of its life cycle and toxicity, and the way it kills fish prey. During fish kills attributed to *P. piscicida* in natural habitats, and in contrast to blooms of many ichthyotoxic flagellates, its lethal stages [i.e., toxic zoospores, planozygotes, large lobose amoebae (at temperatures <15°C)] usually represent only a minor component of the phytoplankton community present in the water column. Blooms of *P. piscicida* are very ephemeral—generally only hours in duration, of relatively modest cellular abundance (>250 cells mL^{-1}), and rarely discolor the water mass (*15*). Of particular interest, *P. piscicida* is the first dinoflagellate and the first harmful bloom species observed to attack prey spanning all trophic levels of estuarine food webs, from bacteria to fish (*16*).

The complexity of the life cycle and unusual behavior ascribed to *P. piscicida* raise the following questions: Are the reported ecophysiology and trophic impact of this apparently novel dinoflagellate compromised by as yet imprecise (inconclusive) methodology and investigation? Or is *P. piscicida* a particularly problematic species, one that warrants continued special research attention and public health concern? In asking these questions, the panel recognizes that the context of the evidence in which they are asked comes from only a decade of research.

Several scientific challenges to the validity of some key ecophysiological features advocated for *Pfiesteria* can be raised in relation to the peer-reviewed and non–peer-reviewed literature, including:

- Some of the proposed "stages" in the complex life cycle proposed for *P. piscicida* are actually other organisms ("contaminants") present in the *P. piscicida* cultures.
- Field events, toxicity features, and fish kills attributed to *P. piscicida* have been confused with the behavior of look-alike species, or are based on inadequate field sampling.
- *P. piscicida* outbreaks are consequences, rather than causes, of fish kills.

These challenges are addressed in the following sections.

The Life Cycle Issue

The life cycle of *P. piscicida* (Figure 2) is based primarily on documented transformations of isolated cells and populations studied in the laboratory and exposed to various combinations of temperature, salinity, nutrient addition, prey availability, and live and dead fish exposure (*11,13,14*). Life-cycle transformations appear to be highly sensitive to habitat conditions, often occurring rapidly and producing structurally similar stages despite different life cycle pathways. Most dinoflagellates that spend part of their life cycle in the water column and part in bottom sediments are biphasic, with swimming and resting stages. *Pfiesteria* is notable because it is apparently triphasic, including flagellated, amoeboid (three distinct types), and resting (encysted) stages and as many as 24 distinct life-form variants.

P. piscicida is not the first dinoflagellate shown to produce amoebae that dominate its life cycle or the first to exhibit a triphasic life cycle (*17,18*). *Haidadinium ichthyophilum,* a freshwater dinoflagellate ectoparasitic on three-spined stickleback, has four distinct amoeboid stages, cysts, and "gymnodinioid" swarmers (*19*). The 24 stages presently recognized in *P. piscicida*'s life cycle are considerably less than the 38 stages recorded for *H. ichthyophilum*, the previously mentioned freshwater dinoflagellate that is also characterized by the nutritional versatility exhibited by *P. piscicida* (*15*).

Contamination of laboratory cultures of *P. piscicida* by other organisms cannot be ruled out and it is possible that some stages have been incorrectly identified. Nonetheless, such complexity is neither unique nor exclusive to *P. piscicida* among dinoflagellates. Its complexity is not therefore an a priori reason to doubt the validity of the life cycle as now described. Two additional bits of evidence support this: During multiple *Pfiesteria*-linked fish kills, several of the life stages depicted in Figure 2 have been recorded within the water column. The dominant amoeboid stages, which are particularly abundant on sediments regardless of fish availability, were ubiquitous during the fish kill events (*20*). More quantitatively, analyses of ribosomal DNA sequences demonstrated that four dinoflagellate species (*P. piscicida*, a *Pfiesteria* look-alike species, a cryptoperidiniopsoid sp., and the obligate fish parasite *Amyloodinium ocellatum*) are closely related members of an early ancestral group (Blastodiniphyceae) having parasitic tendencies (*21*). Litaker et al. (*22*) also reported sequence data for *P. piscicida*, but uncertainty surrounds this GenBank entry.

Problem of Identification and Look-Alike Species

Two species currently form the *Pfiesteria* complex: *P. piscicida* and a new species to be named *P. shumwayae*. A *Pfiesteria* sp. has been implicated in a fish kill in a marine home aquarium (*23*). This fish kill cannot be attributed definitively to *Pfiesteria* because other parasitic dinoflagellates were identified in the tank and because the *Pfiesteria*-like organism disappeared before it could be examined and classified taxonomically. A combination of microscopic techniques and fish bioassay is required to detect *P. piscicida* in its various life stages (see below, "Measurement Tools for Detecting the *Pfiesteria* Organism"). Many small gymnodinioid dinoflagellate species look like *P. piscicida* zoospores under light microscopy. Taxonomic identification requires scanning electron microscopy (SEM) (*12,18*) (see below, "Scanning Electron Microscopy").

P. piscicida is easily overlooked in fish kills that it may have induced, for several reasons. Its toxic zoospore stages closely resemble other small, nontoxic species. Its small flagellated forms tend to preserve poorly in the fixatives routinely used in phytoplankton analyses. When preserved, its amoeboid stages often resemble organic debris, and its

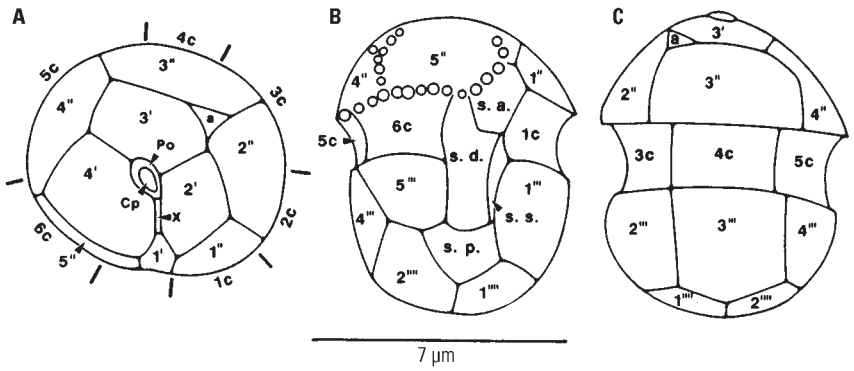

Figure 3. The dinoflagellate *P. piscicida*. (*A*) Apical view with epithecal plate tabulation and pattern. (*B*) Oblique ventral view showing four sulcal plates; the s.a. plate ties under the 1′ and 5″ plates. (*C*) Dorsal view. Views *B* and *C* show the complete hypothecal plate tabulation and pattern. Scale bar = 7 µm. Reproduced by permission from *Journal of Phycology* (*12*).

colorless heterotrophic cells blend into "counting chamber" surroundings (*15*). Proper identification requires SEM, an approach generally not used in field monitoring. The optimal period for detecting lethal *Pfiesteria* stages is during the fish kill (*10*), but sampling is usually done after the fish kill is reported and rarely conducted during the active die-off period.

Cryptic occurrences prior to fish kills are another problem. *Pfiesteria*'s presence may be overlooked because of its low abundance or life cycle stage. A Neuse River, North Carolina, experimental study was hampered by chronic low abundance of the biflagellate zoospore stage (frequently <6 cells mL^{-1}). Abundance of other phytoplankton organisms of similar size ranged from 10^3 to 10^4 cells mL^{-1} (*24*). Dead fish often are carried to regions outside of the active kill area by tidal and other currents, blurring interpretations based on analyses of watermass properties in samples collected in the "dead zone."

The rapid stage transformations and population dynamics that characterize *P. piscicida* contribute to these detection and identification difficulties. Time constants of life-cycle transformations are consistent with the ephemeral, rapid response, "sudden death" syndrome described for ichthyotoxic *Pfiesteria* events. Upon stimulus, zoospores can transform within minutes into filose amoeboid stages (Figure 2) (*18*). Flagellated stages subjected to shear can encyst within minutes to hours (*11*). Nontoxic zoospores become lethal within 3–4 hr upon exposure to live fish (*15*), and they are capable of growth rates that double the population in 15–21 hr (*25,26*). Recently formed cysts (hours to days in age) can excyst and yield toxic zoospores within 15–20 min after exposure to live fish (*11*). In culture, toxic zoospores of *P. piscicida* and two tested *Pfiesteria*-like species killed healthy fish within minutes after exposure (*15*). Toxic zoospores sharply declined in abundance within 1–2 hr after fish death as a result of their transformation into cysts or amoeboid cells (*10*).

P. piscicida is also adapted to withstand prolonged periods of unsuitable growth conditions, a feature that further compounds the problems of its detection and association with putative fish kills. The lag period for excystment upon stimulation by live fish varies from minutes to days to months, and increases with cyst age. Cysts dormant for two years required 6–8 weeks to produce toxic zoospores; less than 2 weeks if dormant for 2–8 weeks, and less than 30 min if only hours or several days old (*11*). Lobose amoebae held in culture one year produced nontoxic zoospores when supplied with a microalgal prey, and a Pamlico Sound, North Carolina, water sample rich in amoebae and kept in darkness for 4 months transformed into toxic zoospores when live fish were added (*11*).

In summary, traditional methods of analysis typically cannot be applied to detecting and monitoring *P. piscicida*'s involvement in fish kills for three 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 ecophysiologically distinctive *Pfiesteria*-like species. These complications, and discrepancies in fish bioassay results used to quantify *P. piscicida* involvement, have fueled controversy. A rigorously standardized fish bioassay process has only recently been used to replicate and confirm key findings concerning the ichthyotoxicity of *P. piscicida* (*6*).

These gaps and limitations of the available evidence are a legitimate rationale for questioning the proposition that *Pfiesteria* transforms and then releases a fish-killing toxin. Published comments include rejection of the idea that *P. piscicida* blooms cause fish kills, and strenuous denials that the organism may pose significant threats to natural fish stocks or human health (*24,27,28*). Some of these objections (*27*) are basically polemical arguments flawed by scientific misinterpretations that have been pointed out by Lewitus et al. (*29*) and Oldach (*30*). The more substantive points raised by Pinckney et al., and Stow are addressed in subsequent sections of this report.

Distribution and Ecophysiology of *P. piscicida*

P. piscicida is distributed in estuarine waters throughout the mid-Atlantic and southeastern United States, from Delaware Bay to Mobile Bay, Alabama, and may be found as far north as Long Island, New York (*31*). It is best known for its association with fish kills in the Albemarle–Pamlico Estuarine System (Pamlico and Neuse Rivers, North Carolina) (*13,15,32*), where it was implicated as the causative organism in 50% of 35 major fish kills (10^3–10^9 fish) observed during 1991–1993 (*15*). Within the Chesapeake Bay, it has been recorded from the Choptank and Patuxent River estauries in Maryland and the York River Estuary in Virginia; and it and/or a related species is found in 7 of 10 eutrophic Florida embayments where fish kills have occurred (*15,33*). Within this distribution, *P. piscicida* behaves as a warm-temperate estuarine species that occurs across a wide range of temperature and salinity but displays a distinct preference for relatively shallow, turbid, slowly flushed, nutrient-enriched, moderately saline habitats (5–18 ppt). An exception to this may be its recent discovery (via fish bioassay) in the pristine North Inlet Estuary of South Carolina (*26*).

Pfiesteria-linked fish kills occur over a broad range of salinity (0–35 ppt) but predominate in moderately saline habitats (5–18 ppt). Laboratory and field data suggest that 15 ppt is the optimum salinity for toxicity of *P. piscicida*, with toxic outbreaks (as zoospores) most frequent at ≥26°C within its bloom temperature window, which ranges from 10 to 33°C (*15,20*). Low temperature has also been shown to induce toxicity of amoeboid stages. Addition of live fish or shellfish (flounder and scallop) to experimental aquaria held at 10–15°C triggered ichthyotoxicity in the lobose amoebae that are the predominant life cycle stage of *Pfiesteria* in that temperature range (*11*).

Laboratory and field results indicate that *P. piscicida* is highly sensitive to water-column mixing and shear (*11*). In the laboratory, its flagellate stages encyst within minutes to hours when exposed to rapid filtration or agitation. Even gentle swirling of cultures can induce transformation. Field populations tend to be evenly distributed within the water column during calm weather unless tracking targeted fish. During wind-induced mixing, zoospore populations collect into lenses and cluster at or near the bottom.

Toxic *Pfiesteria* zoospores have no apparent optimal irradiance level and do not exhibit diurnal activity in the laboratory. They actively kill fish at night or in full sunlight (*11*). Given their heterotrophic nature, this apparent indifference to irradiance is not surprising.

Although the life history stages of *P. piscicida* are controlled primarily by the availability of fresh fish secretions/excretions or fish tissue, the availability and utilization of alternate prey and dissolved nutrient energy sources are also important nutritionally (*11,13,29,34*). The nutritional versatility of *P. piscicida* is remarkable. Zoospores consume bacteria, microalgae, microfaunal ciliates, erythrocytes, and fish tissue; amoebae are equally versatile. When deprived of fish tissue, zoospores and amoebae (in culture) can survive on a diet of microalgal prey but then exhibit prey selectivity and differing growth rates based on the microalgal species ingested [see Table 1 in Burkholder and Glasgow (*15*)]. Zoospores consume microalgae, either by phagotrophy or by attachment of their peduncles to prey, followed by sucking out cellular contents in saprophytic manner.

P. piscicida also can function as a phototroph, carrying out photosynthesis by utilizing the chloroplasts captured from microalgal prey and inorganic and organic nitrogen and phosphorus. Laboratory observations indicate that this expropriation and retention of the captured chloroplasts (termed kleptoplastidy) can last for at least 9 days (*29,34*).

The nutritional versatility of *P. piscicida* is consistent with the polymorphism and differing habitat requirements of its life cycle

stages. It is a "prey generalist" that can behave as an ichthyoparasite and survives periods of fish deprivation by means of a complex life cycle and nutritional versatility.

Research has focused on the toxic "ambush predator" features of *P. piscicida*; there is very limited information on the extent to which it is itself predated. Mallin et al. (*35*) have shown that a rotifer and the common estuarine copepod *Acartia tonsa* readily grazed upon *P. piscicida* in short-term experiments. There is no information on the long-term effects of *Pfiesteria* blooms on these or other grazers. Bay scallops and oysters did not strongly stimulate toxic activity of *P. piscicida*, and scallops remained viable for 9- to 14-day test periods while filtering low concentrations of toxic zoospores (~60 cells mL^{-1}) (*20*). At higher toxic zoospore levels and in the presence of dying fish, scallops died. These data suggest that filter feeding shellfish may help control the abundance of *P. piscicida*, particularly when population densities are low.

Given the complex life cycle of *P. piscicida*, the problems of detecting its presence, its impacts on fish, and the limited availability of quantitative field studies, little can be said about the ecological drivers of its population dynamics, ichthyotoxicity, or spreading potential. Attention has been focused on the hypothesis that nutrient loading of waters by man's activities is one determinant of *Pfiesteria*'s presence and abundance. Lewitus et al. (*29*) warned that continued and/or increased nutrient loadings of coastal and estuarine habitats may lead to an increase in the magnitude and geographical range of *P. piscicida* toxic events. About 75% of the toxic outbreaks of *Pfiesteria*-like dinoflagellates have occurred in nutrient-enriched waters (*20*), with *Pfiesteria*-linked fish kills often clustering in North Carolina estuaries near sites exposed to discharge from wastewater treatment facilities, fish processing plants, domestic animal operations, and phosphorus mining (*16*).

Mean nontoxic zoospore abundance (1,240 cells mL^{-1}) at four wastewater sites was 6-fold higher than at control sites (*14*). In the Neuse–Pamlico estuaries, where total phosphorus averaged ≥200 μg L^{-1} and total Kjeldahl nitrogen levels ≥580 μg L^{-1}, *Pfiesteria* was abundant.

Nutrification of a small estuary by accidental discharge of swine waste effluent was followed three weeks later by a fish kill ($>10^4$), during which toxic flagellate and amoeboid stages of *P. piscicida* were present (*36*). This fish kill cannot be definitively attributed to nutrient induced stimulation of *P. piscicida* because concurrent disruption of habitat conditions and altered bloom responses in the normal microbial and microalgal communities also occurred.

Pinckney et al. (*24*) concluded from mesocosm experiments that *Pfiesteria*-like organisms in the Neuse River do not respond to inorganic nitrogen and phosphorus nutrification, nor is their growth negatively influenced by water column mixing. The authors' interpretation of their findings directly contradicts laboratory experience with these ecophysiological factors (*11,16*), which Pinckney et al. dismiss in extrapolating their results to *P. piscicida*. Their study is 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.

In laboratory culture, *P. piscicida* responds both via increased abundance and life-cycle transformation to enrichment with inorganic and organic nutrients (*10,11,14,26*). Under some laboratory conditions, phosphorus-containing substances released by fish or direct phosphorus enrichment stimulate growth of zoospores.

Zoospores that have "stolen" chloroplasts from microalgal prey take up inorganic and organic nitrogen at rates equaling ingestion of particulate nitrogen (prey N) by phagotrophic stages. In natural settings, nutrient enrichment often stimulates the growth of microalgae and other microbial loop components on which *P. piscicida* can feed. The capacity of *P. piscicida* for both direct uptake (phototrophy) and indirect uptake via ingestion of stimulated prey (phagotrophy) suggests that the response of natural populations of *Pfiesteria* to nutrient enrichment is very complex. The specific pathway of any stimulation depends upon the nutritional state and life cycle stage of *P. piscicida*. The specific pathway of nutrient stimulation may be through enhancement of microalgal prey abundance, or direct stimulation by nutrient uptake from ingested prey. *Pfiesteria* may require a specific chemical "water quality" factor in its habitats—the bottom sediments and water column—to complete its life cycle, undergo stage transformation or emerge from dormancy. This factor may also be influenced by degree and type of nutrient loading.

The various field and laboratory results, paucity of quantitative data, and nutritional versatility of *P. piscicida* complicate field and laboratory efforts to establish a definitive relationship between blooms of the organism and degree of anthropogenic nutrient enrichment. The recent discovery (*26*) of *P. piscicida* in the highly flushed, pristine, and low-nutrient North Inlet Estuary of South Carolina (where no major fish kills have been reported) suggests that the effects of nutrients on *Pfiesteria* may be site specific. Water samples from North Inlet were introduced into fish aquaria, and fish died. *P. piscicida* was identified in these cultures by SEM.

It seems reasonable to conclude that *P. piscicida* is highly tolerant of nutrient-enriched waters, which appear to be a preferred habitat, but neither laboratory nor field research has identified specific conditions within eutrophicated habitats that are responsible for ichthyotoxic blooms. To conclude that eutrophication, per se, will lead to geographical spreading and increased magnitude of its bloom events would be premature.

Fish Kills: Cause or Consequence of *P. piscicida* Blooms?

That *P. piscicida* can become ichthyotoxic upon exposure to live fish under appropriate laboratory conditions appears to be clearly established. The reported threshold density of toxic zoospores required to kill fish is 250–300 mL^{-1}. During 26 estuarine/coastal fish kills linked to *P. piscicida* (*20*), observed zoospore population densities ranged from 270 to 35,360 cells mL^{-1} during fish kills. Zoospore densities in another fish kill were below the reported threshold. The various life-cycle stages of *P. piscicida* were also present during fish kills (*11,32*).

Some authors have argued that *P. piscicida* is not the cause of, but a consequence of, these die offs (*28,37*). Stow's reservations (*28*) are based primarily on his misinterpretation that field sample data on cell densities and presence of *Pfiesteria* were the sole basis for inferring its active role in the observed fish kills. His largely statistical argument ignores the laboratory-based experimental evidence that established the organism's toxicity in the first place (*11*), confirmatory fish bioassays, and the presence of toxic life stages during the fish kills [see Tables 1 and 2 in Burkholder et al. (*20*)], and may be moot in light of subsequent confirmatory fish bioassay results by others (*7,34*).

Blazer et al. (*37*) challenged the assertion that *Pfiesteria* is responsible for the ulcerative skin lesions purported to be diagnostic of *P. piscicida* involvement in fish kills. Their histological analyses suggested to them that fungal pathogenicity can induce the same ulcerative lesions in juvenile menhaden. While fish skin lesions may not be a useful proxy for the presence of *Pfiesteria* or its toxins, Blazer et al. do not dispute the ichthyotoxicity of *Pfiesteria*, or do they dispute the evidence that *P. piscicida* has caused major fish kills. This is also discussed in the section "Effects of *Pfiesteria* on Marine Organisms."

The preponderance of evidence from laboratory and field investigations supports the proposition that *P. piscicida* has caused fish kills in estuaries of Chesapeake Bay and the southeastern coastal regions of the United States. The evidence supporting the argument

that blooms of the organism are consequences, rather than causes, of fish kills is considerably less persuasive. The behavior reported for *P. piscicida* is consistent with the considerable global experience with other ichthyotoxic algal bloom species, their impacts, and established procedures applied by harmful algal bloom researchers.

Conclusions

Compelling evidence indicates that, in experimental enclosures, exudates, and/or excretions from fish stimulate blooms of *P. piscicida*, which then attack the fish by producing an exotoxin, although the toxin has not been isolated or characterized. Field observations, together with the laboratory evidence, indicate that *P. piscicida* has been responsible for major fish kills in mid-Atlantic estuaries. Laboratory experiments have revealed *P. piscicida* to have a very complex life cycle consisting of toxic and nontoxic stages, which exhibit great nutritional diversity. These traits suggest a multiple-niche requirement and dependency on several trophic levels. Laboratory nutrient enrichment experiments have yielded growth responses consistent with the results of field studies that suggest a preference for nutrient-enriched habitats.

The limited availability of quantitative ecophysiological observations from laboratory and field studies, together with taxonomic complexities, has impeded general acceptance of the ichthyotoxic affects, distinctive life cycle, and ecophysiology ascribed to *P. piscicida*. Some critical scientific initiatives that would help to resolve such uncertainty include the following:

- Establishment of research-quality cultures for wide distribution among the scientific community for life cycle, physiological, and ecological study
- Development of more effective and quicker methods to distinguish *P. piscicida* in each of its life cycle stages from look-alike species, and to quantify their abundance, distribution, seasonality, and toxicity
- Laboratory and field investigations, using appropriate controls, to clarify the role of organic versus inorganic nutrients, including those in sewage and in wastes from agro-industrial sources, in stimulating growth and toxicity of *P. piscicida*, and the role of these and nonnutritional factors, including prey and predatory dynamics, in regulating its *in situ* growth, population dynamics, and seasonal cycles.
- Rigorous evaluation of the linkage between the stimulation of blooms of *P. piscicida*, induction of toxicity, and substances produced by targeted fish, including chemical characterization of the putative growth/toxicity stimulant(s) and the ichthyotoxin
- Assessment of the role of environmental signals in controlling loss, or gain, in toxin-producing capability, life-cycle transformations, and functional phenotype occurrences, along with determination of the accompanying physiological characteristics of the life-cycle stages and population strains

Such ecophysiological studies will help to resolve current ecological enigmas, knowledge of which is essential to clarify the fisheries and human health issues attributed to *P. piscicida*'s bloom events.

Effects of *Pfiesteria* on Marine Organisms*

In both laboratory bioassays and field situations, the toxic stages of *P. piscicida* and *Pfiesteria*-like dinoflagellates affect every species to which they have been exposed, at least 33 species of finfish and four species of estuarine invertebrates (*10,11,20,38*). In this section, we focus our review on studies dealing with the toxicologic and pathologic effects of *Pfiesteria* spp., particularly in finfish. *Pfiesteria* is toxic to shellfish such as blue crabs (*Callinectes sapidus*), bay scallops (*Argopecten irradians*), and eastern oysters (*Crassostrea virginica*) as well (*20*), but detailed reports on those species are not available. Whether in aquaria or in the wild, *Pfiesteria* principally elicits neurotoxic effects and skin lesions in exposed fish in patterns that are considered to be characteristic of the dinoflagellate toxins (*10,11*).

Effects of *Pfiesteria* Toxin on Fish in the Wild

The link between *Pfiesteria* and kills of wild fishes was made when water from a site of fish kills involving Atlantic menhaden (*Brevoortia tyrannus*) was bioassayed with tilapia (*Oreochromis aureus* and *O. mossambicus*) held in aquaria (*10*). Subsequently, *Pfiesteria* has been implicated in fish kills and epizootic disease in estuarine fishes along the Atlantic coast (*10,20,39*). The presence of fish, usually large schools of menhaden, stimulates *Pfiesteria* to excyst and transform into toxic stages. The toxic stages of the dinoflagellate release toxins to the water and the toxins cause disorientation, respiratory distress, and death in fish and invertebrates (*11,39*). Skin lesions attributed to *Pfiesteria* may be acute or chronic. Typically, gross chronic lesions are well circumscribed ulcers with necrotic centers, or round raised, friable red nodules (*40*). Microscopically, the lesions are marked by a chronic inflammatory infiltrate, granulomas and exposed necrotic muscle. Fungal hyphae often were present in the granulomas and gram negative, rod-shaped bacteria were present in the lesions. Pathological lesions in other organs and tissues from wild fish-kill specimens have not been reported.

Ulcerative skin lesions like those described above have been considered to be caused primarily by fungal infections and termed "epizootic ulcerative syndrome" or "ulcerative mycosis," common lesions of fish worldwide and attributed to the fungal pathogens *Aphanomyces invadans* and *Saprolegnia* sp. (*37,41,42*). However, after the discovery of *Pfiesteria* and the association of the dinoflagellates with fish kills, fungal infections seen in menhaden and other fishes from mid-Atlantic estuaries were considered secondary, opportunistic infections occurring subsequent to epithelial damage caused by exposure to *Pfiesteria* toxins (*14,37,39*). Blazer et al. (*37*) have challenged this assertion after histologically examining about 150 ulcerated menhaden from sites in the Chesapeake Bay area where fish kills and ulcerated fish were attributed to *P. piscicida*. Fungal hyphae were present both in deeply penetrating ulcers and in raised lesions with or without epithelial erosion. The authors contended that the menhaden lesions from the Chesapeake area corresponded to similar lesions reported worldwide and caused by the *A. invadans*. An *Aphanomyces* sp. was cultured from the menhaden lesions, but Koch's postulates have yet to be fulfilled for this fungus (*37*). Thus, the roles of *Pfiesteria* and various fungi in the pathogenesis of skin lesions in fish from fish kills along the mid-Atlantic coast remain uncertain.

Effects of *Pfiesteria* Toxin in Laboratory Exposures

Pfiesteria toxins, rather than contact with or ingestion of the dinoflagellate cells, are responsible for effects in fish and invertebrates (*20*). Laboratory studies have shown that toxin elaboration depends on the presence of live finfish or their secretions that stimulate transformation of *Pfiesteria* cysts or other nontoxic life stages to toxin-producing stages. Large numbers of live fish are required to stimulate excystment and toxin production in *Pfiesteria* in the laboratory (*14*). Burkholder and Glasgow (*14*) supplied *Pfiesteria* cultures in 40-L aquaria with 15–20 live, 5- to 7-cm tilapia per day to maintain densities of more than 2,000 cells/mL of toxic *Pfiesteria* stages. Even in swarming schools of menhaden, such densities rarely occur in the field. The lag period for excystment of *Pfiesteria* cysts increased with the age of the cysts; cysts that had remained inactive for more than about 170 days could not be activated in the laboratory by exposure to live fish. Yet in the field, the

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organisms presumably remain encysted for more than 170 days every winter. Evidently, additional stimuli or environmental conditions that have not yet been elucidated must be required to stimulate toxic *Pfiesteria* blooms and maintain high densities of toxic stages in the field.

Some evidence indicates that *Pfiesteria* produces more than one toxin (see "Chemical Properties of *Pfiesteria* Toxins").

As discussed above, similar effects have been observed in both the laboratory and the field (*43*). Bioassays of *Pfiesteria* with aquarium fish are difficult to conduct. Because purified toxins are not available, bioassays depend on culturing or acquiring the organism and transforming the cysts to toxic stages that release toxins. Furthermore, there are considerable human safety concerns as well as the need to add fresh, live fish during the bioassay to ensure toxin production.

To date, histological effects of *Pfiesteria* in fish have been characterized and documented only for the skin (*39*). Damage to "myelinated neural tissue" has been suggested but not substantiated (*33*). Noga et al. (*39*) reported that in striped bass exposed to *Pfiesteria* skin damage began within 8 hr, resulting in a nearly complete denuding of the epithelium within 48 hr. Bacteria colonized areas denuded of epithelium. In severe cases, the erosion extended to the basement membrane (*39*). The pathogenesis for skin lesions in laboratory fishes is assumed be the same for skin lesions in wild fishes. This was true particularly for menhaden, in which the skin damage combined with systemic immunosuppression, as indicated by splenic lymphoid depletion, resulted in secondary bacterial and fungal infections in the skin ulcers (*20,39*). Fungal infections in menhaden skin lesions examined by Blazer et al. (*37*), however, appeared to be primary lesions and not secondary infections.

Skin lesions often precede severe systemic effects and the appearance of neurological signs of toxicity. With exposure to *Pfiesteria*, fish become lethargic, with episodic hyperexcitability; they also experience respiratory distress, often gulping air at the surface of the water (*10,11,20,38,39*). They exhibit a poor fright response, disorientation, and loss of balance (*44*). The fish lose the ability to osmoregulate; in salt water, blood osmotic pressure increases as concentrations of sodium, chloride, and potassium in serum increase toward those in the ambient seawater (*39*). White blood cell counts may be depressed by 20–40% in fish exposed to *Pfiesteria* toxins (*45*). Menhaden and striped bass eggs do not hatch in the presence of the toxic stages of *Pfiesteria* (*20,45*).

Conclusions

Relatively little is known about specific effects of *Pfiesteria* spp. on marine organisms. Research progress is slowed by lack of purified toxin with which to conduct bioassays and develop antibodies. Despite the complex effects of the toxin, few clear patterns of toxicity emerge, those being general neurological and skin effects and death. The actual cause of death, even in acute exposures, is not known and there is, as yet, no pathognomonic lesion. Extreme caution is needed when attributing particular fish kills, especially fish lesions, to *Pfiesteria*. Whatever the outcome of the ongoing controversy concerning the role of the fungus in the pathogenesis of skin lesions in wild fish, the mere presence of lesions in fish—whether or not the lesions are associated with a fish kill—is not a valid indicator of an ongoing or previous *Pfiesteria* bloom. Until field techniques for identifying the *Pfiesteria* toxin are available, work should be continued to understand the role of *Aphanomyces* spp. and possibly other infectious agents in skin ulcers in wild menhaden.

Neurotoxic effects are considered to be indicative of *Pfiesteria* toxin effects in fish as well as in humans. However, little information is available on correlative pathology of the nervous system in affected fish. There is a need for rigorous, complete (full tissue), time- and dose-associated pathological assessment accompanying laboratory bioassays and, as far as possible, in kills of wild fishes. To resolve diagnostic bias, consideration should be given to having *Pfiesteria*-related lesions in fish reviewed by an independent team of fish pathologists and veterinary pathologists who have expertise in fish pathology. Additional studies are needed of the physiological effects (i.e., osmotic and ionic regulation, respiration, endocrinology, neurophysiology) of *Pfiesteria* exposure to better understand the mechanisms of toxic action of the dinoflagellate toxins in fish.

In summary, additional research should be undertaken to do the following:

- Characterize the role of *Aphanomyces* spp. in causing skin ulcers observed in wild menhaden and the potential interaction of this fungus with *Pfiesteria* toxin in affecting fish
- Conduct full tissue pathological evaluations of fish from laboratory bioassays of *Pfiesteria* and in the wild fish from *Pfiesteria*-associated fish kills
  - Develop a detailed standardized protocol for necropsy, histopathologic examination, and diagnostic criteria for specimens from laboratories conducting fish bioassays or analyzing fish collected from fish kills.
  - Compile findings of these examinations in a centralized database shared among laboratories.
- Investigate the physiological and toxicological effects of *Pfiesteria* toxin exposure in fish, especially neurophysiologic effects and tissue and organ distribution. Pathophysiological studies should be accompanied by chemical analyses aimed at characterizing the toxin and its binding sites (below).

Measurement and Detection of *Pfiesteria* and *Pfiesteria*-like Organisms and Their Toxins*

Measurement Tools for Detecting the *Pfiesteria* Organism

Significant progress has been achieved toward developing methods for specific detection and differentiation of *Pfiesteria* spp. and *Pfiesteria*-like organisms (PLOs). Nevertheless, these methods remain labor intensive and restricted to laboratory facilities and are not yet available for routine field application. Initial field determinations are based on relatively nonspecific monitoring of fish kills, particularly those in which affected fish display behavioral abnormalities and ulcerative lesions. The standard method for associating *P. piscicida* with fish kills involves analysis by light microscopy, followed by fish bioassay and SEM. Morphological analysis by SEM remains the definitive method for speciation of *Pfiesteria*, but it is limited to laboratories that have the requisite equipment and expertise. Recently described methods employing specific gene probes have facilitated more rapid detection and differentiation of *P. piscicida*, the closely related *P. shumwayae* ("species B"), and other PLOs. However, the development of immunochemical assays, which might be used for onsite analysis, has been hampered by researchers not having *Pfiesteria*-specific antibodies. Further efforts will be needed to produce methods capable of selectively detecting toxic *Pfiesteria*, since none of the available assays, except for the fish bioassay, can differentiate toxic from nontoxic *Pfiesteria* strains.

Light Microscopy

Light microscopy is typically used for preliminary identification and enumeration of *Pfiesteria* spp. in water samples. Distinguishing *Pfiesteria* from other species may be difficult because in these samples *Pfiesteria* spp. often comprise a minority of the phytoplankton population (*20*). Although light microscopy lacks the resolution to provide a definitive result (*12*), biologists with sufficient experience can identify and quantify organisms whose morphology is consistent with *Pfiesteria* (*46*). Preserved cell samples are observed at high magnification with bright field, phase contrast, and differential interference optics (*12*). Counts exceeding 300 zoospores/mL in water samples taken from active fish kills are

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considered presumptively positive for possible involvement of *Pfiesteria* in a fish-kill event (*10,20,47*). Staining with fluorescent dyes may increase specificity (*48*) but does not obviate the need to use SEM for definitive identification of the organism (*12*). Light microscopy cannot differentiate toxic from benign *Pfiesteria* but does provide a rapid screening tool to select samples that should be tested for toxicity by fish bioassay (*47*).

Electron Microscopy

Electron microscopy (EM) provides sufficient resolution to classify the species, *P. piscicida*, in a new family (Pfiesteriaceae) and genus of dinoflagellate (*12*). SEM studies have revealed a multiphasic life cycle with polymorphic unicellular flagellated, amoeboid, and cyst stages. Transmission EM of the flagellated form (i.e., zoospore) shows typical dinoflagellate ultrastructure, including mitochondria with tubular cristae, endoplasmic reticulum, lipid bodies, trichocysts, Golgi apparatus, mesokaryotic nucleus with condensed chromosomes, large food vacuoles, microtubular basket associated with a peduncle, and a multimembrane thecal complex with vesicles and thin plates (*12,49*).

SEM is the standard technique for morphological and taxonomic classification of *Pfiesteria*. Culture amplified zoospores are processed for SEM using a membrane-stripping or suture-swelling technique that reveals a characteristic number and organization of thecal plates [see Figure 1 in Steidinger et al. (*12*)]. Recent examination of cultured *P. piscicida* cysts by SEM X-ray analysis indicates that cystic scales contain silica (*6*), suggesting that *Pfiesteria* represents a primitive dinoflagellate species. SEM is used to confirm the presence of *Pfiesteria* zoospores in positive fish bioassays, as well as to reconfirm species identification following cloning by micromanipulation or flow cytometry (*7,47*). SEM plays an essential role in the diagnosis of *Pfiesteria* in water samples but suffers from a number of potential drawbacks. These include the possible overgrowth of competing algae during culture amplification (*50*), the inability to directly distinguish toxic from benign *Pfiesteria*, the need for specialized equipment and facilities, and the requirement for staff with expertise in sample preparation, data acquisition, and results interpretation.

Genetic Diagnostics

Pfiesteria gene sequencing efforts have focused primarily on the ribosomal gene complex. Extensive databases exist for these genes [e.g., GenBank at the National Center for Biotechnology Information, National Library of Medicine (see *http://www.ncbi.nlm.nih.gov/genbank*)] and the phylogenetic relationships can be deduced from these data (*46*). The entire 18S small subunit (SSU) ribosomal DNA gene has been sequenced for the prototypical *P. piscicida* (GenBank accession #AF077055) and *P. shumwayae* (GenBank accession #AF218805) strains (*50*). Partial sequences are available for the 18S SSU gene of a PLO [(*21*); GenBank accession #AF080098] and for *P. piscicida* mitogen-activated protein kinase mRNA (GenBank accession #AF227275). Portions of the internal transcribed spacer regions I and II, the 5′ end of the large subunit ribosomal gene, nontranscribed spacer regions adjacent to the ribosomal genes, and the cyclin box gene have been sequenced but have not been published. These gene sequences offer the means to develop highly specific analytical tools for environmental testing. To date, all reported assays are based on the *Pfiesteria* SSU gene sequences. These include polymerase chain reaction (PCR) assays, a heteroduplex mobility assay/single-strand conformational polymorphism (HMA/SSCP) assay, and a fluorescent *in situ* hybridization (FISH) assay.

Polymerase Chain Reaction

Polymerase chain reaction is a method for exponential DNA amplification that has become the default method for DNA and RNA analysis. PCR uses paired (forward and reverse) gene probe primers in combination with a thermostable DNA polymerase that are cycled through a repetitive process of DNA denaturation, probe annealing, and primer extension. Typically, PCR products are separated by gel electrophoresis and detected with DNA binding dyes.

Rublee et al. (*31*) developed a PCR assay for detecting *P. piscicida* in environmental water samples, based on *P. piscicida*-specific forward primers and a generic eukaryotic-specific 18S SSU ribosomal DNA reverse primer. Four species-specific forward primers (65 For, 110 For, 286 For, 301 For) were evaluated. DNA sequences of the resultant PCR products showed 100% homology with *P. piscicida* (GenBank sequence #AF077055). Environmental sample DNA analyzed by PCR was isolated from filter-concentrated cell samples by maceration in a buffered detergent solution followed by chloroform extraction and isopropanol precipitation.

Rublee and co-authors (*31*) noted potential difficulties associated with assaying field samples, such as cross reactivity against unrelated organisms that share the target sequence, the presence of high levels of nontarget DNA that can interfere with primer hybridization, and decreased assay sensitivity due to *Taq* DNA polymerase inhibitors and/or non specific adsorption of extracted DNA to particulate matter. These concerns were validated, as in some samples only one or two of the primer sets produced positive results. Nevertheless, this assay appears generally robust and was successfully used to screen 170 estuarine water samples, collected from New York to northern Florida, for the presence of *P. piscicida*. Of the 170 samples, 35 were positive, including sites where there was no historical evidence of *P. piscicida*-related fish kills.

Fluorescent *in Situ* Hybridization

Fluorescent *in situ* hybridization techniques employ fluorescently tagged gene probes to label target DNA (or RNA) in intact cells. FISH has been adapted by Rublee et al. (*31*) to detect and enumerate *P. piscicida* in water samples using selected 18S SSU rDNA probe sequences reported by this group for *P. piscicida* PCR. Unlike the fish bioassay, FISH does not require culture amplification prior to analysis, and it may be better suited than PCR to the analysis of dinoflagellate populations in complex matrices, such as sediment samples (*51*), where *Taq* DNA polymerase inhibitors can affect PCR results (*31,46*).

The FISH technique is conceptually straightforward, involving sample concentration by centrifugation, fixation with paraformaldehyde, and hybridization with two 5′-fluorescein–labeled probes. The hybridization reaction takes place in a programmable thermal controller, and, following a washing step, the labeled cell samples are captured on a black polycarbonate filter for observation under a fluorescence microscope equipped with filters that permit detection of green FITC fluorescence as well as red chlorophyll-derived autofluorescence. Due to the potential for nonspecific binding, positive control–confirmed *P. piscicida* and negative control dinoflagellates are also carried through the process. Multiple-labeled probes are used to compensate for compromised accessibility of the probes to targeted sequences, which can result in a failure to efficiently label target cells or in low fluorescent signal strength (*52*). Detection of FISH-labeled cells can be adapted to flow cytometry for quantitative analysis of target gene labeling in combination with additional parameters such as size distribution.

Rublee et al. (*31*) used FISH, in conjunction with a PCR assay described above, to identify *P. piscicida* as far north as Long Island, New York, thereby extending the dinoflagellate's known range from New York to Mobile Bay, Alabama. The FISH probe also enabled researchers to discern *P. piscicida* from look-alike species (for example, *P. shumwayae*, *Gyrodinium galatheanum, Cryptoperidiniopsis* nov. gen.) and to estimate its abundance in water samples from a major fish kill and toxic *Pfiesteria* outbreak that occurred in North Carolina waters in 1998 (*53*). Many of the sites where *P. piscicida* was

detected were not associated with active fish kills or a history of fish health problems, suggesting that *P. piscicida* may usually exist in a benign state (*26*).

Heteroduplex Mobility/Single-Strand Conformational Polymorphism Assay

Oldach et al. (*50*) recently reported a heteroduplex mobility assay (HMA) capable of detecting and differentiating DNA "signatures" of *Pfiesteria* spp. and PLOs. The method is based upon PCR amplification of highly conserved regions within dinoflagellate 18S ribosomal DNA gene sequences. Sequences amplified from target organisms are then combined with a "driver" DNA sequence amplified from *Gymnodinium sanguineum* to form DNA heteroduplexes that are separated by gel electrophoresis. Since DNA heteroduplexes migrate more slowly through the gel matrix than homoduplexes, distinct banding patterns are produced when the target sequence differs from the driver sequence. Homoduplex (single-band) patterns arise when the driver and target DNA share 100% sequence homology or if marked sequence divergence prevents hybridization. In some examples, the technique has been shown to resolve single nucleotide differences between DNA fragments, illustrating its utility for guiding DNA sequence discovery.

By modification of the hybridization conditions, it is possible to concurrently perform the HMA and an SSCP. The SSCP is based upon the principle that the 3-dimensional conformation of a single-stranded DNA molecule has a specific sequence-based secondary structure in a non denaturing gel matrix (*54*). SSCP can often further resolve dinoflagellate strains not differentiated by HMA. In combination, the HMA/SSCP assays provide a high resolution means to distinguish among *Pfiesteria* species and strains. The HMA/SSCP has identified mixed dinoflagellate cultures previously thought to represent clonal cultures.

The banding patterns produced by HMA can be used as a guide for gene sequence discovery. For example, the PCR amplicons yielding an HMA banding signature characteristic of multiple SEM-confirmed *P. piscicida* cultures were cloned, sequenced, and found to be identical (*50*). This sequence was used to design PCR primers yielding a near full-length sequence of the *P. piscicida* 18S SSU ribosomal DNA gene sequence. The remaining sequence was appended by sequencing PCR products amplified using a generic dinoflagellate PCR primer and a universal eukaryotic 3′ SSU primer. For *P. shumwayae*, a similar strategy was employed, in which full-length 18S gene sequences were PCR amplified from a HMA/SSCP-confirmed clonal culture using universal eukaryotic 5′ and 3′ SSU primers. A 1,800 basepair sequence has been deposited with GenBank for *P. piscicida* (accession #AF077055) and for *P. shumwayae* (species B; accession #AF218805).

Based on these sequences, Oldach et al. (*50*) developed highly selective PCR primers for *P. piscicida* (Ppisc108F/Ppisc311R) and *P. shumwayae* (SpecB-forward/SpecB-reverse). PCR assays incorporating these primers have been used to screen environmental water samples and mixed population cultures suspected of containing *Pfiesteria* spp. The *P. piscicida* PCR assay has demonstrated high specificity as evidenced by *a*) negative assay results for more than 400 estuarine water samples in which "generic dinoflagellate" DNA had been detected by PCR; *b*) negative assay results for 33 characterized non-*Pfiesteria* dinoflagellate cultures and a series of 28 *Pfiesteria*-like dinoflagellate cultures that did not share the identical HMA pattern; *c*) negative results for the most closely related dinoflagellate species available, *P. shumwayae* and *Cryptoperidiniopsis* sp.; and *d*) sequence identity among all *P. piscicida* PCR product amplicons examined. The analogous PCR assay for *P. shumwayae*, also subjected to extensive testing, has been shown to cross-react with an as yet uncharacterized organism. Thus, the *P. shumwayae* PCR primer pair is highly selective but does not have absolute specificity.

Chemical Properties of *Pfiesteria* Toxin

The chemical properties of *Pfiesteria* toxin are not well understood, primarily because the toxin has not been purified and characterized. Informal suggestions have been made that at least three toxins exist, including two lipid-soluble toxins and one water-soluble toxin (*55*). A water-soluble toxin that appears to target the nervous system was tentatively assigned an estimated molecular mass of 400–500 atomic mass units (amu) (*56*). One of the lipid-soluble toxins, estimated to have a molecular mass of 390 amu (*55,56*), has been attributed dermonecrotic activity that may contribute to the skin ulcer formation commonly observed in *Pfiesteria*-associated fish kills (*38*). The other lipophilic toxin has been termed "lipid-soluble lethal factor" (*55*).

Purification efforts may have been complicated by attempts to isolate toxin from preparations derived from nonclonal *Pfiesteria* cultures (*57*). Individual *Pfiesteria* strains could produce a unique toxin or complement of toxins and metabolites. Thus, over time the spectrum of toxins harvested might change in concert with the population dynamics of the culture. Toxin instability may also have impeded successful purification. It is not clear, however, whether this instability is due to chemical degradation, adsorption to surfaces, metabolism, or other factors. Sterile aquarium water filtrate has been shown to be nonlethal to fish after 48 hr at ambient room temperature (*11,38*), but water samples stored frozen retain toxic activity in rats through at least one freeze–thaw cycle (*58,59*). Lipophilic toxin fractions adsorbed to C-18 chromatography resin and eluted with acetonitrile retained potent cytotoxicity (*60*).

Methods for Detecting *Pfiesteria* Toxin

Fish bioassay. The batch-culture fish bioassay is the standard method for detecting exotoxin activity associated with *Pfiesteria* (*6,10, 28,30*). The assay is used to test unpreserved environmental water (or sediment) samples taken from sites where fish kill events are in progress after it is determined by light microscopy that presumptive *Pfiesteria* zoospore counts of ≥300/mL are present. The fish bioassay has been described as "a process rather than a rigid procedural 'recipe' to allow the flexibility needed for optimizing detection of toxic *Pfiesteria* from samples collected across a range of environmental conditions" (*6*). Nevertheless, the assay requires a biohazard Biosafety Level 3 laboratory to prevent potential human exposure to *Pfiesteria* toxins.

The assay typically is carried out with juvenile (3–7 cm) tilapia, *Oreochromis* spp., under conditions that favor proliferation of toxic zoospores. For example, this would include replicate tilapia cultures, each with 2–15 fish, kept in covered aquaria maintained at 18–24°C, under a 12-hr light/dark cycle (30 μEin m^{-2} s^{-1}) with 15 ppt Instant Ocean salts (Aquarium Systems, Mentor, OH, USA) and aeration (*6*). Bioassays are initiated by introduction of *Pfiesteria* cultures or environmental samples (water or sediment). Control fish cultures without *P. piscicida* are maintained under otherwise identical conditions. Aquarium water samples are taken at frequent intervals to measure dissolved oxygen and ammonia levels, and to determine *Pfiesteria* and other microbial counts. A minimum live fish density must be maintained for optimal stimulation of toxic zoospores (*11*). Therefore, dead fish are removed from cultures and replaced with live fish to sustain growth of toxic zoospores and inhibit transformation to amoebae or non–toxin-producing cysts.

The time to death of fish exposed to toxic *Pfiesteria* varies, apparently depending on whether toxin biosynthetic pathways have been induced in the dinoflagellate population by (undefined) stimuli elaborated by fish. However, population densities of ≥250–300 toxic zoospores per milliliter can produce lethal concentrations of *Pfiesteria* toxins (*16*). Burkholder has classified *Pfiesteria* toxin production capacity into three phenotypic categories (*6,47,53*):

- Actively toxic (Tox A) strains are producing ichthyotoxic substances that have caused stress, disease, or death in fish. Environmental samples of actively toxic Pfiesteria are "primed" for toxin production and produce a positive fish bioassay (i.e., cause disease or death of test fish) within 21 days (usually in 4–9 days).
- Nontoxic (Tox B) strains are potentially toxic—they can be induced to produce toxins that cause fish stress, disease or death, but they are not actively producing toxins; *Pfiesteria*/PLO species collected as part of surveys (i.e., not in response to reports of fish disease or fish kills) may produce positive fish bioassays, but time to death is 8–10 weeks.
- Noninducible (never toxic) strains are incapable of producing ichthyotoxins in the presence of live fish or their fresh materials.

Actively toxic *Pfiesteria* strains will gradually lose the ability to produce toxin when cultured over a period of months, even when cultured in the presence of fish (*53*). Whether associated microorganisms have a role in toxin biosynthesis is not known. However, intracellular bacteria have been shown to exist in *P. piscicida* flagellated and amoebae stages (*11,12*). The phylogeny of these bacteria has not been reported, but their presence raises the possibility that they play a role in toxin production, as has been proposed for *Alteromonas*/*Pseudomonas*-like bacteria associated with the dinoflagellate *Alexandrium tamarense* (*61*).

The complete process for associating toxic *Pfiesteria* spp. with a fish kill event involves the following (*7,47*): *a*) initial field determination that an active fish kill event is in progress that may be associated with a *Pfiesteria* bloom; *b*) sample collection and transportation to laboratory facility; *c*) enumeration of PLOs by light microscopy and determination that potentially toxic PLO concentrations (≥300 zoospores mL^{-1}) are present; *d*) determination by fish bioassay that cultured PLOs produce ichthyotoxins; *e*) speciation of fish bioassay dinoflagellate population by SEM and molecular assays, confirming the presence of *Pfiesteria*; *f*) cloning and retesting by fish bioassay, SEM, and molecular assays.

Fish bioassays can also be used to detect *Pfiesteria* toxin preparations (as opposed to toxin produced *in situ* during the bioassay). Culture water filtrate (0.22 μm) from actively killing *Pfiesteria* cultures has been shown to retain full toxicity and kill fish within 3 hr (*11*). Concentrated aqueous toxin extracts killed fish within 20 min in a modified fish bioassay system (below) (*62*).

The fish bioassay remains the standard method for detecting toxic *Pfiesteria* and *Pfiesteria* toxins. While intoxicated fish may display characteristic signs (lethargy, erratic swimming behavior, hemorrhage, lesions) that provide a degree of specificity, the assay is time consuming (up to 21 days), requires *Pfiesteria* culture amplification, cannot be used in the field, and lacks specificity with respect to toxin identification. A rapid and specific method that can detect toxic *Pfiesteria* and/or *Pfiesteria* toxins at fish kill sites is needed to expedite management of public health interests.

Modified fish bioassay. Ramsdell and co-workers described a miniaturized 24-well plate version of the fish bioassay that they used in validation studies for a reporter gene cytotoxicity assay (below), and to monitor chromatography fractions during purification procedures (*62*). The assay is conducted with one 7- to 10-day-old sheepshead minnow, *Cypronodon variegates*, added per well containing 1 mL 25 ppt Instant Ocean. Following addition of toxin samples, fish are observed for up to 2 hr for signs of intoxication. Even though this assay has not been thoroughly validated, it may be useful as a rapid screening method to aid in bioactivity-guided purification of ichthyotoxins.

Cellular assays. Cellular assays have been used to test *Pfiesteria* culture water, fish tissue and toxin purification fractions for the presence of toxin activity. These assays include cytotoxicity (viability) assays and a reporter gene assay.

Cytotoxicity assays. Ramsdell and co-workers used a colorimetric cytotoxicity assay for detecting cell survival and proliferation based on the dye MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide], which undergoes reduction in the mitochondria of living cells to form a purple formazan product (*63*). Culture water, toxin purification fractions, and a residual water fraction were tested against a panel of eight cell lines, including three mammalian neuronal cell lines, three finfish cell lines, and two mammalian epithelial cell lines. Assays were carried out in 96-well plates and colorimetric end points were determined in a microtiter plate reader. A rat pituitary epithelial cell line, GH_4C_1, was shown to be the most sensitive to diethyl ether and residual water purification fractions, and was therefore selected for development of a reporter gene assay (below).

Another cytotoxicity assay was developed to detect toxicity in fish tissue in an effort to ascertain whether consumers could be at risk because of consumption of fishery products exposed to toxin during a fish kill (*60*). Live fish representing several species were collected from the site of an ongoing fish kill, although it is unclear that *Pfiesteria* was confirmed to be the causative agent. Tissue samples were extracted with methanol/water and partitioned against hexane, then methylene chloride. The methylene chloride fraction was dried and dissolved in dimethyl sulfoxide (DMSO) for cytotoxicity testing against Caco-2 human colon carcinoma and neuro 2A mouse neuroblastoma cells. Cell counts and morphological changes were used as cytotoxicity assay end points. A partially purified toxin preparation adsorbed to C-18 cellulose resin, eluted with acetonitrile, dried, and resuspended in DMSO was used to establish assay parameters prior to testing fish tissue extracts. This preparation was reported to induce cell rounding, membrane blebbing, cytoplasmic graininess, and membrane lysis at the extraordinarily dilute level of 1×10^{-16} g extract/mL. This represents unprecedented cytotoxicity for a partially purified marine toxin and the authors cautioned that the result must be verified with pure toxin (when available). Extracts of fish tissue from the kill site were mostly noncytotoxic except for some of the Atlantic menhaden samples. However, the authors cautioned that these results were not conclusive because *Pfiesteria* toxin could not be rigorously established as the cause of the fish deaths.

Reporter gene assay. Reporter gene assays rely upon specific gene induction in responsive cell lines stably transfected with reporter gene constructs. A reporter gene assay based on the inducible expression of the immediate early gene, c-*fos*, has been described by Fairey et al. (*62*). The AP-1 transcription factor, consisting of Fos and Jun protein dimer complexes, coordinates cellular responses to growth and stress stimuli, including toxins (*64*). Previously, the same group demonstrated that various neuronal cell lines differentially express c-*fos* in response to toxin exposure, conferring a degree of selectivity to the assay. For this assay, GH_4C_1 and neuro 2A (N2AC) cells were stably transfected with a gene construct consisting of the c-*fos* regulatory region ligated to the coding region of the reporter element, firefly luciferase. The transfected cell lines GH_4C_1-A1 and N2AC were evaluated for *Pfiesteria* toxin-induced expression of the reporter gene.

Reporter gene activity was induced in GH_4C_1 cells exposed to toxic culture water containing live *Pfiesteria* cells, whereas the N2AC cells were unresponsive. In N2AC cells, the marine toxins brevetoxin (PbTx-1) and ciguatoxin (CTX-3C) induced c-*fos*-luciferase expression, while saxitoxin inhibited PbTx-1 induced reporter gene activation. In contrast, GH_4C_1-A1 cells were selectively responsive to *Pfiesteria* toxin preparations, and unresponsive to PbTx-1, CTX-3C, STX, and domoic acid. This selectivity is attributed to a higher complement of voltage-activated sodium channels in the N2AC cell line.

The GH_4C_1-A1 reporter gene assay showed a concentration-dependent induction of c-*fos*–luciferase expression over a range of 30–300 *Pfiesteria* cells/mL and was used to screen environmental water samples for the presence of toxin-producing *Pfiesteria*. This assay was also useful for bioactivity-guided purification of *Pfiesteria* toxins, as water soluble fractions with reporter gene activity coeluted with ichthytoxic activity. Finally, the assay was shown to be useful for detecting *Pfiesteria* toxin activity in biological fluids, including tissue culture medium and human serum.

Despite its demonstrated utility, however, certain features of the GH_4C_1-A1-based assay are problematic. For example, at higher concentrations, *Pfiesteria* toxins inhibited c-*fos*-luciferase expression and resulted in a bell-shaped dose–response curve. Thus, samples must be serially diluted over a wide range to assure that responses are detected within the relatively narrow linear response range. Also, 5-fold concentrated Instant Ocean salts were shown to nonspecifically induce reporter gene expression. This prevents testing of concentrated aqueous samples unless the salts can be removed. The possibility that other agents will nonspecifically induce c-*fos*–luciferase expression in GH_4C_1-A1 cells cannot be ruled out.

Conclusions

Two critical gaps remain in what is known about detecting the dinoflagellate *Pfiesteria* and its toxins. First, despite the development of species-selective PCR assays, gene sequences targeted in current tests do not differentiate toxic from benign *Pfiesteria* populations. Second, the chemical identity of *Pfiesteria* toxins remains unsolved, preventing development of toxin-specific analytical methods and field assays and limiting the ability to undertake systematic toxicological studies.

Efforts to identify genes involved in toxin biosynthesis may be facilitated by the rapidly evolving technologies emanating from the Human Genome Project. For example, since it is known that *Pfiesteria* toxin production is inducible in the presence of fish, it may be possible to compare gene expression in "Tox-A" versus "Tox-B" phenotypes by such methods as differential display reverse transcriptase–PCR, representational difference analysis, or serial analysis of gene expression (*65*). Sequences of inducible mRNA transcripts might then be targeted to develop RT-PCR assays that are selective for toxin-producing *Pfiesteria*.

The development of toxin purification methods and development of well-characterized toxin-specific assays are interdependently linked. Thus, without purified toxin, reliable and valid assays cannot readily be developed; conversely, without the assays to guide purification, toxin cannot readily be purified. The apparent instability of the toxins is another barrier. The methods that have been developed for toxin detection, especially the reporter gene assay, offer potential means to break through this circular conundrum. Development of a high performance liquid chromatography (HPLC) method, based on correlation of reporter gene activity with elution times, could serve both analytical and preparative functions. It seems logical to suggest that such efforts have already been undertaken, and one wonders if the toxins and the "phantom" organism are equally ephemeral.

Without purified toxin, it still may be possible to develop highly sensitive and selective bioassays such as receptor-binding assays. However, this assumes that a specific receptor exists and, if so, that toxins competing for receptor binding can be identified—perhaps requiring a substantial screening effort. With purified toxin available, it should be possible to develop a variety of analytical methods, such as HPLC and capillary electrophoresis, and possibly antibody-based toxin assays. The availability of purified toxin would also enable structural determinations to be made using nuclear magnetic resonance (NMR), mass spectroscopy, and perhaps crystallography. Structural identification could then be used to design haptens for antibody production.

A need clearly exists for a field assay for *Pfiesteria*, its toxins, or both. Antibody-based assays can be adapted to rapid qualitative or semiquantitative devices such as lateral flow "dipsticks." Furthermore, such antibodies can be used in laboratory-based methods such as the enzyme-linked immunosorbent assay or flow cytometry. To date, however, there are no reports of antibodies specific to either the *Pfiesteria* organism or its toxins. A toxin-specific immunoassay would be especially useful, as antibodies to the organism would be unlikely to differentiate toxin-producing and benign *Pfiesteria*.

Finally, recent advances in the development of portable PCR devices make it realistic to expect that PCR assays will soon be carried out with hand-held, battery-powered instruments. The Hand-held Advanced Nucleic Acid Analyzer (HANAA) developed by Dean Hadley and co-workers at Lawrence Livermore National Laboratory (Livermore, CA, USA) measures 5 × 8 × 2 inches and weighs about 2 pounds (*66*). This device is currently undergoing a 6-month validation study by scientists from the U.S. Food and Drug Administration, CDC, University of Maryland, Utah Department of Health, Los Angeles Emergency Operations Bureau, and the Southwest Foundation for Biomedical Research. The device incorporates *Taq*Man fluorescent probes that allow near-real time PCR assays to be carried out. The instrument can simultaneously test four samples for two DNA sequences each and report findings in about 15 min. One can imagine that if PCR probes to "toxicity genes" can be developed that the HANAA will permit on-site detection of both the organism and its toxin-production phenotype.

In summary, to address research gaps, future research should focus on the following:

- Developing and preserving a standard panel of ichthyotoxic water samples and/or extracts derived from clonal *Pfiesteria* cultures
- Promoting collaboration among laboratories developing analytical and purification methods by distributing the standard ichthyotoxic water sample/extract panel
- Standardizing the fish bioassay methodology to facilitate interlaboratory reproducibility
- Developing and/or refining chromatographic methods for toxin analysis and purification using
  a) conventional detection [e.g. diode array ultraviolet (UV) detection] and
  b) bioassay methods (cytotoxicity, receptor-binding assay, rapid fish bioassay) to guide method optimization
- Applying mass spectroscopic detection methods to chromatographic procedures to estimate molecular mass of putative toxins
- Applying NMR methods to (purified) toxin samples for structural determinations
- Producing antibodies and developing immunochemical methods to detect the *Pfiesteria* organism and *Pfiesteria* toxin in the field
- Identifying genes associated with toxin biosynthesis using genomic screening methods and/or analysis of gene expression
- Adapting PCR and/or FISH methods to devices that can be used in the field to detect toxin-producing phenotypes of *Pfiesteria*

Human Health Effects: Epidemiologic and Clinical Studies*

Epidemiologic Observations

Epidemiology comprises methods and principles used to investigate diseases and their causes in human populations. In investigating new syndromes like that caused by *Pfiesteria*, epidemiologists establish case definitions and develop hypotheses about exposures that may have causal roles. The first phases of epidemiological investigation are often descriptive, involving characterization of the affected individuals and obtaining information about exposures they may have received. Building on this initial information,

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epidemiologists next carry out hypothesis-testing studies, typically using studies of either the cohort or case–control design. The cohort study involves follow-up of exposed and nonexposed individuals and observation for the occurrence of the outcome of interest. For example, cohort studies are now under way in Maryland, North Carolina, and Virginia, each involving persons with high potential for exposure to *Pfiesteria* and its toxin because they work on the waterways. In a case–control study, exposures of persons with the outcome of interest are compared with those of similar persons not having the outcome. This design has not yet been applied specifically to *Pfiesteria*-related symptoms and illness. The cross-sectional design (sometimes referred to as a "survey") has been used to characterize persons with *Pfiesteria*-related health problems. In this design, observations are made at one point in time and consequently, causal relationships may not be apparent. This design has been used in describing the health of persons with exposure to *Pfiesteria* and making comparison to the status of unexposed controls.

The epidemiologic method is particularly informative if both exposure(s) and outcome(s) can be sharply specified and measured with little error. Nonspecificity and measurement error typically weaken epidemiological data, most often resulting in a bias toward negative findings. Both potential limitations have already been evident in research on *Pfiesteria*. The exact toxin remains unknown, as does the route of entry into the body—inhalation, percutaneous, or ingestion. The potential for exposure can be described as contact with water at the time of fish kills, but such contact is only a surrogate for the actual exposure to toxin, which cannot yet be measured or quantified. The outcome is also nonspecific and has multiple clinical dimensions, all with many other possible causes. The assessment of neurocognitive functioning, an outcome of concern, requires neurologic evaluation and the use of sophisticated test instruments. To date, a variety of such tests has been used.

Public health researchers have attempted to address these vexing methodologic problems by developing standardized approaches for characterizing exposure and outcome. Participants at a 1997 workshop sponsored by the CDC proposed that exposure to estuarine water might be characterized by *a*) fish with lesions consistent with *P. piscicida* or a morphologically related organism; *b*) a fish kill involving fish with lesions consistent with *P. piscicida* or a morphologically related organism; and *c*) a fish kill involving fish without lesions of *P. piscicida* or related organisms and with no alternative reason for the fish kill (*1*). The workshop participants also offered a description of the clinical features of the associated syndrome (Table 1).

For the purpose of surveillance, the CDC has offered a definition of PEAS (*1,2*). The definition incorporates contact with estuarine water, as well as clinical features (Table 1). These definitions are notable for including exposure as a defining element because the clinical features of the syndrome are nonspecific and not pathognomonic.

In applying these definitions in the context of an epidemiologic study, potential methodologic problems are evident. Participants in a study may be unaware of whether a fish kill has occurred or if there are typical lesions on the fish. Unless they are able to track fish kills and document the presence of the organism, researchers may have difficulty linking activities at different places and times to exposure. Unless they are educated and aware, PEAS may be missed by healthcare providers who may not query persons with possible symptoms as to their exposure.

The nonspecificity of the clinical picture is also a potential methodologic limitation of observational studies of the consequences of exposure to *Pfiesteria*. Although case reports document relatively dramatic and severe acute illnesses among exposed persons, research studies have focused on more subtle and possibly long-term consequences of exposure. These potential effects are typically evaluated using standardized neuropsychological test batteries that address learning and memory. These test batteries are also used, for example, in assessing the health effects of occupational exposure to solvents. Performance on these instruments may be influenced by many factors, including use of alcohol or medications, age, education, and presence of cerebrovascular disease. If exposed and nonexposed persons are not comparable for such potential confounding factors, a study using a neuropsychological test battery may give biased results.

Review of Epidemiologic Evidence

Case reports. A number of case series have been reported; these reports range from anecdotal, popular accounts of affected scientists (*67*) to more formally collected case series (Table 2) (*68*). As would be anticipated, criteria for concluding that the illnesses were related to *Pfiesteria* were variable across the reports and some of the reports provide little clinical information. A series of affected persons was described in *Environmental Health Perspectives* and the *Maryland Medical Journal* in 1997 and 1998 (*70,71,74–77*). From information provided, it is difficult to assess the number of independent cases described across the multiple articles in the issue. The reported cases ranged from dramatic accounts of substantial cognitive impairment in exposed laboratory workers (*16*) and watermen (*71,72*) to symptom episodes in persons with relatively casual contact with water where fish kills took place or may have taken place (*75*). These case series have proved useful for describing the elements needed for a working case definition and as a signal of the need for more formal investigation.

Table 1. Conditions of exposure and symptoms present in possible estuary-associated syndrome.

Symptoms following exposure[a]	Present in 1997 definition	Present in 1999 definition
Memory loss[b]	X	X
Confusion[b]	X	X
Symptoms develop within 2 weeks after confirmed exposure		X
Health provider cannot identify another cause for symptoms		X
Acute skin burning at site of water contact	X	X
Headaches[c]	X	
<2 weeks		
≥2 weeks		X
Skin rash	X	X
Eye irritation		
<2 weeks	X	
≥2 weeks		X
Upper respiratory irritation		
<2 weeks	X	
≥2 weeks	X	
Muscle cramps		
<2 weeks	X	
≥2 weeks		X
Gastrointestinal symptoms (nausea, vomiting, diarrhea, abdominal cramps)	X	
<2 weeks		
≥2 weeks	X	

[a]Conditions establishing exposure: 1997: Exposure to estuarine water characterized by any of the following conditions: *a*) fish with lesions consistent with *P. piscicida* or morphologically related organism (MRO) toxicity (20% of a sample of at least 50 fish of one species having lesions); *b*) a fish kill involving fish with lesions consistent with *P. piscicida* or MRO toxicity; or *c*) a fish kill involving fish without lesions, if *P. piscicida* or MROs are present and there is no alternative reason for the fish kill (*1*). 1999: Development of symptoms within 2 weeks after exposure to estuarine water (*2*). [b]Symptom must be present. [c]For both 1997 and 1999 definitions, three or more of the remainder of the symptoms must be present.

Table 2. Symptoms and clinical conditions among persons with direct contact with waters of Pocomoke Estuary, Chesapeake Bay, Maryland and controls, by estimated exposure.[a]

Symptoms and clinical conditions	High exposure (n = 11)	Moderate exposure (n = 7)	Low exposure (n = 4)	Controls (n = 8)
Neuropsychological symptoms[b]	9 (82%)	6 (86%)	2 (50%)	1 (13%)
Headache	9 (82%)	5 (71%)	2 (50%)	1 (13%)*
Skin lesions	8 (73%)	4 (57%)	1 (25%)	1 (13%)†
Skin burning on contact with water	5 (45%)	6 (86%)	1 (25%)	2 (25%)‡
Diarrhea	5 (45%)	4 (57%)	2 (50%)	1 (13%)
Nausea/vomiting	7 (64%)	4 (57%)	0	1 (13%)
Abdominal cramps	4 (45%)	4 (57%)	1 (25%)	1 (13%)
Joint pain	5 (45%)	2 (29%)	1 (25%)	2 (25%)
Muscle/leg cramps	8 (73%)	2 (29%)	1 (25%)	2 (25%)
Eye irritation	6 (55%)	2 (29%)	2 (50%)	4 (50%)
Sinusitis	5 (45%)	5 (71%)	3 (75%)	3 (32%)
Shortness of breath	2 (18%)	4 (57%)	1 (25%)	2 (25%)
Pneumonia	2 (18%)	1 (14%)	0	0

[a]Data from Grattan et al. (*68*). [b]Confusion, episodes of distortion, new or increasing forgetfulness, or difficulties concentrating. *$p < 0.01$, Fisher's exact test, two tail, comparing high-exposure group with controls; †$p < 0.05$, Fisher's exact test, two-tail, comparing high-exposure group with controls. ‡Control watermen reported seeing sea nettles in association with sensation of burning skin; affected individuals denied sea nettle contact. If two controls with complaints of skin burning are presumed to have had contact with sea nettles and are excluded, $p < 0.05$, Fisher's exact test, two tail.

Epidemiologic studies. Several studies of more formal design than the case series have been carried out. In materials reviewed by the panel, the level of information concerning study design is variable and key aspects of design are not included in all instances. Reviews of the specific studies follow:

GRATTAN ET AL., 1998. This study (*68*) included cross-sectional observations on 24 people having direct contact with the waters of the Pocomoke and other estuaries in the Chesapeake Bay. An additional eight unexposed watermen were recruited for comparison. A structured questionnaire was used to obtain information on exposure and the participants were then placed into strata of high, medium and low exposure (Table 3). Participants were queried about symptoms and a standard neuropsychological test battery was administered that was considered appropriate for exposure to *Pfiesteria*.

SAVITZ ET AL. 1998; SWINKER ET AL. 2000. This study (*69,70*) compared North Carolina workers with and without potential for exposure to *Pfiesteria*. Using maps of locations where distressed or killed fish had been found in 1997, the investigators enrolled gill-net and crab-pot fishermen working the Pamlico, Neuse, and Trent Rivers and tributaries in North Carolina. These workers (n = 19) and an additional four state employees with potential for exposure were enrolled as the exposed group. The unexposed cohort was selected with matching for age, gender, occupation, and educational level but were from the Outer Banks of North Carolina. All participants had a detailed medical evaluation and completed a neuropsychological test battery.

GRIFFITH ET AL., 1999. Griffith et al. (*73*) provide findings of a cross-sectional study of 253 North Carolina crabbers who worked in waters known to be home to dinoflagellates, and of two control populations—115 crabbers working in other areas and 125 nonfishing residents of the communities of the crabbers.

Clinical Assessment and Neurotoxicology

Clinical manifestations. The clinical data found in the various reports vary in their completeness, but they do provide a perspective on symptoms in apparently exposed persons (Tables 3–5). Many of the reports cite nonspecific symptoms of dizziness, eye irritation, and headache (*16,74–77*). Gastrointestinal complaints of diarrhea and abdominal pain were common, as were respiratory complaints of wheezing, coughing, and shortness of breath. Dermatological symptoms were also frequent. Complaints of cognitive deficits were also described, including memory impairments that developed within hours after exposure. The cognitive deficits and memory impairments have been reported to subside spontaneously in some cases but to be aggravated by strenuous exercise in others. Other reports have suggested that treatment with cholestyramine may improve clinical prognosis and hasten recovery, but these observations lack proper controls (*74*).

Neuroimaging studies. Radiological imaging was done in four individuals (*16,74,76*). The magnetic resonance image (MRI) of a waterman examined by Bever et al. (*76*) was reported as normal. A patient with persistent cognitive deficits reported by Shoemaker (*74*) had a computerized axial tomogram (CT) that was normal and a MRI that revealed mild inflammation of the mastoid and a possible polyp in the sinus. Glasgow et al. (*16*) reported that their patient B had minimal changes in the hippocampus that they considered to be of borderline significance. Patient B had cognitive deficits on neuropsychological testing that resolved after 2 months, although the patient continued to complain of episodes of "foggy memory" and irritability lasting about 12 hr and precipitated by strenuous exercise. Findings of a fluorodeoxyglucose positron-emitting tomogram (PET) of patient B were normal (Tables 3–5).

Electrophysiological studies. Peripheral nerve electrodiagnostic tests [electromyogram (EMG) and nerve conduction studies] were done in two subjects (patients A and B) described by Glasgow et al. (*16*) Evoked potential studies were done in Patient B. Findings for Patient A were reported to be essentially normal with mild electromyographic changes and normal sensory and motor conduction velocities consistent with minimal EMG evidence of motor axonopathy and no evidence for Guillain-Barré syndrome. Decreased ankle reflexes were also seen in this patient. The studies in Patient B showed no evidence of either peripheral or autonomic neuropathy. The visual and brainstem auditory evoked potential studies in patient B were normal although neuropsychological testing was reported to support a diagnosis of amnestic syndrome involving verbal more than visual modalities (*16*). The electroencephalogram (EEG) of patient B and that of a Chesapeake Bay waterman examined by Bever et al. (*76*) were assessed as normal.

Neuropsychological studies. Most reports presented descriptions of *Pfiesteria* exposure-associated changes in emotional state such as irritability and lability of mood; cognitive impairments including confusion, poor concentration, disorientation, and memory difficulties; motor impairments including ataxia, dysmetria, and dysarthria; and sensory disturbances such as decreased ability to smell odors and paresthesia.

Shoemaker (*74*) reports findings of neurocognitive studies for four patients. The specific tests used in the evaluation were not described. Findings for a 56-year-old woman were markedly abnormal immediately after exposure to *Pfiesteria* but returned to normal within 1 month after treatment with cholestyramine (2 months after cessation of exposure to *Pfiesteria*). A second patient examined by the group, a 33-year-old man, had memory impairments that persisted 1 year after exposure; these memory problems were reportedly improved by treatment with cholestyramine and multivitamins. Neurocognitive findings in a third patient, a 32-year-old man, were reported as abnormal 3 months after cessation of exposure. Two weeks after beginning treatment with cholestyramine, minimal improvement in his performance on neurocognitive tests had occurred, although the patient was reported able to return to work after initiation of this therapy. Memory impairments were also documented in a 44-year-old man seen by this

Table 3. Clinical manifestations reported among persons with suspected exposure to *P. piscicida*. Suspected source of exposure, neurological and dermatological symptoms, and signs.

ID #	Ref[a]	Age (years)	Sex	Exposure history	Neurological symptoms and signs	Dermatological symptoms
1	(*74*)	23	M	Water skier × 1 hr	Memory, ataxia, dysarthria	1.5 - to 2-cm pruritic lesions with follicular eruption
2	(*74*)	26	M	Swimmer upstream from water skier	None reported	1.5- to 2.5-cm lesions
3	(*74*)	30	F	Swimmer in vicinity of water skier	None reported	None reported
4	(*74*)	56	F	Dept. of Environment worker sorting fish	Memory problems	Burning sensation, rash and desquamation of skin
5	(*74*)	41	M	Sampling shell fish beds	Memory problems	3 lesions with discrete macular desquamation of left hand
6	(*71*)	47	M	Fisherman	Memory problems	None reported
7	(*71*)	33	M	Waterman (commercial fisherman)	Memory problems	None reported
8	(*71*)	32	M	Commercial diver	Memory problems	Skin lesions
9	(*71*)	44	M	Fisheries worker	Memory problems	Skin lesions
10	(*16*)	<40	NR	Marine scientist	Concentration and memory problems, paresthesia, ataxia,	Skin lesions
11	(*16*)	<40	NR	Marine scientist	Memory problems, irritability, emotional lability, disorientation, dysarthria, insomnia, paresthesias	Skin lesions
12	(*16*)	<40	NR	Marine scientist	Disorientation, concentration and memory problems,	None reported
13	(*16*)	NR	NR	5 laboratory personnel and 2 nearby office workers	None reported	None reported
14	(*16*)	>40	M	Waterman (commercial fisherman)	Memory problems, dysmetria, decreased smell in right nostril and positive palmar mental reflex (left) and positive globellar reflex	None reported
15	(*75,76*)	38 (mean)	3 M	Boaters	2/3 disorientation, concentration and memory problems,	2/3 skin lesions; 1/3 burning sensation on contact with contaminated water
16	(*75,76*)	38 (mean)	4 M 3 F	Working with samples of infected water/fish (8–20 hr/week)	6/ 7 disorientation, concentration and memory problems,	5/7 skin lesions; 4/7 burning sensation on contact with contaminated water
17	(*75,76*)	39 (mean)	8 M 1 F	Watermen (commercial fishermen)	9/11 disorientation, concentration and memory problems,	8/11 skin lesions; 5/11 burning sensation on contact with contaminated water
18	(*78*)	45 (mean)	93 M 19 F	Watermen, researchers, recreational water exposures	None reported	None reported
19	(*72,73,77*)	40 (mean)	55 M 11 F	Fishing, handling fish with lesions, boating, swimming	47/55 memory loss, confusion	31/55 rash, burning sensation on contact with contaminated water

Abbreviations: F, female; M, male; NR, not reported. [a]Reference numbers in parentheses correspond to reference numbers in text.

Table 4. Clinical manifestations reported among persons with suspected exposure to *P. piscicida*. Gastrointestinal, respiratory, and nonspecific symptoms.

ID #	Ref[a]	Age (years)	Sex	Gastrointestinal symptoms	Respiratory symptoms	Nonspecific symptoms
1	(*74*)	23	M	None reported	Shortness of breath	Dizziness, headache, stiff neck
2	(*74*)	26	M	None reported	None reported	None reported
3	(*74*)	30	F	Diarrhea, nausea, abdominal cramps	None reported	Headache
4	(*74*)	56	F	None reported	Cough, wheezing, reduced FVC and FEV on pulmonary function testing	Eye irritation
5	(*74*)	41	M	Abdominal pain, diarrhea	None reported	None reported
6	(*71*)	47	M	Abdominal pain	Cough, wheezing,	Headache, eye irritation
7	(*71*)	33	M	Abdominal pain, diarrhea	Cough, pneumonia	Weight loss, pneumonia
8	(*71*)	32	M	Abdominal pain, diarrhea	None reported	Headache
9	(*71*)	44	M	Abdominal pain	None reported	None reported
10	(*16*)	<40	NR	None reported	None reported	None reported
11	(*16*)	<40	NR	Abdominal pain, nausea, vomiting	Shortness of breath, chest tightness	Eye irritation, headache,
12	(*16*)	<40	NR	Abdominal pain, nausea	Shortness of breath, pneumonia, asthmatic bronchitis	Eye irritation, headache, joint pain
13	(*16*)	NR	NR	None reported	None reported	None reported
14	(*76*)	>40	M	None reported	Shortness of breath, chest tightness	None reported
15	(*68*)	38 (mean)	3 M	1/3 diarrhea; 1/3 abdominal pain	1/3 shortness of breath; 3/3 sinusitis	2/3 headache, 1/3 joint pain; 2/3 eye irritation
16	(*68*)	38 (mean)	4M 3 F	4/7 diarrhea, abdominal pain, nausea	4/7 shortness of breath; 5/7 sinusitis; 1/7 pneumonia	5/7 headache, 2/7 joint pain; 2/7 eye irritation
17	(*68*)	39 (mean)	8 M 1 F	5/11 diarrhea; 4/11 abdominal pain; 7/11 nausea	2/11 shortness of breath; 5/11 sinusitis; 2/11 pneumonia	9/11 headache, 5/11 joint pain; 6/11 eye irritation
18	(*78*)	45 (mean)	93 M 19 F	None reported	None reported	None reported
19	(*72,73,77*)	40 (mean)	55 M 11 F	28/55 nausea, vomiting, diarrhea, abdominal pain	37/55 upper respiratory tract irritation	4/55 headache, muscle cramps, eye irritation

Abbreviations: FEV, forced expiratory volume; FVC, forced ventilatory contraction.
[a]Reference numbers in parentheses correspond to reference numbers in text.

Table 5. Clinical manifestations reported among persons with suspected exposure to *P. piscicida*. Findings of neuroimaging, electrophysiological, and neuropsychological tests.

ID #	Ref[a]	Age	Sex	Neuroimaging studies	NCV/EMG/EEG/EP studies	Neuropsychological testing
1	(*74*)	23	M	CT normal, possible MRI sinus polyps and mastoid inflammation	None reported	None reported
2	(*74*)	26	M	None reported	None reported	None reported
3	(*74*)	30	F	None reported	None reported	None reported
4	(*74*)	56	F	None reported	None reported	None reported
5	(*74*)	41	M	None reported	None reported	None reported
6	(*71*)	47	M	None reported	None reported	None reported
7	(*71*)	33	M	Abnormal PET scan	None reported	None reported
8	(*71*)	32	M	None reported	None reported	None reported
9	(*71*)	44	M	None reported	None reported	None reported
10	(*16*)	<40	NR	None reported	Normal NCV and EMG studies	None reported
11	(*16*)	<40	NR	Normal MRI and PET	Normal NCV, EMG, EEG, BAEP, and VEP studies	Verbal memory deficits; returned to normal 2 months postexposure
12	(*16*)	<40	NR	None reported	None reported	None reported
13	(*16*)	NR	NR	None reported	None reported	None reported
14	(*16*)	>40	M	Normal MRI	Normal EEG	Attention, memory, psychomotor impairments
15	(*75,76*)	38 (mean)	3 M	None reported	None reported	3/3 Verbal memory deficits (RAVLT); Stroop; Trails B; Grooved Pegboard; performance improved at follow-up testing (+10–12 weeks)
16	(*75,76*)	38 (mean)	4 M 3 F	None reported	None reported	7/7 Verbal memory deficits (RAVLT); Trails B; performance improved at follow-up testing 10–12 weeks later
17	(*75,76*)	39 (mean)	8 M 1F	None reported	None reported	9/9 Verbal memory deficits (RAVLT); Stroop; Trails B; Grooved Peg board; performance improved at follow-up testing 10–12 weeks later
18	(*78*)	45 (mean)	93 M 19 F	None reported	None reported	Visual contrast sensitivity
19	(*72,73,77*)	40 (mean)	55 M 11 F	None reported	None reported	13/13 with neurological symptoms had RAVLT scores below the 10th percentile

Abbreviations: BAEP, brainstem auditory evoked potential; EP, electrophysiologic testing; NCV, nerve conduction velocity; VEP, visual evoked potential.
[a]Reference numbers in parentheses correspond to references in text.

group. This patient was reportedly unable to remember any of the numbers in a five-number sequence and only one of four words in a list immediately after his exposure to *Pfiesteria*. Three months after cessation of exposure to *Pfiesteria* and 8 days after beginning treatment with cholestyramine, the patient reportedly showed improvement in memory function. The specific neuropsychological tests used to document his improvement were not reported by the authors.

Patient B, in the study reported by Glasgow et al. (*16*), showed verbal memory deficits soon after *Pfiesteria* exposure; his memory function was reported as normal 2 months later. A Maryland waterman examined by Bever et al. (*76*) showed attention, memory, and psychomotor impairments on neuropsychological tests. These authors did not report the specific tests they used, but these tests can be inferred from descriptions provided by Grattan (*75*), who was a co-author of the article by Bever et al. (*76*). Inferences concerning the tests used in the evaluation of this patient are indicated in parentheses in the following discussion. Testing indicated problems with delayed recall of verbal and visual information [Rey Auditory Verbal Learning Test (RAVLT) and Visual Retention Test, respectively]. Selective and divided attention deficits were also seen (Stroop and Trails B, respectively). In addition, this patient's performances on tests of psychomotor speed and dexterity (Grooved Pegboard) were impaired. Performance on a task of clerical speed and accuracy (Digit Symbol) was impaired as well. In contrast, performance on simple attention and concentration (Digit Spans), constructional praxis (block design), verbal fluency (controlled oral word association), naming (Visual Naming Test), reading (Boston Diagnostic Aphasia Exam), visual perceptual abilities (Hooper instrument), remote memory, calculations, and language functions were stated by the examiners to be "within expectation."

Neuropsychological tests were performed on groups of boaters, sport fishermen, and watermen allegedly exposed to waters containing *Pfiesteria* (*75,77,78*). Deficits were seen on the RAVLT (*77*). Compared with an unexposed control group, deficits on the RAVLT and on the Stroop test were more prevalent among persons who were exposed to waters containing *Pfiesteria* (*75*). Visual contrast sensitivity (VCS) deficits were identified in *Pfiesteria*-exposed subjects (*78–80*). Verbal and motor skills, memory, attention, and spatial reasoning were also assessed in some of these subjects (Table 5).

Behavioral changes were reported in rats exposed to extracts from *Pfiesteria* culture water (*81–83*). These behavioral changes suggest impairment of central nervous system functioning in these animals. However, the significance of these findings is difficult to interpret with regard to previously reported changes in human behavior because different parameters of central nervous system functioning (e.g., visual vs verbal learning) are being measured. The animals exposed to *Pfiesteria* culture extracts were assessed for their performance on a radial-arm maze. Human exposures to *Pfiesteria*, in contrast, have been associated with deficits on tests of auditory verbal learning and visual contrast sensitivity.

This experimental system has been proposed for two uses: rapid screening of extracts from cultures of different strains of *Pfiesteria*; and establishing dose–response relationships between the concentration of toxin-producing *Pfiesteria* cells in the culture medium and performance of rats in the radial arm maze. However, the validity of the assay for these purposes has not been established and further research in this area is warranted.

Commentary on Research Findings Reviewed

Human exposure to *Pfiesteria* appears to be associated with effects on functioning of the auditory and visual systems as indicated by performances on the RAVLT and VCS tests. Limited data from performance on other neuropsychological tests indicate that other cognitive domains, including attention and executive function, may also be affected. Although frequencies of symptoms and neuropsychological findings are reported in these group studies, it is nevertheless difficult to discern from the published reports what impairments were seen in which individuals and how those impairments relate to *Pfiesteria* exposure. The subjects in these

groups were not characterized as to individual histories of exposure, time elapsed from cessation of exposure to date of testing, or age at the time of testing. In these cases, the information available from case histories and neurological work-ups is insufficient to determine the neuropathologic basis of the observed neurocognitive deficits. Several reports indicate that the neurological deficits are reversible. The complaints of memory and other cognitive problems, together with formal neuropsychological test results from case studies, suggest that the cognitive disturbances develop within hours after exposure and may subside spontaneously after cessation of exposure but may be exacerbated by strenuous exercise. The few reports of neuroimaging studies and neurophysiological studies do not indicate a neuropathological process; they do suggest that the deficits seen on neuropsychological tests may be due to reversible disruptions of neurotransmission or cellular respiration processes. Cholestyramine has been used to treat cognitive and systemic features of *Pfiesteria* exposure, but no controlled trials have been conducted to support its use for this purpose.

Directions for Future Research—Confirming and Extending Prior Studies

Rey Auditory Verbal Learning Test. Deficits have been reported on the RAVLT among patients exposed to *Pfiesteria* (*77*). The results of the RAVLT should be compared with scores on the California Verbal Learning Test (CVLT) to validate these findings. RAVLT and CVLT scores also should be compared with performances on the Digit Span test for auditory memory function, which is one of seven tests included in the World Health Organization (WHO) Neurobehavioral Core Test Battery (see "Other Neuropsychological Tests"). If an association is found between RAVLT scores and performance on Digit Span tests administered by clinicians, the results could be compared with the computer-administered oral Digit Spans that will be available on the Neurobehavioral Evaluation System-3 (NES-3) once it is fully normed.

Because the RAVLT has been so heavily emphasized, it is essential that any subject evaluated with this test have good hearing. RAVLT, CVLT, and Digit Span scores should be compared with brainstem auditory evoked responses as well as simple auditory tests. Findings from these tests should be validated with other tests of attention and other auditory memory, i.e., the Verbal Paired Associate Learning Task and Logical Memories test. Their performance on those tests should be carefully evaluated using a recognition paradigm to determine if any deficits identified are in encoding or retrieval of verbal information.

The battery neuropsychological tests used by Bever and co-workers (*75,76*) apparently did not include a test such as Logical Memories, which is designed to elucidate the contribution of attention, working memory, and auditory information processing to deficits of the kind reported by these authors. Had their evaluation included such a test, the findings of Bever and co-workers would help to better define the contribution of encoding and retrieval deficits to the findings on the RAVLT reported by these authors and others (*76*). This test battery also lacked a test of vigilance, such as the Continuous Performance Test, which may also have been sensitive to the attention and concentration deficits reported by the patient as difficulty following conversations, driving to familiar places, and managing the finances of his business, and revealed by the formal neuropsychological tests of selective and divided attention.

The Neurobehavioral Evaluation System-2 (NES-2) used by Turf et al. (*78*) does not include an auditory verbal learning test and therefore would not be expected to detect deficits in this cognitive domain. The NES-2 also does not include a computerized counterpart to the VCS test used in studies reviewed here (*78–80*). Hudnell et al. (*84*) have published reports indicating that subjects with VCS deficits might perform below expectation on subsets of the NES-2, including the hand–eye coordination test, when compared with subjects with intact visual function. The potentially confounding effects of such contrast sensitivity deficits should be taken into consideration in evaluating performance on the NES-2.

Visual contrast sensitivity tests. A report by Mergler and Blain (*85*) suggests that the Lanthony D-15 or the Farnsworth-Munsell 100 Hue tests would be more suitable for assessing color vision in putatively exposed patients who are suspected to have visual deficits. The Lanthony-D15 (comprising 15 caps of different colors) is a shorter test, taking approximately 5 min, and would therefore be appropriate for assessing larger groups, while the Farnsworth-Munsell 100 Hue (comprising 85 caps of different colors) should be used for more detailed assessments of individual subjects. By contrast, these authors suggest that the Ishihara Plates are not good for assessing acquired color vision loss, such as may occur in persons exposed to *Pfiesteria*, because this test is sensitive only to red–green color vision loss and thus may not detect blue–yellow color vision loss commonly seen in acquired dyschromatopsia. In addition, acquired dyschromatopsia is complex, may involve one or both eyes, and is age dependent. Therefore, the value of the Ishihara Plate test would be as a screening tool for detecting possible congenital color blindness or retinal problems.

VCS measures the function of retinal cells and their pathways to the cortex and cortical function but does not localize deficits. These findings should be correlated with visual evoked potentials (VEPs), which measure conduction of impulses from the retinal cells through to the optic cortex. VEPs are sensitive to demyelinating processes such as multiple sclerosis (MS). The VEPs of patients with MS are also sensitive to changes in sine wave grating pattern orientation. This finding implies cortical pathology because the receptive field of retinal cells is circular or oval (*86*) and therefore these cells do not have a significant response preference for one orientation or another. The VEPs of patients with macular degeneration are slowed but are not sensitive to sine wave grating orientation (*87*), further supporting the hypothesis that cortical pathology underlies the sensitivity to orientation, as the retina is the site of the lesion in macular degeneration. These findings indicate that VEPs using sine wave grating and visual contrast sensitivity studies can be used to localize pathology in the visual system of patients exposed to *Pfiesteria* and that localization of the pathology will depend on the response to orientation of the grating stimulus.

Other neuropsychological tests. While verbal memory and visual contrast sensitivity deficits are the reported salient features of *Pfiesteria* poisoning in the subjects so far studied, deficits may also be seen on tests of attention and executive function, visual memory, psychomotor speed, and personality and affect. Improvement in cognitive functioning may be seen within 3 months after cessation of exposure, but whether residual deficits can persist indefinitely has not been determined (*16,76*).

Among the persons assessed in this series of reports, memory deficits typically were reported within several days after exposure, indicating that the first neuropsychological assessment of an exposed subject should be done as soon after the exposure as possible. Serial testing will document clinical course and recovery and help to predict prognosis after cessation of exposure.

The WHO Neurobehavioral Core Test Battery (NCTB) assesses central nervous system function. It is composed of seven tests that measure simple motor function, short-term memory, eye–hand coordination, affective behavior, and psychomotor perception and speed. The battery includes Digit Span for auditory memory; Santa Ana manual dexterity; Digit Symbol for perceptual motor speed; the Benton visual retention for visual perception and memory; and Pursuit Aiming II for motor steadiness. The sensitivity of this battery for detecting exposure to neurotoxins

such as *Pfiesteria* is limited because it is too brief and does not include more complex tasks of attention and executive function such as the Trail Making Test (Trails B), Paced Serial Auditory Addition, oral arithmetic, or the Wisconsin Card Sort.

The Boston Extended Neurotoxicologic Battery (Clinical) (*88*) contains omnibus tests such as the Wechsler Adult Intelligence Test Scale—Revised as well as tests selected for their sensitivity to deficits in particular cognitive domains. This battery is extensive and requires a full day to administer but would be very useful in defining well-studied cases with serial assessments to fully elucidate the clinical picture of exposure to *Pfiesteria*. Performances on various tests with salient auditory components could be compared with scores or tests of verbal memory to determine if deficits are in the processing of all auditory information or in auditory memory only. Visual–spatial test performance could be compared with visual contrast sensitivity scores to further localize possible functional deficits. The addition of tests sensitive to attention and executive function will reveal contributions that deficits in these domains make to the clinical profile.

An annotated version of this battery could be developed for research work, based on previous published reports, and could include selected tests of attention and executive function (e.g., Trail Making Tests A and B; Digit Spans, oral arithmetic, Paced Auditory Serial Addition; Stroop Test; Wisconsin Card Sort; Continuous Performance Test); visuopatial and visuomotor function (e.g., finger tapping; Digit Symbol; Block Designs; Object Assembly; Boston Visuospatial Quantitative Battery; Santa Ana Form Board; memory (e.g., California Verbal Learning Test; Peterson task, visual reproductions, Logical Memories, Verbal Pair Associate Learning, Rey Osterreith Complex Figure); verbal and language function (e.g., Boston Naming Test; writing sample, reading comprehension and the information and vocabulary subtests from Wechsler); mood and affect (e.g., Profile of Mood States or Minnesota Multiphasic Personality Inventory).

Clinical Documentation

Further clinical studies should better characterize the temporal course of effects of exposure, documenting the time relationship from exposure to symptom onset and the subsequent course of the clinical symptoms. Formal neuropsychological tests must be done while the patient still has clinical complaints about memory or emotional disturbances. Selected brief but highly sensitive test instruments or clinical tasks should be done to assess attention, cognitive tracking, working memory, and visual and auditory memory. Tandem and fine-motor control must be tested to substantiate the clinical complaints of ataxia and dysmetria. Should positive findings appear within the first 4–6 hr after exposure, serial testing with any measures used is essential so that the time course of effect can be accurately documented. Persistent effects may emerge over days but the longer the time interval until testing, the greater the likelihood that any underlying causal relationship will be obscured. Of course, a well-characterized biological marker of the exotoxin for use in experimental studies is sorely needed.

Pathological Studies

Pathological findings in fish exposed to *Pfiesteria* (*33*) suggest that a demyelinating processes is involved in the clinical neurological manifestations seen in fish. However, species differences in metabolism of and cellular responses to the toxin have not been studied. Therefore, no inferences about the role of demyelination in reported human responses to *Pfiesteria* can be made based on these limited findings in fish. In addition, it is unclear whether the demyelinating process described in this study affects the peripheral and central nervous systems in the same fashion. Demyelination could result secondarily from axonal injury. More pathological studies are needed to see if the reported observations of fish swimming upside down have an anatomical correlate in the balancing mechanisms, such as the vestibular and cerebellar systems of fish. Such information may shed light on the complaints of ataxia and disorientation given by allegedly exposed persons.

Magnetic resonance spectroscopy studies may be useful for documenting the subtle changes in brain chemistry that underlie the neuropsychological findings thus far reported.

Neurotoxicology

Because *P. piscicida* is a dinoflagellate, inferences about its toxicity can be drawn from the behavioral neurotoxicology of toxins produced by other dinoflagellates. To cause the effects described in the literature, the exotoxin must be quite potent: fish are affected within minutes of exposure; reported effects in humans occurred after exposure to what must be minute quantities of the toxin in a contaminated spray of water. The pharmacology of this exotoxin has not yet been elucidated.

If the apparent prompt uptake in fish is from the skin as well as the gill, it can be deduced that the exotoxin is lipophilic. For that reason, the toxin would also be taken up quickly by the nervous system and would probably move easily across the blood–brain barrier to affect the central nervous system. If it is lipophilic, the exotoxin could also affect the peripheral myelin.

With scant descriptive information about the neurological consequences available from the case reports, only speculations can be offered concerning the possible anatomical sites of action. The diarrhea and nausea described in the case reports suggest an effect upon the autonomic nervous system, which would also explain the blurred vision reported by some affected persons. The central nervous system effects of ataxia, dysarthria, and dysmetria would indicate a cerebellar site of effect; the postural instabilities may arise from vestibular imbalance. If memory is the most prominent effect, one can assume that there are neurotoxic effects on either the hippocampal structures (e.g., layer CA1) or the prefrontal cortex area. Central nervous system neurotransmitters, such as glutamate, glycine, and dopamine may be implicated in the mechanism of cognitive impairment.

Conclusions

The best-documented instances of adverse effects in humans are the self-reported cases of laboratory workers. The clinical findings in these cases suggest an association between exposure to *Pfiesteria* and the development of neurological symptoms consistent with nervous system dysfunction that may persist beyond the acute exposure period, suggesting that the toxin possesses significant neurotoxic potential. Few additional studies of individual cases and groups that were thoroughly evaluated within a short time after exposure have been reported thus far. The inconsistencies in the clinical approaches and test batteries used to document the effects of exposure to *Pfiesteria* make it difficult to interpret the significance of the neurological findings. Tests of auditory verbal memory and visual contrast sensitivity appear to be sensitive to the central nervous system effects, but further research is needed to substantiate these early findings. A dose–response relationship cannot be ascertained at this time.

The lack of neuropathological findings will not permit speculation on specific anatomical sites within the human nervous system that may be vulnerable to the effects of *Pfiesteria*. The possible mechanisms of *Pfiesteria* neurotoxicity have not been elucidated. The reports of persistent behavioral changes and clinical neuropsychological findings suggest that, with sufficient exposure to the toxin, more than an acute disruption of neurotransmission occurs.

Directions for Future Research

With regard to effects on human health, we endorse the following research to address data gaps:

- Aggressive surveillance should be maintained in the coastal states where

instances of adverse effects on human health have been reported.
- Any persons considered to have symptoms related to exposure to *Pfiesteria* toxin should be enrolled in a prospective study with the objective of characterizing the time course of adverse effects.
- A standard battery of neuropsychological tests should be adapted and applied uniformly to all persons considered to have possible manifestations of exposure to *Pfiesteria* toxin. A standardized protocol for neurological assessment should be established as well.
  - A questionnaire such as the Boston Occupational and Environmental Neurology Questionnaire (*89*) should be administered to all persons with possible exposure to *Pfiesteria* to ascertain concomitant exposures to industrial chemicals and other factors that could produce similar neurological manifestations.
  - The assessment of effects of exposure should be charted with careful attention to the time relationship with exposure and to track the natural history of any adverse effects.
- Epidemiological studies of persons considered at high risk as a result of occupation are in progress. These studies represent an appropriate step in characterizing risk to the population because they address groups considered to have high exposure potential. These studies should be conducted with sufficient standardization of methods to allow ready cross-comparison of findings and even pooling.

Conclusions and Directions for Future Research

Research on *Pfiesteria* has the ultimate purpose of providing evidence that will guide development of strategies to protect the health of estuarine ecosystems and of the people at risk for exposure to *Pfiesteria* toxin. Researchers must also use the clinical evidence to guide evaluation and management of persons affected by *Pfiesteria* toxin. Recognizing the need for research to provide a basis for protection of the environment and human health, the panel adopted a conceptual framework that extends from the ecological drivers of the presence and abundance of *Pfiesteria* to its effects on fish and people (Figure 1). The panel's review was structured around this framework and its research recommendations are directed at key points of uncertainty.

The panel found that uncertainties still remain throughout this framework, although laboratory-based studies, coming now from several laboratories, and the supporting field observations, provide firm evidence that *Pfiesteria* can cause fish kills. It is more difficult, however, to attribute a particular fish kill to *Pfiesteria*. The experimental evidence is sufficient to meet the modification of Koch's postulates that has been applied by this panel and others. The toxin still remains unidentified and there is even uncertainty as to the number of toxins produced by the organism. Questions remain as to whether there is a pathognomonic ulcerated lesion of the skin caused by *Pfiesteria*. Using the fish bioassay, several laboratories

Table 6. Summary of areas for future research on ecophysiology, toxicopathology, and human health effects of *Pfiesteria*.

Topical area	Future research
Ecophysiology of *P. piscicida*	Establish research-quality cultures of *P. piscicida* for wide distribution among the scientific community for life cycle, physiological, and ecological study Develop more effective and quicker methods to • distinguish *P. piscicida* in each of its life cycle stages from look-alike species • quantify the abundance, distribution, seasonality, and toxicity of *P. piscicida* Conduct laboratory and field investigations, using appropriate controls, to clarify the role of organic versus inorganic nutrients, including those in sewage and in wastes from agroindustrial sources, in stimulating growth and toxicity of *P. piscicida*, and the role of these and nonnutritional factors, including prey and predatory dynamics, in regulating its *in situ* growth, population dynamics, and seasonal cycles Identify and chemically characterize the substances produced by fish that stimulate life-stage transformations, growth, and toxin production in *P. piscicida* Assess the role of environmental signals in controlling toxin-producing capability, life-cycle transformations, and functional phenotype occurrences of *P. piscicida*; determine the accompanying physiological characteristics of its life cycle stages and population strains
Effects on fish and other aquatic organisms	Characterize the role of *Aphanomyces* spp. in causing skin ulcers observed in wild menhaden organisms and the potential interaction of this fungus with *Pfiesteria* toxin in affecting fish Conduct full tissue pathological evaluations of fish from laboratory bioassays of *Pfiesteria* and in the wild fish from *Pfiesteria*-associated fish kills Develop standardized protocol and diagnostic criteria for necropsy and histopathologic examination Compile histopathology and necropsy findings in centralized database shared among laboratories Investigate the physiological and toxicological effects of *Pfiesteria* toxin exposure in fish, especially • neurophysiologic effects • tissue and organ distribution
Methods to detect *Pfiesteria* and toxin	Develop and preserve a standard panel of ichthyotoxic water samples and/or extracts derived from clonal *Pfiesteria* cultures Promote collaboration among labs developing analytical and purification methods by distributing the standard ichthyotoxic samples or extracts Standardize the fish bioassay process to facilitate interlaboratory reproducibility Develop or refine chromatographic methods for toxin analysis and purification: • conventional diode array UV detection • bioassay methods (cytotoxicity, receptor-binding assay, rapid fish bioassay) to guide method optimization Apply mass spectroscopic detection methods to chromatographic procedures to estimate molecular mass of putative toxins Apply NMR methods to determine structure of purified toxins Produce antibodies and develop immunochemical methods to detect the *Pfiesteria* organism and its toxin in the field Identify genes associated with toxin biosynthesis using genomic screening methods and analysis of gene expression Adapt PCR and/or FISH methods to devices that can be used in the field to detect toxin-producing phenotypes of *Pfiesteria*
Epidemiological and clinical studies	Maintain aggressive surveillance for possible estuary-associated syndrome in the coastal states where adverse effects have been reported among persons possibly exposed to waters containing *Pfiesteria* Enroll symptomatic persons identified through surveillance in a prospective study of the natural history of adverse effects of exposure Establish a standardized protocol for neurological assessment and apply it to all persons considered to have clinical manifestations of exposure to *Pfiesteria* toxin; the protocol should include a standard battery of neuropsychological tests adapted for the purpose Using a validated questionnaire, ascertain concomitant exposures to other neurotoxic agents (e.g., industrial chemicals, metals) Conduct repeated neuropsychological and clinical evaluations of symptomatic persons to characterize the clinical course of any adverse neurological effects and establish their time relationship with exposure Standardize methods in epidemiological studies of persons at high risk for occupational exposure to *Pfiesteria* toxin to allow ready cross-comparison of findings or pooled analyses

have independently confirmed that the organism can kill fish.

Laboratory and field observations indicate that the organism has a complex life cycle. The current formulation includes 24 stages, but only a few of the stage transformations have been fully documented with photographic or videographic techniques, and molecular confirmation of the genetic identity of each stage has not been published. The certainty of the panel's conclusions with regard to both the life cycle of *Pfiesteria* and its ability to cause fish kills reflects the convergence of evidence from both the laboratory and the field.

For other key points in the panel's conceptual framework see Figure 1; however, uncertainty remains. The extent and nature of the hazard posed to human health by exposure to *Pfiesteria* toxin remains unknown. The anecdotal reports of affected scientists and individual case reports of persons exposed at times of fish kills are a basis for concern and have appropriately led to a broad program of epidemiological and clinical research. Aside from indicating a potential threat to public health, however, the available clinical and epidemiological data provide neither a clear picture of the consequences of exposure to *Pfiesteria* toxin nor an indication of the magnitude of the problem. A patchwork of approaches has been used to describe symptoms and neuropsychological effects and the panel could not find a cohesive picture on reviewing the evidence. Work in this area is further limited by the need to use surrogates for exposure, since the presence of toxin cannot be directly confirmed and exposure cannot be quantified.

In its review of the ecological evidence, the panel could not reach any firm conclusion as to the role of nitrogen- and phosphate-containing runoffs in determining the extent of *Pfiesteria* contamination of waters. The available data support a hypothesis that discharges from sewage treatment plants, industrial operations, and agriculture may have a role, but further tests of this hypothesis are needed.

This report offers a series of recommendations for addressing these uncertainties. Each section of the report concludes with suggested directions for future research, which are summarized in Table 6. This is an extensive research agenda that spans from broad-scale ecological studies to detailed clinical evaluation and molecular studies. At present, the Panel believes priority should be given to research that will give better insight into the effects of *Pfiesteria* on human health; this understanding is needed to assure that public health is protected to the extent possible and to gauge the urgency with which initiatives to protect against exposure are needed. In this regard, key gaps include:

- Our very limited understanding of the *Pfiesteria* toxin; it has not yet been isolated and its mechanism of action is a matter of speculation.
- The incomplete description of the effects of exposure on humans; we have a patchwork of data and little longitudinal information.
- Finally, the nature and extent of exposures that place people at risk but remain uncharacterized; we need to determine the magnitude of the potential threat to persons exposed to toxin-contaminated waters through work or recreational activities.

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