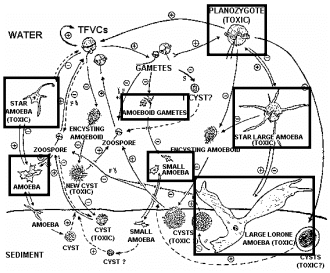
Pfiesteria Hysteria
Fad or Fallacy?

Pfiesteria piscicida was associated with

1. Ambush Predation of fish (complex life cycle)
2. Menhaden with lesions
3. Fish death in Tank Cultures
4. Putative toxins isolated from Tank Cultures
5. Episodes of human rashes or memory loss

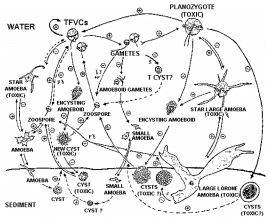
In 1992 was called the "Cell from Hell"

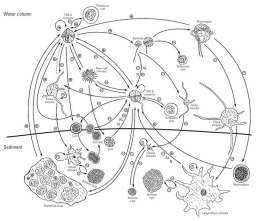
Proposed Complex Life Cycle
for *Pfiesteria piscicida*



- Amoebae - rayed floating forms
- Dinozoites - planozygotes, gametes, cysts

Reported Pfiesteria Life Cycles





1995 Version **2003 Version**

Indicates that microbial consortia is changes depending on culture

Identification of Amoebae Implicated in the Life Cycle of *Pfiesteria* and *Pfiesteria*-Like Dinoflagellates

Patrick M. Gillevet [a], Michael T. Peglar [a], Thomas A Nerad[a], and O. Roger Anderson [b]

[a] George Mason University, Manassas, VA, 20110, USA
[b]Lamont-Doherty Earth Observatory, Columbia University, Palisades, NY, 10964, USA

J. Eukaryot. Microbiol., 51(5), 2004 pp. 542–552

Peglar MT, Zettler LAA, Anderson OR, Nerad TA, Gillevet PM, Mullen TE, Jr. SF, Silberman JD, O'Kelly CJ, Sogin ML. Two New Small-Subunit Ribosomal RNA Gene Lineages within the Subclass Gymnamoebia. J. Euk. Micro. 50 (3) 224-232 2003

Different Approaches used by Phycologists and Protozoologists in Studying Isolates

Amoebae

- Predominantly use clonal monoprotist, xenic cultivation systems.
- Clonal monoprotist, monoxenic systems (with a single bacterial prey).
- Taxonomic requires LM and TEM combined with SSU rRNA gene sequencing.

Dinoflagellates

- Use xenic and clonal dinoflagellate diprotist cultures (with a single protist prey).
- Systems often contain unidentified protist, bacterial, and fungal contaminants.
- Taxonomic assignment requires LM and SEM combined with SSU rRNA sequencing.

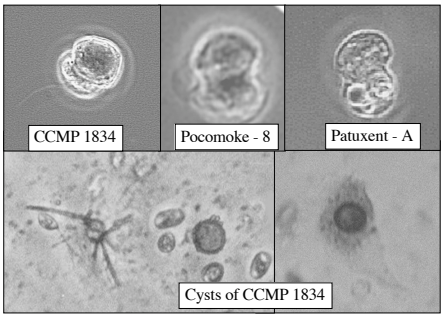
Life Cycle of Pfiesteria and PLD

H_A: Stellate amoebae are not life cycle stages of dinoflagellates.

H_0: The 18S rRNA of stellate amoebae is the same as that of dinoflagellates.

- Cannot prove Hypothesis right
- Only prove the Null hypothesis wrong !
- Falsifiability (Popper)
- Reproducibility (validation of cultures)
- Otherwise it is Pseudoscience

Isolated Dinoflagellates



Some life cycle stages observed in clonal dinoflagellate, diprotist cultivation systems observed for as long as 3 years

Many Amoebae Isolated from VCR LTER Site and Chesapeake Bay Tributaries form Rayed Stages

Can be Confused with:

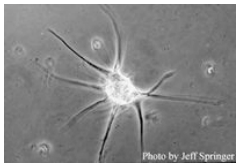
Class Lobosea
- Order Acanthopodida
  - Family Acanthamoebidae
    - *Acanthamoeba*
- Order Leptomyxida
  - Family Flabellulidae
    - *Flabellula*
    - *Paraflabellula*

Order Enamoebida
- Family Paramoebidae
  - *Mayorella*
  - *Paramoeba*
- Family Vannellidae
  - *Clydonella*
  - *Platyamoeba*
  - *Vannella*
- Family Vexilliferidae
  - *Neoparamoeba**
  - *Vexillifera*

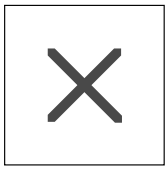
Note the wide taxonomic range of amoebae that form a stellate rayed stage.

*Neoparamoeba pemaquidnesis implicated as causative agent of lobster disease and a crab disease. Also known to cause various fish diseases.

Comparison of *Korotnevella*



Burkholder Web site 2003

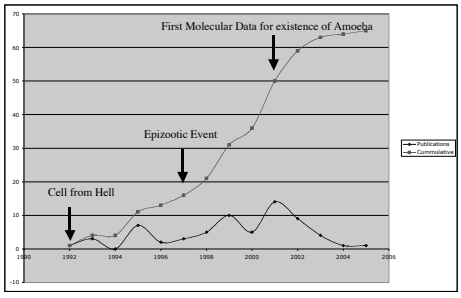


Settled

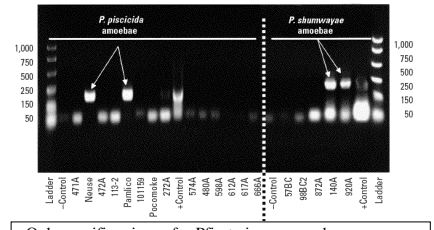
Peglar et. al. 2003

Floating

Burkholder Publications



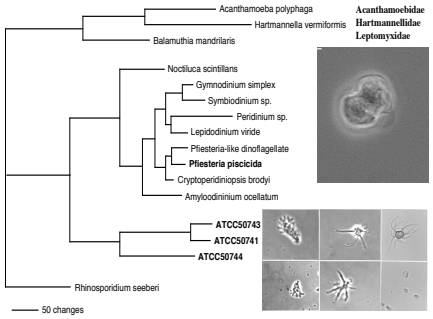
First molecular evidence for amoebae presented 2001:



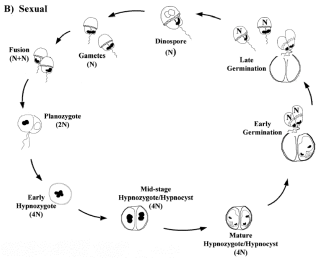
- Only specific primers for Pfiesteria were used.
- No primers for amoebae were used.
- It was simply a mixed Pfiesteria and Korotnevella culture.
- Set up to Prove Hypothesis not disprove Null Hypothesis.

Burkholder JAM, Glasgow HB, Deamer-Melia NJ, Springer J, Parrow MW, Zhang C, et al. Species of the toxic Pfiesteria complex, and the importance of functional type in data interpretation. Environmental Health Perspectives 2001;109, suppl. 5:667-679.

Phylogenetic Analysis of PLOs and Stellate Amoebae



Pfiesteria sp
Typical Dinoflagellate Life Cycle



- Litaker et al J. Phycology 38(3):442-463 2002
- Peglar et al. J. Euk. Micro. 50 (3) 224-232 2003
- Parrow & Burkholder J. Phycology 39(4):697-711 2003

There is no rigorous prove that amoeboid life stages exist.

- Examination of surface features of the amoeboid stages using TEM not SEM.
- SSU rRNA gene sequencing of putative amoeboid stages.
- Demonstration of the transformation from a dinozoite to an amoeba on videotape.
- Submit cultures of amoeboid life stages to culture collections for verification by other scientists.

None of these have been done!
Burkholder Pfiesteria Amoebae cultures do not exist!

In 1992, Pfiesteria piscicida was associated with

1. Ambush Predation of fish (complex life cycle)
2. Menhaden with lesions
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5. Episodes of human rashes or memory loss

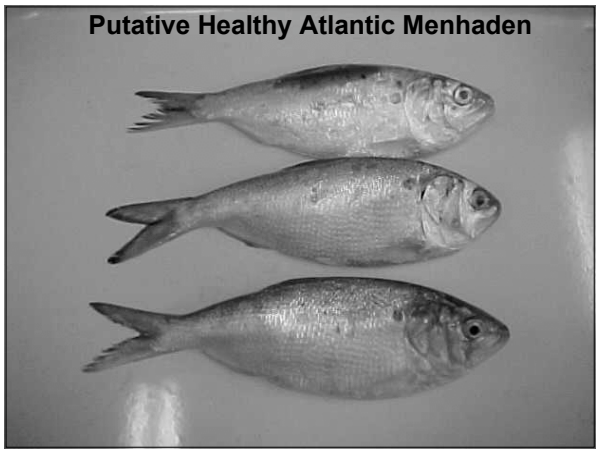
Does P. piscicida Cause Lesions in Fish

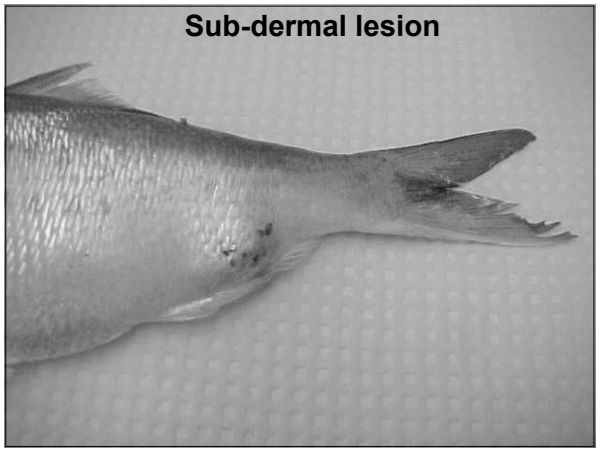
H_A: ***P. piscicida*** **is not directly associated with incidence of fish lesions.**

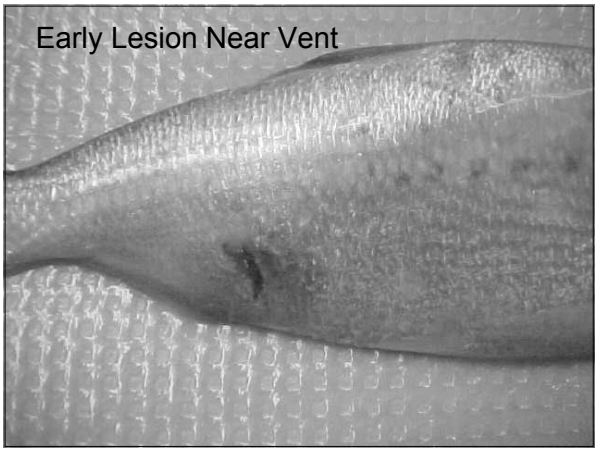
H_0: ***P. piscicida*** **is always associated with incidence of fish lesions.**

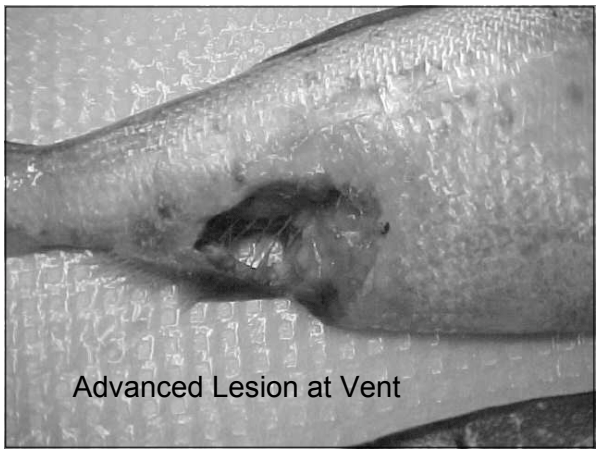
Epizootic Event: October 1999 in the James River, VA

Stanley R. Webb, Gregory C. Garman, Stephen P. McIninch, Thomas A. Nerad, Michael T. Peglar, Patrick M. Gillevet, and Bonnie L. Brown. Etiology of ulcerative lesions of Atlantic menhaden (Brevoortia tyrannus) from James River, Virginia Parasitology Research 2005 in press



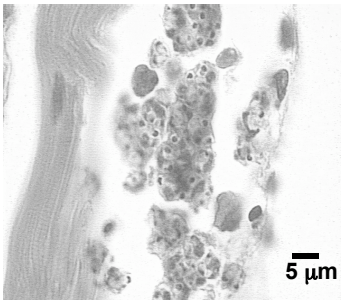




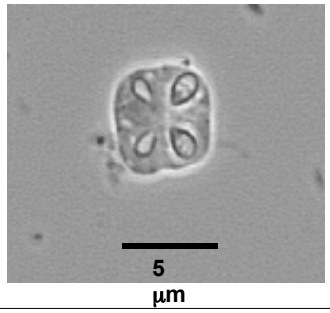


Kudoa plasmodia & maturing spores from sub-dermal lesion

(Hemotoxylin & Eosin stain)



Kudoa clupeidae live spore from Menhaden early lesion



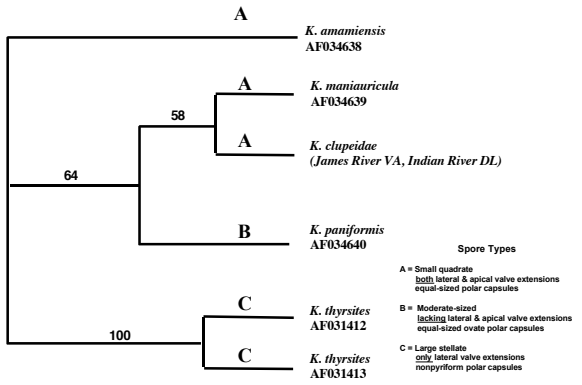
Summary of organisms recovered from lesions

90% of the 200 individuals captured had lesions

Taxa	No lesions (n = 3)	Sub-dermal lesions (n = 3)	Advanced lesions (n = 32)
Kudoa	100 %	100 %	100 %
Bacteria	0 %	0 %	100 %
Fungi	0 %	0 %	100 %
Amoebae	0 %	0 %	33 %
Dinoflagellates	0 %	0 %	0 %

(VCU assayed)

Parsimony Analysis
(100 Bootstraps)



Cause of Menhaden Lesions in these events

- ***P. piscicida*** **is not directly associated with lesions**
- ***Kudoa clupeidae*** **initiates Menhaden "lesions"**
- **Does *Aphanomyces invadans* take over and cause Ulcerative Mycosis????**

Vogelbein WK, Shields JD, Haas LW, Reece KS, Zwerner DE. Skin ulcers in estuarine fishes: a comparative pathological evaluation of wild and laboratory-exposed fish. Environmental health perspectives, 2001 Oct, 109 Suppl 5:687-93 2001.

Blazer VS, Vogelbein WK, Densmore CL, May EB, Lilley JH, Zwerner DE. Aphanomyces as a Cause of Ulcerative Skin Lesions of Menhaden from Chesapeake Bay Tributaries. Journal of Aquatic Animal Health 1999;11(4):340-349

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4. Putative toxins isolated from Tank Cultures
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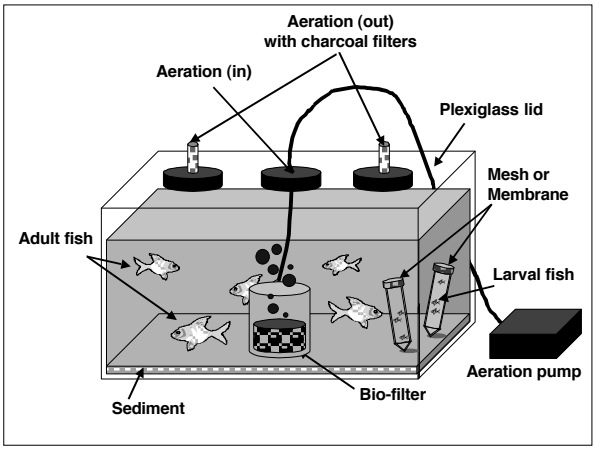
Characterization of Ichthyocidal Activity of *Pfiesteria piscicida* in Fish Bioassays

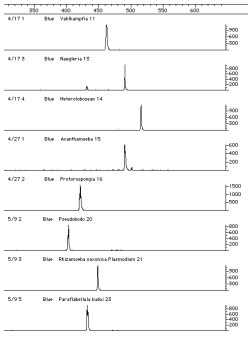
T. Drgon[1], K. Saito[1], P. M. Gillevet[2], Masoumeh Sikaroodi[2], B. Whitaker[3], D.N. Krupatkina[1], F. Argemi[1], and G.R. Vasta[1]

[1]*Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Baltimore, MD 21202, USA,*
[2]*Microbial and Environmental Biocomplexity, Department of Environmental Sciences and Policy, George Mason University, 10900 University Boulevard, Manassas, VA 20110, USA,*
[3]*Department of Animal Health, The National Aquarium in Baltimore, 301 East Pratt Street, Baltimore, MD 21202, USA.*

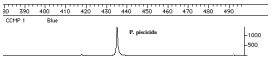
Applied Environmental Microbiology 71(1) 519-525 2005



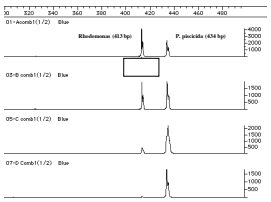
ALH Fingerprints of Isolates



Various Amoebae and Protists

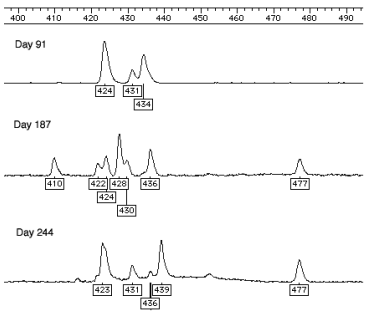


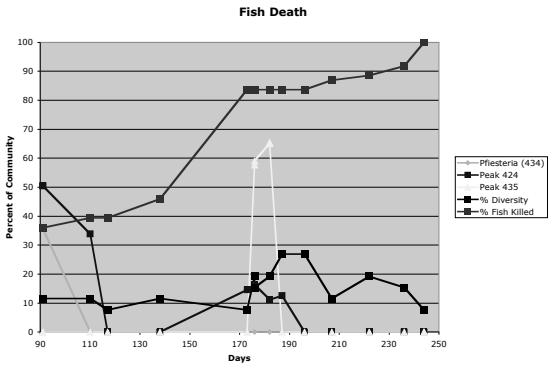
Pfiesteria piscicida (CCMP1834)



Abundance *P. piscicida* + *Rhodomonas*

ALH Fingerprint of Protists in Fish Bioassay





Peak of Pfiesteria at 90 days (38% of community) does not correlate with increased fish death at 180 days

Cloned and Sequenced Protist from various Fish Bioassays

Dinophyceae: *Pfiesteria*
Dinophyceae: *Scrippsiella*

Aconchulinia: *Nuclearia*
Pelobiontida: *Mastigamoeba*

Chlorophyta: *Nanochlorum*
Chlorophyta: *Scenesdesmus*
Chlorophyta: *Tetradesmus*
Chlorophyta: *Ostreococcus*

Chrysophyceae: *Ochromonas*
Chrysophyceae: *Paraphysomonas*

Oomycetes: *Phytophthora*

Alveolata: *Strombidium*
Alveolata: *Novistrombidium*
Alveolata: *Parautonea*
Alveolata: *Lecudina*
Alveolata: *Oxytricha*
Alveolata: *Uronema*

Fungi: *Neocallimastix*
Fungi: *Powellomyces*
Fungi: *Podospora*
Fungi: *Spizeelomyces*
Fungi: *Thalassiosira*

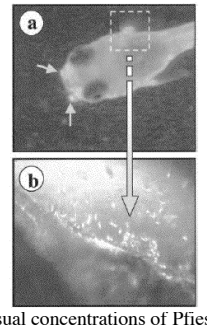
Fish introduce a complex microbial community !
All putative life stages are contaminants introduced by the fish !

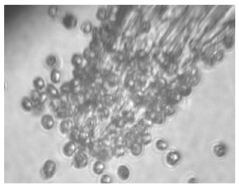
Cloned and Sequenced Bacteria from Various Fish Bioassays

Alpha proteobacter:	*Methylobacterium*
Alphaproteobacter:	*Vibrio*
Gamma proteobacter:	*Pseudomonas*
Gamma proteobacter:	*Aeromonas*
Gamma proteobacter:	*Methylococcus*
Bacteroides:	*Flexbacter*
Bacteroides:	*Cytophaga*
Bacteroides:	*Cyclobacterium*
Bacteroidetes:	*Pedobacter*

Bacteria are from the protist culture, sediments, or from the fish!
Some are potential pathogens !

Why Do Fish Die in Tank Culture?





- Unusual concentrations of Pfiesteria attack epidermis of fish.
- These concentration do not exist in environment.
- There are no toxins involved?

In 1992, Pfiesteria piscicida was associated with

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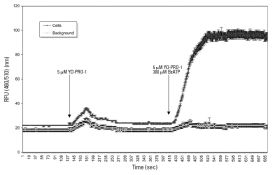
In Vitro Toxin Assay

- Cytotoxicity of GH4C1 rat pituitary cells
- Activation of P2X7 receptors causes uptake of fluor and cell lysis



- Organic extraction from 100s of liters tank water
- 1000 fold concentration
- di(2-ethylhexyl)phthalate = plasticizer from tubing

Fairey ER, Ramsdell JS. Reporter Gene Assays for Algal-derived Toxins. Natural Toxins 1999;7(6):415-421.

Toxin Assay

Burkholder PNAS 102 (9) 3471–3476 2005

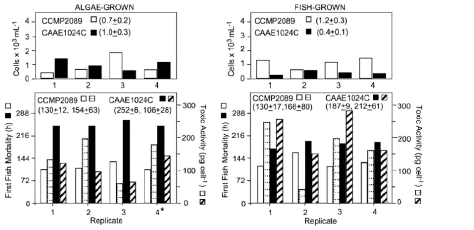


Fig. 2. *Pfiesteria* cell densities and cytotoxic activity in SFBs. (*Left*) SFBs of four subclones of algae-fed *P. shumwayae* CCMP2089 and CAAE1024C, showing water-column cell densities at the time of first fish mortality (*Upper*) and time of first fish mortality (left ordinate) and estimated cytotoxic activity per cell at time of second fish mortality (right ordinate) (*Lower*). (*Right*) Data from SFBs of four subclones previously fed fish (from subclone 4 at left).

- Pfiesteria concentrations higher in Algae fed tanks
- Fish die quicker in fish grown tank ???
- Absolute concentration of putative toxin greater in algal fed tanks
- No correlation between toxin and fish death????????

Toxin Assay

Burkholder PNAS 102 (9) 3471–3476 2005

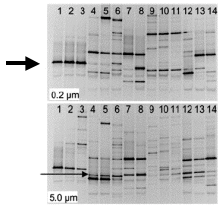


Fig. 1. DGGE images of PCR products, amplified for prokaryotic 16S rDNA by using eubacterial primers (31). DNA template was isolated from size-fractionated media from SFBs [0.2- to 5-μm fraction (*Upper*) and >5-μm fraction (*Lower*)]. Lanes 1–6, toxic *P. shumwayae* (two cultures evaluated, *n* = 3: CAAE1024C in lanes 1–3, CCMP2089 in lanes 4–6); lane 7, cryptoperidiniopsoid CAAE543A-AC1; lane 8, *P. piscicida* CAAE1332T-AC2 (CCMP2361); lanes 9–14, negative controls (media from fish cultures minus dinoflagellates). The presence of band 10 (*arrow*) in lanes 4–6 (toxic *P. shumwayae* CCMP2089) and lanes 9–14 (negative controls) suggests that this bacterium does not play a role in *Pfiesteria* toxicity.

1-4 = CAAE 1024
4-6 = CCMP 2089
7 = Rhodomonas (algae)
8 = CAAE 1332????
9-14= control tanks

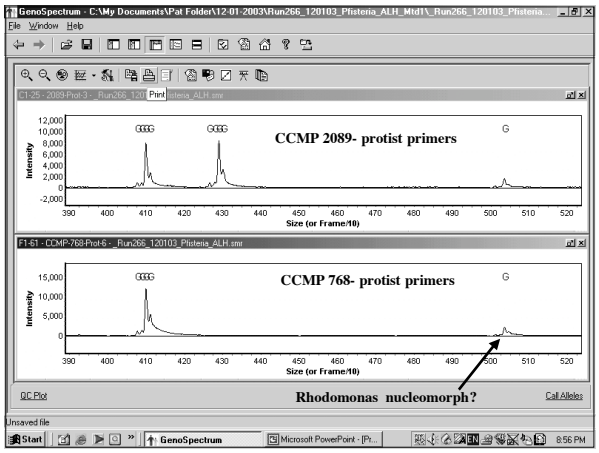
There are bacteria associated with "toxic" cultures of Pfiesteria !

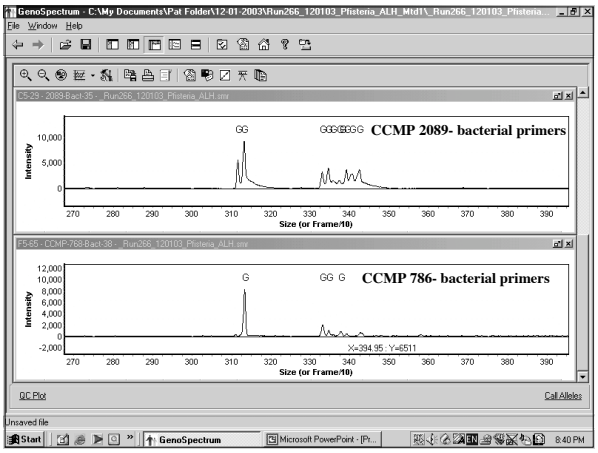
Are Burkholder PLD Cultures Monodinoflagellate, Diprotist Cultures?

- Isolated 5/1/1992
- Submitted June 20, 2003
- Fed CCMP 768

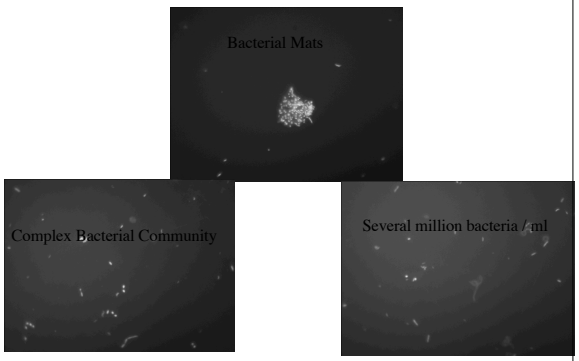
- CCMP 2300 - PLD, North Inlet, SC
- CCMP 2301 - PLD, Florida Bay, FL
- CCMP 2302 - PLD, Rhodes River, MD
- CCMP 2303 - PLD, Aquaculture Facility, NC

- CCMP 2089 - P. shumwayae, Pamlico River, NC

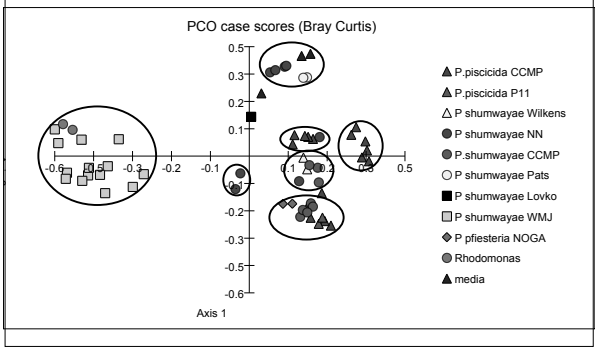




Bacterial Community in Burkholder Cultures



Bacterial Community in VIMS Cultures



SUMMARY

Xenic cultures in fish bioassays

- No direct evidence for toxic and non-toxic strains
- No direct correlation between Pfiesteria and exotoxins (or endotoxins)
- Cannot directly infer Pfiesteria is causal agent for any phenomena

Diprotist cultures

- Contaminated with bacteria
- No amoeboid life stages
- No ambush predation
- Normal Dinoflagellate life cycle

Lesions caused by Kudoa or Aphanomyces

- no rigorous evidence of involvement of Pfiesteria

No Human Health Affects

- Less than two dozen people reported to have had effects of Pfiesteria exposure
- Recent 5 year CDC cohort study: No effects

Is Pfiesteria the Cell from Hell?