

Skin Ulcers in Estuarine Fishes: A Comparative Pathological Evaluation of Wild and Laboratory-Exposed Fish

W.K. Vogelbein, J.D. Shields, L.W. Haas, K.S. Reece, and D.E. Zwerner

Department of Environmental Sciences, Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, Virginia, USA

The toxic dinoflagellate *Pfiesteria piscicida* Steidinger & Burkholder has recently been implicated as the etiologic agent of acute mass mortalities and skin ulcers in menhaden, *Brevoortia tyrannus*, and other fishes from mid-Atlantic U.S. estuaries. However, evidence for this association is largely circumstantial and controversial. We exposed tilapia (*Oreochromis* spp.) to *Pfiesteria shumwayae* Glasgow & Burkholder (identification based on scanning electron microscopy and molecular analyses) and compared the resulting pathology to the so-called *Pfiesteria*-specific lesions occurring in wild menhaden. The tilapia challenged by high concentrations (2,000–12,000 cells/mL) of *P. shumwayae* exhibited loss of mucus coat and scales plus mild petechial hemorrhage, but no deeply penetrating chronic ulcers like those in wild menhaden. Histologically, fish exhibited epidermal erosion with bacterial colonization but minimal associated inflammation. In moribund fish, loss of epidermis was widespread over large portions of the body. Similar erosion occurred in the mucosa lining the oral and branchial cavities. Gills exhibited epithelial lifting, loss of secondary lamellar structure, and infiltration by lymphoid cells. Epithelial lining of the lateral line canal (LLC) and olfactory organs exhibited severe necrosis. Visceral organs, kidney, and neural tissues (brain, spinal cord, ganglia, peripheral nerves) were histologically normal. An unexpected finding was the numerous *P. shumwayae* cells adhering to damaged skin, skin folds, scale pockets, LLC, and olfactory tissues. In contrast, histologic evaluation of skin ulcers in over 200 wild menhaden from Virginia and Maryland portions of the Chesapeake Bay and the Pamlico Estuary, North Carolina, revealed that all ulcers harbored a deeply invasive, highly pathogenic fungus now known to be *Aphanomyces invadans*. In menhaden the infection always elicited severe myonecrosis and intense granulomatous myositis. The consistent occurrence of this fungus and the nature and severity of the resulting inflammatory response indicate that these ulcers are chronic (age >1 week) and of an infectious etiology, not the direct result of an acute toxicosis initiated by *Pfiesteria* toxin(s) as recently hypothesized. The disease therefore is best called ulcerative mycosis (UM). This study indicates that the pathology of *Pfiesteria* laboratory exposure is fundamentally different from that of UM in menhaden; however, we cannot rule out *Pfiesteria* as one of many possible early initiators predisposing wild fishes to fungal infection in some circumstances. **Key words:** *Aphanomyces invadans*, menhaden skin ulceration, pathology, *Pfiesteria shumwayae*. — *Environ Health Perspect* 109(suppl 5):687–693 (2001). <http://ehpnet1.niehs.nih.gov/docs/2001/suppl-5/687-693vogelbein/abstract.html>

Atlantic menhaden, *Brevoortia tyrannus*, from estuaries of the mid-Atlantic United States commonly exhibit a cutaneous ulcerative disease characterized by the presence of deeply penetrating perianal ulcers and associated granulomatous dermatitis and myositis. These lesions were first reported during the mid-1980s in menhaden and other fishes from Virginia and Maryland portions of the Chesapeake Bay and the Tar–Pamlico River Estuary, North Carolina (1–4). Lesion etiology was actively investigated at the time; on the basis of pathology, microbial characterization, and exposure studies, the condition was ascribed to infection by Oomycete fungi including members of the genus *Aphanomyces* (1,2,5–7). In North Carolina the condition was referred to as ulcerative mycosis (UM) (2), but Hargis (1) called it ulcer disease syndrome because he did not consistently observe fungal hyphae in lesions from Chesapeake Bay fishes. Investigators from both regions considered lesion etiology to be complex, with other environmental factors including high levels of precipitation, agricultural runoff, or nutrient

loading playing probable roles in disease expression (6). Massive fish kills were attributed to the disease in North Carolina estuaries (3), and the disease has recurred frequently in subsequent years.

More recently, fungi associated with the menhaden ulcers in North Carolina estuaries were hypothesized to be secondary, opportunistic invaders colonizing lesions caused, in some instances, by exotoxins of *Pfiesteria piscicida* Steidinger & Burkholder (8–10). This hypothesis was based on the observed co-occurrence of the ulcerous lesions in wild menhaden and *P. piscicida* in some acute fish kill events in North Carolina estuaries (8). A recent laboratory exposure study provided some support for these field observations. Striped bass (*Morone saxatilis*) and tilapia (*Oreochromis* spp.) exposed to sublethal concentrations of *P. piscicida* exhibited initial widespread loss of epidermis followed by ulcer development in a few fish (9). This suggested a possible early initiating role for *P. piscicida* in menhaden ulcer development.

Similar cutaneous ulcerative syndromes have been reported in over 100 species of wild

and cultured freshwater and estuarine fishes in the Indo-Pacific region since the 1970s (11). Outbreaks have been referred to respectively as epizootic ulcerative syndrome (EUS) in Asia, red spot disease in Australia, and mycotic granulomatosis in Japan (12). This disease has devastated several major commercial fisheries, both wild and farmed, and is now collectively referred to as EUS (13). *Aphanomyces invadans*, an Oomycete fungus, was recently implicated as the etiologic agent of EUS. Grossly and histologically, EUS in Indo-Pacific fishes and UM in menhaden from mid-Atlantic U.S. estuaries are essentially identical, and a recent investigation indicates that the fungal agent observed in menhaden UM is also *A. invadans* (14,15). Recent work in our laboratory indicates this fungal agent may be a primary pathogen in menhaden, capable of inducing formation of skin ulcers, with other environmental or biological factors, including *Pfiesteria* spp., playing no role whatsoever (16).

The roles that *Pfiesteria* spp. play in development of skin ulcers in wild menhaden and other fishes therefore remain unresolved and controversial. Despite this, menhaden lesions are presently used in conjunction with presumptive counts of *Pfiesteria*-like cells in water samples and toxic fish bioassays as primary criteria for decisions about human health impacts and river closures in Maryland, Virginia, and North Carolina. In an effort to better understand the association between *Pfiesteria* spp. and fish lesions, we

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

Address correspondence to W.K. Vogelbein, Dept. of Environmental Sciences, Virginia Institute of Marine Science, The College of William and Mary, Rt. 1208, Gloucester Point, VA 23062 USA. Telephone: (804) 684-7261. Fax: (804) 684-7186. E-mail: wolf@vims.edu

The authors thank P. Blake for histologic preparations, P. Mason and V. Lovko for SEM identification of *Pfiesteria shumwayae*; C. Squyars, Y. Kiryu, and A. Miller for toxic *Pfiesteria* exposures of fish in the Biosafety Level 3 (BSL-3) facility; V. Foster and L. Ott for dinoflagellate culture support; and N. Stokes and L. Walker for molecular identification of *P. shumwayae*. T. Shedd of the U.S. Army Center for Environmental Health Research provided the prototype BSL-3 facility. This article is contribution 2403 from the Virginia Institute of Marine Science. Supported in part by U.S. Environmental Protection Agency/National Oceanic & Atmospheric Administration ECOHAB grants R-828225-01-0 and R-826791-01-0 and Virginia Commonwealth *Pfiesteria* Initiative.

Received 3 April 2001; accepted 23 July 2001.

compare and contrast UM in wild menhaden and pfiesteriosis in laboratory-exposed fishes. Our recently developed ability to conduct laboratory fish toxicity bioassays with *Pfiesteria shumwayae* Glasgow & Burkholder (17–19) makes this critical comparison possible.

Materials and Methods

P. shumwayae and Fish Sources

Five water and sediment samples from the Neuse (Cherry Point, Slocum Creek, Neuse Creek) and Pamlico (Cove Point, Blounts Creek) rivers were collected on 14 July 2000, shortly after reported fish kills. Quarantined and prophylactically treated tilapia (10 fish, 3.0–5.0 cm) (see below) were immediately placed in 2 L water collected from each source. Fish and additional water samples were then transported to the laboratory. Subsamples from each location were processed for DNA analysis [polymerase chain reaction (PCR) primer amplification of small subunit (SSU) and large subunit (LSU) regions of extracted DNA; see below] and scanning electron microscopy (SEM) to determine the presence of *Pfiesteria* spp. and related dinoflagellates. DNA analysis and SEM verification were used to identify *P. shumwayae* in aliquots from aquaria and clonal isolates from original water samples cultured on algae and from aquaria.

Since 1997 ulcerous and healthy menhaden have been collected by cast or gill nets from numerous sites within Chesapeake Bay and from the Neuse and Pamlico Rivers, North Carolina. Fish were either dissected and fixed in the field immediately following capture or transported on ice to the laboratory for workup within 12 hr of capture.

38-L Biototoxicity Assays

Tilapia (*Oreochromis niloticus*) used in the 38-L format biototoxicity assays were acquired from an aquaculture facility (Southern States tilapia farm, Richmond, VA, USA). Fish used in the assays were maintained in artificial sea water (ASW; Forty Fathoms Marine Mix; Marine Enterprises Intl., Baltimore, MD, USA) and underwent quarantine and prophylaxis of two 1-hr formalin baths (1:4,000) separated by a 3-day interval, followed by a 7-day exposure to Cutrine (chelated copper sulfate, 5 ppm), then an acclimation/recovery period of 1 week. All toxicity assays with tilapia were conducted in a biohazards level 3 containment facility. For the assays, 25–40 tilapia (3.0–5.0 cm total length) were placed in 38 L aquaria held at 20°C, with aeration and filtration through preconditioned nylon Whisper filters containing crushed coral as biological filter media. Water and sediment samples from environmental collections were added to aquaria (at salinities of the respective

collection site, 6–12‰) with tilapia. New bioassays (i.e., subcultures) derived from fish-killing aquaria were initiated by transferring water aliquots from toxic aquaria to new aquaria (ASW at 12‰). Fish were fed a commercial aquaculture feed (Southern States) 3 times per week. Space and logistical considerations prevented replication of the bioassays. Controls consisted of replicate treatments with fish maintained in ASW with no river water or sediment added.

Fish were monitored daily for signs of morbidity, lesions, and mortality. During mortality events, new fish were added daily to replace dead fish and to maintain fish densities. Fish mortality was expressed as the percentage of fish dying over 24 hr, and cumulative mortality was the total number of fish killed related to time. After 80 days any assay not resulting in fish mortality was assessed as nontoxic and destroyed.

Water quality parameters monitored weekly included pH (Thermo Orion, Beverly, MA, USA), temperature, salinity (refractometer), ammonia and nitrite (Hach kits with appropriate dilutions; Hach Inc., Loveland, CO, USA). Dissolved oxygen (Yellow Springs Instruments, Cincinnati, OH, USA) was monitored weekly in several aquaria; it showed little relation to fish mortality and was dropped from the monitoring protocol. Water changes (–60–75% aquarium volume using ASW) were made after mortalities ceased and after ammonia levels were high (>30 ppm).

Water samples to estimate cell densities of *P. shumwayae* were collected weekly prior to assessment of mortalities and daily when fish exhibited morbidity or mortality. A 50-mL aliquot of aquarium water was pipetted into a centrifuge tube, fixed with Lugol's iodine, mixed, and settled for 2–3 hr. Sample volume was reduced to 5 mL using a pipette, and stirred gently to resuspend the cells; aliquots were counted in a hemacytometer (Neubauer, Bright-line; Reichart, Buffalo, NY, USA). The presence of different life history stages was noted, but the estimated densities included all of the cells resembling *P. shumwayae*. Additional aliquots were observed live or fixed in 25-mL tissue culture flasks using Hoffman-contrast and bright-field microscopy, respectively.

Histopathology

Field-collected menhaden and moribund laboratory-exposed tilapia were killed by overdose with tricaine methanesulfonate. Grossly visible skin lesions on wild menhaden and the skin, gills, liver, spleen, pancreas, alimentary tract, kidney, and head (brain and other neural tissues) from laboratory-exposed tilapia were processed by routine methods for paraffin histology (20). Tissues were fixed in 10% neutral buffered formalin for 48 hr, washed

overnight in running water, dehydrated, infiltrated and embedded in paraffin, sectioned on a rotary microtome at 5 µm, mounted on slides and stained with hematoxylin and eosin (H & E) or with Grocott's methenamine silver stain for fungal hyphae (20).

Molecular Verification of *P. shumwayae*

DNA was prepared from 20- to 100-mL samples of clonal cultures, with water collected from the environment, or water from bioassay aquaria. Cells were collected either by centrifugation followed by osmotic shock lysis (21) or by filtration onto 3 µm Nucleopore (Whatman; VWR, Charlotte, NC, USA) filters with DNA preparation using the DNeasy tissue kit (Qiagen, Valencia, CA, USA) and following the manufacturer's protocol. DNA sequences were first determined for the internal transcribed spacer region and portions of both the SSU and LSU genes of the ribosomal DNA complex for clonal dinoflagellate cultures. Sequences were compared among *Pfiesteria*-like organisms (PLOs), including *P. piscicida*, *P. shumwayae*, "Lucy"-like PLOs, and *Cryptoperidiniopsis* spp., and to other dinoflagellates and protozoans. Primers for use in the PCR were designed based on DNA sequences unique to each group of organisms. The primer combination that successfully amplified *P. shumwayae* DNA with specificity and sensitivity was PfBSSU (5' CCAGCTTCTGGATTTTGTGCGC 3') and 16S-like primer B 5' GATCCTTCCGCA GGTTACCTAC 3' (22). The primers were tested for specificity against DNA isolated from our "Lucy"-like cultures, *P. piscicida* and *Cryptoperidiniopsis* spp., as well as DNA from other dinoflagellates including *Amphidinium carterae*, *Proocentrum micans*, and the more distantly related oyster pathogens *Perkinsus marinus*, *Perkinsus atlanticus*, and *Haplosporidium nelsoni*. In addition, primers were tested for their ability to specifically amplify these DNAs from environmental and aquarium water samples by spiking DNA extracts with clonal culture DNAs. Identities of the PCR amplification products from the bioassay aquaria and the environment were confirmed by DNA sequence analysis. Amplification products were cloned into the plasmid vector pCR2.1 (Invitrogen, Carlsbad, CA, USA) using the TA cloning kit (Invitrogen) and following the manufacturer's protocol. Plasmids were sequenced by simultaneous bidirectional cycle-sequencing using the Thermo sequenase sequencing kit (Amersham Life Science, Piscataway, NJ, USA) and infrared-labeled (IRD700 or IRD 800) M13 primers (LI-COR, Lincoln, NE, USA) following the manufacturer's protocol. Resulting sequences were aligned and compared to the homologous DNA sequences of clonal cultures for identification.

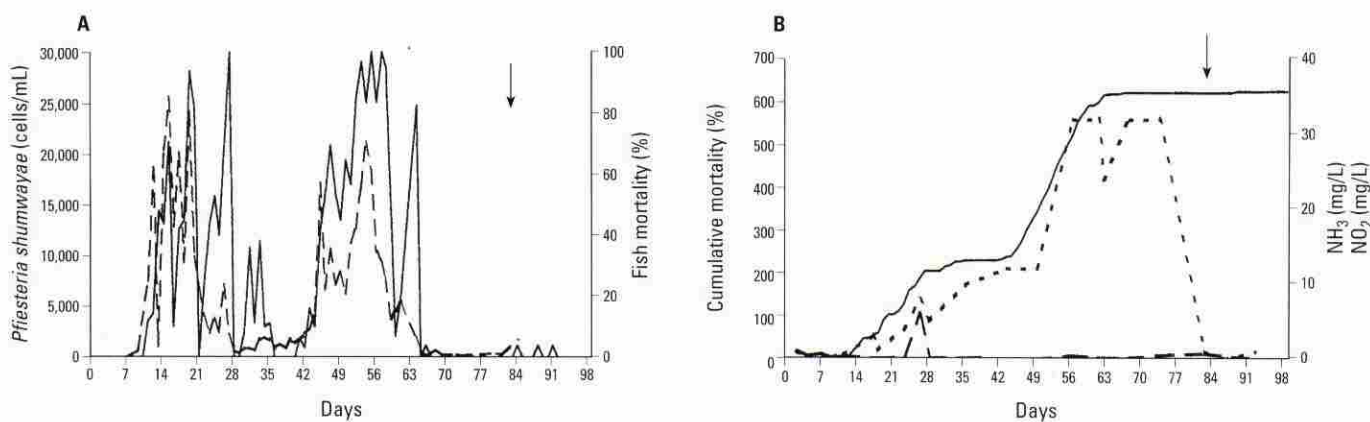


Figure 1. Representative data from long-term 38 L bioassay using water (38 L) and sediments (10 g) from Slocum Creek, North Carolina. (A) Fish mortality (solid) in relation to cell densities of *Pfiesteria shumwayae* (dashed). (B) Cumulative mortality (solid) in relation to total NH₃ (dashed) and nitrite (chain). Arrows: 75% water change.

Results

Pfiesteriosis of Laboratory-Exposed Fish

Long-term bioassays. Three of five bioassays using water samples collected in North Carolina exhibited fish mortalities (Core Point, Cherry Point, Slocum Creek). In all three cases high mortality occurred in direct relation to the density of *P. shumwayae*, which was the only *Pfiesteria*-like dinoflagellate observed in the bioassays. Mortality events typically consisted of two major peaks, each lasting 2–3 weeks with 1–2 lesser sequelae (Figure 1A). During mortalities, densities of *P. shumwayae* often reached high levels (15.0–25.0 × 10³ cells/mL). Heavy bacterial loads (*Vibrio* spp. and *Shewanella putrefaciens*, 10⁶–10⁷ cells/mL) were present in aquaria exhibiting repeated mortalities. Control aquaria experienced negligible mortalities and excellent water quality throughout the study, with low bacterial loads.

Water quality degraded over time, partially in response to fish mortalities (Figure 1B). pH decreased over time to 5.8–6.0, in relation to increased organic wastes and thus kept reactive ammonia low. Daily ammonia levels were not correlated with daily fish mortality. However, ammonia levels increased in response to cumulative mortalities and reached relatively high levels during mortality events, particularly during the second peak in mortalities (Figure 1B). Nitrite cycled quickly in aquaria and had little bearing on fish health. Nitrite is not generally considered toxic at 1–6 ppm with salinities of 6–12‰. Because aquaria were aerated, dissolved oxygen never fell below 5.0 ppm. Thus, water quality factors were not directly related to the observed fish mortalities; rather, water quality degraded in response to fish death.

After the second peak in mortality, aquaria generally lost their fish-killing activity (Figure 1A). During the nadirs in *P. shumwayae* densities, other protozoans were observed feeding on dinospores and cysts of the dinoflagellates.

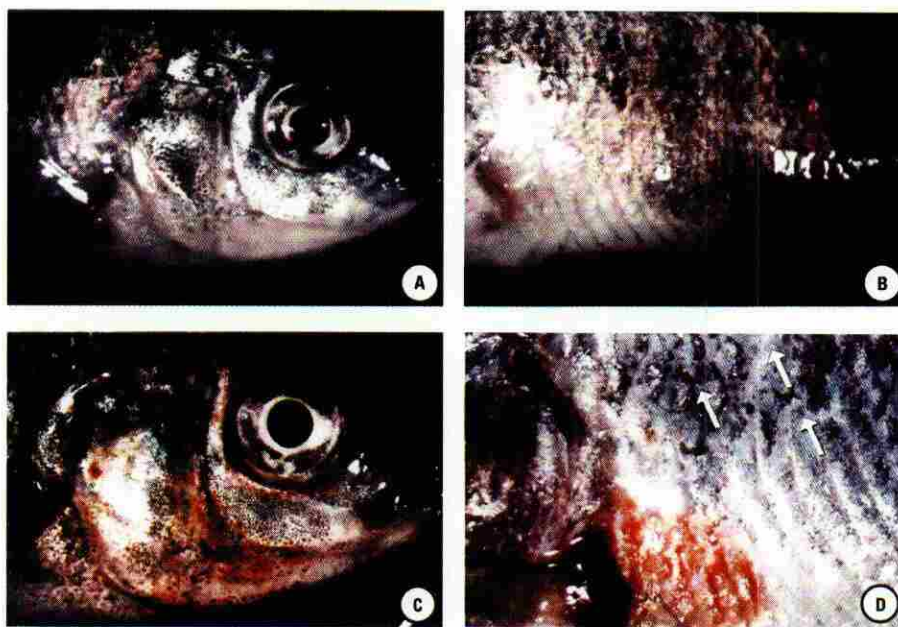


Figure 2. Gross pathology of acute pfiesteriosis in laboratory-exposed tilapia (*Oreochromis* spp.). (A) Normal appearance of control fish, head region. Original magnification ×1.8. (B) Normal appearance of flank. Original magnification ×1.8. (C) Petechial hemorrhage over operculum after acute lethal exposure. Original magnification ×2.3. (D) Focal erythema caudal to pectoral fin (arrowhead), loss of mucus coat, epidermis, and scales along flank (arrows). Original magnification ×2.3.

Several species of amoebae and a large ciliate were the primary predators on the dinoflagellates and may have contributed to the decline in their densities. For example, one large amoeba (~150 μm) contained at least 16 cysts of *P. shumwayae*. Aquaria contained communities of ciliates (holotrichs, hymenotrichs), rotifers, amoebae, and microflagellates (bicosids, bodonids). Except for dinoflagellates, such communities were also abundant in control aquaria.

Molecular identification. DNA were isolated from water samples of all nine aquaria exhibiting mortality contained *P. shumwayae*. PCR amplification products were obtained using the primers designed to specifically amplify *P. shumwayae* DNA. No amplification products were seen with the

P. piscicida, “Lucy”-like, or *Cryptoperidiniopsis* spp. primers. Identity of PCR products was confirmed by DNA sequence analysis. The nucleotide sequences matched those obtained from clonal *P. shumwayae* cultures.

Gross pathology. Gross and histopathological characterization of pfiesteriosis in tilapia were based on 30 moribund individuals exposed to pathogenic cultures of *P. shumwayae*. Unexposed control fish exhibited intact skin with a well-formed mucus coat over all of the body surface (Figure 2A,B) and intact, clear fins. Gross clinical signs of pfiesteriosis in exposed moribund fish were restricted to subtle changes of the skin. The most common observation in exposed moribund fish was a widespread loss of the mucus coat, with associated rapid drying and dulling

of the body surface once fish were removed from the exposure tank. Additionally, mild diffuse hyperemia and petechial hemorrhage, usually on the ventral body surface or the head (Figure 2C) were common on moribund fish. Some fish exhibited a discrete focus of hemorrhage below the pectoral fin and disruption of the epidermal layer over much of the flanks, with extensive loss of scales (Figure 2D). Others exhibited mild fin erosion. None of the exposed tilapia developed lesions that resembled those described for UM.

Histopathology. Unexposed control fish exhibited an intact epidermis comprising

stratified squamous epithelium 15–20 cells thick (Figure 3A). In contrast, tilapia exposed to *P. shumwayae* consistently exhibited widespread partial to complete loss of the epidermal layer of the skin. In moribund fish the epidermis was completely eroded over much of the body surface (Figure 3B). Bacterial colonization of the dermis was common, and *Pfiesteria* cells were frequently seen adhering to the surface (Figure 3B, inset). However, inflammation associated with these alterations was minimal. *Pfiesteria* cells often were observed within exposed scale pockets and accumulated within skin folds such as those

along the edge of the eye (Figure 3C). Epidermal erosion was often associated with the presence of *Pfiesteria* cells in the skin folds (Figure 3D). Similar pathological effects were observed in the oral cavity, which is normally lined by a multilayered mucosa composed of squamous epithelium and goblet cells (Figure 3E). Exposed fish often exhibited complete mucosal erosion (Figure 3F). *Pfiesteria* cells and bacterial contaminants (Figure 3F, inset) were frequently seen adhering to exposed connective tissues within the oral cavity. Commonly observed alterations in the gill tissues included separation and lifting of the respiratory epithelium (Figure 4A,B), accumulation of lymphocytes in secondary lamellae, and accumulation of *Pfiesteria* cells within the branchial chamber (Figure 4B inset). Degenerative changes also occurred within the lateral line canal (LLC) system of the head. The epithelial lining of the LLC was eroded and often completely lost, and colonized with bacterial contaminants and *Pfiesteria* cells (Figure 4C,D). Similar degenerative changes were observed within the nares and olfactory organs (Figure 4E,F). Lesions attributable to *Pfiesteria* exposure were not observed in internal organs or tissues including liver, spleen, kidney, pancreas, alimentary tract, brain, spinal cord, and peripheral nervous tissues.

Ulcerative Mycosis of Wild Menhaden

The pathology of UM in menhaden (5) and EUS of Indo-Pacific fishes (11,12,23) has been previously reported and therefore is only briefly described here. The most characteristic gross clinical sign of the disease in wild menhaden in the present study was a single, deeply penetrating ulcer often located perianally (Figure 5A). However, other affected fish had single or multiple ulcers that could be located anywhere on the body. Histologically these lesions are characterized by the presence of highly invasive, aseptate fungal hyphae penetrating deeply into the body muscles or into the peritoneal cavity, where they actively invade and damage the visceral organs. These deeply penetrating aseptate fungi were a consistent observation in the vast majority (>99%) of the menhaden. Necrosis of skeletal muscle and intense granulomatous inflammation directed at the fungus were prominent features of lesions. Fungal hyphae were encapsulated by inflammatory cells, and tracks of hyphae surrounded by a capsule composed of inflammatory cells extended deeply into the host tissues (Figure 5B), sometimes extending almost completely through the body of the fish. Advanced ulcerous lesions exhibited a zone of surficial tissue necrosis (Figure 5B) that contained abundant bacterial and saprophytic fungal contaminants not associated with

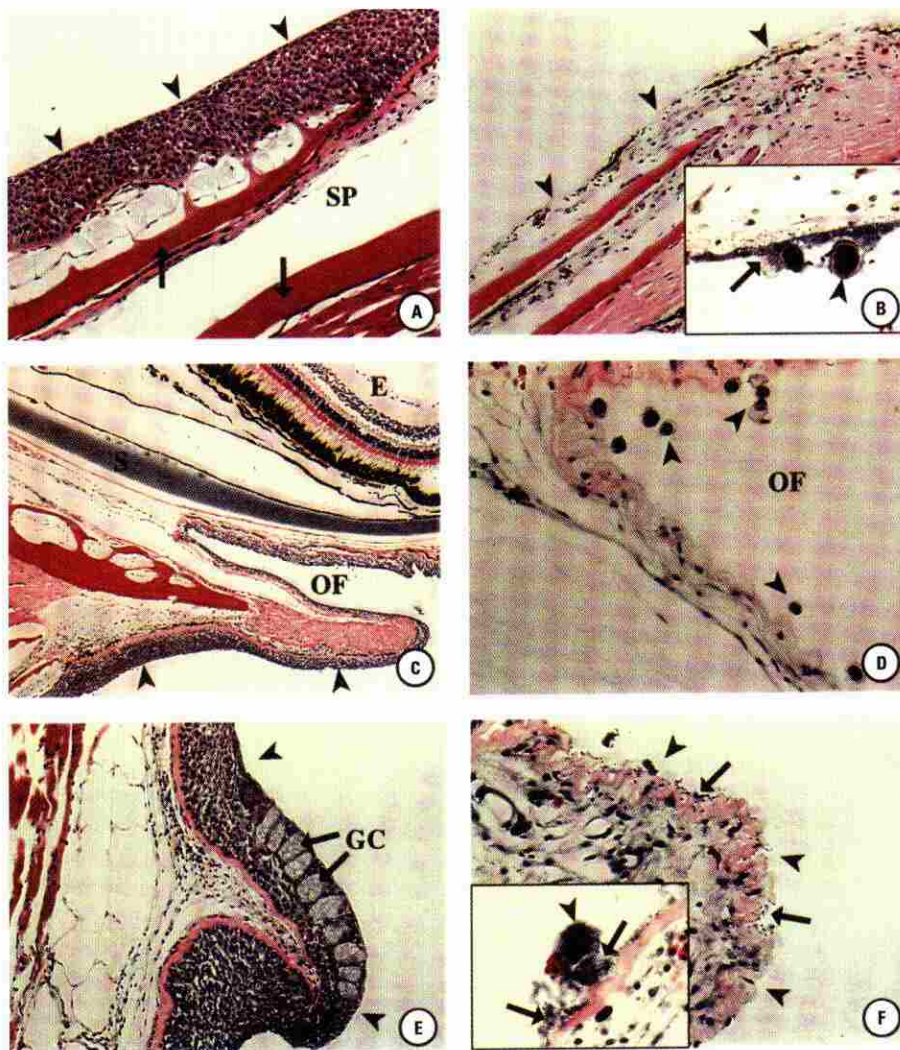


Figure 3. Histopathology of acute *P. shumwayae* exposure in tilapia (*Oreochromis* spp.). (A) Skin section of control fish with normal multilayered epidermis (arrowheads). Note scales in underlying dermis (arrows), SP = scale pocket. H & E. Original magnification $\times 155$. (B) Loss of epidermal layer (arrowheads) in moribund exposed fish. H & E. Original magnification $\times 155$. Inset: surface of exposed dermis with two adherent *P. shumwayae* cells (arrowhead) and bacterial contaminants (arrow). H & E. Original magnification $\times 457$. (C) Section of head through the eye; control fish. Note orbital fold (OF) at edge of the eye (E) lined with normal epidermis. S: sclera, arrowheads: epidermis. H & E. Original magnification $\times 77$. (D) High magnification of orbital fold (OF) in exposed fish; loss of epidermis and numerous adherent *P. shumwayae* cells (arrowheads). H & E. Original magnification $\times 340$. (E) Mucosal lining of oral cavity in control fish with prominent mucosa (arrowheads) containing mucus-secreting goblet cells (GC). H & E. Original magnification $\times 145$. (F) Erosion of oral mucosa in exposed fish with exposure of underlying dermis (arrowheads). Arrows: bacteria. H&E. Original magnification $\times 320$. Inset: Eroded oral mucosa; adherent *P. shumwayae* (arrowhead), bacteria (arrows). H & E. Original magnification $\times 488$.

granulomatous inflammation. Granulomas were well organized and composed largely of macrophages. Fungal hyphae were readily discerned within granulomas in tissue sections stained routinely with H & E (Figure 5C) but were most easily visualized using Grocott's methenamine silver stain for fungal hyphae (Figure 5D). Other than for the focal ulcers, the skin of these wild menhaden was histologically normal.

Discussion

Pathological responses associated with UM in menhaden differ fundamentally from those occurring in acute pfiesteriosis of laboratory-exposed fishes. Whereas the menhaden ulcers are infectious and develop over an extended period (weeks) (14), *Pfiesteria* exposure of tilapia results in rapid development (hours) of acute widespread erosion of the epidermis, followed quickly by death. UM is an infectious disease characterized by one or more focal, deeply penetrating, often perianal ulcers. The causative agent is now known to be an invasive, highly pathogenic Oomycete fungus called *A. invadans* (14,15). This fungus is also the cause of EUS in the Indo-Pacific region and has caused extensive losses to commercial fisheries there since the early 1970s (11–13). However, disease outbreaks are considered a multifactorial problem because a variety of predisposing factors may be involved, the only absolute requirement being the presence of the fungus. In some outbreaks *A. invadans* may be the only factor required for disease to occur, whereas in others, environmental factors, trauma, or other infectious agents are thought to predispose fish populations to outbreaks (11,12). A similar situation has been hypothesized for UM in menhaden, with environmental factors such as high levels of precipitation, agricultural runoff, nutrient loading (6), and recently, exposure to *Pfiesteria* toxins (9,10) playing possible roles in disease initiation and expression. It is reasonable to speculate that exposure to sublethal concentrations of *Pfiesteria* spp. will induce only mild skin damage and provide a portal of entry for infectious zoospores of *A. invadans* and other opportunist viral, bacterial, fungal, or protozoan pathogens. Recent experimental work in our laboratory, however, suggests that *A. invadans* is a primary pathogen able to cause UM in menhaden without other predisposing factors such as poor water quality or *Pfiesteria* spp. playing any role whatsoever (16).

Menhaden ulcers are characterized histologically by the consistent presence of deeply penetrating fungal hyphae, extensive myodegeneration, and a special type of host cellular defensive response called granulomatous inflammation directed at the fungal agent (6,14). This type of response is typically

elicited in vertebrates by infections that are difficult for a host to kill and degrade and is composed predominantly of a mononuclear phagocyte called the macrophage (24). These cells accumulate within the infected tissues and organize into discrete structures called granulomas that effectively encapsulate the foreign invader. This special type of inflammatory response develops over an extended period and represents an attempt by the host to bring to bear a suite of toxic and cytolytic substances produced by the macrophages and designed to inactivate, kill, and degrade the infectious agent. The nature and severity of the inflammatory response in menhaden infected with *A. invadans* indicates that the ulcers are chronic lesions developing over a

period of 1–3 weeks and not within several hours as a result of acute *Pfiesteria* toxicosis, as has been suggested (25,26). If this were the case we would also expect affected wild menhaden to exhibit the acute widespread epidermal erosion occurring in laboratory exposures with *Pfiesteria* spp. (9,17,18). These acute lesions have never been reported in wild menhaden. Additionally, we have recently confirmed the extended time frame required for ulcer development using waterborne exposure of menhaden to *A. invadans* zoospores (27).

Although UM in menhaden has been well characterized histologically, only one report describes the histopathology of *Pfiesteria* exposure in laboratory fishes. Noga et al. (9) exposed striped bass, hybrid striped bass, and

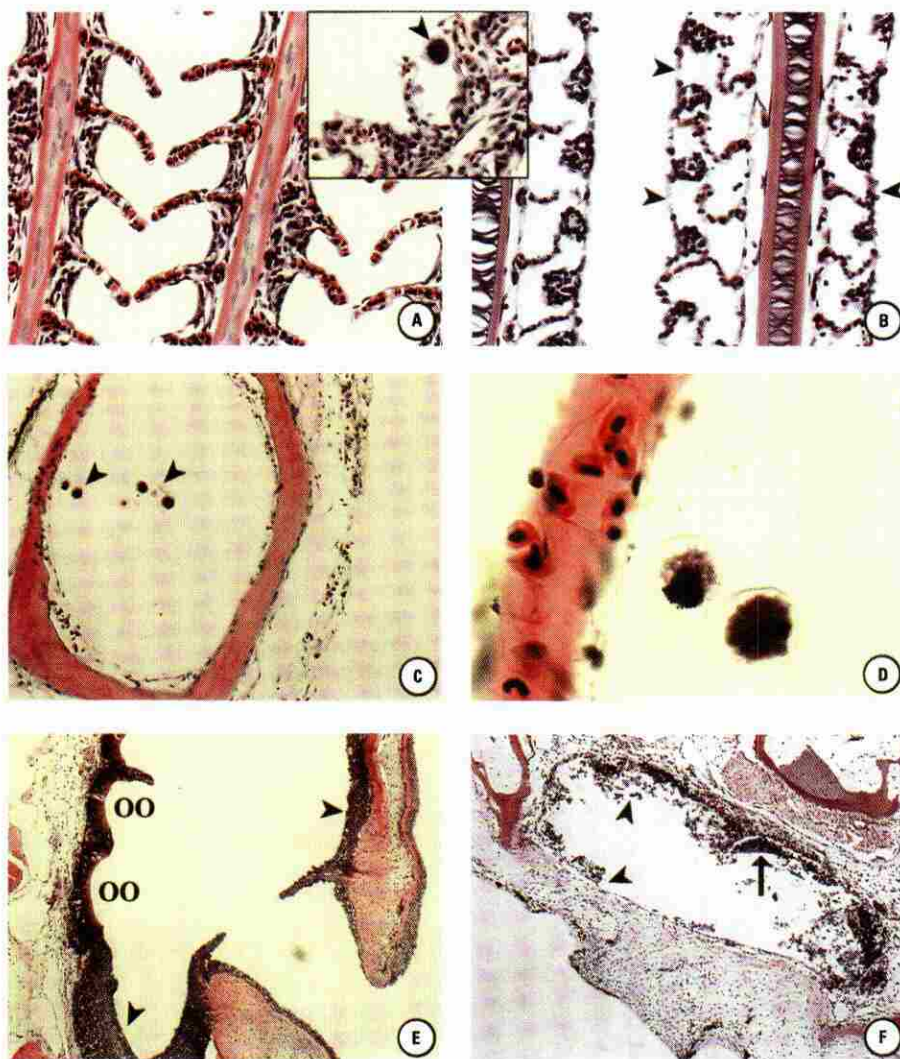


Figure 4. Histopathology of acute *P. shumwayae* exposure in tilapia (*Oreochromis* spp.). (A) Normal gill structure in control fish. H & E. Original magnification $\times 155$. (B) Lifting of respiratory epithelium in exposed fish (arrowheads). H&E. Original magnification $\times 155$. Inset: Gill tissue with adherent *P. shumwayae* cell (arrowhead). H & E. Original magnification $\times 390$. (C) Lateral line canal (LLC) of exposed fish showing loss of lining epithelium and adherent *P. shumwayae* cells (arrowheads). H & E. Original magnification $\times 42$. (D) Closeup of Figure 5C showing eroded epithelial lining of LLC and two *Pfiesteria* cells (arrowheads). H & E. Original magnification $\times 341$. (E) Olfactory chamber in control fish with normal epithelial lining (arrowheads) and sensory olfactory organs (OO). H & E. Original magnification $\times 41$. (F) Olfactory chamber in exposed fish showing erosion of epithelial lining (arrowheads), destruction of olfactory organs (arrows). H & E. Original magnification $\times 39$.

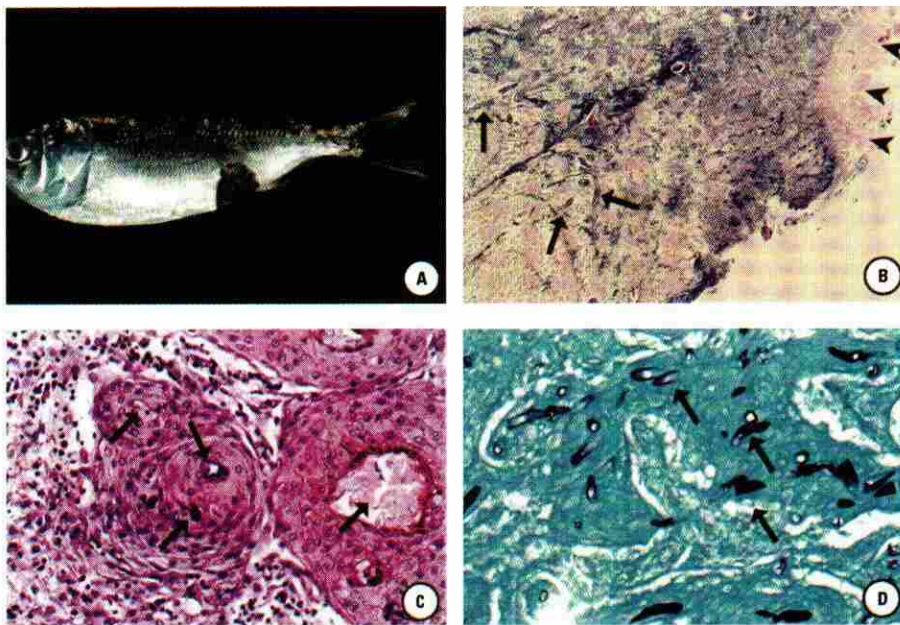


Figure 5. Ulcerative mycosis in wild menhaden, *B. tyrannus*. (A) Gross appearance of perianal ulcer. Original magnification $\times 0.9$. (B) Histologic section of ulcer illustrating fungal agent and associated granulomatous myositis penetrating into body wall of infected fish (arrows). H & E. Original magnification $\times 31$. Note surficial tissue necrosis (arrowheads). H & E. (C) Higher magnification of granulomas around hyphae (arrows). H & E. Original magnification $\times 312$. (D) Histochemical staining of hyphae (arrows). Grocott's methenamine silver stain. Original magnification $\times 312$.

tilapia to *P. piscicida* ($2\text{--}5 \times 10^3$ cells/mL = 48 hr LD₁₀₀) and tracked lesion development over time. Although multiple tissues including skin, gills, liver, pancreas, stomach, intestine, spleen, kidney, and brain were examined histologically, changes were limited to epidermis and were comparable to those described in the present study in exposed tilapia. Exposed fish additionally exhibited highly elevated osmolality and serum sodium, chloride, and potassium levels, likely a direct result of the extensive skin damage and a loss of osmoregulatory ability. None of the moribund fish exhibited ulcers characteristic of UM. Interestingly, neural lesions were not observed in acutely exposed fish or in surviving fish held for an undisclosed extended period, despite the fact that *Pfiesteria* spp. are believed to secrete a potent neurotoxin (10,26). Significantly, a percentage of sublethally exposed fish (24 hr) held in clean water for an unspecified period developed more advanced ulcerations. Only one fish developed a fungal infection associated with ulceration, whereas the majority developed bacterial infections only. None developed the granulomatous inflammatory response typical of *A. invadans* infections (9). Exposure of tilapia to similar concentrations ($2\text{--}12 \times 10^3$ cells/mL) of *P. shumwayae* in this study resulted in identical pathological alterations as those observed for *P. piscicida* (9). The predominant lesion in moribund fish was a widespread, often extensive erosion of the epidermis. However, lesions not previously

reported in *P. piscicida* exposures but observed in this study included extensive epithelial necrosis within the LLC, olfactory organs, gills, and oral mucosa. Additionally, the consistent presence of attached *P. shumwayae* cells at the site of the epidermal lesions has not been previously reported.

The significance of the adherent *Pfiesteria* cells within epidermal lesions of exposed fish is somewhat unclear. In the presence of fish, zoospores of *Pfiesteria* spp. are believed to secrete potent exotoxins responsible for the observed lesions and death (8–10,26). We have, however, recently observed *P. shumwayae* zoospores actively attracted to and attaching in large numbers via the peduncle to anesthetized fishes, followed by direct feeding on epidermal cells via myzocytosis. The histopathology of pfiesteriosis is superficially consistent with an acute toxicosis specific to the epithelial tissues of the skin, gills, and other exposed tissues such as the oral mucosa and linings of the LLC and olfactory tissues. As *Pfiesteria* spp. have been described as predators able to feed on sloughed fish tissues (8,10,26), we suggest that the pathology described here may alternatively be explained as the result of direct predation by the dinospores. Although we do not discount the role of a presumptive toxin in *P. shumwayae*-induced pfiesteriosis, the associated histopathology suggests that active feeding of the dinospores alone can account for the damage to the epidermis and other epithelia observed in this study. A third possibility is that a

toxin(s) acts in concert with dinospore feeding to induce the observed pathology and resulting mortalities. We are currently investigating these possibilities.

Skin ulcers in menhaden, and to a lesser extent other wild fishes, continue to be attributed to the activity of *Pfiesteria* spp. This association is based on the observed co-occurrence of menhaden lesions and *Pfiesteria* in certain acute fish kills in estuaries of North Carolina. During 1997 *P. piscicida* was implicated in several small fish kills, widespread menhaden lesion events, and adverse human health effects in Maryland portions of Chesapeake Bay (28). Several rivers were temporarily closed as a result. Subsequently, ulcers in menhaden have been used in conjunction with presumptive counts of *Pfiesteria*-like cells in water samples and toxic fish bioassays as criteria for river closures in Maryland, Virginia, and North Carolina. Our findings indicate however, that the characteristic ulcerous lesions in menhaden are probably not reliable indicators of local, current *Pfiesteria* activity in Chesapeake Bay, even if *Pfiesteria* spp. ultimately are verified to play an early initiating role in their development. This is because *a*) the etiology of these lesions is consistently infectious, with a primary fungal pathogen always present; *b*) the host cellular response to the fungus indicates the chronic nature of the ulcers and their extended age (1+ weeks of age); *c*) the more diffuse and superficial acute effects described here and elsewhere in fishes exposed in the lab to *Pfiesteria* spp. are absent in afflicted wild menhaden; and *d*) menhaden are highly mobile (29,30). This investigation clearly distinguishes the acute, superficial and widespread pathology of laboratory-induced pfiesteriosis in tilapia from the chronic, focal, and infectious nature of UM in the menhaden. Whereas UM is clearly the result of a highly pathogenic fungal infection, the pathology of acute pfiesteriosis is likely the result of attachment and direct feeding by the dinospores of *P. shumwayae* (27). The role of an exotoxin in this process is presently unclear.

REFERENCES AND NOTES

- Hargis WJ Jr. Quantitative effects of marine diseases on fish and shellfish populations. *Trans N Am Wildl Natur Resour Conf* 50:608–640 (1985).
- Dykstra MJ, Noga EJ, Levine JF, Moye DW, Hawkins JH. Characterization of the *Aphanomyces* species involved in ulcerative mycosis (UM) in menhaden. *Mycologia* 78:664–672 (1986).
- Noga EJ, Dykstra MJ. Oomycete fungi associated with ulcerative mycosis in menhaden, *Brevoortia tyrannus* (Latrobe). *J Fish Dis* 9:47–53 (1986).
- Noga EJ, Wright JF, Levine JF, Dykstra MJ, Hawkins JH. Dermatological diseases affecting fishes of the Tar-Pamlico Estuary, North Carolina. *Dis Aquat Org* 10:87–92 (1991).
- Noga EJ, Levine JF, Dykstra MJ, Hawkins JH. Pathology of ulcerative mycosis in Atlantic menhaden. *Dis Aquat Org* 4: 189–197 (1988).
- Dykstra MJ, Levine JF, Noga EJ, Hawkins JH, Gerdes P, Hargis WJ Jr, Grier HJ, Strake TE. Ulcerative mycosis: a serious

- menhaden disease of the southeastern coastal fisheries of the United States. *J Fish Dis* 12:175–178 (1989).
7. Noga EJ. Fungal diseases of marine and estuarine fishes. In: *Pathobiology of Marine and Estuarine Organisms* (Couch J, Fournie JW, eds). Boca Raton, FL: CRC Press, 1993:85–110.
 8. Burkholder JM, Noga EJ, Hobbs CW, Glasgow HB Jr, Smith SA. New “phantom” dinoflagellate is the causative agent of major estuarine fish kills. *Nature* 358:407–410 (1992).
 9. Noga EJ, Khoo L, Stevens JB, Fan Z, Burkholder JM. Novel toxic dinoflagellate causes epidemic disease in estuarine fish. *Mar Pollut Bull* 32(2):219–224 (1996).
 10. Burkholder JM, Glasgow HB Jr. *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates: behavior, impacts, and environmental controls. *Limnol Oceanogr* 42:1052–1075 (1997).
 11. Callinan RB. A comparative review of *Aphanomyces* species associated with epizootic ulcerative syndrome, red spot disease and mycotic granulomatosis. In: *Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome* (Roberts RJ, Campbell B, MacRae IH, eds). Bangkok, Thailand: The Aquatic Animal Health Research Institute, 1994:248–252.
 12. Lilley JH, Callinan RB, Chinabut S, Kanchanakhan S, MacRae IH, Phillips MJ. Epizootic Ulcerative Syndrome (EUS) Technical Handbook. Bangkok, Thailand: The Aquatic Animal Health Research Institute, 1998.
 13. Lilley JH, Roberts RJ. Pathogenicity and culture studies comparing the *Aphanomyces* involved in epizootic ulcerative syndrome (EUS) with other similar fungi. *J Fish Dis* 20:135–144 (1997).
 14. Blazer VS, Vogelbein WK, Densmore C, Zwerner DE, May EB. *Aphanomyces* as a cause of ulcerative skin lesions in menhaden from Chesapeake Bay. *J Aquat Anim Health* 11:340–349 (1999).
 15. Blazer VS, Lilley JH, Schill WB, Densmore CL. *Aphanomyces invadans* as a cause of ulcerative lesions in menhaden along the east coast. Presented at the CDC National Conference on *Pfiesteria*: from Biology to Public Health, 18–20 October 2000, Atlanta, Georgia.
 16. Kiryu Y, Shields JD, Vogelbein WK, Zwerner DE, Kator H, Blazer VS. Induction of skin ulcers in menhaden by injection and water-borne exposure to zoospores of *Aphanomyces* spp. Presented at the CDC National Conference on *Pfiesteria*: from Biology to Public Health, 18–20 October 2000, Atlanta, Georgia.
 17. Glasgow HB, Burkholder JM, Morton SL, Springer J. A second species of ichthyotoxic *Pfiesteria* (Dinomoebales, Pyrrophyta). *Phycologia* 40:234–245 (2001).
 18. Shields JD, Vogelbein WK, Haas LW, Kiryu Y, Miller AT, Squyers CM, Reece KS, Stokes NA, Kator H. Problems with biotoxicity exposures involving *Pfiesteria*. Presented at the CDC National Conference on *Pfiesteria*: from Biology to Public Health, 18–20 October 2000, Atlanta, Georgia.
 19. Lovko VJ, Vogelbein WK, Shields JD, Kator H, Zwerner DE, Kiryu Y. Development of a larval fish assay for testing *Pfiesteria* toxicity. In: *Proceedings of the CDC National Conference on Pfiesteria: from Biology to Public Health*, 18–20 October 2000, Atlanta, Georgia.
 20. Luna LG. *Histopathological Methods and Color Atlas of Special Stains and Tissue Artifacts*. Gaithersburg, MD: American Histolabs, 1992.
 21. Scholin CA, Herzog M, Sogin M, Anderson DM. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II: Sequence analysis of a fragment of the LSU rRNA gene. *J Phycol* 30:999–1011 (1994).
 22. Medlin L, Elwood HJ, Stickel S, Sogin ML. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71:491–499 (1988).
 23. Wada S, Rha SA, Kondoh T, Suda H, Hatai K, Ishii H. Histopathological comparison between ayu and carp artificially infected with *Aphanomyces piscicida*. *Fish Pathol* 31:71–80 (1996).
 24. Cheville N. *Cell Pathology*. 2nd ed. Ames, IA: Iowa State University Press, 1983.
 25. Burkholder JM. Personal communication.
 26. Burkholder JM. The lurking perils of *Pfiesteria*. *Sci Am* 281(2):42–49 (1999).
 27. Kiryu Y, Shields JD, Vogelbein WK, Zwerner DE, Kator H, Blazer VS. *Pfiesteria* or fungus? Induction of skin ulcers in menhaden with zoospores of *Aphanomyces* spp. Presented at the Symposium on Harmful Marine Algae in the U.S., 4–8 December 2000, Woods Hole, Massachusetts.
 28. Grattan LM, Oldach D, Perl TM, Lowitt MH, Matuszak DL, Dickson C, Parrott C, Shoemaker RC, Kauffman CL, Wasserman MP, Hebel JR, et al. Learning and memory difficulties after environmental exposure to waterways containing toxin-producing *Pfiesteria* or *Pfiesteria*-like dinoflagellates. *Lancet* 352(9127):532–539 (1998).
 29. Friedland KD, Arenholtz DW, Guthrie JF. Influence of plankton on the distribution patterns of the filter-feeding *Brevoortia tyrannus* (Pisces: Clupeidae). *Mar Ecol Prog Ser* 54:1–11 (1989).
 30. Friedland KD, Ahrenholz DW, Guthrie JF. Formation and seasonal evolution of Atlantic menhaden juvenile nurseries in coastal estuaries. *Estuaries* 19:105–114 (1996).