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Mini-review

Pfiesteria piscicida, *P. shumwayae*, and other *Pfiesteria*-like dinoflagellates

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Abstract

Pfiesteria piscicida and *Pfiesteria shumwayae* are estuarine dinoflagellates thought to be responsible for massive fish deaths and associated human illnesses in the southeastern United States. These dinoflagellates are described as having a complex life cycle involving flagellated zoospores, cysts, and amoeboid stages. Although no *Pfiesteria* toxin has been identified, certain strains of these dinoflagellates are thought to produce a water-soluble toxin that can kill fish and cause human illness. Recent reports show no evidence for amoeboid stages and indicate that a much more simplified life cycle exists. In addition, researchers have shown that *P. shumwayae* only kills fish through direct contact that does not necessarily involve the production of one or more toxins. This review summarizes these and other recent findings with an emphasis on establishing basic facts regarding the toxicity and life history of *Pfiesteria* dinoflagellates.

Keywords: *Pfiesteria piscicida*; *Pfiesteria shumwayae*; Harmful algae; Toxin; Life stages; Detection; Microbial community structure

1. Introduction

The dinoflagellate, *Pfiesteria piscicida* (Fig. 1) was first discovered in an aquarium at North Carolina State University in 1988 where it was blamed for an incident of fish deaths [24]. These deaths occurred shortly after the addition of Pamlico River water and were coincident with an increased abundance of the small (ca. 10 μm) dinoflagellates. Interest in *Pfiesteria* was heightened in 1991 and 1992 when massive fish mortalities accompanied by open ulcerations on the bodies of the fish were reported in the Pamlico and Neuse estuaries of North Carolina. Water taken from these areas was tested in a fish bioassay in which the abundance of dinoflagellates resembling *P. piscicida* was coincident with fish death [4]. From these results and in the absence of alternative explanations, *P. piscicida* was identified as a possible cause for the observed fish mortalities in the Neuse and Pamlico estuaries, as well as in the fish bioassay aquaria (reviewed in [11]). Further research conducted in the laboratory of Dr. JoAnn Burkholder (NCSU) suggested that these dinoflagellates produced a putative water-soluble toxin that causes ulcers, disorientation, and eventually death of fish and other marine animals [4,14]. During this period, three scientists reported adverse symptoms attributed to laboratory exposure to *Pfiesteria* [14]. These symptoms included extremity paresthesias, circumoral paresthesias, arthralgia, myalgia, asthemia, headache, nausea, abdominal pain, vomiting, perspiration, tearing/eye irritation, dyspnea/respitory problems, memory problems, emotional changes, and skin lesions [14]. Other scientists also working with *Pfiesteria*, as well as watermen working near areas of *Pfiesteria*-related fish deaths, reported a similar suite of symptoms (tentatively named the "possible—estuary associated syndrome" [22,28]). These reports and intense media coverage enhanced concern over *Pfiesteria* and public safety. The "*Pfiesteria* hysteria" reached its apex in 1997 when the Maryland Department of Natural Resources (MDNR) reported *Pfiesteria*-related fish deaths in the Pocomoke and Chicamacomico Rivers. As a result, several waterways were closed for recreational use by MDNR and news media coverage of the event fueled a growing concern among politicians and the general public over the safety of commercial seafood products for human consumption and in general, the health of the Chesapeake Bay. The "*Pfiesteria* hysteria" instigated funding for the development of better monitoring techniques, both for *Pfiesteria* itself and its putative toxin. A direct result of this research has been a greater under-

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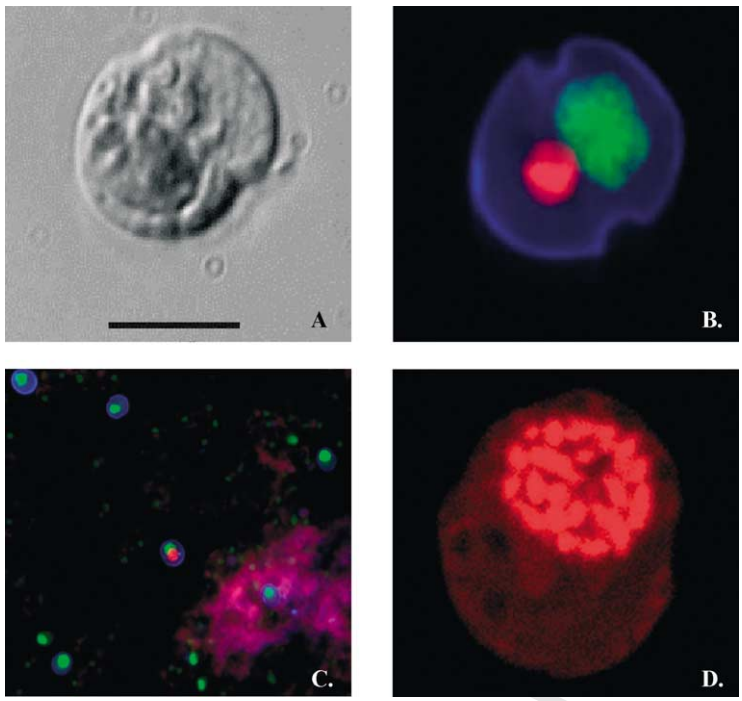

Fig. 1. Micrographs of *P. piscicida*. (A) Phase contrast micrograph (bar = 10 μm). (B) Epifluorescent micrograph in which the dinoflagellate is stained with acridine orange (green) to show the nucleus and calcaflour (blue) to show the thecal plates. Prey chlorophyll is seen in red as autofluorescence. (C) A low magnification view of the field depicted in panel B showing other dinoflagellates without prey chlorophyll. (D) Confocal scanning laser micrograph in which *P. piscicida* is stained with ethidium bromide to show the permanently condensed chromosomes.

standing of mixotrophic dinoflagellates and the complexities of detecting harmful dinoflagellates. Unfortunately, scientists are still in disagreement over issues of toxicity and life stages of *Pfiesteria* species [15].

1.1. General characteristics

Pfiesteria species are phagotrophic dinoflagellates that employ a feeding tube known as a peduncle to feed primarily on phytoplankton, such as unicellular Cryptomonads. This type of feeding is known as myzocytosis [20,33]. One *Pfiesteria* species, *P. piscicida*, has also been reported to absorb nitrogen compounds including ammonia (NH_4), nitrate (NO_3), urea, and even glutamate [18]. To complicate matters, *P. piscicida* may harbor whole, intact chloroplasts obtained after engulfing prey algae in its food vacuole, a phenomenon called kleptochloroplastidy [17]. However, it is unclear how much photosynthesis contributes to the growth of *P. piscicida*, as these dinoflagellates cannot be grown autotrophically [3,5]. Currently, there are two recognized *Pfiesteria* species, *P. piscicida* Steidinger et Burkholder [31] and *P. shumwayae* Glasgow et Burkholder [12]. *Pfiesteria*-like organisms include numerous cryptoperidiniopsoid strains, which may superficially look very similar to *Pfiesteria* spp. but are in fact different genetically and ultrastructurally [19,30]. Studies by Marshall et al. [21] suggest that cryptoperidiniopsoid species share some of the same life stages and feeding behaviors as the two-named *Pfiesteria* spp., but are not toxicogenic. Both *Pfiesteria* species were originally placed in the Dinamoebales because it was thought that the dominant stage was amoeboid [31]. However, recent data questioning whether *Pfiesteria* has amoeboid life stages (Section 1.2. “Life history”) has suggested that a more appropriate taxonomic grouping for these dinoflagellates is in the Peridiniales, considering *P. piscicida* readily feeds upon algal prey and is therefore not an obligate parasite of fish, unlike its closest relative of the Blastodiniales, *Amyloodinium ocellatum* [9].

1.2. Life history

The life cycle originally proposed for *P. piscicida* by Burkholder et al. [5] includes 19 flagellated, encysted, and amoeboid stages. This life cycle has undergone recent revision to include a palmelloid mass (defined as a group of nonmotile cells in a gelatinous matrix) [6]. In contrast, the life cycle described for *P. shumwayae* is somewhat less complex and has fewer stages [6]. For both species, transitions between stages, are reported to require the presence of live finfish, which is an integral aspect of toxicity [6,8]. Functional or physiologically distinct strains (i.e., toxic or nontoxic [6]) have been tested using the fish bioassay to determine ichthyotoxicity. Whether these are truly distinct strains, or the result of substrate limitation for toxin synthesis remains uncertain. To date, no toxin has been purified from *Pfiesteria* dinoflagellates making this assessment even more uncertain [23].

The discrepancies and confusion about the number of life stages inherently results in part from the use of cultures of *Pfiesteria* dinoflagellates growing in mixed assemblages with other microorganisms native to its habitat. For example, the fish bioassay used to test for toxicity and identify amoeboid and toxic *Pfiesteria* life stages contains a large consortia of micro- and macro-organisms, including viruses, bacteria, cyanobacteria, coccoid green algae, chrysophytes, protozoan ciliates, amoebae, rotifers, parasitic copepods, nematodes and opportunistic fungi [8]. The identification of *Pfiesteria* life stages is made difficult by the presence of these other organisms and because *Pfiesteria* zoospores are remarkably similar to the zoospores of many other nontoxic heterotrophic dinoflagellate species. Indeed, the "*Pfiesteria*" amoebae described are very similar to other known true species of amoebae [7]. In addition, newly excysted toxic dinoflagellates that were isolated from *Pfiesteria* fish bioassays have been described as being photosynthetic, a trait not possessed by *Pfiesteria* [4]. For these reasons, the 20 or more life stages in the *Pfiesteria* life cycle have remained controversial. Recent efforts have focused on developing clonal cultures of single cell isolates of *Pfiesteria* isolated by micromanipulators and then confirmed as bone fide *Pfiesteria* sp. by SEM, PCR or Fluorescent In Situ Hybridization (FISH) [3,5,7,25–27].

Recent research indicates that a much simpler life cycle exists for *Pfiesteria* dinoflagellates, one that is more characteristic of other marine dinoflagellates. In this work, Litaker et al. [20] combined the use of standard light, epifluorescence and video microscopy with fluorescent in situ hybridization using peptide nucleic acid probes (PNA) to describe the life cycle of *P. piscicida*, which is presented in two relatively simple phases: the asexual phase and the sexual phase. Within each phase, the dinoflagellates can be either cysts or zoospores (no amoeboid forms). In the asexual phase, there are three cyst types (division cysts, resting cysts, and temporary cysts) as well as a motile flagellated zoospore. In this phase, mitosis always occurs during encystment. The sexual phase of the life cycle involves the fusion of two daughter cells resulting in the planozygote followed by the hypnozygote and germination. Thus, the entire life cycle consists of six stages.

Are amoeboid stages a part of the *Pfiesteria* life cycle? The existence of amoeboid stages in the *Pfiesteria* life cycle was tested by Litaker et al. [20] using cultures of *P. piscicida* grown in the presence of goldfish (*Carassius auratus*) or tilapia (*Oreochromis niloticus*). Amoebae from these cultures were fixed and probed with different fluorescently labeled small subunit (18S) ribosomal RNA-specific PNA probes. These included a universal eukaryote-specific probe which served as a positive control to ensure the RNA targets were intact and that the probes penetrated the cells, a eubacteria-specific negative probe which served as a non-specific binding control, and finally amoeba and *P. piscicida*-specific probes. The universal eukaryotic probe hybridized to both *P. piscicida* zoospores, as well as the amoebae isolated from fish cultures, while PNA probes specific to *P. piscicida* did not bind to amoebae, and probes specific for amoebae did not bind to *P. piscicida* zoospores. Using the amoebae specific probe, 98% of the 300 amoebae examined were hybridization positive, while none were positive using the *P. piscicida* probe. Although further research needs to be done, there is, at this time, no conclusive evidence for an amoeboid stage in the life cycle of any *Pfiesteria* dinoflagellate.

1.3. Toxicity

Much of the *Pfiesteria* literature has been directed at the production of one or more "toxins" that are induced in the presence of fish (reviewed in [7]). We broadly define *Pfiesteria* "toxin" (or more appropriately, toxicity) as the production of one or more bioactive substances with potency towards fish and other animals that does not involve bioaccumulation in shellfish or finfish. Despite what can best be described as Herculean efforts by several prominent laboratories, to date, no *Pfiesteria* toxin has been isolated, purified, nor a chemical structure elucidated [23]. Methods to detect toxicity have primarily relied upon the standardized fish bioassay, as described by Burkholder et al. [8]. In the standardized fish bioassay, a set of guidelines has been developed to help determine if water from an active fish kill event (either natural or laboratory aquaria where fish are dying) contains toxic *Pfiesteria*. The guidelines are:

(Step 1) Measure field parameters (temperature, salinity, wind/current patterns) and confirm that other factors such as low oxygen or other toxic species are not present.
(Step 2) Count dinoflagellates that resemble *Pfiesteria* using light microscopy to establish a minimum required density of more than 300 cells per ml.
(Step 3) Incubate fish with unpreserved water samples with a goal of observing significantly more fish deaths over controls within 21 days.
(Step 4) Continue the bioassay for an incubation sufficient to produce higher cell densities, permitting identification by a combination of SEM and PCR assays, and isolation followed by growth of the dinoflagellates in axenic culture (free from all other organisms except algal prey and bacterial endosymbionts).
(Step 5) Expose fish to the axenic culture as in Step 3 to prove a set of standard hypotheses.

These standard hypotheses are tested as one would test Koch–Henle's Postulates for an infectious organism [8]. If all of these criteria are met then the water sample is assumed to contain *Pfiesteria* dinoflagellates that produce a toxic fish-killing substance. This assay has been the basis for all identifications of toxic *Pfiesteria* dinoflagellates [8].

Research by Moeller et al. and others [23] indicates that a toxin may be partially purified from cultures of *Pfiesteria* dinoflagellates growing in the presence of fish where fish are dying. In these experiments, these scientists used a luciferase reporter assay [10] in which rat Neuro2A and GH_4C_1 cells were transfected with plasmids containing the *c-fos*-luciferase gene fusion such that light produced by the luciferase enzyme was dependent on the activation of the *c-fos* promoter. The *c-fos* gene is induced by a wide range of extracellular stimuli including convulsant drugs such as picrotoxin and pentylenetetrazole, various growth factors, and other pharmacologically active substances [29,32]. Using water from *Pfiesteria* fish bioassay aquaria, luciferase activity from GH_4C_1-A1 cells increased 41% over controls. These data suggest that cytotoxic substances (referred to as putative *Pfiesteria* toxin, pPfTx) in the *Pfiesteria* bioassay water sample activate the *c-fos*-luc.

In a second set of experiments, this same group determined that pPfTx acted upon the $P2X_7$ receptor of GH_4C_1 pituitary cells [16]. Several polar, methanol-soluble fractions of water obtained from a *Pfiesteria* fish bioassay aquarium were purified from a C18 column and tested using the *c-fos*-luc assay. The lipid soluble *c-fos*-luc-active fractions were found to contain a phthalate ester as their principle active component [23]. Such phthalate esters are common contaminants resulting from plastic polymerization processes. In this case, the authors believe that the phthalate ester identified was derived from the manufacturing process to make Instant Ocean artificial sea salt, subsequently used to make up the water in the bioassay aquarium [23]. No compounds were identified from the water-soluble *c-fos*-luc-active fractions. Thus, as of this time, no toxin produced by either *P. piscicida* or *P. shumwayae* has been identified.

Efforts to identify toxic materials produced specifically by *Pfiesteria* dinoflagellates grown in fish bioassay aquaria are fraught with problems. As may be expected, there is abundant evidence that the *Pfiesteria* bioassay aquaria are rife with a thriving community of microorganisms, complicating the identification of the agent responsible for the toxicity (Wang et al., manuscript in preparation). For this reason, the toxicity of *Pfiesteria* dinoflagellates has been rigorously examined using experimental procedures designed to separate *Pfiesteria* dinospores from the other microorganisms, and to determine if fish death is the result of a soluble toxin. In an elegant set of experiments, Vogelbein et al. [33] utilizing low protein binding polycarbonate inserts to create two compartments within a culturing vessel, showed that fish mortality occurred only when *P. shumwayae* was in physical contact with the test fish, and then, only at cell densities exceeding 1000 cells per ml [33]. In these experiments, *P. shumwayae* zoospores were observed attached to fish epidermal cells, which they ultimately consumed. Interestingly, while *P. shumwayae* quickly attached and consumed fish cells, *P. piscicida* never caused fish death and was not associated with fish epidermal tissue when placed in physical contact with fish. Moreover, an analysis of fish deaths in a 38 l fish bioassay aquarium using Tilapia (*O. nilotocus*) as test fish confirmed that only fractions containing whole, intact *P. shumwayae* killed 100% of the larvae within 48 h. In contrast, dinoflagellate cell-free, bacteria enriched, and high ammonia fractions never killed larvae within the 96-h period. Thus, the data indicate that fish deaths require the presence of intact, living *P. shumwayae* cells that then attach to and devour fish epidermal tissues in a process of myzocytosis. Interpretation of these data does not require invoking an extracellular toxic molecule to explain the morbidity and mortality of the test fish.

Most toxic dinoflagellates produce a class of toxins called polyketides, chemically stable molecules synthesized nonribosomally by a polyketide synthase. In a separate study, Berry et al. [2] amplified polyketide synthase (PKS) genes from several known toxin-producing dinoflagellates using PCR and "universal" PKS gene primers, but were unable to amplify PKS genes from *P. shumwayae* bulk DNA or cDNA. In addition, these researchers conducted an 8-month long fish bioassay using *P. shumwayae* and sheepshead minnows (*Cyprinodon variegatus*) in which fish died on a regular basis. Cell free supernatants, dichloromethane/methanol extracts, raw tank water, cell-free lysates, and a clonal culture of *P. shumwayae* grown on algal prey were tested for their ability to kill *C. variegatus* in six-well plates over a seven-day period. Only the clonal culture and raw water samples from the fish bioassay killed fish within the seven-day period. The author's conclusion from these studies is *P. shumwayae* does not kill fish by releasing a toxin into bulk water, but rather consumes fish epidermal tissue by myzocytosis, a phenomenon the authors call micropredation.

In conclusion, the life history and toxicity of *Pfiesteria* species has been called into question. A majority of the 20 or more proposed stages of the *Pfiesteria* life cycle has only been shown in mixed microbial assemblages, a condition prone to the misidentification of life stages. Using advanced molecular tools, researchers have not been able to verify the amoeboid form of *P. piscicida*, even when it is in the presence of fish and other microorganisms. In addition, certain stages of the proposed *Pfiesteria* life cycle are differentiated based on the ability of the dinoflagellate to produce toxins. Thus, it appears that *Pfiesteria* dinoflagellates have six life stages involving three types of cysts, motile zoospores (or dinospores), planozygote, and heterozygote stages. There is little evidence to support amoeboid life stages in this dinoflagellate. Although cytotoxic substances have been partially purified from water containing *Pfiesteria* and other microorganisms, it has not been clearly established that those substances are derived from *Pfiesteria* dinoflagellates, and use of the fish bioassay as a source of toxic material may prove useless in answering questions of toxin production specifically by *Pfiesteria*. More likely, *Pfiesteria* dinoflagellates, as exemplified by *P. shumwayae*, do not produce toxins, but rather kill fish by micropredation. Indeed, there are no data in the peer-reviewed literature that demonstrate toxin production specifically by *Pfiesteria*.

Do these new findings make *Pfiesteria* dinoflagellates uninteresting? No. Quite the contrary, it appears that, while not the "menace" they originally were imagined to be, *P. piscicida* and *P. shumwayae* are excellent representatives of a very ubiquitous group of small, heterotrophic dinoflagellate species that inhabit estuarine and coastal marine habitats. It has been well established that primary production by autotrophic phytoplankton increases due to nutrient loading by non-point source pollution, a process called eutrophication. In comparison, few studies have successfully mapped the response of these small heterotrophic dinoflagellates, such as *Pfiesteria* species, to increased nutrients in estuarine waterways [1,13]. *Pfiesteria* provides an excellent model organism for such studies. In addition, *Pfiesteria* dinoflagellates have a rapid growth rate in culture making them suitable for laboratory studies. They also occupy a unique ecological niche having characteristics of fish parasites, detritivores, and free-living herbivores. What controls the abundance of these dinoflagellates, and how do they affect the overall balance of carbon, nitrogen, and other nutrients in estuarine habitats? How do they sense fish and other types of prey and what molecules are involved? Answers to such questions may provide missing links between fish deaths and the presence of *Pfiesteria*. They may also provide critical information to governmental organizations struggling to manage nutrient overloading in our waterways. Scientists have learned much about the life cycle, physiology, and genetics of *Pfiesteria* dinoflagellates and it is highly likely that many more discoveries will be made. Thus, we suggest that *P. piscicida* and *P. shumwayae* serve as excellent laboratory model organisms for further and future studies designed to understand more about estuarine dinoflagellates.

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