

## *Pfiesteria piscicida*, toxic dinoflagellate

cellular organisms - Eukaryota - Alveolata - Dinophyceae - Peridinales - Pfiesteriaceae - Pfiesteria - Pfiesteria piscicida

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### 1. General informaiton

*Pfiesteria* spp. and *Pfiesteria*-like organisms belong to class *Dinophyceae* (synonym *Pyrrhophyta*), dinoflagellates. Dinoflagellates are organisms whose dominant stage is unarmored or armored flagellated **zoospore**. Armor consists of thecal (wall) plates. Their arrangement is most important morphological characteristic for taxonomic placement of the dinoflagellates. *Pfiesteria*

*spp.* are small (~10-20 µm in length) lightly armored heterotrophic dinoflagellates, typically with a **dinokaryon** (dinoflagellate nucleus) in the **hypocone** (region of a dinoflagellate cell posterior to the girdle) and food vacuoles in the **epicone** (region of a dinoflagellate cell anterior to the girdle).

## 2. Introduction: dinoflagellates

- The dinoflagellates are a major marine phytoplankton group found in high energy aquatic biosystems. They are the major agents causing **Harmful Algal Bloom (HAB)** and are also the symbionts of corals. Despite of the autotrophic nature of many dinoflagellates, the group is phylogenetically affiliated with obligate parasites *Plasmodium falsiparum* (malaria) and *Toxoplasma gondii* (toxoplasmosis).
- The dinoflagellates are characterized by the presence of one or two flagella which propel the organisms in a rotating manner through the water. Each flagellum is located in a groove on the cell surface. One flagellum encircles the cell and is flattened like a ribbon; the other one is whip-like and extends beyond the posterior end of the cell. The action of both flagella results in characteristic spinning motion of the cell, thus, **dinoflagellates** (spinning flagellated).
- Dinoflagellates have characteristics that historically have been considered "botanical", e. g., presence of chloroplasts, as well as "zoological", e.g., flagella and heterotrophic mode of feeding.
- Because of their unique cytological characteristics, dinoflagellates sometimes are referred as **mesokaryotes**, suggesting the group as being intermediate between the prokaryotes and the eukaryotes. Dinoflagellates have:
  - extranuclear spindles
  - no nucleosomes
  - circular chromosomes
  - gigantic genomes ranging from 3 to 200 pg (human genome is ~3.2 pg)
  - smallest chloroplast genomes (14-15 core genes in contrast with 100-250 genes in plants and algae)
  - nuclear DNA in liquid crystal state (which, probably, makes them sensitive to turbulence in the water)
  - "closed" mitosis (mitosis performed without breakdown of nuclear envelope)

## 3. *Pfiesteria* brief facts

- *Pfiesteria spp.* are mixotrophic armored dinoflagellates distributed along the Eastern

coast of the United States. They have been a focus of intense research and controversy due to their reported association with several massive fish kill events as well as a human illness in North Carolina estuaries and in Chesapeake Bay.

- Two main species currently form the *Pfiesteria* complex: *Pfiesteria piscicida* Steidinger & Burkholder and *Pfiesteria shumwayae* Glasgow & Burkholder (more recently, *Pseudopfiesteria shumwayae*). There are at least 10 other organisms that look similar when examined under the light microscope and are known as *Pfiesteria* complex organisms (PCO), *Pfiesteria*-like organisms, or morphologically related organisms (MRO). Collectively, they belong to family *Pfiesteriaceae*.
- The alleged ability of the PCO organisms to launch sudden physical as well toxic attack on fish in response to the finfish presence and its cryptic appearance during "bloom" event earned them such names as **ambush-predator**, **phantom-like**, **cell from hell**, **fish AIDS**, etc.

## 4. *Pfiesteria* discovery

- The fascinating story of *Pfiesteria* species began in 1988, when tilapia added to an aquarium, filled with Pamlico River (North Carolina) water at the NCSU veterinary school, started dying. Fish biologist Edward Noga and JoAnn Burkholder linked the fish morbidity with abundance of small (10  $\mu$ M) dinoflagellates that were identified as a novel species. The species were named in honor of phycologist Lois Pfiester (literally, **Pfiester's fish killer**). Later, when in 1990s, occurrences of massive fish deaths in estuaries of North Carolina were noticed to coincide with the *Pfiesteria* blooms, Burkholder have suggested that the organism was a causative agent of these events. After working for several months with *Pfiesteria* cultures, lab assistant in her lab reported mood swings, memory loss, and other neurological symptoms. In 1997, several fish kills occurred in Chesapeake Bay estuary, which costed Maryland's seafood industry \$50 million. In addition, cases of adversely affected fishermen have been reported. This stirred a considerable public concern and even panic ("Pfiesteria" hysteria), and resulted in substantial governmental money influx into the *Pfiesteria* research. At some point the Center for Disease Control and Prevention (CDC) has issued criteria for **Possible Estuary-Associated Syndrome (PEAS)**. Also, it was mandated that *Pfiesteria* could be studied only using biosafety level 3 (BSL-3) precautions (same as for HIV).
- *Pfiesteria* spp., and especially, *Pfiesteria piscicida*, caused unprecedented controversy in research community. The heated debates focused mainly on the life cycle of the organism, toxin production and potential danger the organism may pose for human health as well as fish population.

## 5. Human health implications

- Initial (197) CDC criteria for adverse consequences of exposure to *Pfiesteria* complex organisms (PCO):
  - Fish with lesions consistent with *Pfiesteria piscicida* or PCO toxicity (>20% of a sample of >50 fish of a single species having lesions)
  - Fish kill involving fish with lesions consistent with *Pfiesteria piscicida* or PCO toxicity
  - Fish kill involving fish without lesions, if *Pfiesteria piscicida* or PCO are present and no other explanation for fish kill can be found
- Any of the following signs and symptoms:
  - Memory loss
  - Confusion
  - Acute skin burning on direct contact with water
  - Three or more of the following:
    - Headaches
    - Skin rash
    - Eye irritation
    - Upper respiratory tract irritation
    - Muscle cramps
    - Gastrointestinal complaints
    - Nausea / vomiting
- *Pfiesteria piscicida* is not infectious and there is no evidence that it can invade human tissue or enter the bloodstream.
- There is no current evidence that PCO or their toxins can enter the food chain (toxins of some other dinoflagellates can accumulate in shellfish and finfish and render them poisonous).
- About 4% of dinoflagellates are known to cause human illness. Many of the signs and symptoms of PCO-related illness are similar to those caused by ingestion of endotoxins produced by well known toxic dinoflagellates such as *Gambierdiscus toxicus* (ciguatera fish poisoning), *Gonyaulax sp.* (paralytic shellfish poisoning), *Karenia brevis* (neurotoxic fish poisoning), and others. However, unlike above mentioned types of poisoning, no one has been known to die of PCO exposure.
- Behavioral and neurological effects of *Pfiesteria* toxins were shown in experiments with rats, which were given intraperitoneal injections of filtrates obtained from toxic cultures (Duncan PM et al. (2005)). In another study, the filtrates were shown to be cytotoxic to mouse Neuro-2A cells and primary human epidermal keratinocytes (HEK). The filtrates

also caused mild irritation and inflammation of ear skin in Balb/C mice (Patterson RM et al. (2007)).

## 6. *Pfiesteria* general biology

- *Pfiesteria spp.* is distributed in estuarine waters throughout the mid-Atlantic and south-eastern United States, from Delaware Bay to Mobile Bay, Alabama, and may be spread as far north as Long Island. Potentially toxic *Pfiesteria spp.* also has been found in northern European waters. The organism can live in a wide range of temperature and salinity with a distinct preference to relatively shallow, turbid, slowly flushed, nutrient enriched, moderately saline habitats.
- *Pfiesteria* is very versatile nutritionally (**mixotrophic, heterotrophic**). They prey on bacteria, microalgae, ciliates, and fish tissue (*Rhodomonas sp.*, *Dunaliella sp.*, and *Cryptomonas sp.* are used in laboratories). Zoospores feed by attaching a special feeding tube (**peduncle**) to a living cell and sucking out cellular contents (**myzocytosis**). Dinospores are able to empty contents of most cells within 1 minute of insertion of the peduncle, and consume five to six cells consecutively. They also can retain intact chloroplasts captured from microalgae in their epithelial food vacuoles (**kleptoplastidy**) for at a few 9 days. However, it is unclear in what degree the photosynthesis contributes to the growth of the *Pfiesteria*, as it cannot grow autotrophically. It was reported that they can uptake and utilize inorganic nitrogen in form of ammonia (NH<sub>4</sub>), nitrate (NO<sub>3</sub>), and urea. Because *Pfiesteria* can sustain its population by feeding on algae and other microorganisms, it is not an obligate parasite of fish, unlike its closest relative of the order *Blastodiniales*, *Amyloodinium ocellatum*.
- It was shown in short-term experiments that some microscopic invertebrates like **rotifers** (phylum *Rotifera*, genus *Rotaria*) and common estuarine copepod *Acartia tonsa* can graze upon *Pfiesteria piscicida*. Filter feeders like scallops and oysters could filter low concentrations of toxic zoospores (~60 cells per mL) and remain viable for period for up to 14 days.
- Currently, there is following model of pathogenicity of the two *Pfiesteria* species:
  - Both can harm finfish during physical contact by attacking fish epithelial cells and consuming them by way of myzocytosis (the phenomenon is called sometimes **micropredation**). In nature, or in bioassays with environmental samples, micro-wounds and lesions resulted from the attacks become quickly infected by other fish pathogens as well as various opportunistic bacteria and fungi. Primary cause of fish deaths in these circumstances is difficult to determine.
  - *Pfiesteria spp.* are considered predators rather than parasites because they prey upon microscopic organisms in the water consuming them whole. While attacking live finfish or other multicellular marine organisms, *Pfiesteria spp.* do not exhibit host specificity and do not change their preferred mode of feeding or their morphology.

- Some strains of *Pfiesteria piscicida*, as well as, probably and/or to a lesser degree, *Pfiesteria shumwayae*, may produce toxin or toxic cocktail, which can kill finfish and several crustacean and mollusk species (shrimp, crabs, bivalves) by poisoning only. Strains vary in toxicity: actively toxic strains (TOX-A) are capable of killing fish in the matter of minutes after exposure, other strains have only negligible toxicity. According to some reports, toxin(s) alone may cause skin lesions.
- For both species the presence of live fish is required to trigger coordinated attacks on flesh and, if strain is toxicogenic, toxicity. Toxicogenicity may be lost in cultures grown for 6-8 months in laboratory. Presence of certain bacteria can facilitate toxins' action.

## 7. *Pfiesteria* life cycle controversy

- The life cycle originally proposed by Burkholder consisted of at least 19-24 flagellated, encysted, and amoeboid stages. Recently, one more form, **palmelloid mass**, was added.
- Many research groups perceived Burkholder's life cycle representation as unreal. Indeed, very few laboratories managed to show that numerous amoeboid forms detected in *Pfiesteria* bioassay are indeed *Pfiesteria* spp..
- Litaker et al. have demonstrated much simpler life cycle for *Pfiesteria*, which is characteristic for majority of dinoflagellates and is lacking amoeboid forms. Amoebas that are present in abundance in the bioassays with live fish were ruled out as being *Pfiesteria* on the basis of fluorescent in-situ hybridization (FISH) and PCR experiments.

## 8. *Pfiesteria* toxin controversy

- Much of the *Pfiesteria* literature has been focused at the organism's alleged toxicity defined as the production of one or more substances that are poisonous to fish and to other marine animals and, in the same time, do not accumulate in their organisms.
- Known toxic dinoflagellates produce two classes of toxins: **polyketides** (e.g. **brevetoxins**) and **alkaloids** (e.g. saxitoxins). Neither of these toxins were found in *Pfiesteria piscicida*.
- For many years *Pfiesteria piscicida* toxicity could not be confirmed reliably under laboratory conditions. Very few labs were able to show that *Pfiesteria piscicida* does not have to be in physical contact with fish in order to kill it.
- Gordon et al. (2002) reported that filtrates from toxic cultures (*Pfiesteria piscicida* CAAAE #2200 and *P. shumwayae* CAAE #101272) retained their toxicity for up to 6 months at -20 °C. Most recent study (Moeller PD et al. (2007)) characterized the toxins as metal-

containing organic substances. The high toxicity is attributed to metal-mediated free-radical production. This mode of activity also explains previously reported difficulties in confirming its existence because of the ephemeral nature of free radical species.

## 9. Life forms by Burkholder JM, Glasgow HB Jr. (1997)

The toxic *Pfiesteria* has 3 distinct polymorphic life cycle stages

- Flagellated free-swimming forms

- zoospore

Also called **dinospore**. Motile flagellated live form of *Pfiesteria piscicida*.

- Toxic zoospore

Zoospore that produces toxin(s) after exposure to live fish.

- Non-toxic zoospore

Zoospore that does not produce the toxin, for example, when feeding on other microorganisms.

- Gametes

Haploid reproductive motile cells with two trailing flagella (longitudinally biflagellate cells) formed as a result division of vegetative zoospore. Fusion between isogamous, as well as between anisogamous gametes, was observed in different studies.

- Planozygote

The free-swimming, longitudinally biflagellate, actively feeding diploid cell formed by fusion of two gametes. Toxic when originates from a toxic strain and in presence of live fish. Sexual

reproduction is usually observed at the presence of live fish; however, Parrow et al. (2002) reported gamete formation and fusion in algae-fed *Pfiesteria piscicida*.

- **Amoeboid form**

Amoeboid forms result from zoospores' transformation upon fish killing.

- **Rhizopodial amoeba**
- **Filose amoeba**
- **Lobose amoeba**

- **Resting stages**

- **Encysted**

Encysted zoospores as well as amoeboid forms of various morphology.

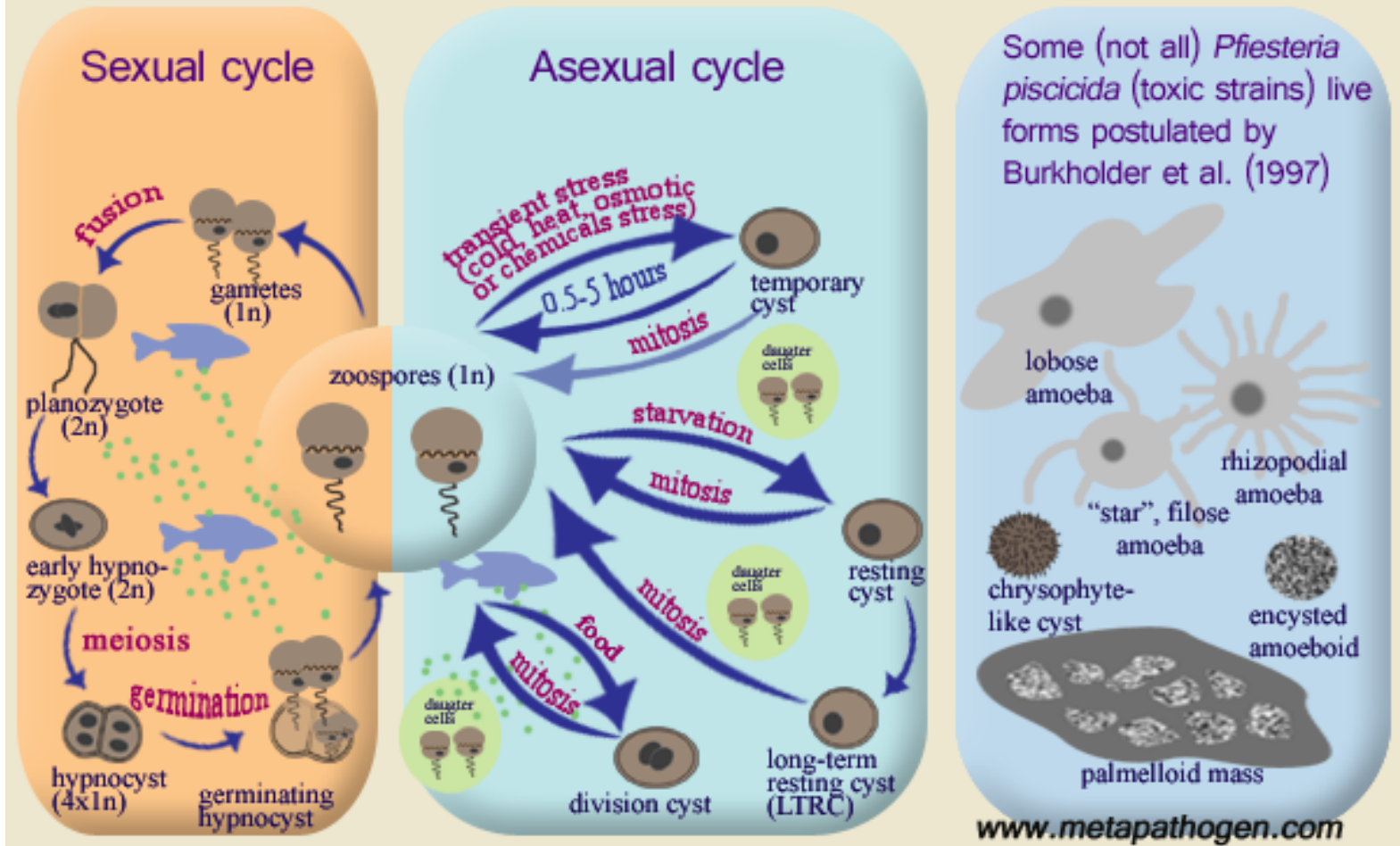
- **Palmelloid mass**

Immobile cells surrounded by gelatinous matrix.

- **Hypnozygote**

Encysted zygote

# Reproduction of *Pfiesteria piscicida*



## 10. Life cycle by Litaker et al., (2002)

Main characteristics:

- **haplontic** (haploid stages dominate)
- **homothallic** (no mating types, all gametes are compatible)
- **isogamous** gametes (all gametes have same morphology)
- vegetative (asexual) reproduction occurs by mitosis inside **division cysts** rather than by fission
- after 2 years of cultures, no amoeboid forms were observed
- no toxicity was demonstrated in this study, which, in opinion of Burkholder, renders it biased and insufficient to disprove her research data on life cycle and toxicity of *Pfiesteria piscicida*

- asexual

- zoospore

Dominant haploid live stage. Flagellated haploid motile cells, also called **dinospores**. Note that both names are misnomers because there is no sporogenesis in the life cycle. Twelve hours doubling time for algae-fed *P. piscicida* during exponential growth phase was reported.

- Division cyst

The division cyst, also called **zoosporangium**, is about 8-14  $\mu\text{m}$  in diameter. Encysted zoospore undergoes mitosis resulting in liberation of 2 asexual daughter zoospores (8.5-12  $\mu\text{m}$ ).

- Temporary cyst

Round thick-walled non-motile cell formed in order to survive under transient adverse conditions (short term lack of nutrients, cold or heat, osmotic or chemical stress, etc.). The temporary cyst's germination can result either in excystment of the single cell (majority of cysts) or liberation of two daughter cells if the cyst entered mitosis.

- Resting cyst and long-term resting cyst (LTRC)

Zoospore encysted in response to nutrients deprivation or cold. The ovoid resting cyst form aggregates on the bottom of about 30 cysts each. Large proportion of cultures deprived of food for 2-3 days is represented by the resting cysts. At 4 °C encystment of all zoospores take about 3 weeks in laboratory. The cysts can enter mitosis under favorable conditions resulting in liberation of two daughter cells upon germination. The cysts lose their viability with time.

- Sexual

- Gamete

Small motile zoospore capable of fusion with another zoospore.

- Fusion

Gametes begin to fuse soon after coming into contact. The fusion involves the formation of **fertilization tubes**. During fusion gametes retain their motility with epicone of the trailing cell fusing with hypocone of the leading cell.

- Planozygote

Motile diploid cell formed as a result of plasmogamy (cytoplasmic fusion) and karyogamy (nuclear) between two gametes takes less than 24 hours). Planozygotes are distinguished from other cells by their heart-shaped appearance and the presence of the two longitudinal flagella. Planozygote swims for a variable amount of time until forming hypnozygote.

- Hypnozygote

Encysted de-flagellated planozygote. When germinating, hypnozygote enters two rounds of meiosis (meiosis I and meiosis II), which results in liberation of 4 haploid asexual zoospores. In laboratory, hypnozygotes germinated 24 to 48 hr after formation of the planozygotes.

- Hypnocyst

The two-celled hypnozygotes can differentiate into long-term resting hypnocysts.

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