

# *Pfiesteria* “Cell From Hell” or Laboratory Artifact? An Update

Wolfgang K Vogelbein

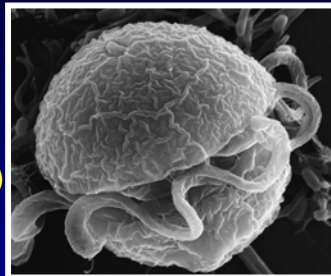


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## Background: The Toxic *Pfiesteria* Complex

Joann Burkholder et al., 1992-2003

- 2 recognized species: (*P. piscicida* & *P. shumwayae*; *Pseudopfiesteria* gen. nov.)
- Secretion of potent exotoxin(s)
- “Ambush predator” dinoflagellates (exotoxin sloughs fish tissues, zoospores feed)
- Cause of fish lesions, fish kills & human disease in mid-Atlantic estuaries
- 10 gal, 3 month “Gold Standard” fish bioassay (Burkholder et al. 92-03)
- Complex life history: (24-29 stages)



Menhaden: “*Pfiesteria*-characteristic” lesion



# Pfiesteria: A Brief History

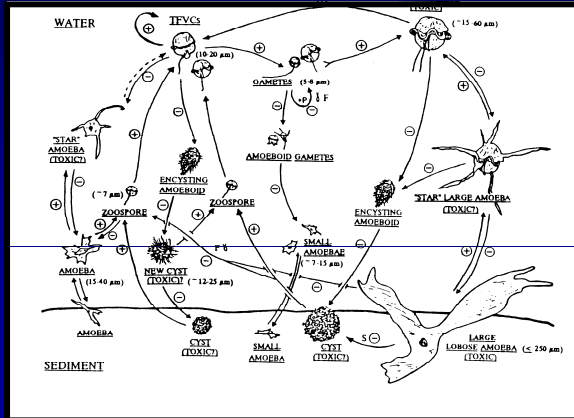
**1992** New “phantom” dinoflagellate is the causative agent of major estuarine fish kills. JM Burkholder, EJ Noga, CH Hobbs & HB Glasgow Jr. *Nature* **358**, 407-410 (1992).

**1995** Multiple papers detailing complex 24-stage life cycle and “Ambush Predator Hypothesis”

**1996** *Pfiesteria* blooms linked to hog waste

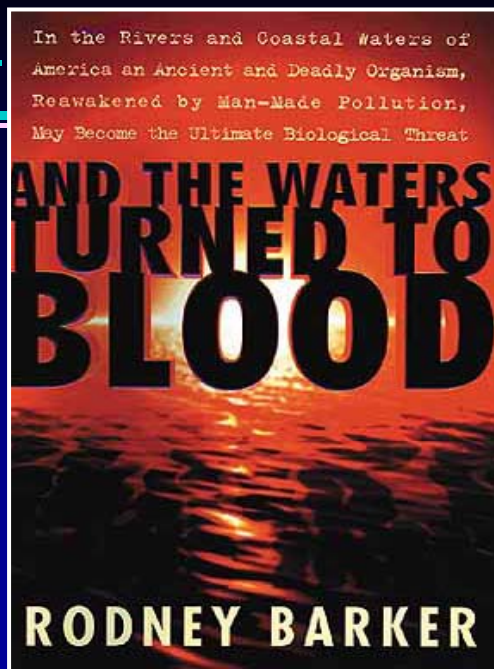
Outbreak of fish lesions in Chesapeake Bay

First alarmist news media reports



## Pfiesteria History contd.

**1997** “Below the smooth surface of North Carolina's eastern rivers lurks a deadly one-celled organism that is eating holes in fish and threatening people. The “cell from hell” is as frightening as the Ebola virus, but disinterested state regulators look the other way.”





### Pfiesteria hits Chesapeake Bay

**Maryland reaction is swift, concerted**

ANNAPOLIS, Md. (AP) — The Maryland General Assembly passed legislation Monday to what has been described as the most comprehensive legislation in the country to combat pfiesteria, a fish-killing microbe that closed some rivers last summer.

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Gov. Glenneeng included several provisions in his budget to help farmers, such as tracking money away from saturated areas and buying manure at a 50% price. He also budgeted to speed up repairs at sewage treatment plants.

Sen. Lowell Slichter, R-Somerset, said even though the final version pushed back the compliance dates proposed in the Senate version, the bill was still better than

farmers. "I think it validates the farmer's independence," he said. "It guarantees access by a government agency to our farms."

The Sen. Thomas Middleton, D-Chesley, said scientific information is conclusive that phosphorus runs into the bay.

"It's a landmark not only for the state of Maryland but for the nation," said Sen. Middleton, a farmer.

"This bill isn't about politics." In the House, rural lawmakers and environmentalists expressed dislike for the bill, but all voted for it anyway.

"We said we wanted mandatory controls by date certain. In exchange for mandatory controls, the date certain was delayed," said Delegate Law Billings, D-Montgomery.

Delegate Kenneth Schuler, R-Talbot, said the final bill was more positive and more businesslike than the House proposal.

Delegate James Hubbard, D-Prince George's, said the bill wasn't radical enough, but conceded it was a step in the right direction to improve water quality.

## Maryland General Assembly OKs anti-Pfiesteria law

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# 1997-98



**CRAB HOUSE & MARKET**

**NO PFIESTERIA HERE!**

**NEW LUNCH BUFFET**


**M-F 11-2 8.95**



**COASTAL HERITAGE**

**Pfiesteria Hysteria: Just When You Thought It Was Safe....**

## Burkholder, JM. 1999. The Lurking Perils of Pfiesteria. Scientific American 42:42-49

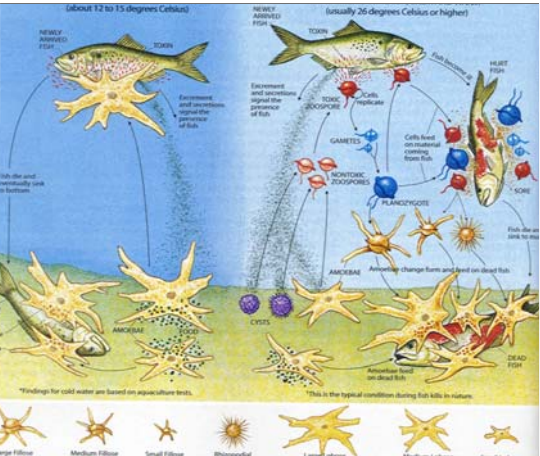


Neither plant nor animal, this tiny microbe has invaded our coastline, killing millions of fish. Now we're fighting back.

By Sharon Gwynn

# The Cell from Hell

### Ambush Predator Hypothesis



**(Below 12 to 15 degrees Celsius)**

**(Usually 25 degrees Celsius or higher)**

The diagram illustrates the 'Ambush Predator Hypothesis' for Pfiesteria. It shows two scenarios based on water temperature:

- Cold Water (12-15°C):** Pfiesteria is shown as a 'NEWLY ARRIVED FISH' that is 'TOXIC' to the fish. The fish are shown 'HURT' and 'DIE'. The diagram notes that 'Environment and nutrients signal the presence of fish' and that 'Cells feed on residual energy from fish'.
- Warm Water (25°C+):** Pfiesteria is shown as a 'NEWLY ARRIVED FISH' that is 'TOXIC' to the fish. The fish are shown 'HURT' and 'DIE'. The diagram notes that 'Environment and nutrients signal the presence of fish' and that 'Cells feed on residual energy from fish'.

At the bottom, a legend identifies different Pfiesteria cell types: Large Filamentous (10-15 micrometers), Medium Filamentous (5-10 micrometers), Small Filamentous (2-5 micrometers), Rhizoidal, Large Globular, Medium Globular, and Small Globular.

# Results of Science & Media Coverage

- Seafood & tourism industries in Chesapeake Bay hurt (> \$100 million in economic losses)
- Legislation enacted in Maryland regulating nutrient inputs
- Increased Federal and State funding for research (> \$25 million allocated to *Pfiesteria* research)

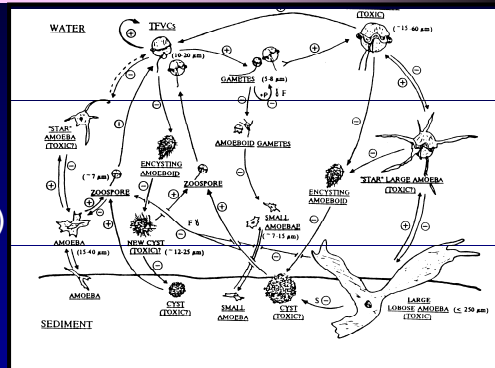


What have we learned from the research funds invested?

# Controversial Issues

## Burkholder et al. (1992-2003)

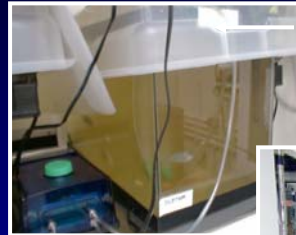
- **Life History** (simple vs. complex; amoebae vs. no amoebae)
- **Adverse Environmental Impacts** (fish lesions, death, human disease)
- **Mechanisms of Pathogenicity** (How does *Pfiesteria* kill fish?)
  - are there toxin-secreting strains?
  - other mechanisms of killing?
  - "Time to fish death", "% mortality"
- **Structure of *Pfiesteria* "Toxin"**
  - >15 years of effort, structure still unknown



# Early Studies: The “Gold Standard” Fish Bioassay

Burkholder et al. (2001)

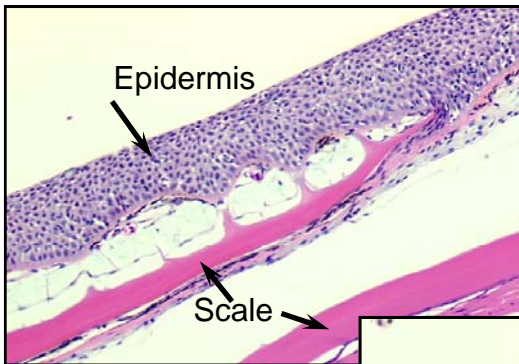
- **Concerns & issues**
  - cultures not available
  - human exposure
  - water quality (NH<sub>3</sub>, DO)
  - assay duration (months)
- **Causes of fish mortality**
  - *Pfiesteria* toxin
  - ammonia toxicity/DO
  - bacterial/parasitic diseases
  - other?
- **Water collected from Pamlico River, NC. (Nov. 1999)**
  - Killed tilapia 46 days later
  - ID *P. shumwayae* (SEM, PCR)



Fish Killing Assay

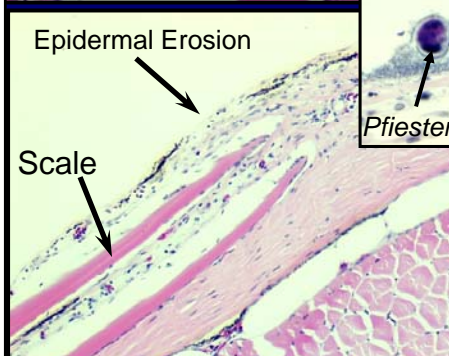


Control Assay



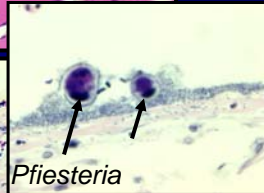
Epidermis

Scale



Epidermal Erosion

Scale



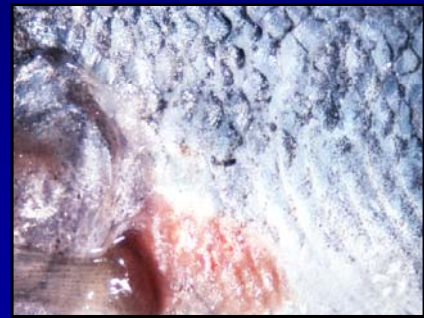
*Pfiesteria*

Exposed

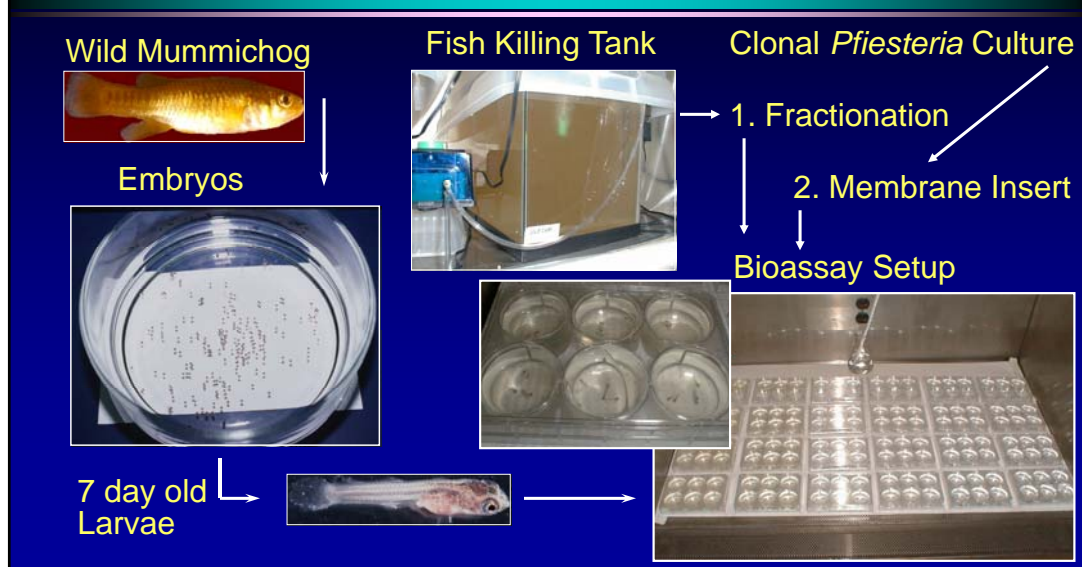
## Tilapia Pathology

Identical to *Pfiesteria* toxin exposure  
(Noga et al., 1996, Law, 2002)

Control



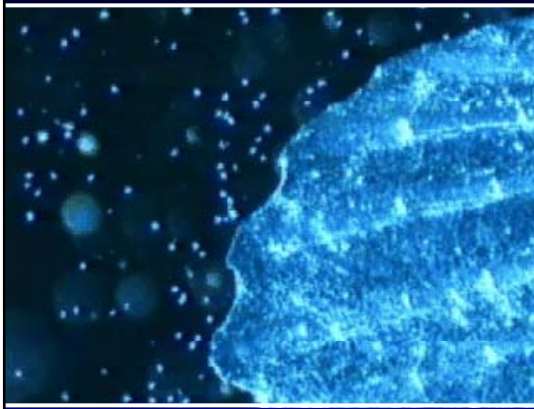
## 96 Hr Larval Fish Toxicity Bioassay



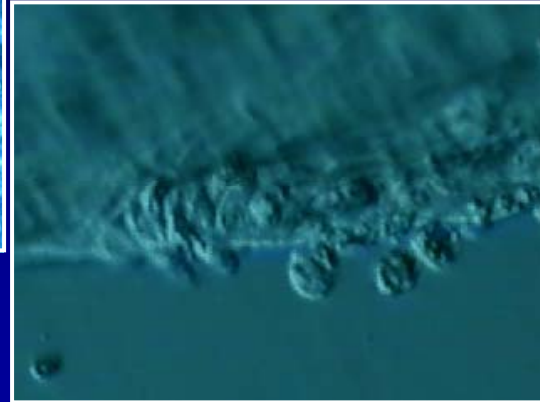
## 96 Hr Larval Fish Assay: Advantages

- Short assay duration (96 hr)
- High statistical power (large sample size, replication, proper controls) and reproducibility
- Reduced water quality issues (fish not fed)
- Reduced human health concerns
- Dinoflagellate/fish interactions readily observable microscopically (pathogenicity & life history studies)
- Lab-reared, parasite-free fish minimize contamination

# Micro-Predatory Feeding on Fish Epithelium (SKIN): The Movie



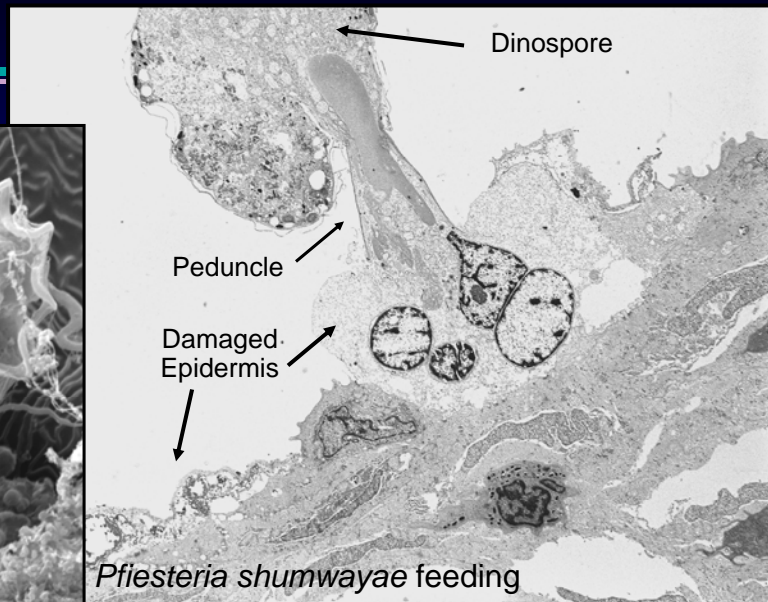
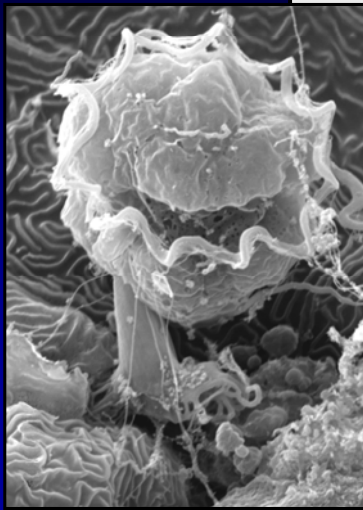
1. Chemoattraction
2. Swarming
3. Physical Attachment



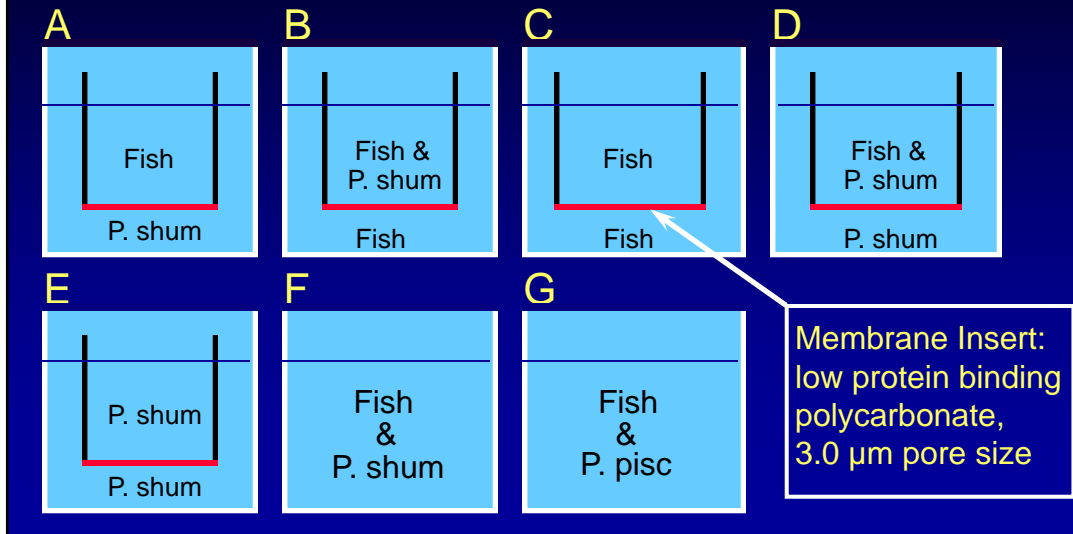
5. Feeding on Epidermis
6. Detachment (1-3 min)



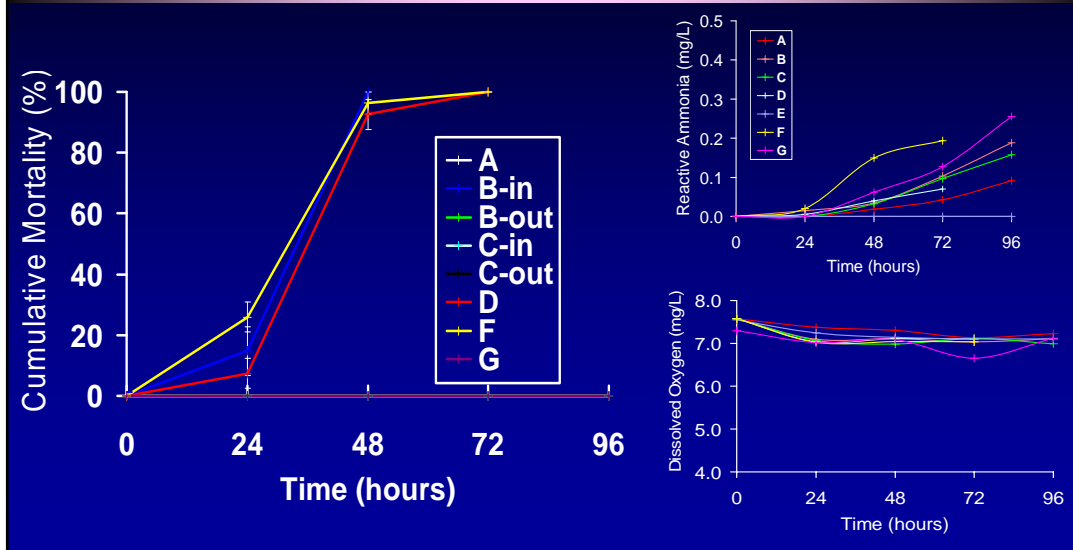
## Electron Microscopy: Fish skin/Zoospore interface



## Experimental Design: Membrane Insert Study



## Membrane Insert Experiment: Fish Mortality And Water Quality



# Experimental Design: Fractionations

Fish Killing 38 L Aquarium



"Raw" Water Sample

Fractionation of Raw Water  
(Filtration/Centrifugation)

Water Quality

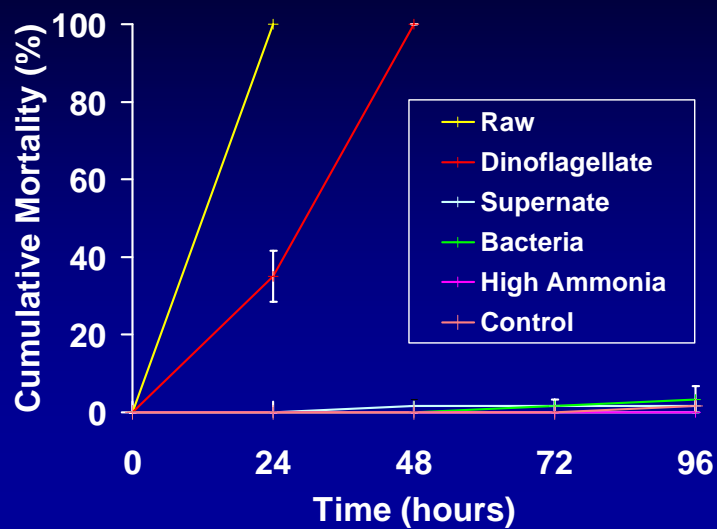
DO    Temp  
pH    Sal  
NH<sub>3</sub>  
NO<sub>2</sub>

1. Dinoflagellate Fraction
2. Bacterial fraction
3. Cell-free supernate
4. "Raw" water
5. NH<sub>3</sub> control
6. Clean water control

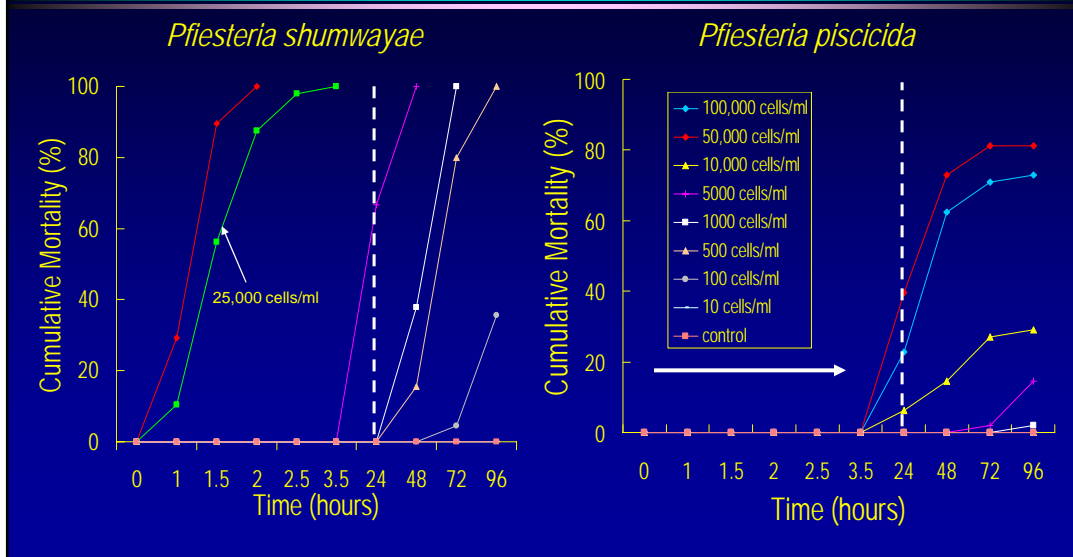
Larval Fish Assay



## Fractionation Study: Fish Mortality



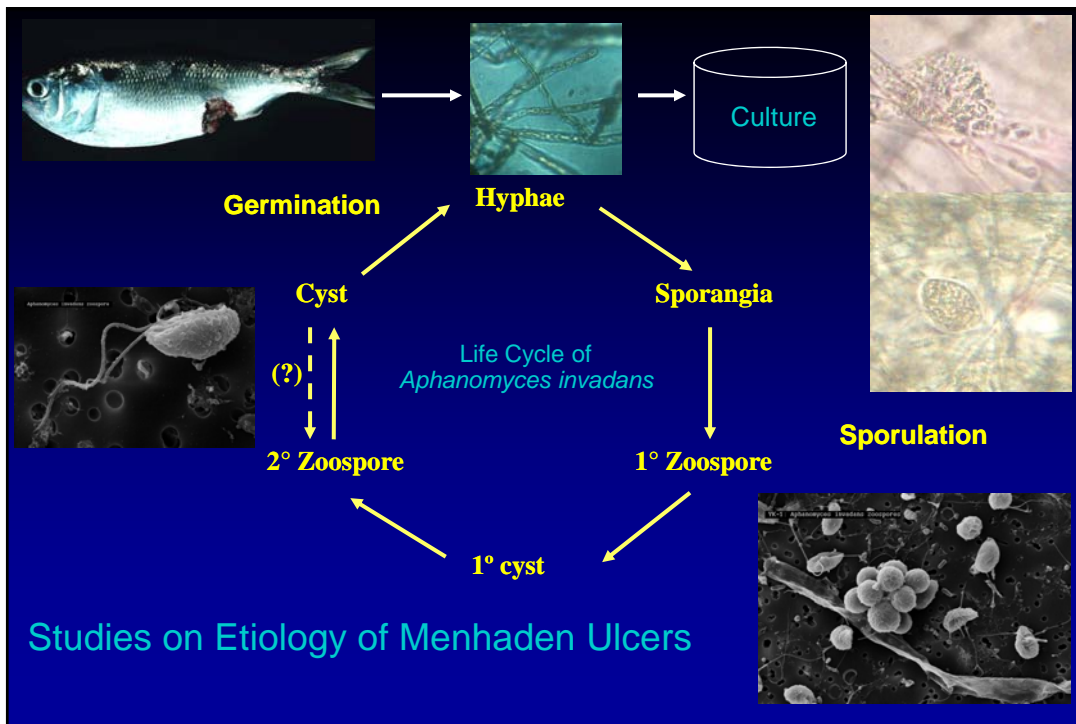
# Dose Response: Fish Mortality



## Summary: An Alternate Mechanism of Pathogenicity

Vogelbein et al. *Nature* 418:967-970 (2002)

- *P. pseudoshumwayae* & *P. piscicida* kill larval, juvenile & adult fish without secreting toxin(s)
- They kill fish by micro-predatory attack; zoospores feed on superficial epithelial tissues
- Do true “toxic” strains of *Pfiesteria* exist?
  - (Moeller et al., 2001)
    - Lipophilic fraction: phthalate ester contaminant
    - hydrophilic fraction: killed *Artemia*, fish but no skin lesions
  - (Moeller et al., 2002)
    - glycoside half sugar half something else unidentified
  - (Moeller et al., 2007. *Environ Sci Technol* 41:1166-72)
    - pPfTx ligated copper compound, free radical toxicity
  - No convincing evidence for toxicity or complete structure to date



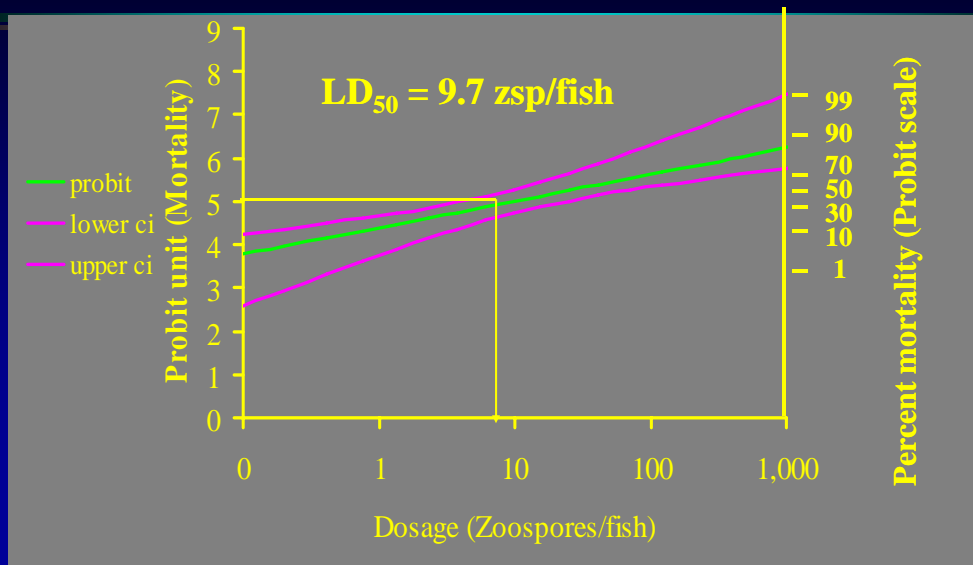
## Bath Challenges

14 D post-challenge

Treatment	Dose (Cells/mL)	Exposure (h)	Morts	Lesions/total (21 days)
Net-stress (high)	700	2	17/17	19/19
Net-stress (low)	70	2	17/18	20/20
Trauma	700	1	16/16	14/16
No-stress	110	5.5	4/17	6/19
Control	0	5.5	1/13	0/14

WIC strain only

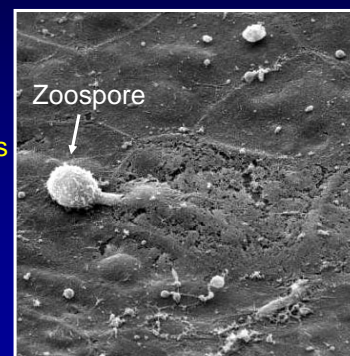
## *Aphanomyces invadans*: a highly virulent 1° pathogen of menhaden



## Results: Menhaden Challenges

- *Aphanomyces invadans*: 1° pathogen in menhaden
- Zoospores infectious via injections & aqueous exp
- Trauma provides a portal of entry
- Koch's Postulates fulfilled
- *A. invadans* is the causative agent of ulcerative mycosis
- *Pfiesteria* plays no role
- Massive NC fish kills attrib by other to hypoxia events

Blazer et al., (1999). *J. Aquat. Anim. Health* 11:340-349  
Vogelbein et al., (2001). *Env. Health Persp.* 109(5):687-693  
Blazer et al., (2002). *J. Aquat Anim. Health* 14:1-10  
Kiryu et al., (2002). *J. Aquat. Anim. Health* 14:11-24  
Kiryu et al., (2003). *Dis. Aquat. Org.* 54:135-146



## Other Critical Findings

- *P. pseudoshumwayae* & *P. piscicida* exhibit simple life cycles typical of other heterotrophic dinoflagellates (Litaker et al., 2002, Parrow & Burkholder, 2003)
- Adverse human health effects not supported by 5 year CDC Human Cohort Study
  - (Morris et al., 2006)
  - Unpublished Virginia & North Carolina data
- *Pfiesteria* spp. occur in Chesapeake Bay (<10 cells/ml: Reece unpublished)