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Pathogenicity of the purportedly toxic dinoflagellates Pfiesteria piscicida and Pseudopfiesteria shumwayae and related species

Vincent J. Lovko
College of William and Mary - Virginia Institute of Marine Science

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Pathogenicity of the Purportedly Toxic Dinoflagellates \textit{Pfiesteria piscicida} and \textit{Pseudopfiesteria shumwayae} and Related Species

A Dissertation

Presented to

The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

by

Vincent J. Lovko

2008
APPROVAL SHEET

This dissertation is submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Vincent J. Lovko

Approved, by the Committee, July 2008

Wolfgang K. Vogelbein, Ph.D.
Committee Chairman/Advisor

Leonard W. Haas, Ph.D.

Kimberly S. Reece, Ph.D.

Jeffrey D. Shields, Ph.D.

Allen R. Place, Ph.D.
Center of Marine Biotechnology
University of Maryland
Baltimore, Maryland
DEDICATION

This dissertation, and the many long hours that it represents, is, in part, dedicated to Jennifer Lynn Miselis, without whom this would not likely have come to fruition, or would at least have been a much less pleasurable experience.

This work is also dedicated to my father, Conrad Charles Lovko, who, I believe, would have been immensely interested in my work and immensely proud of what I have been able to accomplish.
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Figure 5, page 121. Micrograph in panel (f) is credited to Patrice Mason.
The heterotrophic dinoflagellates *Pfiesteria piscicida* and *Pseudopfiesteria shumwayae* (here referred to as *Pfiesteria*) have been reported to secrete potent toxins responsible for inducing lesions in Atlantic menhaden, causing deaths of fishes in natural systems and laboratory assays and impacting human health. Many aspects of *Pfiesteria* biology and ecology have been questioned, including its complicated life-cycle, its role in fish kills, fish lesion events and human health effects as well as its ability to produce toxins. Although the involvement of *Pfiesteria* in major estuarine fish kills and lesion events has been disputed, there is a demonstrated ability of these organisms to cause fish pathology and mortality in laboratory bioassays. Little evidence exists however, to support the conclusion that these effects are due to a potent exotoxin. Many other dinoflagellates are morphologically and genetically similarity to *Pfiesteria* and have been referred to as “*Pfiesteria*-like”, although the pathogenic ability of these other species has not been investigated.

In this work we address various aspects of the behavior and biology of *Pfiesteria* and related dinoflagellates in order to determine their ability to impact the health of fishes and to determine if any adverse effects are caused by a toxin or if of some other pathogenic mechanism is involved. Aquarium-format bioassays commonly used to detect and determine the toxin-producing status of *Pfiesteria* are subject to impediments that can make determination of the cause of fish mortality difficult. We developed a sensitive, 96 hr larval fish bioassay for assessing *Pfiesteria* pathogenicity using 2-10 mL volumes and larval cyprinodontid fishes. This assay was a highly effective method to verify and evaluate ichthyocidal activity in these dinoflagellates. Results from the larval assays, together with histopathological analysis, electron microscopy and direct observations of the interactions of *Pfiesteria* and fish, demonstrated the ability of dinoflagellates of the Pfiesteriaceae (including several *Pfiesteria*-like species) to cause pathology and mortality by feeding on the epidermal tissues of live fish. For some species, this process of micropredatory feeding resulted in fish mortality in laboratory bioassays comparable to that reported for purportedly “toxic” strains, but without the involvement of a toxin.

Thus, the assertion that *Pfiesteria* produces a potent, fish-killing toxin is disputed. In laboratory assays with larval fish, *P. shumwayae* was consistently and significantly more ichthyocidal than *P. piscicida*. This differential pathogenicity was observed to be unrelated to chemoattraction of the dinoflagellates to fish tissues. Instead, as determined with comparative morphometric analyses, differences in ichthyocidal activity between these two species were related to their capacity to grow and reproduce after feeding on fish or algal prey. The results of these studies suggest that the perception of toxicity in these dinoflagellates is erroneous and related to different life history strategies and associated differences in growth rate in response to prey type. There is no direct evidence that these heterotrophic dinoflagellates impact fish health in the natural environment.
Pathogenicity of the Purportedly Toxic Dinoflagellates *Pfiesteria piscicida* and *Pseudopfiesteria shumwayae* and Related Species
SECTION I

LITERATURE REVIEW AND RESEARCH OBJECTIVES
INTRODUCTION

Throughout the 1990’s, dinoflagellates of the genus *Pfiesteria* (now *Pfiesteria* and *Pseudopfiesteria*) were considered to be a serious emerging fish and human health problem in some Mid-Atlantic estuaries including the Chesapeake Bay, the Pamlico Sound and their tributaries (Burkholder et al. 1992, 1995, 2001a,b, Burkholder and Glasgow 1995, 1997a, Glasgow et al. 1995, 2001a,b, Marshall et al. 2000). These dinoflagellates had been implicated as the causative agents of massive fish kills and the formation of deep, ulcerative lesions in fishes, primarily Atlantic menhaden (*Brevoortia tyrannus*) (Burkholder et al. 1992, 1995, 2001a,c, Noga et al. 1993, 1996, Burkholder and Glasgow 1997a, Glasgow et al 2001a). They were also implicated in significant human health effects including gastrointestinal, respiratory and skin abnormalities and neurocognitive impacts (Glasgow et al. 1995, Grattan et al. 1998a,b). These adverse effects were purported to be caused by a potent exotoxin, with production of this toxin tied to aspects of the life-history and nutritional ecology of the dinoflagellate (Burkholder and Glasgow 1997b, Burkholder et al. 2001a). The life history of *Pfiesteria* was reported to be highly complex, consisting of more than 20 different stages involving various flagellated, amoeboid and cyst forms, several of which were thought to be toxin-producing stages (Burkholder et al. 2001a). However, many aspects of *Pfiesteria* toxicity and biology, including the complicated life cycle, the production of a potent toxin and the effects on human and animal health, were poorly documented and, as *Pfiesteria* research intensified, these issues became disputed and highly controversial.
The genus *Pfiesteria* (Steidinger et al. 1996a) has represented a small group of heterotrophic dinoflagellates first discovered in the 1980’s, and collectively referred to as “ambush predators” (Samet et al. 2001). The term “*Pfiesteria*” is often used to include *Pfiesteria piscicida* (Steidinger et al. 1996a) and *Pseudopfiesteria shumwayae*, initially described as *Pfiesteria shumwayae* (Glasgow et al. 2001a), but subsequently reclassified into a new genus based upon morphologic and genetic differences between the two species (Litaker et al. 2005). It is recognized that *Pfiesteria* phylogeny and taxonomy are under continued investigation and debate and that not all investigators agree on their taxonomic position (Litaker et al. 2005, Marshall et al. 2006). Throughout this work, the term *Pfiesteria* will frequently be used to collectively refer to *Pfiesteria piscicida* and *Pseudopfiesteria shumwayae*. However, these species are distinguished from other closely related dinoflagellates that share many morphological, genetic and behavioral characteristics with *Pfiesteria* and are commonly referred to in the literature as “*Pfiesteria*-like” dinoflagellates (PLDs), because, to date, only the two aforementioned species have been purported to produce potent exotoxins implicated in major fish and human health impacts.

**BIOLOGY OF PFIESTERIA**

*Description*

*Pfiesteria* species are small (~10 – 20 µm) heterotrophic, peridinoid dinoflagellates belonging to the family Pfiesteriaceae (class Dinophyceae, division Dinoflagellata, order Peridiniales) (Steidinger et al. 1996a, 2001, Litaker et al. 2005).
Their primary nutritional strategy is heterotrophic feeding using a peduncle (Steidinger et al. 2001). This tube-like structure emerges from a longitudinal groove called the sulcus and is used to attach to and withdraw the contents of prey cells by a process called myzocytosis (Steidinger et al. 2001, Leander and Keeling 2003). However, they have been reported to supplement their nutritional requirements using chloroplasts captured from algal prey and sequestered in food vacuoles, a process called kleptochloroplasty (Burkholder and Glasgow 1995, 1997a, Steidinger et al. 1996a, Lewitus et al. 1999). Like other dinoflagellates, *Pfiesteria* spp. use two flagella for locomotion; a transverse flagellum encircling the cell within an equatorial groove called the cingulum, and a longitudinal flagellum oriented perpendicular to the transverse flagellum and arising from a longitudinal groove called the sulcus (see Taylor 1987 for a review of dinoflagellate morphology). A typical dinokaryotic nucleus, containing permanently condensed chromosomes, is located in the hypotheca (Steidinger et al., 1996a).

*Pfiesteria*-like Species

The family Pfiesteriaceae includes several other closely related species, commonly referred to as “*Pfiesteria*-like”, that share biological and morphological characteristics with *Pfiesteria* spp. and resemble *Pfiesteria* under light microscopy (Burkholder and Glasgow 1997a, Burkholder et al. 2001a,b, Litaker et al. 1999, Marshall et al. 2000, Steidinger et al. 2001). Currently, these additional species comprise 2 genera and include *Cryptoperidiniopsis brodyi*, *Luciella masanensis* and *Luciella atlantis* (Steidinger et al. 2006, Mason et al. 2007). Another species, *Stoeckeria algicida*, is not currently placed in the Pfiesteriaceae although it is closely related to and resembles the
other *Pfiesteria*-like dinoflagellates (Jeong et al. 2005). Although none of these dinoflagellates have previously been demonstrated to kill fish, members of *Luciella* and *Cryptoperidiniopsis* have been considered to be potentially ichthyotoxic (Kane et al. 1998, Steidinger et al. 2001) and *Cryptoperidiniopsis* spp. are thought to contain bioactive substances (Litaker et al. 1999).

*Identification*

Identification and taxonomic placement of dinoflagellates has been based primarily on morphological criteria, although molecular identification methods have more recently been developed to supplement the morphological observations (see Rublee et al. 2005 for review of molecular detection of *Pfiesteria*). Lightly armored dinoflagellates such as *Pfiesteria* spp. and related species contain thin cellulose plates, or theca, within vesicles called amphiesma located immediately under the plasmalemma (Taylor, 1997). Dinoflagellate species are assigned to genus based on the number, shape and arrangement of these thecal plates (Kofoid, 1909). Visualization of thecal plates by light microscopy is difficult because they are very thin and may be obscured by the membranes of the amphiesma and plasmalemma (Taylor 1997, Steidinger et al. 1996b). For these lightly armored species, scanning electron microscopy (SEM) is the preferred method of visualizing thecal plate arrangement (Steidinger et al. 1996b). Prior to imaging, the plasmalemma and amphiesmal membranes must be removed by chemical or mechanical disruption, or the cells must be osmotically swollen so that the sutures between the plates can be visualized through these membranes (Steidinger et al. 1996b, Mason et al. 2003). The plate tabulation for the genus *Pfiesteria* includes: an apical pore
complex (APC) comprised of a pore plate (Po), a closing plate (cp), and a canal plate (X); an apical series comprised of four apical plates (4'); one three-sided anterior intercalary plate (1a); five precingular plates (5'''); six cingular plates (6c); five or more sulcal plates (5+s); and an antapical series with five postcingular plates (5'') and two antapical plates (2'') (Steidinger et al. 1996a). The plate tabulation for the genus *Pseudopfiesteria* differs in having 6 precingular plates instead of 5, and a 4-sided, or diamond-shaped, anterior intercalary plate (Burkholder et al. 2001a,b, Glasgow et al. 2001a, Litaker et al. 2005, AlgaeBase, http://www.algaebase.org/).

*Abundance and Distribution*

*Pfiesteria piscicida, P. shumwayae* and the PLDs are members of the plankton community in shallow, eutrophic estuaries along the mid-Atlantic and southeastern US coasts. They have been detected in samples collected from New York, Delaware Bay, Chesapeake Bay, the Albemarle-Pamlico estuary in North Carolina, South Carolina, the Atlantic and Gulf coasts of Florida and Mobile Bay, Alabama (Burkholder et al. 1995, Lin et al. 2006). The development of species-specific molecular assays for detection of *Pfiesteria* has broadened the distribution to locations around the world (Bowers et al. 2000, Rublee et al., 2005, Rhodes et al. 2006, Lin et al. 2006). Using light microscopy, abundance of *Pfiesteria* spp. was reported to be high in North Carolina estuaries during fish kill events, averaging ~1000-5000 cells ml\(^{-1}\) (Burkholder and Glasgow 1997a). Lower abundances (<20 cells·ml\(^{-1}\)) of *Pfiesteria*-like dinoflagellates have been reported in tidal creeks in Virginia and South Carolina, using light microscopy (Marshall et al. 1999, Lewitus et al. 2002). Quantitative molecular assays have demonstrated measured
*Pfiesteria* concentrations to typically be less than 1-2 cells·ml\(^{-1}\) and seldom exceeding 5 cells·ml\(^{-1}\) in estuaries worldwide (Coyne et al. 2001, Litaker et al. 2003, Reece et al. 2002, 2005, Lin et al. 2006). These concentrations are several orders of magnitude lower than those reported to harm fish (Burkholder and Glasgow 1997a, Burkholder et al. 2001a, Noga et al. 1996).

Although euryhaline, *Pfiesteria* and related dinoflagellates are most commonly found at salinities between 5 and 20 psu, with optimal growth at ~15 psu (Burkholder et al. 1995a, Lin et al. 2006). They tolerate a broad temperature range of 6-31°C, but demonstrate greatest abundance and activity at warmer temperatures (15-30°C) (Burkholder et al. 1995a, Lin et al. 2006) and in nutrient rich waters (Burkholder et al. 1992, 2001a). It has been reported that *Pfiesteria* spp. do not have a light intensity optimum for growth (Burkholder et al. 1995a, Eriksen et al. 2002). Growth of flagellated stages has been reported to be stimulated by addition of inorganic phosphorous (50-400 µg PO\(_4\) L\(^{-1}\)), but not with nitrogen (Burkholder et al. 1992), although it is unclear how such stimulation would occur in an obligate heterotrophic organism. Although cryptophytes (small flagellated phytoplankton) are the preferred prey of *Pfiesteria* (Burkholder and Glasgow 1997a), they can also survive on a variety of microbial and animal prey (Burkholder et al. 2001a). Several species of protists have been shown to prey on *Pfiesteria*, including ciliates (*Strombidium* sp., *Mesodinium pulex*) (Stoecker et al. 2000), rotifers (*Brachionus plicatilis*) (Stoecker and Gustafson 2002) and calanoid copepods (*Acartia tonsa*) (Mallin et al. 1995), suggesting that *Pfiesteria* is readily consumed by grazers.
BACKGROUND OF EARLY PFIESTERIA RESEARCH

**Discovery**

*Pfiesteria piscicida* was discovered in 1988 at the North Carolina State University, School of Veterinary Medicine, when cultured tilapia (*Oreochromis aureus*) died after exposure to water from the Pamlico River, North Carolina (Burkholder et al. 1992, Noga et al. 1993). Water from the affected tanks contained a small dinoflagellate that greatly increased in number prior to fish mortalities, but decreased rapidly when live fish were no longer present (Burkholder et al. 1992). Affected fish displayed shallow, rapid ventilation, dull skin and neurological signs including depression, hyper-excitability and loss of equilibrium (Noga et al. 1993). Histopathological analysis revealed epidermal edema and necrosis, and focal to widespread epithelial erosion of the skin (Noga et al. 1996). Bioassays using juvenile tilapia were conducted in 9-L aquaria inoculated with dinoflagellates (initial densities <100 cells L\(^{-1}\)) from the original fish-killing tank (Noga et al. 1993). Exposed fish demonstrated clinical signs identical to those observed in the original tanks. Fish mortalities of 80-100% were associated with increasing dinoflagellate densities (500 – 10,000 cells ml\(^{-1}\)), suggesting a cause and effect relationship. Sub-lethally exposed fish, transferred to clean water, subsequently developed skin ulcers that became secondarily colonized by bacteria and oomycete water molds (Noga et al. 1996). Tilapia exposed to water that had been filtered (0.22 µm) to remove the dinoflagellates, displayed similar clinical signs and experienced mortalities reaching 60% after 48 hr (Burkholder et al. 1992, Noga et al. 1993). No mortalities occurred in fish exposed to filtrate from control tanks. Researchers concluded that disease
and mortality in tilapia, as well as adverse effects observed in 33 additional finfish and shellfish species, were caused by a potent toxin secreted by the dinoflagellate (Burkholder et al. 1992, 1995a, Noga et al. 1993, Steidinger et al. 1996a).

Based on results of the above studies and the fact that water used in the original fish-killing tanks was derived from the Pamlico River, North Carolina, it was hypothesized that *Pfiesteria* could have been involved in the numerous fish kills occurring in the Albemarle-Pamlico estuary during the late 1980’s and early 1990’s (Burkholder et al. 1992, 1995a, Noga et al. 1993, Burkholder and Glasgow 1995, 1997a, Glasgow et al. 1995). A massive fish kill event involving more than a million fish, primarily Atlantic menhaden (*Brevoortia tyrannus*), was investigated while in progress in the Pamlico River during May 1991 (Burkholder et al. 1992, Noga et al. 1993). Dinoflagellates resembling the contaminant originally found in the fish-killing aquarium were abundant in the fish kill zone and cultures derived from these samples killed juvenile tilapia in laboratory challenges (Burkholder et al. 1992, 1995a,b). To further define the role of *Pfiesteria* species in estuarine fish kills, a standardized approach based on a series of laboratory fish assays was developed to investigate these events. First, field-collected water samples from an in-progress fish kill were evaluated by light microscopy for the presence of *Pfiesteria* (Burkholder et al. 1992, 1995a). Samples with cell counts \( \geq 300 \text{ cells ml}^{-1} \) of “*Pfiesteria*-like” dinoflagellates were considered candidates for potential toxic *Pfiesteria* activity and were subsequently used in “standard” fish bioassays, in which juvenile tilapia were exposed in 9-L aquaria to the environmental water sample (Burkholder et al. 1992, 1995a). Controls consisted of fish in tanks with artificial seawater, but without dinoflagellates. If fish mortality occurred,
which in many assays took weeks to months, tank water would again be evaluated microscopically for the presence of *Pfiesteria* (Burkholder et al. 1995a). If present, dinoflagellates would be enumerated, clonally isolated and cultured for a second tier of fish bioassays. If mortalities occurred in the second set of bioassays and if the clonal culture was positively identified as *Pfiesteria*, then *Pfiesteria* would be implicated as the causative agent of the originally sampled fish kill (Burkholder et al. 2001a,c, Glasgow et al. 2001b). This fish bioassay approach was claimed to fulfill the Henle-Koch postulates modified for toxic, rather than infectious, organisms and purportedly confirmed involvement of toxic *Pfiesteria* in the fish kill (Burkholder and Glasgow 1997a, Burkholder et al. 1992, 1995, 2001a,c). Using this approach, *Pfiesteria* was implicated in ~50% of the major annual fish kill events (≥1000 fish per event), predominantly involving Atlantic menhaden, in the Neuse and Pamlico estuaries between 1991 and 1993 (Burkholder et al. 1995). In many of these events fish exhibited ulcerative skin lesions often penetrating into the musculature and visceral organs (Burkholder et al. 1995). Thus, *Pfiesteria* was presumed to play a causative role in these fish kill events and in the development of ulcerative lesions in wild fish populations in the Pamlico and Neuse estuaries of North Carolina and was postulated to be involved in other unexplained kills and disease events in other similar nutrient-rich and poorly flushed estuaries in the mid-Atlantic region (Burkholder et al. 1992, 1995, Noga et al. 1996). In fact, the association between *Pfiesteria* and ulcerative lesions in fishes was considered strong enough to use lesioned fish as an indicator of actively toxic *Pfiesteria* in the environment (Noga et al. 1996, Burkholder and Glasgow 1997a, Burkholder et al. 2001a,c, Glasgow et al. 2001a, Magnien 2001).
Additionally, a causative link was made between *Pfiesteria* and human health, due to reports of adverse effects in humans that had contact with *Pfiesteria*, either through laboratory exposure while working with *Pfiesteria* (Glasgow et al. 1995), or by recreational or occupational exposure to waterways with purportedly toxic *Pfiesteria* present (Grattan et al. 1998b). In response to the concern over *Pfiesteria*, the Centers for Disease Control and Prevention (CDC) funded a multi-state surveillance program in North Carolina, Virginia and Maryland to investigate what was termed “possible estuary-associated syndrome” or PEAS, so called because of the uncertainty concerning the involvement of *Pfiesteria* (CDC 1997, Moe et al. 2001). Subsequently, multiple cohort studies were undertaken in these states in an attempt to assess the effects on individuals occupationally or recreationally exposed to waterways in which toxic *Pfiesteria* had been reported (Grattan et al. 1998a,b, Turf et al. 1999, Moe et al. 2001, Swinker et al. 2001a,b).

**Life History**

Toxic *Pfiesteria* was reported to have a complex, multiphasic life-cycle involving at least 24 different life stages (size range = 5 - >400 µm) encompassing three basic morphological forms, including flagellated, amoeboid and cyst stages, occupying various niches in the water column and sediments (Burkholder and Glasgow 1997a,b). The proposed life cycle included transformations between lobose, rhizopodial and filose amoebae, free swimming toxic and non-toxic zoospores (bi-flagellated cells), planozygotes, and gametes, and distinct cysts types, including smooth-walled and ornamented forms with a covering of scales with spiny bracts that resemble marine

*Pfiesteria* was described as an “ambush predator” with ephemeral flagellated forms existing for only a few hours following exposure to live fish (Burkholder et al. 1995a,b, Burkholder and Glasgow 1995, 1997b). According to this proposed life-cycle strategy, large reservoirs of *P. piscicida* exist in the sediment as encysted or amoeboid forms. Unidentified substances produced by fish were believed to stimulate these sediment-dwelling forms to excyst or transform from amoeboid to flagellated stages. Zoospores then exhibit directed chemotaxis toward fish and purportedly secrete a potent neurotoxin that stuns fish and causes the epidermis to slough off, which the dinoflagellates then consume phagotrophically (Burkholder et al. 1992, 1995a,b, Burkholder and Glasgow 1997b).

Reproduction was reported to occur either by simple binary fission of flagellated cells (asexual reproduction) or by concentration of cells into a gelatinous mass with repeated cytoplasmic divisions generating multiple "male” or “female” gametes from each parent cell (Burkholder et al. 1995b, Burkholder and Glasgow 1997b). Fusion of these gametes (sexual reproduction) to form a larger, tri-flagellated planozygote was reported to occur only in the presence of live fish, with the planozygotes eventually undergoing division to produce four bi-flagellated cells (Burkholder and Glasgow 1995).

In the absence of live fish, toxic cells and planozygotes reportedly decrease in abundance as they encyst or revert to amoeboid forms, while some flagellated cells may revert to non-toxic forms (NTZs) and remain in the water column if there is an abundant alternative prey source (Burkholder and Glasgow 1995a,b, 1997a,b). Flagellated cells may also attach to fish carcasses and transform into lobose amoebae that continue to feed...
on the fish remains (Burkholder et al. 1995b, Burkholder and Glasgow 1995, 1997a). Without live fish, gametes that have not fused may form temporary cysts or transform into small lobose amoebae, reported to be present in large numbers in the water column and sediment surface at fish kills (Burkholder et al. 1995b). This complex life cycle provided a presumptive mechanism for the “ambush predator” lifestyle wherein a large reservoir of cells in the sediment and water column can be rapidly mobilized to cause massive fish disease and mortality (Burkholder and Glasgow 1997a).

**Functional types and the “Toxic Pfiesteria Complex”**

Much of the early literature that focused on *P. piscicida*, also refers to a second species that is distinct from, yet very similar to, *P. piscicida* (Burkholder et al. 1995a,b, Burkholder and Glasgow 1997a, Steidinger et al. 1996a). This species, *Pseudopfiesteria shumwayae*, was formally described in 2001 and, although morphologically and genetically distinct, was considered identical to *P. piscicida* in regards to toxicity and life history (Glasgow et al. 2001a). Because of the similarities, these two species were collectively referred to as the “Toxic Pfiesteria Complex” (TPC) and are described as having three distinct “functional types” with respect to toxin production (Burkholder et al. 2001b). Non-inducible strains (“NON-IND” functional type) are incapable of producing toxin under any circumstances and are defined as being “unable to cause distress, disease or death in fish” (Burkholder et al. 2001b,c, Glasgow et al. 2001a,b). Temporarily non-toxic strains (“TOX-B” functional type) lose their toxicity after extended culture in the absence of fish (Burkholder et al. 2001a,b). They can, however, regain toxicity when again cultured with fish (Burkholder et al. 2001b,c, Glasgow et al. 2001b,c, Glasgow et al. 2001a,b).
2001a,b). The longer the period away from fish, the greater the time required, to restore toxicity (Burkholder et al. 2001b,c, Glasgow et al. 2001a,b). After a sufficient duration (weeks to months), “TOX-B” strains were reported to permanently lose their ability to kill fish and become “NON-IND” (Burkholder et al. 2001b,c, Glasgow et al. 2001a,b). Actively toxic strains (“TOX-A” functional type) demonstrate a strong chemotactic attraction to fish and fish tissues, exhibit more rapid cell proliferation and cause rapid fish death (hours to days) in bioassays (Burkholder et al. 1992, 2001a,b, Burkholder and Glasgow, 1997a). “Mildly toxic” strains (capable of causing fish death or pathology, but at rates significantly less that “TOX-A” strains) were considered part of the “TOX-B” functional type (Burkholder et al. 2001a,b, Burkholder and Glasgow, 1997a). Fish exposed to “TOX-A” strains exhibit narcosis, hyper-excitability, ataxia, diffuse skin hemorrhage, scale loss and death (Burkholder et al. 1992, Burkholder and Glasgow 1997a,b, Noga et al. 1996). Filtrate from fish-reared cultures that were actively killing fish was reported to have similar effects on fish, supporting the hypothesis that a toxin was responsible for the observed disease effects (Burkholder and Glasgow 1997a,b).

**Controversial Aspects of Pfiesteria Biology**

The existence of toxic *Pfiesteria* has become controversial and many aspects of *Pfiesteria* biology have been disputed, including the complex life-cycle, the effects of *Pfiesteria* on human health and the involvement of *Pfiesteria* in estuarine fish-kills and cutaneous ulcers of Atlantic menhaden (see Vogelbein et al. 2008 for a thorough review of the controversial aspects of *Pfiesteria*). The causative link between *Pfiesteria* and human health effects has been questioned due to the lack of compelling evidence.
(Griffith 1999, Swinker et al. 2002). Although reports have suggested a link between inadvertent laboratory exposure and mild to severe symptoms in laboratory workers (Glasgow et al. 1995, Schmechel and Koltai 2001), this link is based only on isolated incidents and a definitive cause of the symptoms could not be determined (Morris 2001, Schmechel and Koltai 2001). Further, the studies attempting to evaluate human cohorts occupationally or recreationally at risk for *Pfiesteria*-related illness found no connection between *Pfiesteria* exposure and effects on human health (Turf et al. 1999, Moe et al. 2001, Morris 2001, 2006, Shoemaker 2001, Swinker et al. 2002).

The complex life-cycle proposed for *Pfiesteria*, consisting of more than 20 stages of flagellated cells, cysts and amoebae (Burkholder and Glasgow 1997a) has also been disputed (Litaker et al. 2002, Peglar et al. 2004). Litaker et al. (2002) failed to find any amoeboid stages in clonal cultures of a pathogenic *P. piscicida* grown either on algae or fish. Another study tested amoebae collected from the same mesohaline areas where *Pfiesteria* spp. are found as well as those collected from ichthyocidal bioassay aquaria with *P. piscicida* and *P. shumwayae* (Peglar et al. 2004). All amoebae analyzed by morphological and molecular (small subunit [SSU] rRNA) methods fell into known taxa and were not of dinoflagellate origin (Peglar et al. 2004). Further, studies of both *Pfiesteria* species suggest a much simpler life cycle than was previously reported, dominated by only a few cyst and flagellated stages, similar to that of other heterotrophic dinoflagellates (Litaker et al. 2002, Parrow et al. 2002, Parrow and Burkholder 2003).

As previously mentioned, menhaden kills and ulcerative lesions in dead and dying fish were attributed during the 1990’s to the activity of a *Pfiesteria* toxin (Burkholder and Glasgow 1997a,b, Burkholder et al. 2001a, Magnien 2001). However, subsequent studies
have disputed the involvement of *Pfiesteria* in these events and provided alternative explanations for the occurrences of the fish kills and ulcers involving Atlantic menhaden (Paerl et al. 1998, 1999, Blazer et al. 1999, 2002, Vogelbein et al. 2001). Paerl et al. (1998) attributed the majority of fish kills previously ascribed to *Pfiesteria* to oxygen depletion (hypoxia/anoxia) of bottom waters caused by elevated organic loading resulting from eutrophication-stimulated phytoplankton blooms (Paerl et al. 1998). This work was formally challenged based on the detection of non-hypoxic oxygen levels at “in progress” fish kills and the notion that low oxygen typically occurs in bottom water and menhaden, which are surface-schooling fish, are unlikely to be affected by bottom water hypoxia/anoxia (Burkholder et al. 1999). However, in their response, Paerl et al. (1999) described a process known as seiching, which provides a wind-driven mechanism whereby oxygen-depleted bottom water can be pushed to the surface in an upwelling event. A strong event could engulf fish before they have an opportunity to escape. By the time a fish kill is investigated, local conditions may no longer reflect conditions that preceded the fish kill. Seiching has been demonstrated to occur in the Neuse River (Luettich et al. 1999, Paerl et al. 1999).

Prior to intense research efforts on the effects of *Pfiesteria*, cutaneous ulcers observed seasonally in estuarine fishes of the Mid-Atlantic (especially Atlantic menhaden) had been attributed to pathogenic oomycetes and were referred to as ulcerative mycosis (UM) (Noga and Dykstra 1986, Dykstra et al. 1986, 1989, Noga et al. 1988, 1993, Levine et al. 1990). Because of the perceived association between *Pfiesteria* and fish kills and the occurrence of deeply penetrating ulcers on many of the fish found in kills, other researchers attributed these lesions to *Pfiesteria* (Burkholder et al. 1992,
1995a, Noga et al. 1996, Burkholder and Glasgow 1997a). However, the pathology of lesions caused experimentally in *P. shumwayae*-exposed tilapia and those caused by ulcerative mycosis in wild menhaden differed significantly (Blazer et al. 1999, 2000, Vogelbein et al. 2001). *Pfiesteria*-exposed tilapia demonstrated a subtle yet widespread effect including loss of the mucus layer and severe erosion of the epidermis (Vogelbein et al. 2001). This pathology was identical to what was reported by Noga et al. (1996) as an effect of *Pfiesteria* toxin on laboratory exposed striped bass and juvenile tilapia. This differed markedly from the deeply penetrating, usually single, ulcerous lesions in wild menhaden in which hyphae of the oomycete *Aphanomyces invadans* and granulomatous inflammation were consistently found, sometimes penetrating the body musculature, peritoneal cavity or visceral organs of affected fish (Blazer et al. 1999, Law 2001, Vogelbein et al. 2001). Subsequent research demonstrated *A. invadans* as the primary pathogen involved in the formation of these ulcers (Lilley et al. 1998, Blazer et al. 2002, Kiryu et al. 2002, 2003) and clearly demonstrated that certain strains of *A. invadans* can act as the sole etiological agent of the disease (Lilley et al. 1998, Kiryu et al. 2002, 2003). Numerous studies, however, continued to demonstrate the ability of *Pfiesteria* to cause fish mortalities in laboratory assays (Burkholder et al 2001a, c, Glasgow et al. 2001a, Vogelbein et al. 2001, 2002, Berry et al. 2002, Gordon et al. 2002, Springer et al. 2002, Lovko et al. 2003, Drgon et al. 2005), with some studies providing evidence of diffusible ichthyotoxic substances from *Pfiesteria* cultures (Burkholder and Glasgow 1997a, Burkholder et al. 2005a, Moeller et al. 2001, 2007, Gordon et al. 2002, Springer et al. 2002), and others failing to find such evidence (Berry et al. 2002, Vogelbein et al. 2002, Drgon et al. 2005).
The bioassays typically used to test for *Pfiesteria* toxicity are conducted in aquaria inoculated either with environmental samples or laboratory-grown clonal cultures together with fish, typically juvenile tilapia (Burkholder et al. 1995, 2001c, Lewitus et al. 1995, Burkholder and Glasgow 1997a,b, Marshall et al. 2000, Glasgow et al. 2001a). Fish mortality associated with “lethal densities” (>300 cells/mL) of *Pfiesteria* zoospores (identification determined by light microscopy) indicates a positive bioassay (Burkholder et al. 2001b). A positive result in subsequent bioassays, conducted with cells isolated from the first bioassay, confirms the culture as toxic (Burkholder et al. 1995a, 2001a,c, Burkholder and Glasgow 1997b, Marshall et al. 2000, Glasgow et al. 2001a, *Pfiesteria* Interagency Coordinative Working Group). This bioassay procedure has been described as necessary for establishing and maintaining toxic *Pfiesteria* cultures (Burkholder and Glasgow 1997a, Burkholder et al. 1992, 1995a, 2001b, Glasgow et al. 1995, 2001b). However, microbial contaminants in these systems are common, including amoebae, bacteria, chrysophytes, ciliates, diatoms, rotifers and other protists (Burkholder et al. 1995a, 2001a,c Burkholder and Glasgow 1997a,b, Vogelbein et al. 2001, Peglar et al. 2004, Drgon et al. 2005). Drgon et al. (2005) replicating the standard aquarium format bioassay, found potentially pathogenic bacteria, including *Vibrio* sp. and *Aeromonas* sp., among the assemblage of microorganisms that developed over time. The capability of bacteria commonly associated with harmful algal species to produce toxins, or contribute to their production, has been examined (see Doucette 1995 for review). Thus, assignment of causality of mortality and identification of the mechanism of pathogenicity in these types of bioassay systems is problematic.
OBJECTIVES

The overall objectives of this work were to investigate aspects of *Pfiesteria* biology and behavior as related to toxicity/toxin production in order to more definitively address the question of whether *Pfiesteria* spp. and related heterotrophic dinoflagellates are pathogenic to fishes, and if so, to determine the underlying mechanisms of pathogenicity as well as identify the factors that influence the pathogenic capability of these organisms.

Specifically, the following objectives are addressed:

Objective 1: The insufficiencies in the large-format laboratory fish bioassays (9-38 L) that have been used to elucidate much of what is currently known about *Pfiesteria* bring many aspects of *Pfiesteria* biology and pathogenicity into question. The first objective of this work was to develop a smaller-format standardized fish bioassay for evaluating *Pfiesteria* pathogenicity that minimizes human health concerns, minimizes water quality and microbial contamination issues encountered in the larger standard assays, has high statistical power (e.g. large sample size, high number of replicates, suitable controls, and reproducibility) and readily allows for visual and experimental examination of the assay system in order to evaluate possible interactions between *Pfiesteria* and fish. In addition, the relative contributions to fish pathology and mortality of the bacterial, dinoflagellate, and soluble fractions of a fish-killing *Pfiesteria*-culture were determined using this small-scale assay.
Objective 2: Although concerns about the large-format bioassays call into question much of what had previously been concluded about *Pfiesteria* biology, significant evidence exists that demonstrates these organisms as pathogenic, at least in laboratory assays. However, based solely upon fish death in the tank-format bioassays, there is an assumption that *Pfiesteria* pathogenicity is due to secretion of potent exotoxins. Further, it is assumed that *Pfiesteria piscicida* and *Pseudopfiesteria shumwayae* are identical in their pathogenicity/toxicity towards fish. The second major objective of this study was to evaluate cultures of *P. piscicida* and *P. shumwayae* in detail using the newly developed larval fish bioassay, along with histopathological and scanning/transmission electron microscopy analysis of exposed fish to determine the mechanism of pathogenesis involved, if any, and to determine variability in pathogenicity between *P. piscicida* and *P. shumwayae*.

Objective 3: The use of “presumptive cell counts” in early *Pfiesteria* research, the occurrence of several other dinoflagellate species closely related to *Pfiesteria* and the persistent difficulties in distinguishing dinoflagellate species at the level of light microscopy renders the implication of *Pfiesteria* as a causative agent of fish kills as problematic. Further, the speculation of “potential toxicity” in several of these species indicates a critical need to determine the pathogenic capability of these related heterotrophic dinoflagellates. The third objective of this study was to evaluate multiple cultures of several species of *Pfiesteria*-like dinoflagellates to definitively determine if any of these other species exhibit evidence of pathogenicity to fish, and to likewise
determine the mechanism of pathogenicity using the methods developed for the previous objectives.

Objective 4: As detailed above, pathogenicity (purported as toxicity) in *Pfiesteria* has been reported to vary based on several biological and environmental factors. The presence of fish is purported to induce pathogenicity and toxin production in strains considered toxic, while pathogenicity of non-toxic strains is not similarly “activated” by the presence of fish. Lack of access to fish supposedly inhibits toxin production and the ability to cause fish pathology/mortality. Toxic strains are also reported to show a stronger chemotactic response to fish and greater cell proliferation in the presence of fish than non-toxic strains. Additionally, toxic strains are reported to undergo numerous life-cycle transformations, including various flagellated, amoeboid and cyst stages, while benign strains do not exhibit this complex life history. These life-history transformations are also believed to be triggered by the presence or absence of fish. However, toxicity in *Pfiesteria* has not been clearly demonstrated and many of these determining factors of pathogenicity are simply inferred from mortalities and other gross observations in large-format fish bioassays and have not been clearly described or defined. Therefore, the fourth and final objective of this study was to identify, describe and quantify the major underlying determining factors that can account for any observed differences in fish pathogenicity between *Pfiesteria piscicida* and *Pseudopfiesteria shumwayae*. 
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SECTION II

A NEW LARVAL FISH BIOASSAY FOR TESTING THE PATHOGENICITY OF

Pfiesteria spp. (Dinophyceae)†

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ABSTRACT

Water quality, microbial contamination, prior fish health, and variable results have been major impediments to identifying the cause and mechanism of fish mortality in standard aquarium-format Pfiesteria bioassays. Therefore, we developed a sensitive, 96 hr larval fish bioassay for assessing Pfiesteria spp. pathogenicity using 6-well tissue culture plates and 7-d old larval cyprinodontid fish. We used the assay to test pathogenicity of several clonal lines of Pfiesteria piscicida Steidinger and Burkholder and Pseudopfiesteria shumwayae Glasgow and Burkholder that had been cultured with algal prey for 2 to 36 months. The P. shumwayae cultures exhibited 80-100% cumulative mortality in <96 hr at initial zoospore densities of ~1000 cells·mL\(^{-1}\). No fish mortalities occurred with P. piscicida at identical densities or in controls. In a dose-response assay, we demonstrated a strong, positive correlation between dinospore density and fish mortality in a highly pathogenic culture of P. shumwayae, generating a 96-h LD\(_{50}\) of 108 zoospores·mL\(^{-1}\).

Additionally, we applied the assay to evaluate a 38 L P. shumwayae bioassay that was actively killing fish and compared results with those from exposures of juvenile tilapia (Oreochromis niloticus) in a 500 mL assay system. Water from the fish-killing 38 L assay was filtered and centrifuged to produce fractions dominated by dinoflagellates, bacteria or presumed ichthyotoxin (cell-free fraction). After 96 hr, the larval fish assay exhibited 50-100% cumulative mortality only in fractions containing dinoflagellates, with no mortalities occurring in the other fractions. The 500 mL bioassay with tilapia produced inconsistent results and demonstrated no clear correlation between mortality
and treatment. The new larval fish bioassay was demonstrated as a highly effective
method to verify and evaluate dinoflagellate pathogenicity.

INTRODUCTION

*Pfiesteria piscicida* and *Pseudopfiesteria shumwayae* (together here referred to as
“*Pfiesteria*”), members of the “toxic *Pfiesteria* complex” (TPC), are considered
responsible for acute fish kills, fish lesion events, and human disease in estuaries of the
mid-Atlantic United States (Burkholder et al. 1992, Burkholder and Glasgow 1995,
1997b, Glasgow et al. 1995, 2001b, Noga et al. 1996, Burkholder et al. 2001a,b). These
heterotrophic dinoflagellates are reported to have a complex life history with multiple
cyst, zoospore, and amoeboid stages and presumably have the ability to produce potent
ichthyotoxins in response to fish or fresh fish excreta (Burkholder and Glasgow 1995,

The toxigenicity of *Pfiesteria* spp. is reported to depend on life history stage,
functional type (Tox A, Tox B, Non-Inducible as described by Burkholder et al. 2001b),
prey availability, and abiotic factors such as salinity, light, temperature and nutrients
2000). Currently, no cell-specific diagnostic tests exist that can definitively determine the
presence of toxin-producing *Pfiesteria*. Fish bioassays, therefore, are the only method
currently available to detect toxic *Pfiesteria* strains (Burkholder et al. 2001a,c). Bioassays
used to culture and test potentially toxic strains of *Pfiesteria* spp. are typically conducted
in aquaria or other vessels with volumes ranging from 2-40 L, although volumes as low
as 300 mL have been used (Lewitus et al. 1995, Burkholder et al. 1995, 2001c). The vessels are inoculated with either environmental samples (water or sediment) or clonal dinoflagellate cultures, together with fish, most commonly juvenile tilapia, *Oreochromis* spp. (Burkholder et al. 1995, Burkholder and Glasgow 1997a,b, Marshall et al. 2000, Glasgow et al. 2001, Vogelbein et al. 2001).

In bioassays inoculated with environmental samples, mortalities typically begin in 4 to 12 weeks for "non-active" cells, and 4-9 days for "active" cells collected at in-progress fish kills (Burkholder et al. 2001a,c, Glasgow et al. 2001a). Fish death, along with confirmed presence of *Pfiesteria* zoospores at "lethal densities" (>300 cells/ml), constitutes a positive bioassay (Burkholder et al. 2001a,c, Glasgow et al. 2001a, *Pfiesteria* Interagency Coordinative Working Group). Subsequent bioassays, inoculated with cells isolated from the first set of positive bioassays, are conducted to verify "toxicity" (Burkholder et al. 1995, 2001a,c, Burkholder and Glasgow 1997b, Marshall et al. 2000). A positive result in the second set of bioassays confirms the culture as "toxic" (Burkholder et al. 2001a,c, Glasgow et al. 2001a, Marshall et al. 2000, *Pfiesteria* Interagency Coordinative Working Group). These bioassays are indirect and only infer toxicity based upon fish mortality in conjunction with the presence of "relevant" concentrations of *Pfiesteria* zoospores.

Contamination in the aquarium-format bioassays by microorganisms introduced with water, sediment, and fish is common (Burkholder et al. 1995, 2001a, Burkholder and Glasgow 1997a,b, Vogelbein et al. 2001). Due to the extended time it can take for fish death to occur, especially in bioassays with environmental samples, the possibility for degradation of water quality and overgrowth of microorganisms other than the ones being
tested is great. Our 38 L aquarium bioassays, using juvenile tilapia (*Oreochromis niloticus*, 2.5 – 5 cm length) incubated with environmental samples or clonal dinoflagellate cultures, experience degradation of water quality and develop complex microbial communities comprised of micro-flagellates, ciliates, amoebae, and rotifers along with severe bacterial growth (Vogelbein et al. 2001). This obfuscates the determination of the cause of fish mortality and may also complicate the identification of *Pfiesteria* life-history stages. Therefore, a need exists for a rapid, highly sensitive and reproducible bioassay that can clearly identify the cause of fish mortality, allow for investigation of the mechanism of dinoflagellate pathogenicity, and provide a reliable and quantitative method for the observation of *Pfiesteria* biology and behavior.

The objective of this study was to develop a standardized 96 hr larval fish bioassay for evaluating *Pfiesteria* pathogenicity that has high statistical power (e.g. large sample size, high number of replicates, suitable controls, and reproducibility), minimizes the water quality and microbial contamination issues encountered in the larger standard assays, allows for examination of the mechanism of pathogenicity and minimizes human health concerns. Here, we describe a larval fish bioassay approach and its application in determining pathogenicity of several different isolates of *P. piscicida* and *P. shumwayae*. Additionally, we establish a dose-response for a highly pathogenic clonal culture of *P. shumwayae*, and evaluate the contributions of various components of an actively killing large-scale (38 L) culture of *P. shumwayae* to observed fish mortalities.
MATERIALS AND METHODS

Larval fish bioassay design

Bioassays were conducted in covered Falcon® (Becton-Dickson & Co., Franklin Lakes, NJ) 6-well plastic tissue culture plates. A preliminary assay, conducted to determine carrying capacity of individual wells, involved holding 5 to 35 7-d old Fundulus heteroclitus (length 5-6mm) in wells containing 15 mL of 12‰ artificial sea-water (ASW), and monitoring for fish mortality and water quality at 24 h intervals for 96 hr. Water quality (DO, ammonia, nitrate) was poor and mortalities exceeded 10% in wells containing 10 or more fish (data not shown). Water quality in wells with 5 fish remained optimal with no mortalities observed during the 96 hr assay period. Based on these findings, a density of 5 fish per well was used in all subsequent larval fish assays.

All larval bioassays were conducted at room temperature (22.0-25.0°C) in a BSL2 biohazard safety cabinet (see Fig. 1a for photograph of set-up). Personnel conducting the assays wore appropriate personal protection equipment. Clonal cultures of P. shumwayae or fractions derived from aquarium bioassays were placed in 6 well tissue culture plates using disposable graduated pipettes. Each treatment consisted of 12 to 24 wells with 15 mL exposure media (clonal cultures, tank fractions, or clean 12ppt ASW) and 5 fish (7-d old Cyprinodon variegatus, length 4-5mm, or Fundulus heteroclitus, length 5-6mm) per well. Fish were transferred to wells using disposable plastic transfer pipettes (Fisher) with the tips cut to prevent tissue damage. Each assay had an additional treatment consisting of fish in clean, 0.22 µm-filtered and autoclaved York River water or 0.22 µm-filtered
Figure 1. Illustrations of the larval-fish bioassay: (a) photograph of an in progress assay with 6 treatments containing 4 plates (24 replicate wells) per treatment (arranged in rows from front to back), (b) schematic of the larval assay protocol demonstrating the arrangement of a treatment (tank fraction, clonal culture or control) comprised of 24 replicate wells, 15 of which will be used for mortality assessment and 9 for periodic (24 hr) sampling for water quality, zoospore density and histology.
Figure 1. Larval fish bioassay experimental design

a

3 wells sacrificed @ 24, 48, and 72 hours for water quality, zoospore counts and histology.

15 wells used for mortality assessment. 96 hours sampling taken from these wells.

b

Five 7-d old larval fish, *Cyprinodon variegatus* or *Fundulus heteroclitus*

Clonal *Pfiesteria* cultures, fractions derived from 38-L aquarium assay, or clean ASW (control)

3 wells sacrificed @ 24, 48, and 72 hours for water quality, zoospore counts and histology.
ASW that served as a control. Culture plates were observed under an inverted microscope periodically throughout the assay. Every 24 h, 1 to 3 replicate wells (chosen from a random number table) from each treatment were sacrificed and sampled for water quality (DO, NH₄, pH, NO₂), dinoflagellate density and histology (fish). The remaining wells were monitored for fish mortalities. Dead or moribund fish were removed when observed and cumulative mortality was tabulated at 24 hr intervals. Figure 1b provides a detailed schematic of the assay protocol.

Clonal dinoflagellate cultures

Clonal cultures of *P. shumwayae* and *P. piscicida* were maintained at VIMS. A clonal culture of *P. shumwayae* (deposited at the Provasoli-Guillard National Center for Culture of Marine Phytoplankton as strain CCMP-2089), was isolated from a fish-killing 38 L tilapia bioassay inoculated with a field sample collected from the Pamlico River, NC. This culture had been maintained on algae, *Rhodomonas* sp. (CCMP-1319), for approximately 10 months prior to the dose-response study and 18 months prior to the *Pfiesteria* spp. comparison assay. A clonal culture of *P. piscicida* (VIMS-P11) was isolated from a contaminated culture of *Rhodomonas* sp. (CCMP-757) and was subsequently maintained on algae (CCMP-1319) for approximately 3 years prior to the assays. This culture had failed to kill fish in our 38 L tank-format bioassays and was therefore included in certain larval fish bioassay studies as a negative control.

In addition, two cultures were provided by Dr. Patricia Tester (NOAA, Beaufort, NC), a clonal *P. shumwayae* culture (PS-T1) maintained at VIMS on algae for approximately 2 months prior to the assays, and a clonal *P. piscicida* culture (deposited at
the Provasoli-Guillard National Center for Culture of Marine Phytoplankton as strain CCMP-2091), re-isolated at VIMS and maintained on algal prey for approximately 2 years. All cultures were grown in 0.22 µm filtered and autoclaved York River water with penicillin and streptomycin added to inhibit bacterial growth.

Cultures of *P. piscicida* and *P. shumwayae* were identified using a Leo Model 435VP scanning electron microscope to tabulate plate counts on membrane-stripped or suture-swollen cells (see Mason et al. 2003 for details of SEM methodology). Identifications were cross-confirmed using molecular primers developed to specifically amplify *P. shumwayae* or *P. piscicida* rRNA (see online supplementary material from Vogelbein et al. 2002 for details of primer development).

38-L dinoflagellate cultures

Water for fractionation studies was obtained from 38 L bioassays that were actively killing fish (20-50% daily tilapia mortality) and verified to contain high densities (>10,000 cells·mL$^{-1}$) of *P. shumwayae* zoospores (see Vogelbein et al. 2001 for description of 38 L assay methodology). Briefly, this assay involved placing 25 - 40 tilapia (3.0 – 5.0 cm total length), along with water or sediment samples from environmental collections, in 38 L aquaria held at 20° C, with aeration and filtration (Whisper® filter with preconditioned biological filter using crushed coral in a nylon bag). Controls consisted of fish in an identical system containing 12 % ASW without dinoflagellates, sediments, or environmental samples. Fish were monitored daily for signs of lesions, morbidity and mortality. New fish were added daily to replace dead fish, keeping fish densities consistent throughout the duration of the exposures. All tank assays
were conducted in a BSL3 biohazard containment facility at VIMS under strict safety and animal handling protocols (IBC 9906, RASC 9825).

Fish sources

Adult mummichogs, *Fundulus heteroclitus*, were spawned manually as described previously (Armstrong and Child 1965). Fertilized eggs were incubated on moist filter paper at 17-25°C for synchronous hatch in 7-10 days. Hatchlings were held in mesh baskets in a flow-through system receiving 1 µm-filtered York River water (salinity ~ 20 ‰), and fed 24 hr-old *Artemia salina* nauplii once daily *ad libitum* for 6 days. Larvae were held at 12 ‰ for at least 24 hr prior to the start of assays. To minimize waste accumulation in wells during exposure, larvae were fasted for 24 hr prior to initiation and throughout the duration of the bioassays.

Larval *Cyprinodon variegatus*, were obtained from Chesapeake Cultures (Gloucester, VA). Fish arrived at 6 or 7-day post-hatch in 15-20 ‰ artificial seawater and had been fasted for 24 hr prior to start of assay. Fish were held at 12 ‰ and used in the assays within one day of arrival.

Tilapia, *Oreochromis niloticus*, were acquired from a commercial aquaculture facility (Southern States experimental aquaculture facility, New Kent County, VA), quarantined, and treated prophylactically with two 3 hr formalin baths (150 mg·L⁻¹) separated by a 3 day interval, followed by a 7 d exposure to Cutrine™ (chelated copper sulfate, 5 mg·L⁻¹), then held for a depuration period of one week in 12 ‰ ASW.
**Water quality measurements**

Aliquots of water from randomly selected replicate wells were used to measure pH, dissolved oxygen, reactive ammonia and nitrite (nitrite not measured in all assays). Measurements were made initially and at 24 hr intervals in three wells per treatment (only one well per treatment for the fractionation study). Dissolved oxygen was measured using a YSI Model 57 oxygen meter and pH was measured using an Orion Research Expandable ion Analyzer Model EA920. Ammonia and nitrite were measured using Hach Co. (Loveland, CO) colorimetric test kits. Reactive ammonia was calculated from measured ammonia using the equation of Emerson et al. (1975), which compensates for temperature and pH. Air temperature in the BSL2 hood was recorded from a mercury thermometer at each sampling period.

**Zoospore density**

At 24 hr intervals, one 10 mL aliquot of water from each of 3 wells per treatment (only one well per treatment for fractionation study) was pipetted into a centrifuge tube, fixed with Lugol’s iodine (0.5 – 1.0% final solution), mixed, and either allowed to settle for >24 hr or centrifuged @ 3000 rpm/10 min on an IEC Centra CL3R centrifuge at 24°C. Each sample was decanted to 0.5 mL using a pipette, stirred gently with a vortex-type mixer to re-suspend the cells, counted in a hemacytometer (Neubauer, Bright-line) in triplicate, and cell densities calculated.
**Histology**

At 24 hr intervals, fish from the same wells used for water quality measurements were killed by overdose with tricaine methanesulfonate (MS222) and fixed in 10% neutral buffered formalin for histological evaluation. Specimens were decalcified in sodium citrate-formic acid solution, rinsed, dehydrated, embedded in paraffin, sectioned at 5-6 μm, mounted on glass slides, and stained in Harris’ hematoxylin and eosin, following standard procedures for paraffin histology (Luna 1992).

**Pfiesteria spp. comparison assay**

Larval *C. variegatus* were exposed to 2 clonal isolates of *P. piscicida* (VIMS-P11 and PP-CN), 2 clonal isolates of *P. shumwayae* (CCMP 2089 and PS-T1) and 0.22 μm-filtered ASW as a control. Zoospore densities of each culture were determined as described above. Cultures were diluted to 1000 zoospores·mL⁻¹ with 0.22 μm-filtered and autoclaved York River water. Aliquots from each diluted culture were fixed as above to verify actual initial zoospore densities. Twenty-four replicate wells (5 fish per well, 120 fish total) were used for each treatment.

**Pseudopfiesteria shumwayae dose-response study**

An algal-cultured *P. shumwayae* clone (CCMP 2089), verified as pathogenic to fish in both the 38 L and larval fish bioassays and at an initial density of 1000 zoospores·mL⁻¹, was serially diluted (sterile-filtered and autoclaved York River water) to obtain aliquots with initial zoospore densities of 0 (control), 10, 100, 500 and 1000
cells·mL⁻¹. Larval (7-d old) *C. variegatus* were exposed to each dose in 18 replicate wells (5 fish per well, 90 fish total) per treatment.

**Fractionation study**

Water taken directly from a fish-killing 38 L bioassay that was actively killing tilapia (20 – 50% daily tilapia mortality) was used as a positive control (“Raw” fraction). A fraction enriched in dinoflagellates and other protozoa (“Dinoflagellate” fraction) was produced by filtering 3 L of “Raw” water through a 5-µm filter (MSI Magna-R 47 mm nylon filter, at 5” Hg vacuum) followed by three 50 mL rinses with 12 ‰ filter-sterilized ASW to reduce bacteria. Care was taken to ensure that the filters remained wet throughout the procedure. The filter was then immersed in 3 L of 12 ‰ ASW to re-suspend the dinoflagellates. Light microscopy confirmed the presence of free-swimming dinoflagellates in this fraction. The resuspended culture was counted and adjusted to ~1000 zoospores·mL⁻¹. A “Bacteria” fraction was obtained by centrifuging 3 L of aquarium water at 9000 rpm for 45 min at 10°C, resuspending the pellet in 12 ‰ ASW, filtering through a 5 µm filter to remove dinoflagellates and other protozoa, and diluting the filtrate back to 3 L using 12 ‰ ASW. A “Cell-free” (supernatant) fraction was obtained during the production of the “Bacteria” fraction by removing the supernatant from the centrifuged bacterial pellet and filtering through a 5 µm filter followed by a 0.45 µm filter. Filter-sterilized 12 ‰ ASW was used as a control. An additional control consisting of 12 ‰ ASW with ammonia and pH adjusted to that of the “Raw” aquarium water was used to control for the elevated ammonia levels present in the “Raw” and
“Cell-free” fractions. Larval *F. heteroclitus* were exposed to each fraction and control in a total of 12 replicate wells (5 fish per well, 60 fish total) per treatment.

As a comparison to the larval fractionation study, a concurrent fractionation experiment was conducted using juvenile tilapia (*O. niloticus*, 3-5 cm total length) in a small volume (500 mL) assay format. Three replicates of 4 fish each were held in 3.8 L mason jars containing 500 mL of tank fractions (“Raw”, “Dinoflagellate”, “Bacterial” or “Cell-free”) or 0.22 µm-filtered ASW (control). Jars were aerated and held in a BSL2 biosafety cabinet within a BSL3 facility. Jars were examined every 24 hr over 96 hr and dead fish were removed. Cumulative mortality was tabulated at 24 hr intervals. Samples were taken for water quality and cell counts initially and every 24 hr for 96 hr.

**RESULTS**

*Pfiesteria* spp. comparison study

Mortalities occurred only in treatments containing *P. shumwayae*, though there was a difference in the mortality rate between the two isolates (Tarone-Ware Chi-square, p<0.01). Mortalities appeared most rapid between 48 and 72 hr culminating in 100% cumulative mortality in treatments with CCMP2089 and approximately 85% cumulative mortality in treatments with PS-T1 at 96 hr (Fig. 2a). No mortalities occurred in *P. piscicida* treatments or in the controls. Rapid attraction of zoospores to fish was observed only in the treatments containing *P. shumwayae*, with little or no attraction observed in the *P. piscicida* treatments. Initial zoospore densities were approximately 1000 cells·mL⁻¹ in each of the treatments except for PS-T1, which was approximately 1300
Figure 2. Comparison study of *Pfiesteria* spp. in the larval-fish assay: (a) cumulative mortality over 96 hr with standard errors shown (n = 75), (b) dinospore density in each treatment over the 96 hr assay with standard errors shown (n = 3), (c) mean reactive ammonia from each treatment (n = 3), and (d) mean dissolved oxygen from each treatment (n = 3). CCMP-2089 and PS-T1 are *P. shumwayae* cultures; VIMS-P11 and PP-CN are *P. piscicida* cultures.
Figure 2. Results of comparison study of *Pfiesteria* spp in the larval-fish assay
zoospores·mL\(^{-1}\). Zoospore densities fluctuated between approximately 300 and 1000 cells·mL\(^{-1}\) during the assay with the exception of CCMP2089 which reached approximately 3000 cells·mL\(^{-1}\) at 96 h (Fig. 2b). Reactive ammonia was similar in all treatments and did not exceed 0.25 mg·L\(^{-1}\) (Fig. 2c). Dissolved oxygen also was similar in all treatments and did not fall below 5.7 mg·L\(^{-1}\) (Fig. 2d).

**P. shumwayae dose-response study**

Mortalities were greater and occurred more rapidly with increasing cell density (Fig. 3a). No mortalities occurred in the control (0 cells·mL\(^{-1}\)) or 10 cells·mL\(^{-1}\) treatments during the 96-h exposure. Mortality reached approximately 35% by 96 hr at 100 cells·mL\(^{-1}\). Mortality reached 100% at 500 cells·mL\(^{-1}\) by 96 h and at 1000 cells·mL\(^{-1}\) by 72 hours. Survival analysis demonstrated significantly higher survival in controls and 10 cells·mL\(^{-1}\) against all higher doses (T-W \(X^2\): \(p<0.001\), for 100 cells·mL\(^{-1}\) against 500 and 1000 cells·mL\(^{-1}\) (\(p<0.001\)), and for 500 cells·mL\(^{-1}\) against 1000 cells·mL\(^{-1}\) (\(p<0.001\)). The LD\(_{50}\)s based on probit analysis of the nominal cell densities at the beginning of the experiment were 1291 cells·mL\(^{-1}\) at 48 hr (95% CI = 956-3205 cells·mL\(^{-1}\)), 285 cells·mL\(^{-1}\) at 72 hr (95% CI = 226-347 cells·mL\(^{-1}\)), and 108 cells·mL\(^{-1}\) at 96 h (95% CI not estimated).

Zoospore densities decreased during the first 24 hr as zoospores encysted, but then rapidly increased throughout the remainder of the 96 hr exposure as cysts divided and zoospores excysted (Fig. 3b). During the assay, reactive ammonia was near or below 0.10 mg·L\(^{-1}\) (Fig. 3c), DO was near or above 5.0 mg·L\(^{-1}\) and nitrite was undetectable (data not shown) in all treatments.
Figure 3. Dose-response study for *P. shumwayae* in the larval fish assay: (a) cumulative mortality over 96 hr with initial dinospore densities of 0, 10, 100, 500, and 1000 cells·mL$^{-1}$ with standard errors shown (n = 45), (b) change in dinospore density in each treatment over 96 hr (n = 3), (c) mean reactive ammonia from each treatment (n = 3), and (d) mean dissolved oxygen from each treatment (n = 3).
Figure 3. Results of dose-response study for *P. shumwayae* in the larval fish assay
Fractionation study

Mortalities occurred in the “Raw” and “Dinoflagellate” fractions but not in the other treatments (Fig. 4a). Survival analysis demonstrated a significantly higher survival for fish in the “Dinoflagellate” fraction compared to those in the “Raw” fraction (T-W $X^2$, p<0.001). At 96 hours, mortalities reached 100% in the “Raw” fraction and 55% in the “Dinoflagellate” fraction. No fish died in any other treatment or in the controls. Rapid attraction of zoospores to fish was observed in the “Raw” and “Dinoflagellate” fractions. Initial zoospore densities were approximately 12,000 cells·mL$^{-1}$ in the “Raw” fraction and 1000 cells·mL$^{-1}$ in the “Dinoflagellate” fraction. Water quality measurements were similar between different treatments. Mean levels of reactive NH$_3$ in all treatments remained below those measured in the high ammonia control, which reached a maximum level of 0.54 mg·L$^{-1}$ (Fig. 4b). Dissolved oxygen remained above 5.0 mg·L$^{-1}$ in all treatments (Fig. 4c).

In the fractionation assays using tilapia in 3.8 L jars, mortalities occurred in all treatments with no apparent differences between treatments, primarily because of large standard errors (Fig. 5a). Mortalities occurred more rapidly in the “Cell-free” fraction than in the other treatments (50% at 48 h, T-W $X^2$: p<0.003) but data showed large standard errors (+/- 25%). Initial zoospore densities were identical to those in the larval assay. Reactive NH$_3$ varied considerably in all treatments reaching a maximum of 1.8 mg·L$^{-1}$ in the “Cell-free” fraction at 72 h, 1.5 mg·L$^{-1}$ in the “Dinoflagellate” fraction at 96 hr, and 2.8 mg·L$^{-1}$ in the “Bacteria” fraction at 96 hr (Fig. 5b). The other treatments remained below 1.0 mg·L$^{-1}$. Jars were aerated during the assay and therefore dissolved oxygen was not measured.
Figure 4. Fractionation study with the larval fish assay: (a) cumulative mortality over 96 hr with standard errors shown (n = 45), (b) reactive ammonia in each treatment (n = 1), and (c) dissolved oxygen in each treatment (n = 1).
Figure 4. Results of fractionation study with larval fish assay.
Figure 5. Fractionation study with small-scale tilapia assay: (a) percent cumulative mortality over 96 hr with standard errors shown (n = 12), (b) mean reactive ammonia from each treatment (n = 3).
Figure 5. Results of fractionation study with small-scale tilapia assay

(a) Cumulative mortality over time for different treatments:
- "Raw"
- "Dinoflagellate"
- "Cell-free"
- "Bacteria"
- Control

(b) Reactive ammonia concentration over time:
- Time (hours) range from 0 to 96
- Reactive ammonia concentration range from 0.0 to 3.0 mg•L$^{-1}$
Histopathology

The epidermis of larval fish that were not in contact with *P. shumwayae* (including controls, “Bacteria” and “Cell-free” fractions, and treatments with *P. piscicida*) remained intact and normal in appearance for the duration of the assays (Fig. 6a). The epidermis of fish exposed to zoospores of *P. shumwayae* (“Raw” and “Dinoflagellate” fractions and treatments with CCMP-2089 above 10 cells·mL\(^{-1}\)) showed evidence of cell degeneration, necrosis and sloughing of epidermal cells (Fig. 6b). Many fish in these treatments exhibited severe epidermal erosion, often with large areas completely eroded as rapidly as 24 hr into the exposure (Fig. 6c).

DISCUSSION

Any bioassay that purports to characterize the pathogenic potential of a species or strain of *Pfiesteria* must definitively determine both the identity of the pathogen, if more than one potential pathogen exists, and the mechanism of pathogenicity, if more than one potential mechanism exists. The standard aquarium-format bioassay serves an important role in isolation of *Pfiesteria* from environmental samples and may be necessary for establishment and maintenance of toxic strains of *Pfiesteria* and for initial assessment of *Pfiesteria* collected from in-progress fish kills (Burkholder et al. 1992, Burkholder et al. 1995, Glasgow et al. 1995, Burkholder and Glasgow 1997a, Burkholder et al. 2001b, Glasgow et al. 2001b). However, these bioassays can only indirectly infer toxicity based upon fish death and cannot, on their own, reliably identify the cause or the mechanism of fish mortality.
Figure 6. Representative micrographs demonstrating disruption and loss of fish epithelium resulting from *P. shumwayae* exposure in the larval fish assays. (a) Skin of *F. heteroclitus* from a control treatment (48 hr). (b) Skin of a *P. shumwayae* exposed *F. heteroclitus* (72 hr) showing severely disrupted epidermis and sloughing of epithelial cells. (c) Skin of *P. shumwayae*-exposed *F. heteroclitus* (24 hr) showing complete erosion of epidermis.
Figure 6. Histological micrographs of control and *Pfiesteria*-exposed fish epidermis.
Due to microbial contamination and water quality issues, the aquarium-format bioassay systems are not adequate for definitive determination of causality of fish mortality. Microbial contaminants introduced with live tilapia are a consistent feature in any long-term assay we have conducted involving live fish. Bacterial loads as high as $10^7$ cells·mL$^{-1}$ and phytoplankton loads as high as $10^4$ cells·mL$^{-1}$ have been reported for field-collected *Pfiesteria* samples (Burkholder et al. 2001c). Burkholder and Glasgow (1997b) report that tilapia used in their aquarium bioassays are treated for contamination by rinsing with distilled water. This is not adequate quarantine or prophylactic procedure for fish health maintenance and cannot prevent assay contamination. Even with prophylactic formalin and copper treatments of tilapia used in our aquarium assays, fish still bring a variety of microbial contaminants into our 38 L assays including ciliates, rotifers, amoebae, chrysophytes, small flagellates, and bacteria. These contaminants are consistently present in controls as well as the experimental treatments of our assays. Additionally, the community composition in the bioassays changes over time, especially as fish die. The possibility that one or more of these contaminants could contribute to fish mortality must be reconciled.

In our studies, the fractionation procedure in conjunction with our larval fish bioassay has clearly demonstrated that only the fractions containing dinoflagellates were able to kill fish. Bacteria were ruled out as a likely cause of fish mortality. The lack of mortalities in the soluble fraction, in addition to implications concerning a soluble toxin, ruled out dissolved chemical components such as elevated ammonia or nitrate. Further, water quality parameters remained optimal for fish health throughout the duration of all our larval fish bioassays. Reactive ammonia remained well below the LC$_{50}$ level (1.6
mg·L⁻¹) reported by Burton and Fisher (1990) for juvenile mummichog and nitrite was undetectable throughout the duration of our earlier assays and was subsequently dropped from the protocol. The larval fish assay did not require aeration to maintain optimal DO levels, eliminating aerosols and, in conjunction with the small volumes used, reducing potential health risks to laboratory personnel. In comparison, the 500 mL bioassay with juvenile tilapia could not definitively discern the cause of fish mortality and experienced degradation of water quality, even within the short span of the assay, suggesting that this type of assay is ineffective in resolving the causality issue.

Fish mortality in aquarium-format tilapia assays has been assumed to be due to a toxin based upon association with exposure to *Pfiesteria* spp. (Burkholder et al. 1992, Burkholder et al. 1995, Glasgow et al. 1995, Marshall et al. 2000, Glasgow et al., 2001). Fulfillment of the Henle-Koch postulates as modified for toxic rather than infectious organisms (see Glasgow et al. 2001a for description of postulates as applied to *Pfiesteria*) has been purported to be adequate for definitive confirmation of *Pfiesteria* spp. toxicity (Burkholder et al. 2001a,c, Glasgow et al. 2001a). However, this protocol relies solely on positive results in fish bioassays, in association with “lethal” densities of zoospores, as the criteria for toxicity. Using a combination of aquarium-format and larval fish bioassays, we have fulfilled the modified Henle-Koch postulates with *P. shumwayae* strain CCMP-2089. This culture was isolated from an actively killing 38 L tilapia bioassay tank containing an environmental sample collected from the Pamlico River, NC. Pathogenicity of this clonal isolate has been verified repeatedly using the larval assay as well as re-inoculating the culture into our 38 L bioassay system. Confirmation of *P.*
*shumwayae* in the water samples, assay tanks, and in the clonal cultures was made using SEM and molecular probes.

However, fulfilling the modified Henle-Koch postulates in this way does not confirm presence or action of a toxin. To verify toxigenicity, it is necessary to isolate the toxin from a fish-killing culture (i.e. by filtration or by separation of dinoflagellates from fish) and induce mortality with the cell-free aqueous fraction. Other researchers have induced fish mortalities using filtrates derived from fish-killing *Pfiesteria* spp. cultures (Burkholder and Glasgow 1997a, Moeller et al. 2001, Gordon et al. 2002). However, in the larval fish bioassay, we have been unable to induce fish mortality with the cell-free filtrate from a fish-killing *P. shumwayae* assay, while treatments in which dinoflagellates were in physical contact with fish resulted in rapid mortality. Other researchers were also unable to elicit fish mortality from similarly fractionated samples or when fish were separated from *P. shumwayae* zoospores (CCMP-2089) by a semi-permeable membrane (Berry et al. 2002, Vogelbein et al. 2002). To assess the mechanisms by which *Pfiesteria* spp. kill fish and to fulfill the modified postulates, an assay must be able to discriminate between “toxigenicity” and other possible pathogenic mechanisms. Only protocols that routinely involve the use of cell free-filtrates and/or separation by a membrane, can determine the existence of genuinely toxic strains.

Time to fish death and magnitude of fish mortality has also been used as primary criteria for discerning toxic from non-toxic *Pfiesteria* cultures and highly toxic from less toxic cultures (Marshall et al. 2000, Burkholder et al. 2001c, Glasgow et al. 2001a). Such assessments, however, must take into account variations in zoospore density. Replacement of dead fish with live fish over a span of time in bioassays with *Pfiesteria*
spp. results in fish mortality as rapidly as minutes to hours (Marshall et al. 2000).

Although this effect has been attributed to increased toxicity of the *Pfiesteria* culture as a result of having been fed live fish (Burkholder et al. 2001b, Glasgow et al. 2001b), some of these studies also report an increase in zoospore concentrations over this time (Marshall et al. 2000). Our larval assay studies have shown that, when exposed to fish, *Pfiesteria* zoospore densities rapidly increase after a short (~24 hr) period of decline. Burkholder et al. (2001a), using larval fish in a 10mL assay system, demonstrated a difference in time to fish death and magnitude of mortality presumably based upon the toxicity status of the cultures used (“Tox-A”, “Tox-B”, “Non-inducible”). However, the range of zoospore densities among the various strains reported in that study varied by nearly 80%. Our dose-response study demonstrates that time to death and magnitude of mortality are both a function of zoospore density. Zoospore density must be accounted for in comparison studies between *Pfiesteria* strains or species and can be readily standardized using the methods described for the larval fish bioassay.

Additionally, the larval fish bioassay provides the capability to discern important differences among *Pfiesteria* species and strains. Loss of fish-killing activity in actively toxic (Tox-A) *P. piscicida* cultures removed from contact with live fish for periods ranging from 48 hr to 2 months has been reported (Burkholder and Glasgow 1997a,b, Burkholder et al. 2001c). *P. shumwayae* is also believed to require live fish to produce toxin and cause fish mortality (Burkholder and Glasgow 1997, Burkholder et al. 2001a,b,c, Glasgow et al. 2001b) and also exhibits loss of fish-killing activity if cultured without fish for extended periods (6 weeks in Glasgow et al. 2001b). Our *P. piscicida*...
cultures maintained without fish for 2 – 3 years consistently fail to cause fish mortality in both our 38 L and larval fish bioassays. However, repeated applications of the larval fish bioassay using *P. shumwayae* cultures maintained on algae for up to 18 months, consistently result in rapid fish mortality in a dose-dependent fashion, suggesting that the pathogenicity of *P. shumwayae* may differ fundamentally from that of *P. piscicida*.

**CONCLUSION**

Microscale testing of toxicants on a wide range of fishes, invertebrates and microorganisms has become an effective alternative to larger-scale assays (see Blaise et al., 1998 for a review of microscale testing). These assays are more cost-effective, generally take less time to set-up, maintain and monitor, and take up less space, allowing for higher replication. Small-volume toxicity microassays have been shown to yield results comparable to larger volume assays. Toxicity tests with embryos of shellfish have shown no significant difference between EC$_{50}$ values generated for sodium azide in 3 mL or 400 mL assay systems or for zinc toxicity to abalone in 10mL and 200mL assay systems (Hunt et al. 1998). Early life-stages of cyprinodontid fishes have been successfully used for a variety of toxicity and mortality assays (for examples see Able and Palmer 1988, Burton and Fisher 1990, Nacci et al. 1998, Weis and Weis 1998). The mummichog and sheepshead minnow are well studied, having been used in aquatic toxicology studies for more than three decades (Weis and Weis 1998). These fish are amenable to laboratory culture and experimental manipulation and are also readily available from commercial sources. Larval fish and larval shellfish in small-volume
bioassay formats have also had limited use in *Pfiesteria* research (Burkholder et al. 2001a, Springer et al. 2000, Berry et al. 2002, Vogelbein 2002), though the methods have not been fully described or the protocol standardized.

We have developed and described a sensitive, highly reproducible and relatively inexpensive method to accurately test pathogenicity of *Pfiesteria* spp. and *Pfiesteria*-like organisms. Unlike larger aquarium bioassays, the small volumes and tissue-culture plate format allow for microscopic examination of the entire system, facilitating detailed observations of *Pfiesteria* spp. and their interactions with fish. Additionally, high numbers of replicates are possible facilitating rigorous statistical analyses. The ability to generate an LC$_{50}$ allows for differences in pathogenicity between species, strains, and life-history stages to be discerned. Additionally, the small scale and the defined duration of the assay limits the growth of introduced contaminants regularly observed in the large-scale systems. In addition, the use of laboratory-reared larval fish greatly reduces foreign contaminants by allowing for the use of aseptic procedures during fertilization, incubation, and hatching of fish. Fish are fasted for 24 hr prior to assay initiation and not fed for the duration of the assay, thereby minimizing the water quality issues that can severely compromise aquarium-format bioassays.

Furthermore, this larval fish bioassay has the potential for application to a wide range of dinoflagellate and harmful algae research through the use of known algal toxins for comparative studies, standardized methods for generating LC$_{50}$ values for major algal toxins, and further examination of direct interaction of pathogenic dinoflagellates and their hosts.
REFERENCES


SECTION III

MICROPREDATORY FEEDING AS A MECHANISM OF PATHOGENESIS IN

PFIESTERIA PISCICIDA AND PSEUDOPFIESTERIA SHUMWAYAE
Species of *Pfiesteria* are reported to secrete potent toxins responsible for inducing lesions in Atlantic menhaden and causing deaths of fishes in natural systems and laboratory assays. Although the involvement of *Pfiesteria* in major estuarine fish kills and lesion events has been disputed, many researchers have demonstrated the ability of these organisms to cause fish pathology and mortality in laboratory bioassays. However, little corroborative evidence exists to support the conclusion that these effects are due to a toxin and several researchers have provided evidence against toxin production in *Pfiesteria*. We used a larval fish assay to examine the mechanism of fish pathogenicity in *Pseudopfiesteria shumwayae* and *Pfiesteria piscicida*. At densities of \(~1000\) cells·mL\(^{-1}\), *P. shumwayae* caused pathology and mortality in our 96 hr larval fish bioassay, but only when in direct contact with fish, while fish separated from the dinoflagellates with a permeable membrane, remained healthy. *Pfiesteria piscicida*, under similar conditions, failed to cause significant mortality in larval fishes. A dose-response study demonstrated that *P. piscicida* induced significant fish mortality, but only at cell densities \(\geq 5000\) cells·mL\(^{-1}\), while *P. shumwayae* was able to cause significant fish mortalities at initial densities \(\geq 10\) cells·mL\(^{-1}\). These data, together with direct observations of the interactions of *Pfiesteria* and fish, demonstrate the ability of these organisms to cause pathology and mortality by feeding on the epidermal tissues of live fish. This process of micropredatory feeding results in fish mortality in laboratory bioassays comparable to that reported for toxic strains, but without the involvement of a toxin. Thus, the assertion that *Pfiesteria* produces a potent, fish-killing toxin is questioned.
INTRODUCTION

The heterotrophic dinoflagellates *Pfiesteria piscicida* and *Pseudopfiesteria shumwayae* purportedly produce a potent ichthyotoxin responsible for mortalities and the formation of deep, penetrating lesions in wild and laboratory exposed fishes (Burkholder et al. 1995, 2001a, Glasgow et al. 2001a). Previous investigations, however, have attributed the lesions, commonly found in wild Atlantic menhaden (*Brevoortia tyrannus*) and often referred to as ulcerative mycosis (UM), to the highly pathogenic oomycete *Aphanomyces invadans* (Blazer et al. 1999, 2002; Vogelbein et al., 2001). Lesions identical to those found in wild menhaden have been induced in laboratory exposure studies with *A. invadans* without the involvement of *Pfiesteria* or other stressors (Kiryu et al. 2002, 2003). Further, lesions attributed to UM are focal, deeply penetrating and necrotic and are frequently accompanied by granulomatous inflammation and secondary colonization by a variety of microbial contaminants (Noga and Dykstra 1986, Noga et al. 1988, Dykstra et al. 1989, Blazer et al. 1999, Vogelbein et al. 2001). This indicates a chronic (days to weeks) pathology for these lesions, rather than an acute pathology as purported for toxin-induced lesions associated with *Pfiesteria* (Burkholder and Glasgow 1997a, Burkholder et al. 2001a,c). Additionally, a majority (80-85%) of the massive fish kills occurring in the Pamlico Sound and associated estuaries during the 1990s, 50% of which had been previously purported to be the result of toxic *Pfiesteria* activity (Burkholder et al. 1992, 1995a, Burkholder an Glasgow, 1997a), have instead been attributed to hypoxia/anoxia events caused by organic loading resulting from nutrient-
stimulated phytoplankton blooms (Paerl et al. 1998). Thus, the major pathogenic effects and mortality events in fishes purportedly induced by *Pfiesteria* are in question.

In contrast to the above field investigations, numerous laboratory exposure studies have demonstrated the ability of these dinoflagellates to cause fish pathology and mortality (Burkholder et al. 2001a, c, Glasgow et al. 2001a, Vogelbein et al. 2001, 2002, Berry et al. 2002, Gordon et al. 2002, Lovko et al. 2003, Drgon et al. 2005, Shumway et al. 2006). Our previous studies with a strain of *Pseudopfiesteria shumwayae* (CCMP 2089) isolated from the Neuse River, NC, has consistently resulted in acute fish mortalities in 38L aquarium bioassays with juvenile tilapia (*Oreochromis niloticus*) (Vogelbein et al. 2001) and in our 96 hr larval fish bioassays with *Fundulus heteroclitus* and *Cyprinodon variegatus* at initial densities as low as 10 cells·mL$^{-1}$, but without evidence of toxin (Lovko et al. 2003). This strain has been defined by other researchers as toxic, based primarily upon fish death in laboratory bioassays (Burkholder et al. 2005, Gordon and Dyer 2005). Although many studies indicate that *Pfiesteria* can cause fish pathology and mortality in laboratory assays, there is no convincing evidence that supports the involvement of a toxin and several studies have questioned the ability of *Pfiesteria* to produce toxin (Berry et al. 2002, Vogelbein et al. 2002, Lovko et al. 2003, Drgon et al. 2005). Filtrates, lysates and organic extracts of actively ichthyocidal cultures of *P. shumwayae* strain CCMP 2089 were unable to cause fish mortality in larval *C. variegatus* (Berry et al. 2002, Lovko et al. 2003). Similarly, filtrate from an actively fish-killing culture of a *P. piscicida* strain was unable to kill adult *Cyprinodon variegatus* over a 10-day period (Drgon et al. 2005).
Vogelbein et al. (2001) described the pathology of *P. shumwayae*-exposed *O. niloticus* to include a widespread diffuse petecchial hemorrhaging, and mild to widespread loss of mucous coat, epidermis and scales. In contrast to what has been described for the chronic ulcers in wild menhaden attributed to *A. invadans*, this pathology was consistent with that reported for exposure of fish to purportedly toxic *Pfiesteria* (Noga et al. 1996). However, the Vogelbein et al. (2001) study describes an association of *P. shumwayae* zoospores with various tissues of exposed fish, including the skin, lateral line canal, olfactory organ and oral mucosa, occurring concomitant with damage to those same tissues. A direct association of *Pfiesteria* with fish had not previously been considered to occur or was considered unimportant to the observed pathological effects (Burkholder et al. 1992, 2001a, Samet et al. 2001).

Our 96 hr larval fish bioassay (Lovko et al. 2003) allows direct observation of the fine-scale interaction between dinoflagellates and live fish and provides a reliable way to test cultures for fish pathogenicity. In previous studies with *P. shumwayae* strain CCMP 2089, cells were observed to rapidly swarm towards fish, attach to the fish epidermis via the peduncle, and apparently feed on the epidermal tissues. We hypothesize that this behavior contributes to, or is entirely responsible for, the pathogenicity and mortality observed in our fish bioassays with this strain. The objective of the current study is to use modifications of our larval fish bioassay, along with histopathology and electron microscopy, to further examine the necessity of physical contact between *Pfiesteria* and fish to elicit pathogenicity and to more clearly define and describe the mechanism of pathogenesis in *P. shumwayae* and *P. piscicida*. 
MATERIALS AND METHODS

_Dinoflagellate cultures_

_Pfiesteria piscicida_ strain 9-02 culture was obtained from the laboratory of Dr. Wayne Litaker, NOAA, Beaufort, NC, (subsequently deposited by Dr. Litaker to the Provasoli-Guillard National Center for Culture of Marine Phytoplankton as strain CCMP-2091) and was used in the membrane insert study within ~24 hr of receipt. It was subsequently re-isolated and maintained for approximately 14 months prior to the dose-response assay. The source and history of _Pseudopfiesteria shumwayae_ was as previously described (Lovko et al. 2003). This culture was maintained for approximately 12 months prior to the membrane insert study and 30 months prior to being inoculated into a 38-L tilapia bioassay tank from which the material used in the dose-response study was derived. The source and history of _P. piscicida_ strain VIMS-P11 was as previously described (Lovko et al. 2003). Unless otherwise noted, all cultures were grown in 0.22 \( \mu \text{m} \)-filtered and autoclaved seawater (York River, VA) diluted to 12 ppt with DI water with _Rhodomonas_ spp. (CCMP 1319 or CCMP 767) as prey. Culture identities were established and confirmed with electron microscopy and _P. shumwayae_ or _P. piscicida_-specific rRNA primers (as in Vogelbein et al. 2002 and Lovko et al. 2003).

_Fish sources_

For all assays, larval _Cyprinodon variegatus_, 7 or 8-d post-hatch, were obtained from Aquatic Bio Systems Inc., Ft. Collins, CO. Fish were held at 12 ppt and used in the assays within 24 hr of arrival. To minimize water quality issues, fish were fasted for 24 hr
prior to and for the duration of the assay. Fish were randomly distributed into covered tissue culture plates as specified for each individual experiment. All bioassays were conducted within a BSL2 cabinet.

Membrane insert studies

A modified version of the larval fish bioassay described by Lovko et al. (2003) was used to examine whether diffusible toxins produced by *Pfiesteria* are responsible for fish mortalities or if direct physical contact was necessary to elicit mortality. This assay used low protein-binding, semi-permeable polycarbonate membrane inserts (Millipore millicell-PC inserts, 30mm dia., 3µm pore size) that fit into the wells of 6-well tissue culture plates (Falcon, polystyrene, non-tissue culture treated) (Fig.1). This experimental design prevents physical contact between the fish and dinoflagellates while allowing the passage of toxins and other diffusible substances. This allows for treatments where fish and dinoflagellates are together on the same side of the membrane (direct contact) as well as treatments where the dinoflagellates and fish are separated by the membrane (indirect contact). We have previously demonstrated that both lipophilic (brevetoxin) and hydrophilic (saxitoxin) toxins readily permeate this membrane (Vogelbein et al. 2002).

Two separate assays were conducted. The first assay (reported in Vogelbein et al. 2002) tested *P. shumwayae* strain CCMP 2089 (isolated from a fish-killing tank assay containing water collected from the Neuse River, NC) at a target density of ~1,000 cells·mL⁻¹. Various combinations of dinoflagellates and fish were placed either inside the membrane insert (“in”) or in the well exterior to the insert (“out”) to create various
Figure 1. Experimental design for the membrane insert study (adapted from Vogelbein et al. 2002). The semi-permeable membrane inserts allowed separation of fish from dinoflagellates. For the *P. shumwayae* study, treatments A, B and D contained *P. shumwayae* strain CCMP 2089 and treatment E contained *P. piscicida* strain VIMS P11. For the *P. piscicida* study, treatments A, B and D contained *P. piscicida* strain 9-02 and treatment E contained *P. shumwayae* strain CCMP 2089.
Figure 1. Schematic of the experimental design for the larval fish bioassay incorporating semi-permeable membrane inserts.
“direct contact” and “indirect contact” treatments (see Fig. 1 for a description of the experimental design). A culture of *Pfiesteria piscicida* (strain VIMS P11) that has consistently proven non-pathogenic in our laboratory assays and has not demonstrated an attraction to fish tissues was used in direct contact with fish as a control to account for potential particle effects (treatment “E”). A treatment without dinoflagellates (treatment “C”) was used as a control for experimental conditions. The assay consisted of 9 replicate wells per treatment with 3 fish (7-d old *C. variegatus*) per well (either inside insert, outside of insert, or both, as dictated by experimental design).

The second assay was conducted similarly using a culture of *P. piscicida* (strain 9-02) that demonstrated a strong taxis toward narcotized *C. variegatus* but did not elicit mortalities in a previous larval fish bioassay (at ~1000 cells·mL⁻¹ initial density) (Lovko et al. 2003). Initial density for this assay was ~5600 cells·mL⁻¹. CCMP 2089 (@ ~2000 cells·mL⁻¹) in direct contact with fish was used as a positive control (treatment “E”). The assay consisted of 16 - 18 replicate wells per treatment with 6 fish (7-d old *C. variegatus*) per well (3 on each side of membrane in treatments with fish on both sides of insert).

In both assays, 9 additional replicates were used for fish mortality, histopathology (see histopathology methods), cell density (samples fixed in 1% Lugol’s iodine solution) and water quality (dissolved oxygen [DO], pH, NH₄ and temperature [T]). These parameters were assessed at 24 hr intervals for 96 hours.

*Dose-response assays*

Initial assays with *P. piscicida* strain 9-02 did not result in significant mortality, although our observations suggested that, at high densities, this species demonstrates an
attraction to fish tissues and can attach to fish epidermis. To clearly define the relationship between fish mortality and density of *Pfiesteria* dinospores, we conducted a comparative dose-response larval fish assay between *P. shumwayae* strain CCMP 2089 and *P. piscicida* strain 9-02. This assay used 24-well tissue culture plates with one fish per well. This configuration increased the statistical power of the assay and eliminated problems associated with maintaining multiple fish in each well, including the decline in water quality that typically occurs following onset of mortalities and aggressive fish behavior which was observed to increase over time and possibly contributes to observed pathology and mortality. In both assays, 2 mL culture and 1 fish (8-day old *C. variegatus*) were added to each of 48 wells per treatment (plus 9 additional wells for monitoring cell density, water quality and histopathology). Fish and water samples were taken from designated wells at 8-24-hr intervals for processing for water quality (DO, pH, Temp, NH₄), cell density ( aliquots fixed in 1% Lugol’s iodine) and histopathology (see histopathology methods).

For *P. shumwayae*, 6 L of CCMP 2089 from a 38 L tilapia bioassay (@ ~18,500 cells·mL⁻¹) was concentrated by gentle filtration (glass fiber filter [GFF] @ 12 psi vacuum). Filters were placed into two 50 mL centrifuge tubes with 35 mL 12 ppt artificial seawater (ASW) (Instant Ocean) and vortexed to remove cells from filter. Resuspended cells were added to tissue culture flasks with 35mL 12 ppt ASW. After several hours, heavy cyst formation was evident in flasks. Supernatant was poured into a new flask and 25 mL fresh 12 ppt ASW added to the flasks containing cysts. The flasks were incubated overnight to allow excystment. Flasks containing supernatant were allowed to settle, and then carefully decanted into new flasks to minimize carryover of
debris and sessile organisms. High concentrations of motile cells were observed in all flasks the following day. All flasks were combined to create a stock concentrated culture of ~200 mL with a density (± σ) of $2.8 \pm 0.18 \cdot 10^5$ cells·mL$^{-1}$. This stock culture was serially diluted with 12 ppt ASW to produce target exposure densities of 0, 10, 100, 500, 1,000, 5,000, 25,000 and 50,000 cells·mL$^{-1}$. Triplicate samples were measured from each dilution to obtain actual starting cell densities. All treatments were monitored for mortalities at 6 hr intervals for 96 hr (high density treatments, $\geq 25000$ cells·mL$^{-1}$, were monitored at 30 min intervals until mortalities reached 100%). For P. piscicida, an algal-fed clonal culture of strain 9-02 was grown to high density ($\sim 100,000$ cells·mL$^{-1}$) and serially diluted (with 12 ppt ASW) to produce target densities of 0, 10, 100, 500, 1,000, 5,000, 10,000, 50,000 and 100,000 cells·mL$^{-1}$. All treatments were monitored for mortalities at 8 hr intervals for 96 hr.

**Histopathology**

Fish exposed to either P. shumwayae strain CCMP 2089 (initial density = 4700 cell·mL$^{-1}$) or P. piscicida strain 9-02 (initial density = 9600 cells·mL$^{-1}$), as well as unexposed control fish, were killed by overdose with tricaine methanesulfonate (MS222) and fixed in 10% neutral buffered formalin for histological evaluation. Fish collected at designated sampling times from the insert and dose-response assays were also processed for histological evaluation. Whole larval fish were processed for paraffin histology by routine methods (Prophet et al., 1992). Briefly, fish were decalcified in a solution of sodium citrate and formic acid, dehydrated in a graded ethanol series, infiltrated in paraffin using an Excelsior ES Tissue Processor (Shandon), embedded using an EC 350-2
tissue embedding center (Microm), sectioned at 5 µm on an Olympus Cut 4055 rotary microtome, mounted on glass slides, and stained in Harris’ hematoxylin and eosin using a Varistain 24-3 automatic slide stainer (Shandon). Slides were evaluated for epidermal damage and imaged with an Olympus AX70 compound microscope fitted with an Olympus DP70 digital camera.

Electron microscopy

Fish (larval *C. variegatus* or *F. heteroclitus*) were narcotized with tricaine methanesulfonate (MS-222) (50-100 mg/L), thoroughly rinsed (3x with 12 ppt ASW), then exposed to either *P. shumwayae* strain CCMP 2089 or *P. piscicida* strain 9-02 (5-15 min exposure time, dinoflagellate density @ ~1000-5000 cells·mL⁻¹). Fish were fixed whole in 4% glutaraldehyde /5% paraformaldehyde in 0.1M sodium cacodylate buffer (pH 7.2), at room temperature for ~2 hr. Samples were gently washed with three changes of 0.1M sodium cacodylate buffer, (15 - 30 minutes each) and stored in buffer overnight at 4°C. Samples were post-fixed with 1% OsO₄ in 0.1M sodium cacodylate buffer (pH 7.2), at room temperature, for 1 hr and then washed in three changes of 0.1M sodium cacodylate buffer (pH 7.2), for 15-30 min each. The caudal peduncle was removed from each fish and processed for transmission electron microscopy (TEM) while the body of each fish was processed for scanning electron microscopy (SEM). For TEM, tissues were dehydrated in a graded ethanol series, *en-block* stained with 1% uranyl acetate in 70% ethanol, followed by three changes (30 min each) of 100% propylene oxide, then infiltrated in Spurr's resin over a four-day period and embedded. Blocks were sectioned at 90 nm on a Reichert-Jung Ultracut E ultramicrotome, stained with Reynold's lead citrate
and analyzed on a Zeiss CEM 902 TEM. For SEM, samples were dehydrated in a graded ethanol series, critical point dried in a Polaron E3001 critical point dryer, mounted and coated with 20 nm of Au:Pd in an Anatech Hummer VII sputter coater and then analyzed with a LEO 435VP SEM.

Statistical Analysis

Mortality data from the insert and dose-response bioassays was modeled with SPSS statistical software using the Kaplan-Meier estimator for survival analysis. Survival curves were compared using the Tarone-Ware chi-square statistic ($\chi^2$) to determine significance of differences in survival between treatments. In the dose-response studies, a 24, 48, 72 and 96 hr LD$_{50}$ was calculated for each species based on probit analysis of the measured cell densities at the beginning of the experiment (using SPSS statistical software).

RESULTS

Membrane insert studies

Results from the membrane-insert study with P. shumwayae have been published previously (Vogelbein et al. 2002) and are reported here for comparison purposes with P. piscicida. In the assay with P. shumwayae (CCMP 2089), fish mortalities occurred only in treatments that allowed direct physical contact between dinoflagellates and fish (treatments B-in and D, 100% and 96%, respectively, in 48 hr, Fig. 2a). Survival analysis indicated no significant differences between these two treatments (Tarone Ware $\chi^2$,
Figure 2. Results from larval fish bioassays with *Cyprinodon variegatus* exposed to *Pseudopfiesteria shumwayae* strain CCMP 2089 or *Pfiesteria piscicida* strain 9-02 using semi-permeable membrane inserts. **a** and **b**, Cumulative fish mortalities in treatments with *P. shumwayae* (**a**) and *P. piscicida* (**b**). Error bars represent the standard errors of the mean among replicate wells in each treatment (n=9 in all treatments with *P. shumwayae*, n=16-18 in treatments with *P. piscicida*). Only treatments with direct contact between *P. shumwayae* and fish resulted in mortalities (**a**, treatments “B-in” and “D” in; **b**, treatment “E”). Low-level mortalities occurred in some treatments with *P. piscicida* strain 9-02 (**b**, treatments “A-in” and “B-in”), but these were not significantly above mortalities in fish-only controls (**b**, treatments “C-in” and “C-out”). **c** and **d**, Dinospore densities of *P. shumwayae* (**c**) and *P. piscicida* (**d**) in treatments containing dinoflagellates. Error bars represent standard deviations among counts in replicate wells (n=3 for all treatments and time points).
Figure 2. Results of larval fish assays with *Pseudopfiesteria shumwayae* and *Pfiesteria piscicida* using semi-permeable membrane inserts.
p>0.05). No fish mortalities occurred when _P. shumwayae_ was physically separated from larval fish using semi-permeable membrane inserts (treatments “A-in” and B-out”) or in the control (treatment “E” using _P. piscicida_ strain VIMS P11). Initial dinoflagellate densities were approximately 850 cells·mL⁻¹ in all treatments with dinoflagellates. In treatments with direct physical contact with fish (treatments “B-in” and “D”), dinospore densities decreased during the first 24 hrs (likely due to extensive encystment post-feeding, as numerous cysts were observed on the bottom of the well), but increased rapidly over the remainder of the assay (Fig. 2c; treatment “D” increased from 830 ± 64 cells·mL⁻¹ @ 48 hr to 9080 ± 562 cells·mL⁻¹ @ 72 hr; treatment “B-in” increased from 300 ± 180 cells·mL⁻¹ @ 24 hr to 3150 ± 289 cells·mL⁻¹ @ 48 hr). Treatments in which fish and dinoflagellates were separated by a membrane exhibited only a marginal increase in cell densities at 24 hrs, without any appreciable cyst formation, followed by a decline in dinospore numbers over the assay duration (Fig. 2c). Water quality parameters (DO, NH₃, pH, temp) remained within acceptable parameters throughout the duration of the assay (DO among all treatments over duration of assay = 6.6-7.6 mg·L⁻¹; reactive NH₃ among all treatments at 96 hr = 0.09-0.25 mg·L⁻¹ @ 96 hr).

Results of the assay with _P. piscicida_ differed from those with _P. shumwayae_. Only the control treatment with _P. shumwayae_ (treatment “E”: direct physical contact) exhibited 100% mortality in 48 hr (Fig. 2b). Treatment “B-in”, with fish in direct contact with dinoflagellates, experienced 9.3% mortality (not significant compared to controls “C-in” and C-out”, Tarone Ware $\chi^2$, p>0.05) at 96 hr while fish on the other side of the insert (“B-out”, indirect contact) had no mortalities throughout the assay. One treatment with indirect contact (“A-in”) experienced a small number of mortalities (3.7% at 96 hr),
although not above mortalities in controls (C-in and C-out, 11.1% and 13.0% respectively at 96 hr). In treatments containing *P. piscicida* dinospores (“A-out”, “B-in” and “D”), dinoflagellate densities increased appreciably only in treatment “B-in”, from an initial density of 5600 cells·mL⁻¹ to more than 18,000 cells·ml⁻¹ at 24 hrs, and subsequently decreasing steadily over the remainder of the assay to 7600 cells·ml⁻¹ at 96 hrs (Fig. 2d). Cell densities in the remaining treatments remained relatively constant. DO remained ≥ 7.0 mg·L⁻¹ in all treatments and at all time periods measured. Reactive NH₃ was elevated (>0.50 mg·L⁻¹) in some treatments, exceeding 1.0 mg·L⁻¹ in treatments A (1.08 mg·L⁻¹), B (1.34 mg·L⁻¹) and D (1.34 mg·L⁻¹) at 96 hr. None of the measured values exceeded the reported LC₅₀ of 1.6 mg·L⁻¹ for juvenile *F. heteroclitus* (Burton and Fisher, 1990).

*Dose-response studies*

Fish mortalities occurred in *P. shumwayae* exposures at all tested densities except 0 cells·mL⁻¹ (control). Mortalities occurred more rapidly with increasing initial dinospore densities, reaching 100% in the 50,000, 25,000, 5,000 and 1,000 cells·mL⁻¹ treatments at 2, 3.5, 42 and 78 hr, respectively (Fig. 3a). Survival analysis indicated significant differences between all treatments (Tarone Ware $\chi^2$, p<0.001 for all pair-wise comparisons). The estimated LD₅₀s based on probit analysis of initial cell densities were 5128 cells·mL⁻¹ at 24 hr (95% confidence interval [CI] not estimated), 1365 cells·mL⁻¹ at 48 hr (95% CI, 1218-1498 cells·mL⁻¹), 517 cells·mL⁻¹ at 72 hr (95% CI, 450-605 cells·mL⁻¹), and 287 cells·mL⁻¹ at 96 hr (95% CI not estimated). When plotted against log₁₀ dinospore densities, these values demonstrate a strong log-linear relationship with initial cell density ($R^2 = 0.97$). Dinospore densities decreased in most treatments over the
Figure 3. Results from dose-response larval fish bioassays with *Cyprinodon variegatus* exposed to initial densities of *Pseudopfiesteria shumwayae* strain CCMP 2089 or *Pfiesteria piscicida* strain 9-02 ranging between 10 and 100000 cells·ml⁻¹. a,b, Cumulative fish mortalities in treatments with *P. shumwayae* (a) and *P. piscicida* (b). Note the change in the scale of the x-axis for *P. shumwayae* (a). c,d, Log₁₀-transformed dinospore densities of *P. shumwayae* (c) and *P. piscicida* (d) in treatments containing dinoflagellates. Error bars represent standard deviations of counts in replicate wells (n=3 for all treatments and time points).
Figure 3. Results of dose-response larval fish bioassay with *Pseudopfiesteria shumwayae* and *Pfiesteria piscicida*

**Pseudopfiesteria shumwayae**

- % Cumulative Mortality
- Log Dinospores•ml⁻¹

**Pfiesteria piscicida**

- % Cumulative Mortality
- Log Dinospores•ml⁻¹

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*Pseudopfiesteria shumwayae* and *Pfiesteria piscicida* are depicted in the graphs. The graphs show the percentage of cumulative mortality and the logarithm of dinospores per milliliter over time.
first 24 hr, concomitant with post-feeding encystment and accumulation of dinoflagellate cysts on the bottom of the wells. This was followed by a rapid, log-linear increase in cell density throughout the remainder of the assay as excystment occurred ($R^2 = 0.88 - 0.96$ for treatments $10 - 1000$ cells·mL$^{-1}$ for data points from 24 -96 hrs) (Fig. 3c). Cell densities were no longer measured in treatments once mortality had reached 100%. Measured DO ranged between 5.61 - 6.48 mg·L$^{-1}$ in all treatments over the duration of the assay. Reactive NH$_3$ was < 0.25 mg·L$^{-1}$ in all treatments over the duration of the assay.

In the assay with *P. piscicida*, significant mortalities (> 2.5%) occurred only in treatments with initial dinospore densities ≥ 5000 cells·mL$^{-1}$ (Fig. 3b). Mortalities increased gradually over time, eventually reaching a plateau at 64 hr at approximately 80%, 70% and 27% in the 50,000, 100,000 and 10,000 cells·mL$^{-1}$ treatments, respectively. Mortality reached a maximum of approximately 14% in the 5000 cells·mL$^{-1}$ treatment at 96 hrs. A single mortality occurred in the 1000 cells·mL$^{-1}$ treatment at 96 hr. Survival analysis indicated no significant difference between treatments with 100,000 and 50,000 cells·mL$^{-1}$ (Tarone Ware $\chi^2$, $p=0.115$). A significant difference in survival was indicated between all other treatments with mortalities ($p<0.05$). The estimated LD$_{50}$s based on probit analysis of initial cell densities were 40,200 cells·mL$^{-1}$ at 48 hr (95% CI, 24,936-73,647 cells·mL$^{-1}$), 12,180 cells·mL$^{-1}$ at 72 hr (95% CI, 10,779-16,369 cells·mL$^{-1}$), and 12,999 cells·mL$^{-1}$ at 96 hr (95% CI, 10,746-17,945 cells·mL$^{-1}$). A 24 hr LD$_{50}$ could not be estimated. Cell density increases were log-linear over the duration of the assay in treatments with initial cell densities of ≤5000 cells·mL$^{-1}$ ($R^2 = 0.94-0.99$ for treatments ≤5000 cells·mL$^{-1}$; $R^2 < 0.30$ for treatments >5000 cells·mL$^{-1}$) (Fig. 3d). Treatments with greater initial densities exhibited a relatively flat growth rate or a
gradual decline over the duration of the assay. No treatment exhibited appreciable cyst formation. Measured DO ranged between 5.44 - 7.02 mg·L⁻¹ in all treatments over the duration of the assay and was generally at or above 6.5mg·L⁻¹ in all treatments at 96 hr. Reactive NH₃ was < 0.28 mg·L⁻¹ in all treatments over the duration of the assay.

_Fish Skin Histopathology/SEM/TEM_

When analyzed with paraffin histology, SEM and TEM, the epidermis of unexposed control fish at 96 hr remained intact with no obvious signs of pathology (Fig. 4a-c). Histologically, the epidermis was comprised of squamous epithelial cells in a layer 2-4 cells in thickness. Surficial cells contained deeply basophilic, elongated nuclei and scant eosinophilic cytoplasm. Dermis was minimal at this stage of development. Scales were obscure or absent (Fig.4 a). Ultrastructurally, surfaces of outer epithelial pavement cells exhibited characteristic patterns of micro-ridges and occasional goblet cell pores (MC) (Fig. 4b). TEM showed intact epidermis with typical cellular and nuclear morphology and surficial mirco-ridges typical of fish epidermis (Fig. 4c). Similarly, fish indirectly exposed (separated by semi-permeable membrane) to either _P. shumwayae_ or _P. piscicida_ did not demonstrate any obvious pathology and exhibited an epidermal morphology similar to that of fish from control treatments.

In contrast, fish in direct physical contact with _P. shumwayae_ demonstrated varying degrees of skin pathology including sloughing of epidermal cells, cellular degeneration and the formation of shallow focal lesions, often associated with attached dinoflagellates (Fig. 5). Fish in direct physical contact with _P. shumwayae_ exhibited disruption of the epidermis ranging from small focal erosions occurring in as little as 15
Figure 4. Micrographs of epidermis of unexposed (control) *Cyprinodon variegatus*. a, Hematoxylin and eosin (H&E) stained paraffin histology micrograph demonstrating the smooth, intact appearance and elongated nuclei (N) of undisrupted pavement cells. Scale bar = 10µm b, Scanning electron micrograph (SEM) of fish epidermis demonstrating the typical “whorl-like” pattern of epithelial microridges on healthy pavement cells (PC). Note the presence of mucous cells (MC). Scale bar = 5µm c, Transmission electron micrograph (TEM) of healthy, intact fish epidermis demonstrating prominent epithelial microridges (arrow heads) and the elongated, euchromatic appearance typical of pavement cell nuclei (N). Scale bar = 2µm
Figure 4. Histology, SEM and TEM micrographs of epidermis of unexposed larval Cyprinodon variegatus
min. at ~3000 cells·mL$^{-1}$ to widespread loss of epidermis. Even at initially low-level *P. shumwayae* exposures (10 cells·mL$^{-1}$), foci of eroded epidermis were observed by 96 hr. Histologically, by 8 hr post-initiation, at initial dinospore densities of <5000 cells·mL$^{-1}$, the epidermis of exposed fish exhibited signs of necrosis including nuclear pyknosis and karyorrhexis and cellular separation, blebbing and sloughing (Fig. 5a). Dinospores of *P. shumwayae* were frequently observed closely associated with the epidermis in histological sections of fish from larval bioassays (Fig. 5b). Under SEM, cells were observed closely juxtaposed to the epidermis of narcotized larval fish (Fig. 5c,d). Based on ultrastructural analyses, attachment occurred via an extensible feeding structure called a peduncle (Fig. 5d-f), often exhibiting small, filopodial extensions on the distal end (Fig. 5e). Several *P. shumwayae* cells were observed to contain small, electron-dense granules within the distal end of the peduncle and within the filopodial extensions (Fig. 5e inset). Material from fish epidermal cells was observed in the peduncle (Fig. 5f) as well as in large food vacuoles in the epicone of the attached dinoflagellates (Fig. 5b). The food vacuoles occupied a large portion of the cell volume, often extending into the hypocone and displacing the nucleus to the margin of the cell.

Epidermal damage was less commonly observed in fish exposed to *P. piscicida* and was evident only after significantly longer exposure times and/or greater dinoflagellate densities (Fig. 6). Damage most commonly observed involved a thickened appearance of the epidermis along with the presence of numerous granulocytic leukocytes (Fig. 6a). Occasionally, widespread epidermal erosion was observed in fish exposed to high dinoflagellate concentrations (>5000 cells·mL$^{-1}$) for a longer duration (96 hr) (Fig. 6b). However, the epidermis of most fish was indistinguishable from that of
Figure 5. Attachment, feeding, and damage to epidermis of larval *Cyprinodon variegatus* by *Pseudopfiesteria shumwayae* dinospores. a, paraffin histology micrograph of severely damaged *C. variegatus* epidermis after 8 hr exposure to *P. shumwayae* dinospores at an initial density of 4700 cells·ml$^{-1}$. Note complete disruption of epidermis, enlargement of epidermal cells and nuclei (arrows), nuclear pyknosis (P) and karyorrhexis (arrowhead) and cellular blebbing (B). Scale bar = 10µm (H&E). b, micrograph of *P. shumwayae* dinospore (D) attached to pectoral fin of larval fish (F). Note the larval food vacuole (V) occupying much of the cellular volume and marginalizing the nucleus (N). Scale bar = 5µm (H&E). c, SEM of *C. variegatus* epidermis after 15 min. exposure to *P. shumwayae* dinospores (~3000 cells·ml$^{-1}$). Note sloughing epidermis and numerous attached dinospores (arrowheads). Intact epidermal cells can be observed at extreme upper right-hand and lower left corners of frame. Scale bar = 10µm. d, SEM of a *P. shumwayae* dinospore (D) with a broad peduncle (P) appearing to attach to an otherwise undisrupted area of fish epidermis (E). Scale bar = 10µm. e, SEM of the peduncle (P) of a dinospore (D) associated with a disrupted area of fish epidermis. Note the presence of multiple filipodial-like extensions at distal end of peduncle (white arrows). Inset: TEM of peduncular filipodia in cross-section (black arrows) revealing the presence of electron dense granules. Scale bars = 1µm. f, TEM of peduncle (P) of a dinospore ingesting cellular material (C) from fish epidermal cells (E) through the peduncle. Scale bars = 1µm.
Figure 5. Damage to fish epidermis and attachment and feeding by *Pseudopfiesteria shumwayae* dinospores on epidermis of larval *Cyprinodon variegatus*
Figure 6. Attachment, feeding and associated epidermal damage in larval *Cyprinodon variegatus* by *Pfiesteria piscicida* dinospores.  

**a**, Photomicrograph of *C. variegatus* epidermis after 56 hr exposure to *P. piscicida* dinospores at an initial density of 9600 cells·ml$^{-1}$. Note irregular appearance of epithelial surface, thickened appearance of epidermis and presence of numerous granulocytes (white arrows). Scale bar = 20µm (H&E).

**b**, *C. variegatus* epidermis after 96 hr exposure to *P. piscicida* dinospores at an initial density of 9600 cells·ml$^{-1}$. Note complete loss of epidermis and presence of pyknotic nuclei (black arrows). Scale bar = 20µm (H&E).

**c**, SEM of several dinospores (arrow heads) attached to disrupted fish epidermis (E) via extensible peduncle (arrows). Scale bar = 5µm.

**d**, SEM of *P. piscicida* dinospore (D) attaching to fish epidermis (E) via peduncle (P). Note presence of multiple filipodia at distal end of peduncle (white arrows) and perforated appearance of fish epidermis along with loss of fine epithelial microstructure. Scale bar = 1µm.

**e,f**, TEM of dinospore (D) ingesting cellular material (black arrows) from damaged fish epidermal cells through peduncle (P) and into a large food vacuole occupying majority of the dinoflagellate cell (frame e). Note presence of numerous lipid droplets (L) in food vacuole (frame f). Scale bars = 1µm.
Figure 6. Damage to fish epidermis and attachment and feeding by *Pfiesteria piscicida* dinospores on epidermis of larval *Cyprinodon variegatus*
controls (1 of 14 fish observed exhibited severe pathology). As with *P. shumwayae*,
dinospores of *P. piscicida* were observed to attach to fish epidermis by the peduncle (Fig.
6c), which also exhibited the filipodial extensions observed in *P. shumwayae* (Fig. 6d).
The electron-dense “granules” observed in the distal end of the *P. shumwayae* peduncle
were not observed in *P. piscicida*. Cytoplasm and organelles from fish epidermal cells
were present in the tube-like peduncle (Fig. 6e) as well as in food vacuoles present in the
epicone of attached cells (Fig. 6f). The food vacuoles often occupied a large volume of
the dinoflagellate cell and frequently contained numerous lipid droplets (Fig. 6f).

**DISCUSSION**

Toxicity in *Pfiesteria* spp. (including both *Pfiesteria piscicida* and
*Pseudopiesteria shumwayae*) has consistently been defined as the ability of cultures to
cause disease or death in exposed fishes (Marshall et al. 2000, Burkholder et al. 2001a,c,
Glasgow et al. 2001a,b, Burkholder et al. 2005). These studies support the assertion that,
under certain laboratory conditions, both *P. piscicida* and *P. shumwayae* can cause fish
pathology and mortality. However, both species are demonstrated here to attach to and
feed on the epidermis of live fish by myzocytosis. Given sufficient cell density and
exposure time, this feeding process can result in epidermal damage extensive enough to
cause fish death, presumably by disruption of osmoregulation. We consider this behavior
to constitute micropredation, a feeding strategy in which a predator feeds upon prey
considerably larger than itself (Dorland’s Medical Dictionary, available on-line at
http://www.merckmedicus.com/).
Previously, cultures of *P. piscicida* and *P. shumwayae* have been classified as weakly to highly toxic based on the time and cell densities required to cause fish pathology and mortality (Marshall et al. 2000, Burkholder et al. 2001a,c, Glasgow et al. 2001a). This variability in toxicity has been classified into three “functional types” encompassing strains that are considered actively toxic (TOX-A), strains considered to be weakly toxic or temporarily non-toxic (TOX-B) and strains that are considered incapable of producing toxin (NON-IND or non-inducible) (Burkholder et al. 2001a,b,c, Glasgow et al. 2001a,b). According to a study by Burkholder et al. (2001a), “TOX-A” cultures caused 100% mortality (n=90) in larval *C. variegatus* in 9.5 hrs at an initial mean dinospore density of $5.8 \cdot 10^4$ cells·ml$^{-1}$. In that same study, a “TOX-B” culture killed 47% of fish (n=82) in 48 hr at an initial mean dinospore density of $4.1-6.2 \cdot 10^4$ cells·ml$^{-1}$. Noga et al. (1996) explicitly define toxicity as cell densities sufficient to cause fish mortality within a specified time period ($2-5 \cdot 10^3$ cells·ml$^{-1}$ results in 100% mortality of juvenile striped bass and tilapia in 24 - 48 hrs). Non-toxic cultures are repeatedly referred to as cultures that do not cause fish pathology or mortality (Burkholder et al. 2001a,b,c, 2005a, Glasgow et al. 2001a,b). Our culture of *P. shumwayae* strain CCMP 2089, demonstrated here to kill fish by micropredatory feeding without involvement of toxin, easily fits the above criteria for an actively and highly “toxic” (TOX-A) culture, with initial dinoflagellate densities of $\sim 5 \cdot 10^4$ and $5 \cdot 10^3$ cells·ml$^{-1}$ resulting in 100% fish mortality in 2 hr and 42 hr, respectively. By these same criteria, our *P. piscicida* culture 9-02 would be considered a less toxic (TOX-B) culture.

Loss of toxicity in *Pfiesteria* cultures is reported to occur after extended culture (weeks to months) without fish prey (Burkholder and Glasgow 1997a,b, Marshall et al.
2000, Burkholder et al. 2001a,c, Glasgow et al. 2001b). One study reported that 95% of *Pfiesteria* sp. strains previously tested as toxic completely lost their ability to produce toxin (and kill fish) after 8 weeks without fish prey (Burkholder et al. 2001a). Another study demonstrated a loss of fish-killing ability in a *P. shumwayae* culture that encysted after isolation and could subsequently be grown on algal prey but could not be induced to kill fish (Glasgow et al. 2001b). The strains and species used in the studies presented here have been in continuous culture on algal prey for 12-30 months, which, according to the above criteria, should render them incapable of killing fish.

Thus, it is clear that determination of toxicity based solely on fish mortality and dinoflagellate density, is inadequate and cannot distinguish between mechanisms of pathogenicity. It should be noted, however, that the culture of *P. shumwayae* used in the current dose-response study was derived from a fish bioassay while the *P. piscicida* was cultured with algae. Cultures of both species used in all other studies presented here were algal-cultured.

The presence of a fish-killing *Pfiesteria* toxin has been challenged (Berry et al. 2002, Vogelbein et al. 2002, Lovko et al. 2003, Drgon et al. 2005) but continues to be debated (Burkholder et al. 2005, Moeller et al. 2007). Thus far, after nearly two decades of research, a toxin structure has only been partially described (Moeller et al. 2007). This putative toxin, derived from a non-axenic *P. piscicida* culture, was reported as a ligated copper compound with a free-radical-mediated toxicity (Moeller et al. 2007). Several other studies have provided limited evidence of a potent, diffusible toxin. Springer et al. (2002) demonstrated 100% mortality in scallop larvae (*Argopecten irradians*) contained within dialysis tubing and placed in petri dishes with *Pfiesteria piscicida* for 60 minutes.
Others have successfully induced fish mortalities using cell-free filtrates from fish-killing *Pfiesteria* spp. cultures (Burkholder and Glasgow 1997a, Burkholder et al. 2005, Moeller et al. 2001, Gordon et al. 2002). Additionally, other studies have identified “bioactive” fractions in *Pfiesteria* cultures that demonstrated neurotransmitter inhibition in rat brain cells, cytotoxicity to rat pituitary cells and learning impairment in rats (Levin et al. 1999, 2000, El-Nabawi et al. 2000, Moeller et al. 2001). However, these “bioactive” effects were not correlated with activity of a potent fish-killing toxin, as had originally been described for *Pfiesteria* spp. (Burkholder et al. 1992, 1995, 2001a, Glasgow et al. 1995, Noga et al. 1996). Further, many of these studies used fractions derived from fish-exposed cultures. These types of cultures are subject to the growth of a wide variety of microbial contaminants (Vogelbein et al. 2001, Lovko et al. 2003, Drgon et al. 2005), some of which may be pathogenic (Doucette 1995, Drgon et al. 2005). Drgon et al. (2005), replicating the standard aquarium format bioassay, found potentially pathogenic bacteria, including *Vibrio* sp. and *Aeromonas* sp., among the assemblage of microorganisms that developed over time. Thus, it is difficult to rule out the involvement of microbial contaminants in observed toxicity or bioactivity in studies using non-axenic cultures.

“Neurocognitive” abnormalities have been reported in *Pfiesteria*-exposed fishes, including hyper/hypo-ventilation, depressed activity, hyper-excitability and a loss of equilibrium (Noga et al. 1993, 1996). Although these effects were considered signs of central nervous system damage due to action of the *Pfiesteria* toxin, the same study also demonstrated elevated serum osmolality along with concentrations of Na, Cl and K approaching that found in the ambient water, indicating that these behavioral symptoms
may have instead been due to osmoregulatory failure, as might occur due to significant epidermal damage associated with micropredatory feeding.

In the current work, neither *P. shumwayae* nor *P. piscicida* were capable of causing fish mortality or pathology when separated from fish by a porous membrane. Both cultures, however, did demonstrate an ability to feed on fish and cause fish mortality when allowed direct physical contact, although the *P. piscicida* used here required considerably higher cell densities and exposure times to kill fish. Calculated LD_{50} values generated for *P. shumwayae* in the current dose-response study were similar to the values reported in our previous studies with an algal-reared culture (Lovko et al. 2003) and demonstrate a clear dose-dependent relationship with fish mortality in *P. shumwayae* exposures. This relationship was less clear in exposures with *P. piscicida*, where mortalities appeared to level off after approximately 60 hr in all treatments exhibiting fish mortality and never exceeded ~80% even at cell densities of 100,000 cells·mL$^{-1}$.

Other reports have described direct, physical attack by *Pfiesteria* spp. on larval fish (*Cyprinodon variegatus*) (Burkholder et al. 2001a,b, 2005), bay scallop (*Argopecten irradians*) and eastern oyster (*Crassostrea virginica*) (Springer et al. 2002). However, these reports consider a potent ichthyotoxin to be the cause of death and dismiss micropredation as a minor or irrelevant contributor to observed mortality. In one study, physical attack by *Pfiesteria* spp. on larval *C. variegatus* contributed to mortalities by cultures considered to be highly toxic (100% mortality in 9.5 hr) as well as cultures believed not to produce a toxin (12% mortality in 48 hr) (Burkholder et al. 2001a,b). Direct attack was considered responsible for mortalities in the “non-toxic” culture but
was considered insignificant in the “highly toxic” culture. However, dinoflagellate
densities varied widely within and between treatments (range = 1.5-9.8 \cdot 10^4 \text{ cells} \cdot \text{ml}^{-1}
among both cultures) and the methodology used made no provisions to distinguish
between direct and indirect contact, making attribution of fish mortality to a toxin
arbitrary. More recently, micropredation by \textit{P. shumwayae} has been confirmed for
shellfishes (Shumway et al. 2006) and finfishes (Gordon and Dyer 2005) and was
determined to be the most important contributor to mortality in laboratory assays, even in
a strain of \textit{P. shumwayae} previously thought to be highly toxic (Gordon and Dyer 2005).
However, these studies asserted that an ichthyotoxin produced by \textit{Pfiesteria} contributed
to observed mortalities.

Although both \textit{P. shumwayae} and \textit{P. piscicida} are shown to be capable of
micropredatory feeding on fish epidermis and causing mortality by this mechanism, a
significant difference in pathogenicity was observed between the two species.
Widespread epidermal damage was readily evident in \textit{P. shumwayae}-exposed fish while
only minor epidermal damage was consistently observed in \textit{P. piscicida}-exposed fish. In
exposures with both species, areas of intact, undamaged epidermis were frequently
observed immediately adjacent to attached dinoflagellates, indicating that the damage
caused by attachment is localized rather than widespread. Therefore, it may be assumed
that areas of widespread epidermal erosion are due to numerous cells attaching and
feeding. Thus, it is possible that the observed differences in pathogenicity between \textit{P.}
\textit{shumwayae} and \textit{P. piscicida} are due to some limitation in attachment and or feeding
ability by \textit{P. piscicida}. However, these studies are based on only one strain of each
species. The amount of variability in pathogenicity due to micropredatory feeding among strains within each of these species is currently unknown.

Both tested species attached to the fish epidermis via peduncle and exhibited peduncular filopodial extensions. However, electron-dense granules within the distal end and filopodial extensions of the peduncle were only observed in *P. shumwayae*. Although the function of these granules is not known, similar structures have been observed in other dinoflagellates. Electron-dense “rod-shaped bodies” have been found in the peduncle of *Gyrodinium lebourae* (Lee 1977) and similar granules have been found in the pallium of *Protoperidinium spinulosum* (Jacobson and Anderson 1992), but the possible function of these structures was not addressed. Light-refractive granules, presumed to contain lipids and proteins, in *Paulsenella vonstoschii*, are secreted along the feeding tube during host cell penetration (Drebes and Schnepf 1988). The granules in *P. shumwayae* are typically not observed in attached cells, indicating they might be excreted during or just prior to attachment of the cell. It could be speculated that the granules observed in *P. shumwayae* contain substances that may function in adhesion to, or penetration of prey cells.

Many heterotrophic dinoflagellates have been shown to feed by myzocytosis via peduncle on a variety of prey including algae, ciliates, diatoms and nematodes (Spero and Morée 1981, Spero 1982, Drebes and Schnepf 1988, Simon et al. 1992, 1993, Ucko et al. 1994). Certain feeding strategies, such as direct engulfment, impose a limit on prey size dependent upon the size of the predator (Jakobsen and Hansen 1997). However, myzocytotic feeding through a peduncle does not impose such limitations because the predator can consume only a portion of the prey (Spero and Morée 1981, Calado and
Moestrup 1997) thereby allowing it to feed on organisms much larger than itself. Micropredation, as described for *Pfiesteria shumwayae*, is simply feeding by myzocytosis on the epidermal cells of fish (Vogelbein et al. 2002). Other examples of micropredation by heterotrophic dinoflagellates have been reported. *Peridiniopsis berolinensis* feeds on injured nematodes (Calado & Moestrup 1997), *Oxyrrhis marina*, a peduncle feeder, attacks molting amphipods (Gaines and Elbrächter 1987), *Katodinium* (formerly *Gymnodinium*) *fungiforme* feeds via peduncle on injured or dying ciliates (Spero and Morée 1981, Spero 1982) and other species feed on organisms such as diatoms and even other dinoflagellates, which are often considerably larger than themselves (Drebes and Schnepf 1988, Ucko et al. 1994). Thus, micropredation in heterotrophic dinoflagellates is not a novel observation. However, the mechanism of fish mortality resulting from the mechanical disruption of fish epidermis via feeding by *Pfiesteria* represents the first (to the author’s knowledge) account of fish mortality as a result of micropredation by non-parasitic heterotrophic dinoflagellates. Thus, we provide here an alternative interpretation for the observation of fish mortalities in laboratory *Pfiesteria* assays which does not invoke the action of a potent ichthyotoxin. It is imperative that studies aimed at determining the “toxicity” of *Pfiesteria* cultures, consider the role of micropredatory behavior in any observations of ichthyocidal activity. Further, such studies should incorporate appropriate methodologies, such as those used here, in order to accurately distinguish between the occurrences of potentially disparate mechanisms of pathogenicity, including toxin production and micropredation, in these heterotrophic dinoflagellates.
CONCLUSION

Prior to our studies on micropredation, toxicity in *Pfiesteria* has primarily been an assumption based on fish death in laboratory assays correlated to the presence and density of *Pfiesteria* (Burkholder et al. 1992, 2001a,c, Noga et al. 1993, Glasgow et al. 2001a,b). However, because of the large-scale fish bioassays (10 gal aquaria) used in these earlier studies, investigators did not observe direct attachment and micropredatory feeding by the dinoflagellates, and attributed fish pathology and mortality to a potent exotoxin. The results presented here demonstrate that, under bioassay conditions, *P. shumwayae* and, to a lesser extent, *P. piscicida*, cause fish pathology and mortality by micropredation in a dose-dependent fashion, at rates identical to those previously reported for purported “toxic” cultures (Noga et al. 1996, Burkholder et al. 2001a). These findings provide an alternative mechanism of pathogenicity in *Pfiesteria* that can account for fish mortalities without involvement of a potent ichthyotoxin, as had been previously reported (Noga et al. 1996, Burkholder and Glasgow 1997a, Burkholder et al. 2001a, 2005).
REFERENCES


SECTION IV

MICROPREDATORY FEEDING IN DINOFLAGELLATES OF THE
PFIESTERIACEAE (DINOPHYCEAE)
The dinoflagellates *Pfiesteria piscicida* and *Pseudopfiesteria shumwayae* have been demonstrated to kill fish by the process of myzocytosis rather than through the production of a potent ichthyotoxin, as had been previously reported. Several genetically related heterotrophic dinoflagellates closely resemble *Pfiesteria* under light microscopy and exhibit similar biological and ecological characteristics. Although none of these other species have thus far been demonstrated to produce toxin or cause fish mortality, the pathogenic potential of these organisms has not been thoroughly addressed. Here, the comparative pathogenicity of several “*Pfiesteria*-like” dinoflagellates, including multiple strains of *Cryptoperidiniopsis* spp., *Luciella* spp. and *Stoeckeria* sp., is evaluated against *P. piscicida* and *P. shumwayae* using larval fish bioassays, histopathological analysis and electron microscopy. All tested species and strains demonstrated attachment to and feeding on the epidermis of live fish epidermis via an extensible peduncle. At relatively high cell densities, several of these dinoflagellates caused severe damage to the fish epidermis resulting in fish mortality, presumably by disruption of osmotic regulation. Cultures of *P. shumwayae*, *Cryptoperidiniopsis* spp. and *Stoeckeria* sp. caused significant mortality in larval fish while cultures of *P. piscicida* and *Luciella masanensis* caused only minor mortality at the cell densities tested. This study suggests that many species of heterotrophic dinoflagellates that feed on a variety of prey by myzocytosis using a peduncle can cause fish pathology and mortality in laboratory assays in the manner previously described for *P. shumwayae* and *P. piscicida*. 
INTRODUCTION

Heterotrophic dinoflagellates of the family Pfiesteriaceae include the ichthyocidal *Pfiesteria piscicida* and *Pseudopfiesteria shumwayae* (here together referred to as “*Pfiesteria*”) (Burkholder et al. 2001a, Glasgow et al. 2001a, Burkholder and Glasgow 1997a,b, Marshall et al. 2000) as well as several other morphologically and behaviorally similar dinoflagellates that have only recently been formally described (Jeong et al. 2005b, Steidinger et al. 2006, Mason et al. 2007). *Pfiesteria piscicida* and *P. shumwayae* have previously been reported to secrete a potent exotoxin considered to be responsible for adverse health effects in humans and aquatic animals (Burkholder et al. 1995, 2001a, Noga et al. 1996, Marshall et al. 2000, Glasgow et al. 2001a). However, several studies have provided evidence against the presence of a *Pfiesteria* toxin (Berry et al. 2002, Vogelbein et al. 2002, Lovko et al. 2003, Drgon et al. 2005).

Our previous research demonstrated that, in laboratory bioassays, *P. shumwayae* (strain CCMP2089) physically attacked juvenile tilapia (*Oreochromis niloticus*) and larval *Cyprinodon variegatus* and *Fundulus heteroclitus* by attaching to and feeding on the epidermis via the process of myzocytosis through an extensible peduncle (Vogelbein et al. 2001, 2002). Exposed fish exhibited erosion of the epidermis ranging from minor disruption with no fish mortalities to severe epidermal erosion resulting in 100% mortality of test fish in as little as 24 hrs (Vogelbein et al. 2002, Lovko et al. 2003). Fish death was speculated to be due to widespread epidermal loss and resultant loss of osmotic regulation (Vogelbein et al. 2001, 2002). However, based on fish mortality and time to fish death in laboratory bioassays with juvenile tilapia and larval cyprinodontid fishes
ichthyocidal activity of CCMP2089 was equivalent to what had previously been reported for strains of *Pfiesteria* purported to produce toxin (Burkholder et al. 2001a, Marshall et al. 2000, Noga et al., 1996). Myzocytotic feeding on fish epidermis was considered to constitute micropredation and was proposed to represent an alternative mechanism of pathogenesis in *P. shumwayae* that accounted for fish mortalities in laboratory assays without invoking the action of a potent exotoxin (Vogelbein et al. 2002). More recent studies by other researchers have confirmed micropredation as the most significant and consistent mechanism of pathogenicity to finfish and shellfish in bioassays with cultures of *P. shumwayae* previously considered to kill fish with toxin (Gordon and Dyer 2005, Shumway et al. 2006).

In addition to *P. piscicida* and *P. shumwayae*, the family Pfiesteriaceae includes several other small (10-20 µm), lightly armored, heterotrophic, peridinoid dinoflagellates. These other species occupy ecological niches similar to *Pfiesteria* spp. and morphologically resemble *Pfiesteria* under light microscopy (Burkholder et al 1998a, Steidinger et al. 2001). Additionally, they are morphologically and genetically related to *Pfiesteria*, having Kofoidian plate tabulations with only very minor differences, making identification difficult, even with electron microscopy techniques (Jeong et al. 2005b, Steidinger et al. 2006, Mason et al. 2003, 2007). They have, therefore, been variously referred to as *Pfiesteria*-like (specifically, *Pfiesteria*-like species, *Pfiesteria*-like organisms [PLOs] or *Pfiesteria*-like dinoflagellates [PLDs]) (Burkholder and Glasgow 1997a, Burkholder et al. 1998a, Burkholder et al. 2001a,b, Litaker et al. 1999, Marshall et al. 2000, Steidinger et al. 2001). Two additional genera have recently been added to the
Pfiesteriaceae including *Cryptoperidiniopsis* (currently comprising only one named species, *C. brodyi*, but as many as 5 additional putative species have been reported) (Steidinger et al. 2006) and *Luciella* (including *L. masanensis* and *L. atlantis*) (Mason et al. 2007). A third genus, *Stoeckeria* (currently including only *S. algicida*), is not currently placed in the Pfiesteriaceae although it is genetically closely related to and morphologically and behaviorally resembles the other *Pfiesteria*-like dinoflagellates, (Jeong et al. 2005b).

The feeding behavior of several of these species has been briefly described and *Luciella* spp. and *Cryptoperidiniopsis* spp. have been reported to attach to (and presumably feed on) fish tissues (Parrow and Burkholder 2003, Steidinger et al. 2006, Jeong et al. 2005a, 2007, Mason et al. 2007). Further, members of *Luciella* and *Cryptoperidiniopsis* have been speculated to potentially produce ichthyotoxic or “bioactive” compounds (Kane et al. 1988, Litaker et al. 1999, Steidinger et al. 2001). However, none of these newly described species are reported to cause fish mortality and some studies have given evidence against ichthyocidal activity in these species (Marshall et al. 2000). The ability of these dinoflagellates to cause fish pathology or mortality and their capacity as micropredatory feeders on fishes or other organisms has not been thoroughly investigated. Using our larval fish bioassay (Lovko et al. 2003) together with histopathological examination and electron microscopy analysis, we examined the capability of several heterotrophic, peduncle-feeding “*Pfiesteria*-like” dinoflagellates to act as micropredators on live fish and to induce pathology and mortality by this mechanism.
Pfiesteria-like dinoflagellates

For this study, we distinguish the above-mentioned PLD species from other small, gymnodinoid athecate, mixotrophic dinoflagellates, such as Gyrodinium spp., Gymnodinium spp. and the toxin-producing Karlodinium veneficum (formerly Gyrodinium galetheanum), that superficially resemble Pfiesteria under light microscopy and have been referred to as “Pfiesteria-like” in the literature (Burkholder et al. 1998, Kane et al. 1998, Marshall et al. 2000). Instead, the focus will be on those lightly armored, heterotrophic dinoflagellates that are members of, or very closely related to, the Pfiesteriaceae.

Table 1 lists the species, strain, source and culture information for the various “Pfiesteria-like” dinoflagellates used in this study. Cultures were provided by Drs. Patricia Tester and Wayne Litaker (National Oceanic and Atmospheric Administration [NOAA], Beaufort, NC), purchased from the Provosoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP, Bigelow Laboratories) or isolated at the Virginia Institute of Marine Science (VIMS), Gloucester Point, VA from environmental samples. All dinoflagellate cultures were grown at VIMS according to established protocols (Lovko et al. 2003) and maintained on cryptophyte prey (Rhodomonas salina strain CCMP-1319 and Rhodomonas sp. strain CCMP-767). Cultures were maintained at 22°C with a 12L:12D light regime (\( @ \sim 130 \text{ mmol m}^{-2} \text{ s}^{-1} \)). Culture media consisted of autoclaved and sterile-filtered Gulf Stream water (GSW) with salinity adjusted to 12 ppt by diluting with DI water. Rhodomonas spp. were grown in 6-well tissue culture plates.
using standard f/2 media (12 or 35 ppt, depending on species). Weekly to monthly transfers of all cultures (dinoflagellates and cryptophytes) were conducted to maintain culture quality and viability. Dinoflagellate species identifications were confirmed by scanning electron microscopy (SEM) of membrane stripped cells (Mason et al. 2003) and by the use of species specific molecular probes (Litaker et al. 1999, 2003, Reece et al. 2002, Vogelbein et al. 2002, Steidinger et al. 2006, Mason et al. 2007).

Fish sources

Larval Fundulus heteroclitus were spawned manually from adults collected in the wild, as described previously (Lovko et al. 2003). Hatchlings were held in mesh baskets in a flow-through system receiving 1-µm filtered water from the York River (salinity ~ 20 ‰), and fed 24-hr old Artemia salina nauplii once daily ad libitum for 6 d. Larvae were held at 12 ‰ for at least 24 hr prior to the start of assays. Larval (7-day post-hatch) Cyprinodon variegatus, were obtained from Aquatic Bio Systems Inc., Ft. Collins, CO. Fish were held at 12 ‰ and used in the assays within 24 hr of arrival. For all assays, fish were randomly distributed into covered tissue culture plates as specified for each individual assay. To minimize waste accumulation in wells during exposure, larvae were fasted for 24 hr prior to initiation and throughout the duration of the bioassays. All bioassays were conducted within a BSL2 cabinet.

Larval Fish Bioassays

**Fundulus heteroclitus and Cyprinodon variegatus comparative assays.** As an initial assessment of pathogenic capability, two separate assays were conducted in 6-well
Table 1. Source, age and identification (including common strain identification designations) of various *Pfiesteria*-like dinoflagellates used in the present studies.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species designation</th>
<th>Source</th>
<th>Culture Age*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCMP2089</td>
<td><em>Pseudopfiesteria shumwayae</em></td>
<td>VIMS, Gloucester, VA</td>
<td>1-4 yrs</td>
</tr>
<tr>
<td>Ps-T1</td>
<td><em>Pseudopfiesteria shumwayae</em></td>
<td>NOAA, Beaufort, NC</td>
<td>&gt;3 yrs</td>
</tr>
<tr>
<td>CCMP2359</td>
<td><em>Pseudopfiesteria shumwayae</em></td>
<td>Bigelow Laboratories</td>
<td>&gt;7 months</td>
</tr>
<tr>
<td>9-02</td>
<td><em>Pfiesteria piscicida</em></td>
<td>NOAA, Beaufort, NC</td>
<td>2-4 yrs</td>
</tr>
<tr>
<td>cell-N</td>
<td><em>Pfiesteria piscicida</em></td>
<td>NOAA, Beaufort, NC</td>
<td>2-6 yrs</td>
</tr>
<tr>
<td>P-11</td>
<td><em>Pfiesteria piscicida</em></td>
<td>VIMS, Gloucester, VA</td>
<td>~1 yr</td>
</tr>
<tr>
<td>P-28</td>
<td><em>Cryptoperidiniopsis brodyi</em></td>
<td>NOAA, Beaufort, NC</td>
<td>1-6 yrs</td>
</tr>
<tr>
<td>CCMP1828</td>
<td><em>Cryptoperidiniopsis sp.</em></td>
<td>Bigelow Laboratories</td>
<td>~8 months</td>
</tr>
<tr>
<td>VIMS314</td>
<td><em>Stoeckeria sp.</em>**</td>
<td>VIMS, Gloucester, VA</td>
<td>&gt;3 yrs</td>
</tr>
<tr>
<td>VIMS1085</td>
<td><em>Stoeckeria sp.</em>**</td>
<td>VIMS, Gloucester, VA</td>
<td>~1 yr</td>
</tr>
<tr>
<td>CCMP1835</td>
<td><em>Luciella masanensis</em></td>
<td>Bigelow Laboratories</td>
<td>~6 months</td>
</tr>
<tr>
<td>P-27</td>
<td><em>Luciella masanensis</em></td>
<td>NOAA, Beaufort, NC</td>
<td>1-5 yrs</td>
</tr>
<tr>
<td>VIMS1041</td>
<td><em>Luciella masanensis</em></td>
<td>VIMS, Gloucester, VA</td>
<td>1-5 yrs</td>
</tr>
</tbody>
</table>

* Length of time cultured on algae from receipt of culture from source.
** These cultures share plate tabulation with *Stoeckeria algicida* (Jeong et al. 2005) but these specific isolates have not been formally evaluated for specific designation so are here referred to only as *Stoeckeria* spp.
tissue culture plates (Falcon®) using larvae (7-day post hatch) of two fish species, either Fundulus heteroclitus or Cyprinodon variegatus. Each treatment consisted of 9-15 replicate wells with 5 fish per well in 10 mL media (clonal dinoflagellate cultures or clean culture media) for 96 hr. In the first assay, larval F. heteroclitus were exposed (9-12 wells per treatment) to one strain each of P. shumwayae (CCMP2089), P. piscicida (P-11), C. brodyi (P-28) and L. masanensis (P-27) at initial dinoflagellate densities of ~500-1000 cells·mL^{-1}. In the second assay, larval C. variegatus were exposed (15 wells per treatment) to one strain each of P. shumwayae (CCMP2089), P. piscicida (cell-N), C. brodyi (P-28), Stoeckeria sp. (VIMS1085) and L. masanensis (VIMS1041) at initial dinoflagellate densities of ~1000-3500 cells·mL^{-1}. The use of different strains of certain species in the two assays was due to availability of cultures at the time the individual assays were conducted. Control treatments consisted of each fish species with fresh culture media without dinoflagellates. Mortalities were monitored at 24-hr intervals for 96 hrs. In both assays, samples were collected at 24-hr intervals from additional wells (not included in mortality assessment) for measurement of dinoflagellate densities and water quality (DO, pH, Temp, NH₃) and for histopathological analysis.

**Membrane insert assay.** To test for ichthyotoxicity in PLD species, we used a simplified version of the membrane insert studies developed previously with P. shumwayae and P. piscicida (see Vogelbein et al. 2002 and Section III of this work). This assay consisted of treatments with and without semi-permeable membrane inserts fitted into the wells of 24-well culture plates (see Vogelbein et al. 2002 for use of membrane inserts). One strain each of P. shumwayae (CCMP2089), C. brodyi (P-28), L. masanensis
(P-27) and *Stoeckeria* sp. (VIMS314) and two strains of *P. piscicida* (9-02 and cell-N) were assayed at initial densities of 5000-10,000 cells·mL\(^{-1}\). In the treatments with membrane inserts (“indirect contact”, fish separated from dinoflagellates by a semi-permeable membrane that allows dissolved substances to cross), one fish (*C. variegatus*, 7-day post-hatch) and 600 µL clean 12 ppt culture media were placed inside the insert and 800 µL dinoflagellate culture was placed in the well exterior of the insert (\(n=12\)). In treatments without inserts (“direct contact” between fish and dinoflagellates), one fish with 2 mL dinoflagellate culture was added to each well (\(n=48\)). An additional 36 wells per treatment were used for periodic water quality and cell density measurements. Control treatments consisted of fish in fresh culture media without dinoflagellates, with and without inserts. Mortality was assessed at 8-hr intervals and cell density and water quality were assessed at 24-hr intervals for 96 hrs.

**“Dose-response” assays.** A set of larval fish bioassays (3 separate assays conducted over a 6-month period) was conducted to evaluate pathogenicity in “high-dose” and “low-dose” treatments of multiple strains of each species. The assays were conducted in 24-well plates (48-54 replicates per treatment) without membrane inserts. Larval fish (*C. variegatus*, 7-day post-hatch) were exposed to two strains each of *P. shumwayae* (CCMP-2359, Ps-T1), *P. piscicida* (9-02, cell-N), *Cryptoperidiniopsis* spp. (P-28, CCMP-1828) and *L. masanensis* (P-27, VIMS-1041) and one strain of *Stoeckeria* sp. (VIMS-314) at initial densities of 1000 and 10,000 cells·mL\(^{-1}\) (except CCMP-1828 which was run only at ~6000 cells·mL\(^{-1}\)). Mortalities were assessed at 12-hr intervals for 96 hrs. A control treatment consisting of fish in wells (48-54 replicate wells) with clean media and without dinoflagellates was used for each of the 3 assays.
**Water quality measurements.** Water quality in the larval fish bioassays was monitored by periodically measuring pH, dissolved oxygen (DO) and reactive ammonia in replicate wells at time intervals specified for each assay. Dissolved oxygen was measured using a Corning Checkmate II™ dissolved oxygen sensor. pH was measured using an Oakton WP pHTestr2™ pH meter. Ammonia was measured using a colorimetric test kit (Hach). Calculations of reactive ammonia were made using the equation of Emerson et al. (1975), which compensates for temperature and pH. Air temperature in the BSL2 hood was recorded from a mercury thermometer at each sampling period.

**Statistical Analysis.** Mortality data from all bioassays were modeled with SPSS statistical software using the Kaplan-Meier estimator for survival analysis. Survival curves were compared using the Tarone-Ware chi-square statistic ($\chi^2$) to determine significant differences in survival between treatments, species, and strains.

*Fish Epidermis Histopathology and Electron Microscopy (SEM/TEM)*

For histopathological analysis, fish collected at designated sampling times from the bioassays, as well as unexposed control fish, were killed by overdose with tricaine methanesulfonate (MS-222; 200-300mg/L), fixed in 10% neutral buffered formalin and processed by routine methods for paraffin histology (Prophet et al., 2002). For electron microscopy, *C. variegatus* (14-24 day post-hatch) were narcotized with MS-222, thoroughly rinsed (three changes of 12ppt ASW), then exposed to either *Stoeckeria* sp. strain VIMS-314 (SEM only), *L. masanensis* strain P-27 or *C. brodyi* strain P-28 (5-15 min exposure time, dinoflagellate density @ ~1000-5000 cells·mL$^{-1}$). This time frame allowed attachment of sufficient numbers of the dinoflagellates to the epidermis of fish.
Exposed fish were fixed whole in 4% glutaraldehyde /5% paraformaldehyde in 0.1M sodium cacodylate buffer (pH = 7.2) and processed for scanning and transmission electron microscopy by routine methods as covered in detail in Section III of this work.

RESULTS

General Observations

As observed using light microscopy, all tested species and strains exhibited taxis towards, attachment to, and apparent feeding on the epidermis of live fishes, although the intensity of this behavior varied greatly between species and, in some cases, among strains. Qualitatively, the greatest degree of attraction and attachment occurred in all tested strains of *P. shumwayae*. All tested strains of *Cryptoperidiniopsis* spp. and *Stoeckeria* sp. also demonstrated strong attraction and attachment, but to a lesser degree than observed for *P. shumwayae*. In all of the above strains, cells were observed to move rapidly towards fish, aggregating in numerous groups of >50 cells, primarily on the caudal peduncle and fin, pectoral fins, gill covers, and various areas of the head including the mouth and eyes. Cells moved rapidly in and out of these aggregations until contact was made with the fish epidermis. Upon attachment to the epidermis (via an extended peduncle), cells became relatively motionless, although flagellar movement was still apparent. Swelling of cells occurred after several seconds following attachment, with some cells increasing in diameter by ~2x. Movement of fish cell cytoplasm into the peduncle and epitheca of feeding cells was observed in some instances. After ~<1min., swollen cells began to spin, detaching themselves from the fish epidermis and slowly
swimming away. In some instances, cells were observed to settle on the bottom of the culture vessel and round up, losing their peridinoid shape. Within 24 hr, numerous cysts with dark inclusions were present on the bottom of the assay vessel, often in large masses. Within 8-24 hours, exposed fish exhibited agitation, rapid ventilation and gross signs of pathology including epidermal sloughing and fraying of the fins. Similar attachment and pathology was evident in *P. piscicida* strain 9-02 as early as 8 hr, but only in the “high” cell density exposures (>10,000 cells·mL⁻¹). No appreciable cyst formation was observed in any experiments conducted with this strain.

Strong taxis and attachment of individual *L. masanensis* cells or small aggregates of <10 cells (strain P-27) were frequently observed throughout the duration of the bioassays (96 hr), along with apparent sloughing of fish epidermis, although no mortalities occurred in exposures with this isolate and no pathology was observed by histological analysis. Aggregates of <20 cells (or more rarely 50 cells) were observed infrequently in *L. masanensis* strains VIMS-1041 and CCMP-1835, but little taxis was observed. As with *P. piscicida*, no appreciable cyst formation was observed in any of the *L. masanensis* strains. No obvious taxis, little attachment to fish epidermis (usually individual cells or small aggregates of <10 cells only), no observable pathology and no visible cyst formation occurred in assays with *P. piscicida* strains cell-N or P-11.

*Larval fish assays*

*Fundulus heteroclitus* and *Cyprinodon variegatus* comparative assays.

*Fundulus heteroclitus* exhibited significant mortalities only in the presence of *P. shumwayae* (100% at 72 hr) (Fig. 1a). Low level mortalities occurred with *L. masanensis*
Figure 1. Results of comparative larval fish assays with Fundulus heteroclitus and Cyprinodon variegatus exposed to multiple “Pfiesteria-like” species. a and b, Cumulative mortalities of F. heteroclitus (a) and C. variegatus (b). Error bars represent the standard errors of the mean among replicate wells in each treatment (n=9-12 for F. heteroclitus treatments, n=15 for C. variegatus treatments). Mortalities of F. heteroclitus in treatments with L. masanensis and C. brodyi (panel a) were not significantly different from control (Tarone-Ware $\chi^2$, p>0.10). Mortalities in C. variegatus exposed to L. masanensis and P. piscicida (panel b) were not significantly different from control (Tarone-Ware $\chi^2$, p>0.05). c and d, Mean dinospore densities in treatments with F. heteroclitus (a) and C. variegatus (b). Error bars represent standard deviations among counts in replicate wells (n=3 for all treatments and time points).
Figure 1. Fish mortality and dinoflagellate density results of comparative larval fish assays with *F. heteroclitus* and *C. variegatus* exposed to multiple “Pfiesteria-like” species.
(20% at 96 hr) and *C. brodyi* (21.6% at 96 hr) but these were not significantly different from controls (11% at 96 hrs) (Survival analysis, Tarone-Ware $\chi^2$, p>0.10). No mortalities occurred in exposures with *P. piscicida*. Significant mortalities (>10%) of *C. variegatus* occurred in exposures with *P. shumwayae* (100% at 24 hr), *C. brodyi* (100% at 96 hr) and *Stoeckeria* sp. (76% at 96 hr) (Fig. 1b), but initial cell densities were ~2-4x greater than in the *F. heteroclitus* assay (Fig. 1c,d). Low level mortalities of *C. variegatus* occurred in *L. masanensis* (<10% at 96 hr) and *P. piscicida* (2.75% at 96 hr). These mortalities were significantly different from survival in controls for *L. masanensis* (Tarone-Ware $\chi^2$, p=0.032) but were not significantly different from controls for *P. piscicida* (Tarone-Ware $\chi^2$, p>0.50).

Dinospore densities declined dramatically in *P. shumwayae* in both assays during the first 24 hr as many free-swimming cells encysted after feeding on the fish (Fig. 1c,d). Cell densities in *C. brodyi* increased ~6-fold by 48 hr in the presence of *F. heteroclitus* before declining (Fig 1c) but increased steadily throughout the duration of the *C. variegatus* assay (Fig. 1d) (from 2110 cells·mL$^{-1}$ initially to 6950 cells·mL$^{-1}$ at 96 hr). *Stoeckeria* sp. demonstrated a similar increase in cell density in the presence of *C. variegatus* (Fig. 1d). *Luciella masanensis* exhibited a similar increase/decline in the presence of *F. heteroclitus*, but did not demonstrate an increase in cell density in the presence of *C. variegatus* (Fig. 1c,d). *Pfiesteria piscicida* did not exhibit an increase in density in either assay (Fig. 1c,d). Water quality remained adequate in all treatments in both assays and was similar between experimental treatments and controls. Dissolved oxygen remained above 5.8 mg·L$^{-1}$ and reactive NH$_3$ remained below 0.40 mg·L$^{-1}$ throughout the duration of both assays. pH was generally ~7.5 but increased to 8.0 in
some treatments after fish mortalities occurred. Ambient temperature fluctuated between 23.5 and 25°C.

**Membrane Insert Assay.** No mortalities occurred in any treatment when membrane inserts were used to prevent direct contact between the larval fish and the dinoflagellates (Indirect contact: Fig. 2a). With direct physical contact, significant mortalities occurred with *P. shumwayae* (100% at 32 hr), *C. brodyi* (94% at 96 hr), *Stoeckeria* sp. (96% at 96 hr) and, to a lesser degree, *P. piscicida* strain 9-02 (21% at 96 hr) (Fig. 2b). No mortalities occurred with *L. masanensis* or *P. piscicida* strain cell-N (Fig. 1b). Survival among all treatments exhibiting mortalities was significantly different (Tarone-Ware $\chi^2$, p<0.001) except for *Stoeckeria* sp. and *C. brodyi* which were essentially identical (Tarone-Ware $\chi^2$, p>0.90).

When not in direct contact, dinospore densities in all treatments remained steady or declined (Fig. 2c). With direct contact, density of *P. shumwayae* dinospores declined sharply in the first 24 hr (from 5400 cells·mL$^{-1}$ initially to 700 cells·mL$^{-1}$ at 24 hr), presumably due to post-feeding encystment (Fig. 2d). Cell density was not measured in *P. shumwayae* after 24 hr because all fish had expired. When in direct contact with fish, mean densities of *C. brodyi*, *L. masanensis* and *P. piscicida* dinospores generally increased over the duration of the assay (~2-3 fold) although variance among replicates was large (Fig 2d). Density of *L. masanensis* dinospores did not increase when in direct contact with fish (Fig 2d). Water quality parameters were similar to those reported for the previous assays and remained adequate throughout the duration of the assay.

**“Dose-response” Assays.** No fish mortalities occurred at either density in exposures with *P. piscicida* strain cell-N, *Cryptoperidiniopsis* sp. strain CCMP-1828, *L.
Figure 2. Results from larval fish bioassays with *Cyprinodon variegatus* exposed to multiple species of *Pfiesteria*-like dinoflagellates with membrane inserts to prevent direct contact between fish and dinoflagellates (indirect exposure; a and c) and without inserts (direct exposure; b and d). a and b, Cumulative fish mortalities in treatments with membrane inserts ($n=12$) (a) and without membrane inserts ($n=48$) (b). No mortalities occurred in any treatment with membrane inserts (a). c and d, mean dinospore densities in treatments without direct contact between fish and dinoflagellates (c) and with direct contact (d). Error bars represent standard deviations among counts in replicate wells ($n=3$ for all treatments and time points).
Figure 2. Results of 24-well plate larval fish assays with *C. variegatus* exposed to multiple “*Pfiesteria*-like” species with and without the use of membrane inserts to prevent direct contact between dinoflagellates and fish.
Figure 3. Results from “low” dose (a) and “high” dose (b) larval fish bioassays with *Cyprinodon variegatus* exposed to initial densities of “*Pfiesteria*-like” dinoflagellates of 1000 and 10000 cells·ml\(^{-1}\). Only treatments resulting in fish mortality are represented on graph. No mortalities occurred at either density in *P. piscicida* (strain “cell N”), *L. masanensis* (strains VIMS1041 and CCMP1835), *Cryptoperidiniopsis* sp. (strain CCMP1828, only assayed at 6,000 cells·ml\(^{-1}\)) or controls (no dinoflagellates). \(n=48\) for all treatments except \(n=46\) for CCMP1835 @ 10,000 cells·ml\(^{-1}\).
Figure 3. Fish mortality and dinoflagellate density results of “high-dose” and “low-dose” larval fish assays with *C. variegatus* exposed to multiple “*Pfiesteria*-like” species.
*masanensis* (both strains), or in controls (these treatments are not represented in Fig. 3). All other treatments exhibited mortalities at both 1000 and 10,000 cells·mL⁻¹ (Fig. 3).

Both strains of *P. shumwayae* resulted in similar patterns of fish mortality [100% fish mortality in 72 hr at 1000 cells·mL⁻¹ (Fig 3a) and in 24 hr at 10,000 cells·mL⁻¹ (Fig. 3b)], although differences in fish survival between the two *P. shumwayae* strains were significant at both densities (Tarone-Ware $\chi^2$, $p\leq0.002$). Mortalities of fish exposed to *Stoeckeria* sp. and *C. brodyi* (strain P-28) were similar at both densities with 23% and 40% mortality (respectively) at 96 hr at 1000 cells·mL⁻¹ (Fig. 3a) and 83% and 89% (respectively) at 96 hr at 10,000 cells·mL⁻¹ (Fig. 3b). Survival in *Stoeckeria* sp. and *C. brodyi* (strain P-28) exposures was not significantly different at 1000 cells·mL⁻¹ (Tarone-Ware $\chi^2$, $p>0.05$) but was significantly different at 10,000 cells·mL⁻¹ (Tarone-Ware $\chi^2$, $p>0.001$). Fish mortalities in *P. piscicida* strain 9-02 were minor at both densities (4.2% at 96 hr at 1000 cells·mL⁻¹, 16.7% at 96 hr at 10,000 cells·mL⁻¹) (Fig. 3) and survival was significantly higher than in all other treatments with mortalities within either density (Tarone-Ware $\chi^2$, $p\leq0.008$). Water quality parameters were similar to those reported for the previous assays and remained adequate throughout the duration of the assay.

**Histopathology and Electron Microscopy (SEM/TEM)**

Histologically and ultrastructurally, the epidermis of unexposed fish appeared intact and undamaged (Figs. 4a,b, 5f,g). In larval *F. heteroclitus*, it was comprised of squamous epithelial cells in a layer 4-8 cells in thickness (Fig 4a), whereas the epidermis of *C. variegatus* larvae was more delicate and only 2-4 cells in thickness (Fig. 4b). In both fishes, pavement cells contained elongated, basophilic nuclei (Fig 4a,b). The dermis
Figure 4. Photomicrographs demonstrating damage to epidermis of larval *Fundulus heteroclitus* and *Cyprinodon variegatus* exposed to several *Pfiesteria*-like dinoflagellates. Scale bars in all panels = 10µm (H&E). 

a and b, epidermis of unexposed (control) fish demonstrating the smooth, intact appearance and elongated nuclei (N) of undisrupted epidermal cells. 

a, epidermis of unexposed larval *F. heteroclitus* demonstrating a relatively thick epidermis (4-6 cells) above a delicate basement membrane (arrow heads). 

b, epidermis of unexposed larval *C. variegatus* demonstrating a thinner epidermis (2-3 cells thick) and lack of dermis at this early stage of development. Arrow heads show the basement membrane delineating the lower boundary of the epidermis. 

c, epidermis of *F. heteroclitus* exposed to *Cryptoperidiniopsis brodyi* (72 hr exposure, initial density = ~500 cells·ml⁻¹) demonstrating a relatively large (>100µm) epidermal erosion extending into the epidermis but not reaching the basement membrane (arrowheads). Note the presence of a more developed dermis beneath the basement membrane. 

d, epidermis of *C. variegatus* exposed to *C. brodyi* (96 hr exposure, initial density = ~2000 cells·ml⁻¹) demonstrating a small (~20µm) erosion extending down to the basement membrane (arrowhead). 

e, severely damaged epidermis of larval *C. variegatus* exposed to *Stoeckeria* sp. strain VIMS314 (24 hr exposure, initial density = 9800 cells·ml⁻¹). Note the disruption of the epidermis with enlargement and sloughing of cells (arrowhead) and evidence of nuclear pycnosis (arrows). 

f, epidermis of *C. variegatus* exposed to *Luciella masanensis* strain P27 (96 hr exposure, initial density = 6300 cells·ml⁻¹). Observable pathology was limited to localized areas of mild erosion (arrow) and separation of epidermis from basement membrane (arrowhead).
Figure 4. Histological micrographs demonstrating damage to epidermis of *F. heteroclitus* and *C. variegatus* exposed to species of *Pfiesteria*-like dinoflagellates.
was minimal at this stage of development and scales were reduced or absent (Fig. 4 a, b), although a more developed dermis was occasionally observed in *F. heteroclitus* specimens (Fig. 4c). The epidermis of fish in direct contact with *Pfiesteria*-like dinoflagellates exhibited varying degrees of erosion ranging from small focal lesions (~20-100μm) surrounded by intact epidermis (Fig. 4c,d) to widespread sloughing of epidermal cells (Fig. 4e). Similar damage was frequently observed in exposures with all evaluated strains of *P. shumwayae*, *C. brodyi* and *Stoeckeria* sp., with severity depending on exposure time and dinoflagellate cell density. Severe pathology was occasionally observed in fish exposed to *P. piscicida* strain 9-02, but only at higher cell densities and/or longer exposure times (see previous section). Only minor pathology was observed in exposures with *P. piscicida* (strain cell-N) and *L. masanensis* (strains P-27 and VIMS-1041), often consisting of only scant areas of eroded epidermis and mild vacuolization and lifting or separation of epidermal layers (Fig. 4f). The epidermis of most fish from these treatments appeared intact and undisrupted.

With scanning and transmission electron microscopy, dinospores of all species attached to fish epidermis via the peduncle (Figs. 5-7). Ultrastructurally, the peduncle was occasionally observed tightly opposed to the epidermis (Fig. 5c) but was also often associated with sloughed cellular debris (especially in *L. masanensis*) (Fig. 5d). Large food vacuoles, frequently containing large lipid droplets (Fig. 5c,d) were observed in attached cells. Some cells appeared greatly enlarged (15-20 μm dia. in cells with large food vacuoles vs. 6-10 μm in “normal” cells) with food vacuoles occupying most of the cell volume (Fig 5c,d). Additionally, granular material (possibly ribosomes and other
Figure 5. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of attachment and feeding by dinospores of several “Pfiesteria-like” species on epidermis of larval Cyprinodon variegatus. Scale bars in all panels = 3 µm. a, SEM showing a small aggregation of C. brodyi dinospores (arrowheads) attached to fish epidermis (E). b, Dinospore (D) of Stoeckeria sp. strain VIMS314 attached to fish epidermis (E) via peduncle (P). c, Two dinospores (D) of Luciella masanensis strain P27 attached and feeding on fish epidermis. The peduncle of the cell on the left (arrowhead) tightly apposed to the fish epidermis (E). The base of the peduncle of the cell on the right can be seen emerging from the cell at the peduncular opening (arrow). Note the extreme difference in size between the two dinospores. The cross section of the cell on the right (~18µm) is approximately 3 times the length of the cell on the left (~6µm). Nearly the entire cross section of the larger cell is occupied by food vacuoles containing large lipid droplets (L). d, L. masanensis dinospore with extended peduncle associated with some sloughed cellular material. Note the large food vacuole (V) occupying much of the cellular volume and marginalizing the nucleus (N). e, a C. brodyi dinospore feeding on sloughed fish epidermis. Note the presence of granular-appearing fish cell material (arrows) present in the peduncle (arrowhead) as well as in a large food vacuole (F) occupying much of the feeding cell. f, SEM of unexposed fish epidermis demonstrating defined margins (arrows) and pattern of epithelial microridges on intact epidermal cells (E). g, TEM of unexposed fish epidermis demonstrating prominent epithelial microridges (arrow heads) and the elongated nuclei (N) of an intact epidermal cell.
Figure 5. Attachment and feeding of dinospores of several “Pfiesteria-like” species on epidermis of C. variegatus
cellular material from fish epidermal cells) was frequently observed in the tube-like peduncle as well as in the food vacuoles (Fig. 5e), indicating ingestion of epidermal material.

The nature of attachment of the dinoflagellate peduncle to the epidermis of fish varied between species (Figs. 6 and 7). The peduncle of *L. masanensis* widened at the distal end (~3-5 times the width at the proximal end) and appeared veil-like, spreading along the surface of fish epidermis, often over areas of damaged or degenerating epidermis (Fig. 6a and 7a). Filipodial extensions were present at the distal edge of the peduncle (Fig. 6a), much like that described for *Pseudopfiesteria shumwayae* in the previous section. In contrast, the peduncle of *C. brodyi* was straight and more tube-like and appeared to penetrate the epidermal surface when viewed under SEM (Fig. 6b). Ultrastructurally, the distal end of the attached peduncle of *L. masanensis* was observed to include membrane-bound extensions small, electron-dense “granules” (Fig. 7a), similar to what has been observed in *P. shumwayae* (Vogelbein et al. 2002, Section III of this work). Membrane-bound peduncular extensions were also present in *C. brodyi*, but appeared more “rhizoid-like” and penetrated into the epidermal cells (Fig. 7b). Electron-dense “granules” were not observed in *C. brodyi*. Although the epidermal surface immediately surrounding the site of attachment of *C. brodyi* sometimes appeared perforated or otherwise damaged (Fig. 6b), these areas, or the areas adjacent to attachment, typically appeared undamaged (Figs. 6b and 7b). In contrast, attached cells of *L. masanensis* were typically associated with areas of damaged, sloughed or degenerating epidermis.
Figure 6. Comparison of morphology of peduncle attachment to larval *Cyprinodon variegatus* epidermis between *L. masanensis* (a) and *C. brodyi* (b) with SEM. Scale bars in both panels = 1µm. a, peduncle (P) of *L. masanensis* dinospore (D) appearing “veil-like”, enveloping an area of damaged fish epidermis (E). Filopodial cytoplasmic extensions (arrows) are present on the distal end of the peduncle. b, the tube-like peduncle (P) of a *C. brodyi* dinospore (D) appearing to penetrate the fish epidermis (E). Note the perforated appearance of the fish epidermis (arrows) and the loss of the fine epithelial microstructure in the area immediately adjacent to the site of attachment/penetration. The epithelial microridges remain intact just a short distance away from the attachment site (bottom right corner of panel).
Figure 6. SEM comparison of peduncle attachment to fish epidermis by *L. masanensis* and *C. brodyi*.
Figure 7. Comparison of ultrastructure of peduncle attachment to larval *C. variegatus* epidermis between *L. masanensis* (a) and *C. brodyi* (b) with TEM. Scale bars in both panels = 1µm. **a**, peduncle (P) of *L. masanensis* appears to spread along the surface of degenerating fish epidermis (E). Membrane-bound extensions (arrow heads) are present on the distal end of the peduncle. Also present are electron-dense “granules” (arrows). **b**, membrane-bordered extensions (arrow heads) of the peduncle (P) of a *C. brodyi* dinospore are shown to penetrate the fish epidermal cell (E). Note the preservation of the fine ultrastructure (arrows) on the surface of the fish epidermis immediately adjacent to the site of attachment and penetration.
Figure 7. TEM comparison of peduncle attachment to fish epidermis by *L. masanensis* and *C. brodyi*. 
DISCUSSION

The assertion of ichthyotoxicity in *Pfiesteria* has been primarily based on fish death in laboratory bioassays (Burkholder et al. 1992, 2001a,c, Noga et al. 1993, Glasgow et al. 2001a,b) which has continued to be used by some researchers as a defining criterion to characterize a culture as toxic (Marshall et al. 2000, Burkholder et al. 2001a,c, Glasgow et al. 2001a,b, Burkholder et al. 2005). Our previous work demonstrated the ability of *P. shumwayae* (strain CCMP2089) and *P. piscicida* (strain 9-02) to cause mortality in fishes in laboratory bioassays by micropredatory feeding on the epidermis of live fish without involvement of a toxin, although ichthyocidal activity in these two cultures is significantly different (Vogelbein et al. 2002, Section III of this work). Here, we demonstrate the ability of many “*Pfiesteria*-like” dinoflagellates to act as micropredators on live fish by the mechanism of myzocytosis. However, the ability to cause pathology and mortality in fishes by this mechanism varies greatly among the species and strains evaluated, with some strains incapable of inducing any pathology whatsoever.

The *Pfiesteria*-like dinoflagellates described thus far (Jeong et al. 2005b, Steidinger et al. 2006, Mason et al. 2007) demonstrate feeding behavior on algae similar to what has been reported for *Pfiesteria* and *Pseudopfiesteria*, including myzocytotic feeding through an extensible peduncle, directed chemotaxis towards prey and formation of large aggregations around prey (Seaborne et al. 1999, 2006, Steidinger et al. 2001, 2006, Jeong et al. 2005a, 2007). Earlier reports speculated that some of these species may be ichthyotoxic or contain bioactive compounds, but no additional information was given.
to support these assertions (Kane et al. 1998, Litaker et al. 1999, Steidinger et al. 2001). A few studies report the ability of several of these species to feed on fish epidermis but indicate that they do not cause fish pathology or mortality (Steidinger et al. 1996, Mason et al. 2007). Here, we demonstrate that *P. shumwayae* (CCMP 2089, CCMP 2359, Ps-T1), *C. brodyi* (strain P-28) and *Stoeckeria* spp. (strains VIMS-314 and VIMS-1085) all fit within the published criteria that have defined cultures as highly pathogenic (actively toxic, or “Tox-A”) (Burkholder et al. 2001a,b,c, Glasgow et al. 2001a,b). However, survival of fish exposed to *C. brodyi* and *Stoeckeria* spp. was significantly higher than for *P. shumwayae* at comparable dinospore densities. Even at 10,000 cells·mL\(^{-1}\), *C. brodyi* and *Stoeckeria* sp. were unable to cause 100% mortality in fishes within 96 hrs whereas *P. shumwayae* (strain CCMP2089) consistently caused 100% mortality within 96 hrs at densities >500 cells·mL\(^{-1}\) (Vogelbein et al. 2002, Lovko et al. 2003, Sections II and III of this work). This study, as well as our previous work (see Section III), has demonstrated the ability of *P. piscicida* strain 9-02 to cause fish pathology and mortality, but only at initial dinoflagellate densities ≥ 5000 cells·mL\(^{-1}\). No other tested strain of *P. piscicida* (strains P-11 and cell-N) and no tested strains of *L. masanensis* elicited fish mortality, even at initial densities of 10,000 cells·mL\(^{-1}\). As demonstrated previously with *P. shumwayae* and *P. piscicida* (Vogelbein et al. 2002, Section III of this work), mortalities in fishes did not appear to involve soluble toxin as evidenced by the lack of pathology or mortality when fish were separated from dinoflagellates by a semi-permeable membrane.

The present study demonstrates significant differences in fish mortality between the *F. heteroclitus* and *C. variegatus* assays. Although the strains of *P. piscicida* and *L. masanensis* used between the two assays were different, neither of these species
demonstrated appreciable capability of inducing significant fish mortality regardless of the fish species used. Identical *P. shumwayae* and *C. brodyi* strains were used in the assays with both fish species and both dinoflagellates caused greater fish mortality in assays with *C. variegatus*. Although this could have been due to the disparity in dinoflagellate densities used in the two studies, it is also feasible that the overall larger size and greater epidermal thickness in *F. heteroclitus* larvae may account for the disparity. Differences in cumulative mortality between *F. heteroclitus* and *C. variegatus* exposed to *P. shumwayae* appear related to a dose-effect, due to the disparity in dinospore densities between the two assays. However, the reason for the disparity in mortality in the *C. brodyi* exposures is less obvious. In the *F. heteroclitus* assay, *C. brodyi* densities increased in the first 24 hrs to levels identical to *T₀* densities in the *C. variegatus* assay, and continued to increase up to 48 hrs (with a subsequent decline) but with no significant mortalities for the duration of the assay (Fig. 1). Histological analysis revealed that small (~20µm), shallow epidermal lesions caused by *C. brodyi*, were capable of penetrating through to the basement membrane of *C. variegatus* (Fig. 4d). However, much larger (>100µm) lesions caused by *C. brodyi* penetrated only partially through the thicker epidermis of *F. heteroclitus* (Fig. 4c). Thus, the ability to cause mortality in fishes could, in part, be dependent on the species and developmental stage of the fishes tested, as well as the ability of the dinoflagellate to feed on the prey species.

Aspects of taxis, attachment, feeding and cell growth varied among species and strains but appeared to be roughly correlated to pathogenicity. Generally, the cultures that caused higher fish mortality (>20%) (*P. shumwayae, C. brodyi, Stoeckeria* spp.) also exhibited a greater degree of attachment, with larger and more numerous aggregations of
cells and easily observable pathology. These cultures also exhibited massive encystment after feeding, although only *P. shumwayae* demonstrated a significant decline in cell density just after initiation of assays and just before, or concomitant with, massive fish mortalities. This occurred consistently regardless of initial cell density but did not occur in treatments where dinoflagellates were physically separated from fish by a semi-permeable membrane. Cultures resulting in little or no fish mortalities (≤20%) (*P. piscicida* strain cell-N, *L. masanensis* strains CCMP-1835 and VIMS-1041) exhibited little taxis, attachment and feeding, and induced no observable pathology. *Luciella masanensis* strain P-27 exhibited chemotaxis, attachment and feeding, as confirmed by SEM, TEM and direct observation, but did not cause any pathology (as confirmed by histopathology) or mortality. Similarly, *P. piscicida* strain 9-02 exhibited taxis, attachment and feeding but caused observable pathology and low-level mortality only at much higher cell densities. Additionally, no appreciable cyst formation was observed in any of these cultures. Dinospore densities of pathogenic cultures generally increased in the presence of fish, but not in the non-pathogenic strains. *Pfiesteria piscicida* strain 9-02 demonstrated an increase in cell density in the presence of fish, although it was only mildly pathogenic. In all tested cultures, cell densities generally did not increase when dinoflagellates were separated from fish by a semi-permeable membrane.

The peduncle as a feeding organelle, as observed in the *Pfiesteria*-like dinoflagellates investigated here, is essentially identical to feeding structures reported for many other free-living heterotrophic dinoflagellates (Lee 1977, Spero 1982, Ucko et al. 1994, Hansen and Calado 1999) as well as similar structures present in some parasitic dinoflagellates such as *Amyloodinium ocellatum*. The free-swimming dinospore stage of
*A. ocellatum* contains a rhizoid and peduncle complex that emerges from the sulcus and is involved in infection (Landsberg et al. 1994). The rhizoid functions as an attachment organelle and a peduncle-like structure, which may also be associated with a feeding apparatus, functions to inject lytic enzymes into the host (Cachon and Cachon 1987, Landsberg et al. 1994). The penetrating “rhizoid-like” structure described here for *C. brodyi* and the electron dense granules described for *L. masanensis* and *P. shumwayae* may function in attachment, penetration and ingestion of prey. A close genetic relationship between *A. ocellatum* and the Pfiesteriaceae has been reported, and there has been argument for including the Pfiesteriaceae in the Blastodiniales, a group composed of highly variable marine ectoparasites (Litaker et al. 1999, Seaborne et al. 2006). Thus, it could be speculated that certain aspects of peduncle function and structure could be representative of evolutionary relationships between free-living and parasitic dinoflagellates.

The structure of the peduncle differed between the species evaluated in the current study. It could be speculated that a “penetrating” type of peduncle, such as that observed in *C. brodyi*, is capable of more acutely damaging fish epidermis than the spreading “veil-like” peduncle of *L. masanensis*, thus accounting for the differences in pathology and mortality observed between these two species. However, previous work with a highly pathogenic strain of *P. shumwayae* (Vogelbein et al. 2002, and Section III of this work) describes a broad, spreading peduncle, similar to that described here for *L. masanensis* strain P-27, with similar filipodial extensions and electron dense granules. Both *P. shumwayae* and *L. masanensis* strain P-27 also demonstrated strong chemoattraction towards fish and fish tissues, attachment to fish epidermis, and the formation of
extremely large, engorged cells. Despite this however, *L. masanensis* strain P-27 did not cause observable pathology or mortality to fish while *P. shumwayae* consistently results in severe fish mortality and pathology.

**CONCLUSION**

Much of the early work that focused on the relationship of *Pfiesteria* spp. to fish kills used “presumptive *Pfiesteria* cell counts” based on evaluation of water samples under light microscopy, as an initial causative determinant (Burkholder et al. 1992, 1995, 2001c, Burkholder and Glasgow 1997a, Glasgow et al. 2001, Magnien 2001). This is problematic because numerous other small dinoflagellates were likely common in estuaries where *Pfiesteria* was present (Burkholder et al. 1998, Steidinger et al. 2001). Many of these organisms are indistinguishable under light microscopy and can even be difficult to distinguish with electron microscopy, due to the similarities in plate tabulations and the difficulties in visualizing plate patterns and morphology (Burkholder et al. 1995, 1998, Steidinger et al. 2001, 2006, Mason et al. 2003, 2007, Jeong et al. 2005b). Therefore, accurate identification to genus requires more advanced EM or molecular methods, many of which were not well developed until well after the initial implication of *Pfiesteria* in fish kills (Bowers et al. 2000, Oldach et al. 2000, Steidinger et al. 2001, Litaker et al. 2003, Mason et al. 2003, Tengs et al. 2003). As demonstrated here, many of these organisms are capable of causing pathology and mortality to varying degrees in laboratory assays. Thus in addition to the use of fish mortality in bioassays as
a sole criterion for determining putative toxicity, the use of presumptive cell counts further complicates attempts at defining a relationship between *Pfiesteria* and fish kills.

The results of the studies presented here clearly demonstrate that other members of the Pfiesteriaceae (as well as the “*Pfiesteria*-like” *Stoeckeria*) can cause pathology and mortality in fishes at cell densities and at rates similar to what we have previously reported for *P. shumwayae*. Other researchers have demonstrated attachment and feeding on fish tissues by *Cryptoperidiniopsis brodyi* and *Luciella masanensis*. Our current studies confirm these observations and demonstrate that this behavior can result in fish mortality given sufficient exposure duration and dinoflagellate density. Vogelbein et al. (2002) suggested that micropredatory feeding must be considered when using fish mortality as the sole determinant of “toxicity” with *Pfiesteria* and *Pfiesteria*-like dinoflagellates. The results presented here clearly indicate that many co-occurring heterotrophic dinoflagellates, similar in morphological appearance and behavior to *P. piscicida* and *P. shumwayae*, can demonstrate ichthyocidal activity in laboratory fish bioassays although with wide variability among species and strains. This information suggests that toxicity and pathogenicity in the Pfiesteriaceae must be reconsidered. It is important to note that the environmental relevance of the mechanism of pathogenicity described herein is unknown. Although these heterotrophic dinoflagellates can cause pathology and kill fish in a closed system such as a laboratory bioassay, we have no evidence that this actually occurs in the natural environment.
REFERENCES


SECTION V

FACTORS INFLUENCING PATHOGENICITY IN THE MICROPREDATORY DINOFLAGELLATES PFIESTERIA PISCICIDA AND PSEUDOPFIESTERIA SHUMWAYAE
ABSTRACT

Cultures of *Pfiesteria piscicida* and *Pseudopfiesteria shumwayae* exhibit profound differences in their pathogenicity to fishes. These differences are reported to be due to variability in toxin production, primarily influenced by nutritional history of a culture, especially as related to its exposure history with live fish. Our previous work has demonstrated that both *P. piscicida* and *P. shumwayae* are ichthyocidal, but without involvement of a potent exotoxin. However, in laboratory assays with larval fish, *P. shumwayae* is consistently and significantly more ichthyocidal than *P. piscicida*. Using larval fish bioassays, a chemotaxis assay, and comparative morphometric analyses (including electronic particle characterization and differential cell counts), we investigated the mechanisms underlying these differences. Comparative assays with multiple strains of each species demonstrated that variability in pathogenicity among strains was comparable to the variability found within a single reference strain (assayed multiple times) for each species. These differences were unrelated to the attraction of the dinoflagellates to fish tissues. Instead, differences in ichthyocidal activity between these two species were related to their capacity to grow and reproduce after feeding on fish or algal prey. A greater increase in cell size and a larger degree of encystment and reproductive growth was observed in *P. shumwayae* than in *P. piscicida* when both species were cultured with fish prey. Conversely, *P. piscicida* demonstrated a greater increase in cell size and subsequent dinospore proliferation than *P. shumwayae* when both species were fed on algal prey. The results of these studies indicate that differential ichthyocidal activity is modulated by biological and life history factors other than toxin
production and suggest that historical perception of toxicity in these dinoflagellates was erroneous and related to growth rate in response to prey type.

INTRODUCTION

The heterotrophic dinoflagellates *Pfiesteria piscicida* and *Pseudopfiesteria shumwayae* are purported to produce a potent ichthyotoxin responsible for massive fish lesion and mortality events in estuarine systems of the mid-Atlantic and southeastern United States (Burkholder et al. 1995a, 2001b, Lewitus et al. 1995, Glasgow et al. 2001b). Although genetically distinct, these two species were believed to be identical in their biology, ecology, life history, toxicity and behavior towards fishes (Burkholder et al. 2001a, Glasgow et al. 2001b). Toxicity in *P. shumwayae* and *P. piscicida* has been reported to vary based on a number of factors including the age of the culture and its nutritional history, primarily its previous exposure, or lack of exposure, to live fishes or fish secreta/excreta (Burkholder et al. 1992, Burkholder and Glasgow 1997a, Burkholder et al. 2001a, Glasgow et al. 2001b). Purportedly toxic strains, which can range from “highly toxic” to “weakly toxic”, have been reported to require the presence of live fish to induce toxicity (Burkholder and Glasgow 1997a, Burkholder et al. 2001a, Glasgow et al. 2001). Strains previously exhibiting “toxicity” (as inferred from pathogenicity to fishes in a laboratory bioassay) were reported to lose the ability to cause fish mortality when cultured without fish for a sufficient duration (usually weeks to months) (Burkholder and Glasgow 1997a,b, Marshall et al. 2000, Burkholder et al. 2001a,c, Glasgow et al. 2001b). Additionally, numerous “naturally occurring” strains that are
incapable of producing toxin and killing fish have been reported (Burkholder et al. 2001a).

A series of “functional types” (“TOX-A”, “TOX-B” and “NON-IND”), used to describe the toxicity status of cultures of *P. piscicida* and *P. shumwayae*, had been developed (Burkholder et al. 2001a,b,c, Glasgow et al. 2001a,b). “Highly toxic” (“Tox-A”) strains of *P. piscicida* and *P. shumwayae* are reported to exhibit a stronger response to fish, in terms of directed swimming behavior and chemosensory attraction, compared to strains that are less toxic (“Tox-B”) or non-toxic (“Non-Ind”) (Burkholder et al. 2001a, Cancellieri et al. 2001). For example, attraction towards fish or fish tissues, as measured with a microcapillary tube chemoattraction assay, is reported to be up to 5 times stronger in “toxic” than in non-toxic strains (Burkholder et al. 2001a, Cancellieri et al. 2001). Similarly, asexual cell reproduction in the presence or absence of fish has also been reported to vary among presumptively toxic strains, depending on their “toxicity” status, with “highly toxic” (“Tox-A”) strains demonstrating greater cell production than less or non-toxic strains when grown in the presence of fish (versus without fish or with algae) (Burkholder et al. 2001a,b, Burkholder and Glasgow 1997a) while the opposite occurred when cultured with algal prey (Burkholder et al. 2001a,b). Parrow and Burkholder (2003) demonstrated that dinospores of *P. shumwayae* feeding on fish tissue exhibited a greater increase in cell size and a higher production of daughter cells than cells feeding on algae. Although the cultures studied were capable of feeding on fish tissue, the toxicity or pathogenicity of these cultures was not reported.

Our previous work indicates that *P. piscicida* and *P. shumwayae*, as well as other related dinoflagellates (*Pfiesteria*-like dinoflagellates, or PLDs) can cause pathology and
mortality in laboratory-exposed fishes through micropredatory feeding without involvement of a toxin (Vogelbein et al. 2002, this work). Although all tested species could attach to and feed on fish epidermis, there was wide variation in the ability to cause pathology and mortality. Whereas *P. shumwayae* readily and consistently caused fish mortalities at densities that fell within the reported criteria for “TOX-A” cultures (100% mortality of fish in ≤48 hr at initial dinospore densities of $2-5 \cdot 10^3$ cells·mL$^{-1}$) (Noga et al. 1996, Burkholder et al. 2001a,b,c, Glasgow et al. 2001a,b), *P. piscicida* caused low-level fish mortalities (<20%), and only at considerably higher cell densities (>10,000 cells/ml) and longer exposure times (>48hrs). This suggested a fundamental difference in the pathogenicity to fishes between these two species.

The overall purpose of this study was to investigate the underlying biological factors that are responsible for the differences in ichthyocidal activity observed between *P. shumwayae* and *P. piscicida*. Specifically, the goals of this study were to determine the extent of variability in pathogenicity among multiple strains of each of these two species in relation to variability observed within a single “reference” strain of each species, to quantify the chemotactic response of several strains of each species to fish mucus, and to quantify the “ingestion capability” of each of these species in the presence of fish or algal prey, as measured by morphometric changes and increases in cell volume over time. The results of these studies are considered in the context of what has been reported for “toxic” cultures of *P. piscicida* and *P. shumwayae* to determine if micropredatory feeding provides an alternative explanation for the variability in pathogenicity observed for purportedly toxic cultures.
Larval fish bioassays on multiple strains of Pfiesteria and Pseudopiesteria

Larval fish bioassays were used to test and compare the ichthyocidal activity of multiple strains of *P. shumwayae* (19 strains) and *P. piscicida* (11 strains). Table 1 lists the identification, species and source information for each of the tested strains. Because of the large number of cultures, multiple assays were conducted over a period of ~6 months with 2-3 cultures assayed at a time. Each assay included either *P. shumwayae* strain CCMP-2089 or *P. piscicida* strain 9-02 as a “reference” strain since ichthyocidal activity in these cultures has been thoroughly evaluated in our larval fish bioassays. Our results from dose-response studies with *P. piscicida* demonstrated that this species causes mortality of larval fish only at relatively high initial dinospore densities (>5000 cells·mL\(^{-1}\)), whereas *P. shumwayae* regularly causes significant mortalities at initial densities <1000 cells·mL\(^{-1}\). As a result, initial dinospore densities of 10,000 cells·mL\(^{-1}\) were used for assays with *P. piscicida* cultures and 1000 cells·mL\(^{-1}\) for *P. shumwayae* cultures. Assays were conducted in 24-well tissue culture plates with one fish (*C. variegatus*, 7-day post-hatch) and 2 mL of culture media per well (*n*=48 replicate wells). Additional wells were fitted with membrane inserts (Millipore millicell-PCF inserts, 12 mm dia., 3 \(\mu\)m pore size) to examine for evidence of exotoxin production by determining if direct contact is necessary to induce mortality. For insert treatments, one fish (*C. variegatus*, 7-day post-hatch) plus 600 µL 12 ppt culture media were placed inside the insert and 800 µL of dinoflagellate culture were placed in the well exterior to the insert (*n*=12 replicate wells). Control treatments, with and without inserts, consisting of fish without
Table 1. Source and identification (including common strain identification designations) of various *P. shumwayae* and *P. piscicida* isolates used in the present studies.

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<td>Neuse River, N.C.</td>
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<tr>
<td>Pp-8</td>
<td><em>P. piscicida</em></td>
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<td>Calibogue Sound, S.C.</td>
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<td>Pp-9</td>
<td><em>P. piscicida</em></td>
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<td>2423</td>
<td>Neuse River, N.C.</td>
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Chemotaxis Assay

Assay design. A modified version of the chemotaxis assays used by Spero (1985) and Cancellieri et al. (2001) was used to quantify the attraction of *P. shumwayae* and *P. piscicida* dinospores to fish mucus. Assays were conducted in 4-well rectangular plates (Nunc) with 8 mL of culture media per well. Cultures were diluted as necessary (with 12 ppt sterile filtered culture media) to achieve densities of ~2000 cells·mL⁻¹. For each tested dinoflagellate culture, a control was prepared by sterile filtering (Millipore Millex-GV 0.22 µm syringe filters) 5 ml of diluted culture. This control media was used for mucus dilution (see below) and in control tubes. Immediately prior to assaying a particular well, 1mL was removed and fixed in 1% Lugol’s iodine for cell counts. Each well represented a replicate with 3-4 replicate wells per treatment. For the assay, microcapillary tubes (calibrated to hold 3.33 µL, Drummond Scientific) were filled via capillary action with prepared fish mucus (see below), or control media. Tubes were gently wiped with a lint-free tissue (Kimwipes) prior to insertion into wells. Two tubes filled with mucus and 2 tubes filled with control media were added to each well. After 7 min., tubes were removed from wells and the contents carefully ejected onto a hemacytometer (Brightline), using a 1-mL syringe, into 10µL 3% Lugol’s iodine. For each well, pairs of
mucous tubes or control tubes were combined for counting. All cells were counted. A chemoattraction index value (CIV) was calculated per replicate as follows:

\[
    \text{CIV} = \frac{(X_E - X_C)}{(D_w \cdot V_T)}
\]

Where \(X_E\) = the total number of cells entering the experimental (mucus) tubes, \(X_C\) = total number of cells that enter the control tubes, \(D_w\) = density of culture in well, and \(V_T\) = total volume of experimental capillary tubes. The CIV is essentially the net number of cells entering the experimental tubes adjusted to account for attraction to control tubes and normalized to culture concentration and volume of wells and tubes.

An initial chemotaxis assay was conducted comparing multiple strains of \(P.\) shumwayae (strains CCMP-2089, Ps-T1, CCMP-2359) and \(P.\) piscicida (strains 9-02, CCMP-2091, cell-N). All tested cultures had been cultured solely with algal prey.

Generally, assays were conducted only with cultures that appeared to be “starved”, as indicated by a lack of cryptophyte prey in the culture vessel and the dominance of small cells that appeared, under light microscopy, homogeneously translucent without evidence of prominent food vacuoles. Additionally, \(P.\) piscicida strain 9-02 and \(P.\) shumwayae strain CCMP-2089 were comparatively assayed to determine if there was a competition effect due to the presence of other prey (cryptophyte algae). Cultures were assayed as above before adding cryptophytes (\(Rhodomonas\) sp. CCMP 767 at a predator:prey ratio of 1:10) and then assayed again immediately (\(T_0\)), at 1 hr post-addition of prey and at 24 hr post-addition. At 48 hr post-initiation of assay, cells were fed again at the same ratio as previously, and assayed again after 24 hr (72 hr post initiation of assay).
**Fish mucus preparation.** Fish mucus was collected from wild, adult *Fundulus heteroclitus* (males and females, caught with crab-baited minnow traps) held in aquaria with sand-filtered 20 ppt York River water for ~1 week. Surfaces of fish were gently scraped with a sterilized rubber policeman onto a sterile tissue-mincing dish. Mucus was collected with a sterile disposable pipette, transferred to sterile microcentrifuge tubes and centrifuged @ 3000 rcf for 5 min. Supernatant was sterile-filtered into sterile tubes and immediately placed in a -80°C freezer until needed. For assays, mucus was thawed, sterile-filtered and diluted 1:100 with sterile-filtered culture water.

**Electronic Particle Characterization (EPC)**

Cell size distributions and cell concentrations of undiluted samples were determined using an electronic particle characterizer (Coulter Multisizer II). Sample volume was 10 mL with an analytical volume of 500 µL. The aperture was 100 µm and the orifice of the measuring capillary was 75 µm. Calibration beads (10 µm and 20 µm) were run before and after each sampling session to verify accuracy. Data were collected in 256 channels. Cell number and estimated spherical volume (ESV) were estimated using Coulter AccuComp software. Using the Coulter AccuComp software, selected portions of the distribution could be analyzed independently of the rest of the distribution curve. This was critical due to the high level of noise at the lower end of the size range that would otherwise greatly skew the mean volume and particle density calculations. The lower and upper limits for analysis were based on initial (T₀) samples that had less noise, or by determining the point at which noise was sufficiently low so that the distribution of interest was evident.
Cultures of *P. shumwayae* strain CCMP-2089 and *P. piscicida* strain 9-02 (both grown in 12 ppt culture media) were exposed to either *Rhodomonas* sp. (CCMP-768) (“algal prey” treatment), a fish fin plug (“fish fin” treatment) or left unexposed (“no prey” treatment). For the “fish fin” treatment, wild *Fundulus heteroclitus* (caught with crab-baited minnow traps) were narcotized with tricaine methanesulfonate (MS-222; 200-300 mg·L$^{-1}$) and rinsed thoroughly with DI water. Fin plugs were cut with a 3mm dia. cork borer and placed in a petri dish with DI water. After collection, fin plugs were thoroughly rinsed in 3 changes of DI water followed by 3 changes of 12 ppt culture media before being added to the assay wells. Plugs were randomly distributed into wells of a 6-well tissue culture plate (3 fin plugs per well, 9-wells per dinoflagellate species) with 12 mL dinoflagellate culture. Cultures were not diluted prior to addition to the wells. For the “algal prey” treatments, 9 wells per dinoflagellate species received 12 mL of dinoflagellate culture and *Rhodomonas* sp. (CCMP-768) at a density approximately 10x that of the dinoflagellate culture (CCMP-768 = $\sim$3.0·10$^6$ cells·mL$^{-1}$, *P. shumwayae* CCMP-2089 = $\sim$2000 cells·mL$^{-1}$, *P. piscicida* 9-02 = $\sim$3000 cells·mL$^{-1}$). For the “no prey” treatment, 9 wells per dinoflagellate species received 12 mL culture only, with no prey. To limit feeding and minimize contamination in wells, all fish fin plugs were removed from “fish fin” wells 8 hr post-assay initiation. “Algal prey” wells were observed at this time and found to contain only trace densities of *Rhodomonas* sp. At 8hr, 24 hr and 48 hr, 10 mL were removed from each of 3 wells per treatment and analyzed separately on the particle characterizer as specified above.

A mortality bioassay was conducted concurrently with the above assay to demonstrate pathogenicity in the tested cultures. This assay used the 24-well plate format
with one fish + 2mL culture media (undiluted) per well ($n=24$ replicate wells per culture). Fish mortalities were assessed at 8hr, 24 hr and 48 hr. Cell densities were not measured over time in the mortality bioassay.

**Differential Cell Enumeration**

In the above EPC studies, Lugol’s-fixed samples were collected at 0, 8, 24 and 48 hr to verify the cell density data generated by EPC. Upon initial observation of Lugol’s-fixed cells with light microscopy, cells were observed to stain differentially, based on the content of food vacuoles, with the larger, darker staining cells presumably containing more food. Subjectively, small, pale-staining cells dominated in number at 0 hr whereas at later times large, dark-staining opaque cells tended to dominate, especially in treatments where intense feeding activity occurred. Therefore, based on size and staining characteristics with Lugol’s iodine, cells were partitioned into 5 size categories (Fig. 1). These categories are presumed to reflect the stage of feeding in individual cells. Category 1 cells were small (~5-7 µm), pale-staining and translucent with no inclusions or other evidence of food vacuoles and were presumed to be “starved” cells, although they may also represent newly excysted cells. The peridinoid shape of these cells was sometimes difficult to discern. Category 2 cells were slightly larger (~8-12 µm) and stained darker with few inclusions and a more defined peridinoid shape. These cells typically comprised a large proportion of the population and were considered to be “normal” cells, having recently, though not immediately, fed. Category 3 cells were larger still (~10-15 µm) and stained much more heterogeneously with many dark inclusions indicating the presence of food vacuoles. They were often somewhat rounded, having partially lost their peridinoid
Figure 1. Presumed “feeding stages” of cells used for differential cell counts based on staining characteristics with Lugol’s iodine. 

a, Category 1, “starved” cells (5-7 µm), appearing translucent and with a pale-staining cytoplasm devoid of food vacuoles. The peridinoid shape of these cells can be difficult to discern. 

b, Category 2, “normal” cells, appearing slightly larger and more darkly staining with few inclusions (food vacuoles) visible and a more defined peridinoid shape. 

c, Category 3, “feeding” cells, larger and more heterogeneously staining with many dark inclusions visible. May appear rounded with a less obvious peridinoid shape. 

d, Category 4, “engorged” cells, appear much larger and very darkly staining. Appear more rounded. 

e, Category 5, “cysts”, complete loss of peridinoid shape, very dark staining (nearly black) with a readily visible cyst “envelope”. Rarely observed due to adherence to the bottom of the culture vessel. Scale bar = 10 µm and applies to all panels.
Figure 1. Cell-size ("feeding stage") categories for differential cell counts, based on differential staining characteristic of feeding cells when stained with Lugol's iodine. Bar = 10µm
shape. Cells in this category are considered to be actively feeding and are referred to as “feeding” cells. Category 4 cells were much larger (~15-20 µm) and very darkly, almost homogeneously stained. The cells were rounded up and had lost their peridinoid shape and are considered to be “engorged” cells. Category 5 cells were “cysts” and were large (~15-20 µm) and rounded, having completely lost their peridinoid shape, and were very dark (nearly black) and evenly staining. A transparent cyst “envelope” was usually clearly visible. Because encysted cells strongly adhered to the bottom of the culture vessel, they were rarely observed in cell counts. All Lugol’s fixed samples were counted (n=3 per treatment) and the relative contributions of the above five cell categories were calculated and compared among “no-prey”, algae and fish fin treatments as described above. These data were also compared to data generated from EPC to cross-validate the various methods used.

RESULTS

Larval fish bioassays on multiple strains of P. piscicida and P. shumwayae

Six of the 11 tested strains of P. piscicida resulted in fish mortalities with a cumulative mortality range of 2%-17% over the 96 hr assay duration in any of the tested strains (Fig. 2a). None of the tested strains caused fish mortality when separated from fish by the membrane insert and no mortalities occurred in controls in any assay. Mortality in the multiple runs of strain 9-02 varied, and occurred in four of five assays (range = 0%-8% at 96hr). Average mortality in strain 9-02 was 4.8% at 96 hrs. Initial mean cell densities (± σ) ranged from 9.4± 0.320 · 10³ to 1.8 ± 0.224 · 10⁴ cells·mL⁻¹. Dinospore
Figure 2. Results of 96 hr larval fish bioassays comparing pathogenicity of multiple strains of *P. piscicida* against *P. piscicida* strain 9-02. **a**, Cumulative mortalities of 7 day old *F. heteroclitus* exposed to 11 strains of *P. piscicida*. Mortality data for strain 9-02 is an average of 5 separate runs. Error bars shown for strain 9-02 represent the standard error of the mean (*n*=5). Key refers to strain IDs listed in Table 1. **b**, Mean dinoflagellate densities (cells·mL$^{-1}$) over time for each strain tested. Data for strain 9-02 is an average of 5 separate runs. Error bars represent standard deviations (*n*=3).
Figure 2. Cumulative fish mortality and dinoflagellate cell density results of comparative larval fish bioassay with multiple strains of *P. piscicida*.
densities fluctuated greatly among strains and within the multiple runs of strain 9-02 over the duration of the assays (Fig. 2b). Final mean cell densities (± σ) among tested strains ranged from $6.5 \pm 2.275 \cdot 10^3$ to $2.2 \pm 0.178 \cdot 10^4$ cells·mL$^{-1}$.

All tested strains of \textit{P. shumwayae} resulted in significant mortalities at the cell concentrations tested (Fig. 3a). As with \textit{P. piscicida}, none of the \textit{P. shumwayae} strains caused fish mortality when separated from fish by the membrane inserts and no mortalities occurred in controls in any assay. No mortalities occurred in any strain, or in the multiple runs with CCMP-2089, during the first 48 hrs. Eight of 19 strains caused 100% fish mortality by 72 hrs, and 4 additional strains killed 100% of the fish by 96 hrs. Average mortality in CCMP-2089 was 99.7% at 96 hrs. Fish mortality in the remaining 6 strains ranged from 60%-98% at 96 hrs. Mean cell densities among the tested strains exhibited a general pattern of decline during the first 24 hrs followed by a steady increase over the remainder of the assay (Fig. 3b). Initial mean cell densities (± σ) among tested strains ranged from $451 \pm 42.5$ to $972 \pm 96.4$ cells·mL$^{-1}$. Final mean cell densities (± σ) ranged from $1.4 \cdot 10^3 \pm 743$ to $1.3 \cdot 10^4 \pm 1398$ cells·mL$^{-1}$.

Variability in ichthyocidal activity among strains within each species and among multiple runs of the “reference” strain for each species (\textit{P. shumwayae} strain CCMP-2089 and \textit{P. piscicida} strain 9-02) was determined by survival analysis (Kaplan-Meier analysis using SPSS statistical software). When compared pair-wise using Tarone-Ware Chi-square analysis ($\alpha = 0.05$), fish survival among the multiple strains of \textit{P. piscicida} and among the multiple runs of \textit{P. piscicida} strain 9-02 was not significantly different (p $\geq 0.05$). Fish survival among the multiple strains of \textit{P. shumwayae} and among the multiple runs of \textit{P. shumwayae} strain CCMP-2089 demonstrated significant variability.
Figure 3. Results of 96 hr larval fish bioassays comparing pathogenicity of multiple strains of *P. shumwayae* against *P. shumwayae* strain CCMP2089. **a**, Cumulative mortalities of 7 day old *F. heteroclitus* exposed to 19 strains of *P. shumwayae*. Mortality data for strain CCMP-2089 is an average of 10 separate runs. Error bars shown for strain CCMP-2089 represent the standard error of the mean (*n*=10). Both shaded areas denote outliers before integrating the 48 hr survival data into the 72 hr data. The shaded area marked with (*) denotes outliers after integrating the 48 hr survival data into the 72 hr data. **b**, Mean dinoflagellate densities (cells·mL$^{-1}$) over time for each strain tested. Data for strain CCMP-2089 is an average of 10 separate runs. Error bars represent standard deviations (*n*=3). Key refers to strain IDs listed in Table 1.
Figure 3. Cumulative fish mortality and dinoflagellate cell density results of comparative larval fish bioassay with multiple strains of *P. shumwayae*.
However, the greatest amount of variability was apparent at the 48 hr time point, with cumulative mortality ranging from 0 to 94%. When the 48 hr data from the intra-strain comparison (multiple runs of CCMP-2089) was integrated with the 72 hr data (cumulative mortality = 88% to 100%), pair-wise Chi-square analysis indicated no significant differences in fish survival among all 10 assays (p > 0.08). Similarly, variability in fish survival among the different strains of *P. shumwayae* was reduced by integrating the 48 hr mortality data into the 72 hr time-point for survival analysis. This reduced the number of outliers from 8 to 3 (shown by shaded area in Fig. 3a).

To compare intra and inter-strain variability of both survival and dinoflagellate density of each species, the means from multiple runs of *P. piscicida* strain 9-02 and multiple runs of *P. shumwayae* strain CCMP 2089 were compared against the averaged data from all tested strains within each species (Fig. 4). Survival analysis of mortality data indicated no significant difference between the average survival of *P. piscicida* strain 9-02 (n=5) vs. the average of all other tested *P. piscicida* strains (n=11) (Tarone–Ware $\chi^2$, p<0.005) (Fig. 4a). Similarly, no significant differences in average survival was observed between *P. shumwayae* strain 2089 (n=10) and the average of all other tested *P. shumwayae* strains (n=18) (Tarone–Ware $\chi^2$, p<0.01) (Fig. 4b). Analysis of variance (ANOVA) indicated no significant differences in mean dinoflagellate densities, over time, between the average of multiple runs of *P. piscicida* strain 9-02 and the average means of all other tested *P. piscicida* strains (p<0.01) (Fig. 3a) or between *P. shumwayae* strain CCMP-2089 and all other tested *P. shumwayae* strains (p<0.01) (Fig. 4b).
Figure 4. Comparison of variability in mortality and dinoflagellate density exhibited among multiple strains of *P. piscicida* and *P. shumwayae*. **a**, Variability in cumulative mortality (open symbols) and dinoflagellate density (solid symbols) exhibited within *P. piscicida* strain 9-02 (blue lines) and among 10 different strains (red line). Error bars represent standard errors of the mean for cumulative mortality and standard deviation for dinoflagellate density (n=5 for strain 9-02 only, n=10 for multiple strain data). Survival analysis indicated no significant difference in survival between the mean of strain 9-02 and the mean of the other tested strains (Tarone–Ware $\chi^2$, p<0.01). Analysis of variance (ANOVA) indicated no significant difference in mean dinoflagellate densities, over time, between strain 9-02 and the other tested strains (p<0.01). **b**, Variability in cumulative mortality (open symbols) and dinoflagellate density (solid symbols) exhibited within *P. shumwayae* strain CCMP-2089 (blue lines) and among 18 different strains (red line). Error bars represent standard errors of the mean for cumulative mortality and standard deviation for dinoflagellate density (n=10 for strain 9-02 only, n=18 for multiple strain data). Survival analysis indicated no significant difference in survival between mean of strain CCMP-2089 and mean of the other tested strains (Tarone–Ware $\chi^2$, p<0.005). Analysis of variance (ANOVA) indicated no significant difference in mean dinoflagellate densities, over time, between strain CCMP-2089 the other tested strains (p<0.01).
Figure 4. Comparative analysis of variability within strains of *P. piscicida* and *P. shumwayae* vs variability exhibited within a single strain of each species.
Chemotaxis Assays

Comparative chemoattraction index values (CIV) among multiple strains of *P. shumwayae* and *P. piscicida* demonstrated considerable variability both among different strains of the same species and between species (Fig. 5). CIV of *P. piscicida* strain CCMP-1921 was significantly greater than highly ichthyocidal cultures of *P. shumwayae* (strains CCMP-2089 and Ps-T1) (ANOVA, p<0.05). Strains of non-pathogenic *P. piscicida* (cell-N, 9-02) demonstrated attraction to fish mucus similar to the highly pathogenic *P. shumwayae* strain CCMP-2089. Similarly, the CIV of strain cell-N, previously demonstrated as non-pathogenic and observed to exhibit little attraction or attachment to live fish, was significantly higher than that of the pathogenic *P. piscicida* strain 9-02 (ANOVA, p<0.05) and was not significantly different from two strains of *P. shumwayae* previously demonstrated as highly pathogenic to fishes (sections III and IV of this work).

Starved *P. piscicida* cells demonstrated a significantly greater attraction to fish mucus (as measured with CIV) than did starved *P. shumwayae* cells (CIV ± σ = 15.4 ± 3.4 for *P. piscicida* and 5.9 ± 0.87 for *P. shumwayae*, ANOVA, p<0.01, n=4 per treatment) (Fig. 6, “Initial”). However, when cryptophyte algae was added, the CIV in both cultures dropped significantly, with no significant difference in CIV between either species or between time points at 0hr, 1 hr or 24 hr post-cryptophyte addition (except *P. shumwayae* at 24 hr) (ANOVA, p>0.05) (Fig. 6). CIV again increased after 72 hrs (additional cryptophytes added at 48 hr) with *P. shumwayae* demonstrating a significantly greater attraction than *P. piscicida* (CIV ± σ = 26.6 ± 3.0 for *P. piscicida* and 15.1 ± 1.5 for *P. shumwayae*, ANOVA, p<0.01, n=4 per treatment) (Fig. 6, “Initial”).
Figure 5. CIV for several strains of *P. shumwayae* and *P. piscicida*, Error bars represent the standard deviation (*n*=4). Letters represent significance groups (ANOVA, α=0.05).
Figure 5. Chemotactic response (represented by CIV) to fish mucous by three strains of *Pfiesteria piscicida* and *P. shumwayae*.
Figure 6. Results of chemoattraction assays evaluating the chemotactic response to fish mucus of *P. shumwayae* strain CCMP-2089 and *P. piscicida* strain 9-02 before and after the addition of cryptophyte algae (*Rhodomonas salina* CCMP-767 added at “0hr” at a 10:1 prey:predator ratio). Attraction is represented by a Chemoattraction Index Value (CIV). Error bars represent the standard deviation (n=4). Letters over the bars represent significance groups (ANOVA, α =0.05).
Figure 6. Chemotactic response to fish mucus by *P. piscicida* and *P. shumwayae* dinospores before and after addition of cryptophyte prey.

![Graph showing chemotactic response](image-url)
Electronic Particle Characterization (EPC)

Mean estimated spherical volume (ESV in μm³, hereafter volume) of dinospores as well as dinospore densities, over time, averaged over replicate treatments (n=3) for *P. piscicida* and *P. shumwayae* exposed to cryptophyte algae, fish fin or no prey are shown in Figure 7. Differences between the two species in the volume of starved cells (T₀) were small but *P. shumwayae* cells were significantly larger (mean ± stdev = 443.3 ± 13.9 μm³) than *P. piscicida* cells (391.5 ± 3.32 μm³) (ANOVA, p<0.01, n=3 per treatment) (Fig. 7a). *Pseudopezziasteria shumwayae* exposed to fish fin demonstrated a significant increase in mean spherical cell volume at 8-hr post initiation (to 696.0 ± 36.0 μm³) followed by a steady decrease to 353.4 ± 27.1 μm³ at 96 hr (Fig. 7a). In contrast, *P. piscicida* exposed to fish fin demonstrated a significant decrease in mean volume at 8 hr (to 275.0 ± 20.5 μm³) increasing again to 419.4 ± 6.2 μm³ at 24 hr (Fig 7a). However, as indicated by the large amount of noise in the distribution and the disparity between the cell counts generated by EPC and those generated by manual counts (Figs 7 and 10), it is likely that this is an artifact due to contamination by small particles, possibly degradation products from blood or epidermal cells from the fish fin. This anomaly was not observed to such a large degree in other treatments, and density measurements between manual counts and EPC counts generally agreed within ~20%.

Algae and “no prey” *P. shumwayae* treatments exhibited a steady decrease in mean volume throughout the experiment (to 321.7 ± 12.5 μm³ and 282.2 ± 5.2 μm³, respectively, at 96 hr). *Pfiesteria piscicida* exposed to algae and “no-prey” also exhibited a decline in volume over the duration of the experiment (to 350.0 ± 2.9 μm³ and 345.3 ± 7.2 μm³, respectively, at 96 hr), though the final volume was smaller in the *P. shumwayae*
Figure 7. Summary results of electronic particle characterization (EPC). a, Mean estimated spherical volume for *P. shumwayae* and *P. piscicida* dinospores exposed to cryptophyte algae (*R. salina*), fish fin plug (*Fundulus heteroclitus*) or no prey over 48 hrs. Error bars represent 2 standard deviations (*n*=3). b, Mean cell density (cells·mL⁻¹) of *P. shumwayae* and *P. piscicida* exposed to cryptophyte algae (*R. salina*), fish fin plug (*F. heteroclitus*) or no prey over 48 hrs. Error bars represent standard deviation (*n*=3).
Figure 7. Summary results of electronic particle characterization (EPC) showing estimated spherical volume and dinospore density of *P. shumwayae* and *P. piscicida* exposed to algal prey, fish fin or no prey over 48 hrs.
algae and “no-prey” treatments (p<0.05 for pairwise comparisons between *P. shumwayae* and *P. piscicida* algae and “no prey” treatments). Dinoflagellate density increased dramatically in the *P. piscicida* fish fin treatment at 8hr (to $1.57 \times 10^4 \pm 3551$ cells·mL$^{-1}$) followed by a decrease to $8.7 \times 10^3 \pm 769$ cells·mL$^{-1}$ at 24hr and $6.6 \times 10^3 \pm 546$ cells·mL$^{-1}$ at 48hr (Fig. 7b). Density of *P. shumwayae* exposed to fish fin decreased to $1268 \pm 387$ cells·mL$^{-1}$ at 8 hr subsequently increasing to $3961 \pm 1765$ cells·mL$^{-1}$ at 48hr. Densities of *P. piscicida* exposed to algae or “no-prey” were unchanged at each time period compared to $T_0$ (ANOVA, p>0.05). Similarly, densities of *P. shumwayae* exposed to algae or “no-prey” were unchanged at each time period compared to $T_0$ (ANOVA, p>0.05), except for “algae” treatment at 48hr which demonstrated a significant increase to $3473 \pm 349$ cells·mL$^{-1}$ (ANOVA, p<0.01).

Full distributions of mean volume (average of 3 replicates) for *P. shumwayae* and *P. piscicida* from the above experiments are shown in Figures 8 and 9. The distribution of the volume of *P. shumwayae* dinospores exposed to fish fin exhibited a dramatic shift to the right from the initial distribution (Fig. 8a) towards larger cells at 8hr, with an volume range of ~1000-3500 µm$^3$ (Fig. 8b). This cell population shifted towards smaller volumes by 24 hr (Fig. 7c) and by 48 hr a large proportion of the cells was of a considerably smaller volume (range of ~200-800 µm$^3$) (Fig. 8d). The algal and “no-prey” treatments were approximately equal and shifted slightly to the left to a narrower and smaller-volume distribution throughout the 48 hr experiment (Fig. 8b-d).

Distributions of the volume of *P. piscicida* dinospores exposed to algae or “no-prey” remain essentially identical to that at time zero throughout the duration of the experiment (Fig. 9a-d). Volume of *P. piscicida* dinospores exposed to fish fin exhibited a
Figure 8. Mean distributions (n=3) of the estimated spherical volume (ESV) of *P. shumwayae* dinospores exposed to fish fin (*F. heteroclitus*, 3mm plug), cryptophyte algae (*R. salina*, 10:1 prey:predator ratio added at T₀) or no prey over a 48 hr period. **a**, Distributions at time zero. **b**, Distributions in the three experimental treatments at 8hr. Note the dramatic shift to larger cell volumes in the fish fin treatment. **c**, Distributions in all three treatments at 24 hrs. Note the shift from 8hr to smaller volumes in two of the treatments and the distinctly larger cell volume in the fish fin treatment. **d**, Distributions at 48 hrs. Note the dramatic decrease in cell volume distribution in the fish fin treatment. Error bars represent the standard errors of the mean.
Figure 8. Distributions of mean estimated spherical volume (ESV) for *P. shumwayae* exposed to fish fin, algae or prey-free treatments over a 48 hr period.
Figure 9. Mean distributions (n=3) of the estimated spherical volume (ESV) for *P. piscicida* exposed to fish fin (*F. heteroclitus*, 3mm plug), cryptophyte algae (*R. salina*, 10:1 prey:predator ratio added at T₀) or no prey over a 48 hr period. **a**, Distributions at time zero. **b**, Distributions in the three experimental treatments at 8hr. Note dramatic shift to larger cell volumes in the fish fin treatment, although with much smaller maxima than observed in *P. shumwayae*. **c**, Distributions in all three treatments at 24 hrs. Note the shift to smaller volumes in the fish fin treatment. **d**, Distributions at 48 hrs. Note the lack of any noticeable shift in either the “no prey” or “algal prey” treatments throughout the duration of the study. Error bars represent the standard errors of the mean.
Figure 9. Distributions of mean estimated spherical volume (ESV) for *P. piscicida* exposed to fish fin, algae or prey-free treatments over a 48 hr period.
dramatic shift at 8hr similar to that seen for *P. shumwayae*, although the range of the distribution was narrower and the maximum was smaller (range ≈500-1400 µm³) (Fig. 9b). However, the errors for this treatment (particularly at the lower end of the volume range) were very large. These errors were much reduced in the subsequent time periods and a clear shift towards a narrower distribution of dinospores with smaller volume is evident at 24 and 48 hrs (range ≈200-700 µm³ at 48hrs) (Fig. 9c,d).

No mortalities occurred during the 48-hr assay in the mortality assays conducted concurrently with the EPC studies with *P. piscicida* or controls (no dinoflagellates). Mortality reached 75% in the *P. shumwayae* treatment at 48 hr.

**Differential Cell Enumeration**

Relative proportions of the five “feeding stages”, as defined previously, remained fairly constant in *P. piscicida* exposed to algal prey or no prey (Fig. 10, *P. piscicida* “no prey” and “algal prey”). These populations were comprised largely of “normal” cells (~60-80%) with smaller proportions of “starved” and “feeding” cells (~5-25% of each) and only few “engorged” cells (<10%). No “cysts” were observed in these treatments. Similarly, cell densities remained relatively constant in these treatments, fluctuating between 3000-4000 cells·mL⁻¹.

Relative proportions of “feeding stages” in *P. shumwayae* with no prey or algal prey shift towards smaller “starved” cells by 24 to 48hr, with these smaller cells comprising > 50% of the population at 48 hr (increased from ~15% at T₀) (Fig. 10, *P. shumwayae* “no prey” and “algal prey”). Cell densities in these treatments remained
Figure 10. Differential cell counts of *P. shumwayae* and *P. piscicida* exposed to algae, fish fin or no prey over 48 hrs. Blue line depicts total cell densities (cells·mL$^{-1}$) in each treatment.
Figure 10. Differential cell counts over time, of *P. shumwayae* and *P. piscicida* exposed to algae, fish fin or no prey, from EPC study using fish fin plug and *Rhodomonas* sp. CCMP 768
relatively constant throughout the assay, fluctuating around \(~1500 \text{ cells} \cdot \text{mL}^{-1}\) in the “no prey” treatment and between 1000-2000 cells \cdot \text{mL}^{-1}\) in the “algal prey” treatment.

Relative proportions of feeding stages in the fish fin treatment with *P. piscicida* demonstrated a temporary shift in the population to “feeding” cells (~60%, with a reduction in “normal” cells from ~80% to ~30%) at 8hrs and shifting back to previous (T₀) levels by 24 to 48 hrs (Fig. 10, *P. piscicida “fish fin”*). Cell densities in this treatment increased after 8hrs from ~3000 to >7000 cells \cdot \text{mL}^{-1}\), decreasing again by 48 hrs to ~6000 cells \cdot \text{mL}^{-1}\). This contrasted with data generated for this treatment from the EPC study which demonstrated a larger increase in cell density to > 15,000 cells \cdot \text{mL}^{-1}\) at 8 hr (Fig. 7b). Proportions of feeding stages at 8hr in the fish fin treatment with *P. shumwayae* demonstrated a large decrease in “normal” cells (from ~75% to <15%) along with an increase in “engorged” (~35%) and “starved” cells (from ~15% to ~27%) (Fig. 10, *P. shumwayae “fish fin”*). Proportions of feeding stages of *P. shumwayae* at 24hr were generally similar but with a slight decrease in “starved” and “engorged” cells, a slight increase in “normal” and “feeding” cells and the occurrence of a small proportion of “cysts” (<10%). At 48hrs, the proportions of the five cell types for *P. shumwayae* were similar to T₀. Cell density decreased in the *P. shumwayae* fish fin treatment over the first 8hrs (from ~1500 to ~1000 cells \cdot \text{mL}^{-1}\) and subsequently increased to ~2800 cells \cdot \text{mL}^{-1}\) at 48 hrs.
DISCUSSION

Analysis of multiple strains of *P. shumwayae* and *P. piscicida*

Ichthyocidal activity by micropredatory feeding was significantly greater in strains of *P. shumwayae* than in strains of *P. piscicida*. Whereas multiple strains of *P. shumwayae* rapidly and consistently killed larval fish (> 90% cumulative mortality by 96 hr) at relatively low initial cell concentrations (<1000 cells/ml), cumulative fish mortality in *P. piscicida* bioassays was significantly lower (8.7% by 96 hr) and required cell densities at least an order of magnitude higher (> 10,000 cells/ml). Inter and intra-strain variability in survival was relatively low in both species. Further, intra and inter-strain mean survival and mean cell density was comparable within each species. This indicates that the use of *P. piscicida* 9-02 and *P. shumwayae* CCMP-2089 as “reference” strains for further analysis is justified and that they reasonably represent the two species in terms of biology, behavior and ichthyocidal activity. These findings contrast with the wide variability in pathogenicity (toxicity) that has typically been reported in the literature for purportedly toxic strains of *P. piscicida* and *P. shumwayae* (e.g., Burkholder et al. 2001a) and instead suggests that ichthyocidal activity, resulting from micropredation, is relatively consistent within and fundamentally different between these two species. We have been unable to demonstrate any evidence of the production of potent exotoxins in either species.

The observed disparity in ichthyocidal activity between *P. shumwayae* and *P. piscicida* in the current studies is difficult to reconcile with the existing literature. Most prior studies suggest that these two species are essentially identical in their ability to
produce toxin and kill fish, although with wide variability reported among different strains of each species, largely based on their history of exposure to fish (Burkholder et al. 1995a,b, 2001a, 2005, Steidinger et al. 1996a, Burkholder and Glasgow 1997a, Glasgow et al. 2001b). Although we have found no evidence of toxin production in either species (Section III of this work), in *P. shumwayae* we have clearly demonstrated pathology and mortality identical to that reported for purportedly “toxic” strains (Tox-A) (e.g., Noga et al. 1996, Burkholder et al. 2001a). In contrast, even the most pathogenic of our tested strains of *P. piscicida* only fit the criteria of a weakly toxic (“Tox-B”) culture (Burkholder et al. 2001a). These data, from multiple strains of each species, as well as our previous observations with *P. shumwayae* strain CCMP-2089 and *P. piscicida* strain 9-02, indicate that these two species differ fundamentally in their capacity as micropredators on fish tissue and in their ichthyocidal activity resulting from micropredatory feeding.

**Chemoattraction**

Chemoattraction to prey has been demonstrated in numerous dinoflagellates including *Pfiesteria* spp. (Burkholder and Glasgow 1997a, Vogelbein et al. 2002), *Paulsenella* spp. (Drebes and Schnepf 1988), *Protoperidinium spinulosum* (Jacobson and Anderson 1986), *Katodinium fungiforme* (Spero 1985) and *Crypthecodinium* spp. (Ucko et al 1989) and is characterized by a change in swimming behavior resulting in directed movement towards the prey item (Jacobson and Anderson 1986, Gaines and Elbrächter 1987, Burkholder and Glasgow 1997a, Burkholder et al. 1998, Vogelbein et al. 2002). Purportedly toxic strains (“Tox-A”) of “*Pfiesteria*” are reported to demonstrate a
chemotactic response towards fish or fish tissues 1.3-3 times greater than that exhibited by “Tox-B” strains and 4-5 times that of “Non-Ind” strains (Burkholder et al. 2001a, Cancellieri et al. 2001). In our studies, chemotactic response to fish mucus varied among and within all tested species with non-pathogenic or “less-pathogenic” strains (P. piscicida strains cell-N and 9-02) demonstrating a response equal to or greater than that of the more pathogenic strains (i.e. P. shumwayae strain CCMP-2089). Thus, chemotaxis, based on our results, is not a prominent factor influencing ichthyocidal activity in 

Pfiesteria.

A prominent “competition” effect was observed, dependent on how recently a culture had been exposed to algae prior to chemotaxis assay. Recently fed (satiated) cultures exhibited a chemotactic response that was reduced by more than 10-fold compared to the response of unfed cells. This effect was evident immediately upon addition of cryptophytes. It is not clear, however, what influence this effect would have on pathogenicity of a culture, although it is probable that, at least in the case of P. shumwayae, a culture recently fed on algal prey may be less pathogenic that a starved one due to a decreased propensity for moving towards and feeding on fish epidermis. However, our cultures are not necessarily free of algal-prey when they are assayed for pathogenicity in our larval fish bioassay system, meaning that this scenario could be common in our assays. Although the effect of competition was strong on the chemotactic response to fish mucus, the consistency of the results from the strain comparisons presented here suggests that any variability in ichthyocidal activity caused by competition with other prey may be relatively small. However, such an effect could be responsible for
the reduced ichthyocidal activity in the cultures identified as outliers in the strain comparison assay.

*Cell morphometry comparison between algal-fed and fish-fed cultures*

**Considerations regarding life-history.** Asexual zoospore production in several dinoflagellates, including *P. shumwayae*, has been tied to increases in cell volume resulting from feeding (Spero and Morée 1981, Parrow and Burkholder 2003a). Asexual reproduction in the omnivorous non-toxic heterotrophic dinoflagellate *Katodinium* (formerly *Gymnodinium* *fungiforme*) is also reported to be directly related to feeding with a non-motile reproductive stage occurring only after cells have become engorged with food (Spero and Morée 1981, Spero, 1982). That study identified two morphologically distinct cell populations, one consisting of small, clear cells observed to “attack” prey, and another comprised of large (up to 20x larger than the smaller cells), nearly immobile cells containing dark inclusions and presumed to be engorged cells prior to encystment and entry into a non-motile reproductive stage. Parrow and Burkholder (2003) demonstrated that *P. shumwayae* cells feeding on fish tissues exhibited a large increase in cell size followed by repeated divisions (up to 8 cells resulting from a single feeding cell) occurring in a non-motile reproductive cyst stage. In that study, cells that fed less, or that fed on algae, were reported to undergo fewer divisions and produce smaller daughter cells than cells that fed more (as determined by relative size of cell after feeding). However, the ichthyocidal status of the cultures used in that study was not reported.

Our results demonstrated a similar dramatic increase in cell volume and density in *P. shumwayae* exposed to fish fin vs. algal prey or no prey (Figs. 7 & 8). The EPC data
demonstrated a similar, although smaller shift to larger cell volumes at 8hr in *P. piscicida* exposed to fish fin (Fig. 9b), although the mean cell volume was considerably lower than at T₀ (Fig. 7a). It is likely that the small cell volume is an artifact due to a preponderance of small, contaminating particles, as mentioned earlier. By 24 and 48 hr post-initiation, *P. shumwayae* fed on fish fin demonstrated a large increase in cell density concomitant with a decrease in mean cell volume and a shift in volume distribution toward smaller cells, with a large increase in small cells at 48 hr. This increase in the number of small cells is a direct result of post-feeding encystment, asexual multiplication and excystment of a new generation of daughter cells. A similar shift is observed in *P. piscicida*, although it is much less pronounced, with cell volume distributions similar to those of un-fed cells. As with *P. shumwayae*, there was an increase in the density of small *P. piscicida* cells by 24 hr. However, it was not preceded by a decrease in cell density that would indicate post-feeding encystment, a prominent feature of our assays with *P. shumwayae*. In fact, we rarely observe encysted cells in our bioassays with *P. piscicida*, suggesting that the observed increase in cell numbers immediately post-feeding is not the result of asexual multiplication within cysts, as is the case for *P. shumwayae*. The subsequent decrease in both the numbers and volume of *P. piscicida* cells at 48 hrs (Fig. 9c) thus most likely indicates shrinking and/or encystment of cells due to starvation, as the food source (fish fin plug) is removed after 8 hr.

These observations strongly suggest that the reproductive strategies of *P. shumwayae* and *P. piscicida* are fundamentally different and that they are related to feeding capability. It is also very likely that these differences in life history strategy are an important underlying cause of the differential ichthyocidal activity observed in these
two species. *Pseudopfiesteria shumwayae* exhibits extensive encystment after feeding, with reproduction occurring within the cysts (Parrow and Burkholder 2003, Vogelbein unpublished data), whereas *P. piscicida* does not form large numbers of cysts after feeding on fish tissues and likely proliferates within our bioassays by sexual reproduction.

The ability of *P. shumwayae* to ingest a comparatively large amount of material when feeding on fish epidermis could be the mechanism that provides this species with the nutritional resources to undergo such prolific reproduction within the non-motile cysts. Whether it is due to peduncle structure differences or some other biological difference, the EPC data clearly shows that *P. shumwayae* is capable of ingesting more material when feeding on fish epidermis than is *P. piscicida*. In addition to providing an explanation for the life-history differences, this also provides an explanation for the differences in ichthyocidal activity. By consuming more epidermal material, a feeding *P. shumwayae* cell is causing more damage than a feeding *P. piscicida* cell under the same circumstances. Although this is likely not the only factor involved in the differential pathogenicity, it clearly provides an explanation for the observed disparities.

**Considerations regarding perception of toxicity.** The prevailing notion in the literature contends that “toxic” *Pfiesteria* cultures require fish or fish excreta/secretas in order to elicit toxicity (Burkholder and Glasgow 1997a, Burkholder et al. 2001a, 2005). This has been determined by exposing cultures of *Pfiesteria* to fish and algae in comparative fish bioassays (Burkholder et al. 2001a, 2005). Time and dinospore densities required to kill fish are used to determine toxicity status (Burkholder et al. 2001a). According to the reported results, fish-fed cultures are consistently more “toxic” (as
determined by ichthyocidal activity) than algal-fed cultures (Burkholder and Glasgow 1997a, Burkholder et al. 2001a, 2005). We provide an alternative explanation to this scenario that does not require the action of a toxin. In the aquarium-format bioassays, which were commonly used in much of the early *Pfiesteria* research with ichthyocidal cultures (Burkholder et al. 2001c, 2005), dinoflagellate cell densities fluctuated over time with peak cell densities corresponding to fish mortality (Vogelbein et al. 2001). As cells feed on fish and fish tissue available in the aquarium (micropredation), they likely increase greatly in size before migrating to the bottom of the vessel, encysting and undergoing vegetative reproduction (time from feeding to encystment $\approx 8\text{-}24$ hr, time from encystment to excystment $\approx 12\text{-}24$ hr). Our results demonstrate an increase of $>30\%$ in the relative number of engorged (pre-encystment) *P. shumwayae* cells after only an 8-hr exposure to fish fin (Fig.10). Cells feeding on algae do not demonstrate this marked increase in cell volume and increase in the number of large, engorged cells (Figs. 7-10). Other research on *Pfiesteria* as well as other heterotrophic, peduncle-feeding dinoflagellates, indicates a correlation between cell-size increase during feeding and asexual zoospore production (Spero and Morée 1981, Parrow and Burkholder 2003). Thus, when a population of cells from a fish-fed culture and a population of cells from an algal-fed culture are both introduced into a fish bioassay, it is possible that the population from the fish-fed culture contains many large cells ready to encyst and undergo division, thereby increasing population density more rapidly, relative to algal-fed cultures. Similarly, fish-fed *P. piscicida* cells also increase in size relative to algal-fed cells. However, they do not appear to readily form the large, engorged, “pre-encystment” cells observed for *P. shumwayae*. Although the life cycle for *P. piscicida* has been reported to
be very similar to that of *P. shumwayae* (Litaker et al. 2002, Parrow and Burkholder 2003), consisting primarily of haploid and diploid (planozygote) flagellated stages and two or more cyst stages (resting cysts and reproductive cysts) our studies indicate that this species does not undergo encystment to the degree that *P. shumwayae* does, and we rarely observe dividing cysts in this species when exposed to fish or fish tissue. However, we have consistently observed an increase in cell density in fish-exposed *P. piscicida* (current study and Section III of this work), indicating that its primary reproductive stage is either a motile or a very ephemeral non-motile form.

**CONCLUSIONS**

The results of the studies presented here demonstrate that *P. shumwayae* and *P. piscicida* are both capable of actively feeding on fish tissues and causing fish mortality by myzocytosis with no evidence of any involvement of a toxin. Both species also demonstrate a relative consistency in their ichthyocidal activity, in contrast to what has been reported in the literature (Burkholder et al 2001a, Burkholder and Glasgow 1995, 1997a, Glasgow et al. 2001b). However, there is an apparent wide disparity in ichthyocidal capability between the two species, with *P. shumwayae* consistently causing high mortality of larval fish while *P. piscicida* is far less pathogenic.

Although chemoattraction to fish tissue does not appear to influence fish pathogenicity, our results indicate that distinct differences in the life history strategies of *P. shumwayae* and *P. piscicida* are responsible for the observed differences in ichthyocidal activity between these two species. *Pseudopfiesteria shumwayae* is capable
of ingesting a larger volume of material than *P. piscicida* (as reflected in dinospore size and in much greater volume increase after feeding on fish). This results in a greater degree of damage to fish epidermis per feeding cell. In contrast, *P. piscicida*, although highly attracted to fish tissue, has a lower capacity to ingest fish tissues (as reflected in a more limited cell volume increase) and, consequently, does less damage to fish epidermis per feeding cell. This differential capability of ingestion is likely related to reproductive strategy of these two species. In *P. shumwayae*, the greater capacity to feed presumably provides the nutritional resources necessary to undergo multiple divisions within a non-motile reproductive stage that greatly increases population density. In contrast, *P. piscicida* does not undergo multiple divisions in a non-motile reproductive stage and, therefore, likely does not require the same degree of nutritional input as *P. shumwayae*. 
REFERENCES


SECTION VI

CONCLUSIONS
Ichthyotoxicity in dinoflagellate species of the Pfiesteriaceae has been assumed based primarily on the association of *Pfiesteria* (and *Pseudopfiesteria*) with fish mortalities in laboratory bioassays (Burkholder et al. 1992, 2001a,c, Noga et al. 1993, Marshall et al. 2000, Glasgow et al. 2001a,b). However, the aquarium-format bioassays used in earlier studies with *Pfiesteria* suffered from major impediments, including microbial contamination and poor water quality, which made assignment of causality difficult. Further, the use of these assays did not allow for the observations of direct physical interactions between *Pfiesteria* and fish, as is evidenced in the early literature which clearly states that no such direct interactions occur (Burkholder et al. 1992).

In contrast to the larger aquarium-format bioassays, the small-volume format and short duration of our larval fish bioassay minimized contamination and water quality issues and, most importantly, provided for microscopic examination of the dinoflagellates and their interactions with fish. Using this assay, we have verified the capability of *P. piscicida* and *P. shumwayae* to cause fish mortality in closed systems from which fish cannot escape. However, we have also definitively demonstrated that ichthyocidal activity results from micropredatory feeding on fish epidermis rather than from involvement of a potent ichthyotoxin, as had been previously reported (Noga et al. 1996, Burkholder and Glasgow 1997, Burkholder et al. 2001a, 2005). We have clearly shown that dinoflagellates physically attach to fish epidermis and feed on contents of the epidermal cells by myzocytosis via an extensible peduncle. With adequate duration and dinoflagellate density, epidermal damage occurred sufficient enough to result in fish death, presumably due to loss of osmotic balance. No tested species was able to cause mortality or observable pathology when separated from fish by a membrane.
demonstrated as permeable to lipid- and water-soluble algal toxins (brevetoxin and saxitoxin) (Vogelbein et al. 2002). All tested strains of *P. shumwayae* demonstrated ichthycidal activity identical to what has been reported for strains of *Pfiesteria* thought to be highly toxic (Marshall et al. 2000, Burkholder et al. 2001a,c, Glasgow et al. 2001a). This represents (to the author’s knowledge) the first account of fish mortality resulting from mechanical disruption of fish epidermis via feeding by a non-parasitic heterotrophic dinoflagellate. More recently, micropredation has been confirmed as the most significant mechanism of fish pathogenicity in *P. shumwayae* cultures previously considered toxic (Gordon and Dyer 2005, Shumway et al. 2006). Although evidence of bioactivity in certain *Pfiesteria* strains continues to be examined (Burkholder et al. 2005, Moeller et al. 2007), the relevance of such substances to the manifestation of health deficits to humans or aquatic organisms is unclear. Further, since tested strains have not been grown in the absence of microbial contaminants, it is overreaching to conclude that any substances found in these cultures are, in fact, produced by *Pfiesteria* rather than by one of the many contaminants present in the cultures.

Additionally, we have clearly demonstrated that micropredatory behavior towards fish is not unique to *P. shumwayae* and *P. piscicida*, but is ubiquitous among the dinoflagellates of the Pfiesteriaceae. By applying our larval fish bioassay to multiple strains of *Cryptoperidiniopsis* spp., *Luciella* spp. and *Stoeckeria* spp., we have demonstrated that all tested species are capable of micropredatory feeding on larval fish as described for *P. shumwayae* and *P. piscicida*. These data confirm the observations by other researchers of attachment and feeding on fish tissues by *Cryptoperidiniopsis brodyi* and *Luciella masanensis* (Steidinger et al. 2006, Mason et al. 2007), but also demonstrate
that, in some species and strains, this behavior can result in significant fish mortality given sufficient exposure duration and dinoflagellate density. This is in contrast to previous studies that consider these other species to be incapable of causing fish mortality (Burkholder et al. 1998a,b, 2001b, Marshall et al. 2000, Parrow and Burkholder 2003).

Ichthyocidal activity varied between species with *P. shumwayae*, *Cryptoperidiniopsis* spp. and *Stoeckeria* spp. being highly pathogenic and strains of *P. piscicida* and *Luciella* spp. demonstrating little or no ichthyocidal activity. Many of these species are indistinguishable from *Pfiesteria* under light microscopy and are found in similar environments (Burkholder et al. 1995, 1998, Steidinger et al. 2001, 2006, Mason et al. 2003, 2007, Jeong et al. 2005). Much of the early work that focused on the relationship of *Pfiesteria* spp. to fish kills used “presumptive *Pfiesteria* cell counts” based on evaluation of water samples under light microscopy, as an initial causative determinant (Burkholder et al. 1992, 1995, 2001c, Burkholder and Glasgow 1997, Glasgow et al. 2001a, Magnien 2001). This is problematic because, as is shown in these studies, many of these species are also capable of ichthyocidal activity in confined bioassay systems. This further complicates the identification of *Pfiesteria* as a toxic organism and its implication in fish kill and disease events.

Our results demonstrate that *P. shumwayae* consistently causes high mortality of larval fish while *P. piscicida* is far less pathogenic. However, as determined with comparative larval fish bioassays on multiple strains of each species, ichthyocidal activity in relatively constant within each species, with only minimal strain variability apparent. This is in contrast to the wide degree of variability that has been reported in the literature, relating to such factors as time away from fish, life-history characteristics, nutrients and
other biotic and abiotic factors (Burkholder et al. 1995, 2001a, Burkholder and Glasgow 1997, Glasgow et al. 2001b). The disparity in ichthyocidal activity between *P. shumwayae* and *P. piscicida* is in contrast to studies that consider these two organisms to be essentially identical in respect to their biology, behavior, and ichthyocidal capability (Burkholder et al. 2001a, Glasgow et al. 2001b).

We explored the differences between *P. piscicida* and *P. shumwayae* using comparative larval fish bioassays, chemotaxis assays and comparative morphometric analyses. Although chemoattraction to fish tissue does not appear to influence the pathogenicity of these dinoflagellates, our results indicate that distinct differences in the life history strategies of *P. shumwayae* and *P. piscicida* are responsible for the observed differences in ichthyocidal activity between these two species. As demonstrated with morphometric analysis, *P. shumwayae* is capable of ingesting a larger volume of material, when feeding on fish, than *P. piscicida*. This results in a greater degree of epidermal damage resulting from a single feeding event. In contrast, *P. piscicida*, although attracted to fish tissue, is a smaller cell with a lower capacity to ingest fish tissues and, consequently, does less damage to fish epidermis. This differential capability of ingestion is intimately tied to the reproductive strategies of these two species. After feeding, *P. shumwayae* forms a non-motile reproductive cyst that undergoes multiple divisions, producing 8 daughter cells from a single parent cell, allowing for a rapid increase in population density. In contrast, *P. piscicida* does not undergo multiple divisions in a non-motile reproductive stage and, therefore, likely does not require the same level of nutritional input as *P. shumwayae*. 
Prevailing evidence suggests that *Pfiesteria* and related species are not toxigenic organisms and do not represent a credible threat to humans or marine organisms. Instead, it is likely that these dinoflagellates were mistakenly implicated in fish kill and lesion events in natural systems primarily because of their ichthyocidal activity in laboratory assays and their presence during natural fish kill and lesion events. The results of the studies presented in this work make it clear that early observations of pathogenicity by *Pfiesteria*-like dinoflagellates in laboratory assays were misinterpretations of micropredatory feeding. Micropredation by heterotrophic dinoflagellates is unlikely to result in a significant impact on fish health in the natural environment. However, it is possible that natural or aquaculture conditions could occur where duration of contact or dinoflagellate density could reach levels sufficient to cause adverse health impacts. In the absence of significant evidence of a potent exotoxin, impacts on human health by these dinoflagellates are also considered unlikely. Results of the current studies as well as those of other researchers suggest that dinoflagellates of the Pfiesteriaceae, including *P. piscicida* and *P. shumwayae*, are benign heterotrophic dinoflagellates that do not pose any relevant threat to human or aquatic animal health.
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VITA

Vincent John Lovko