ABSTRACTS OF ORAL PRESENTATIONS

PFIESTERIA – DE, MD, VA SESSION

INTENSIVE MONITORING FOR *PFIESTERIA* AND RELATED HAB EVENTS

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Intensive monitoring efforts, a collaboration between the University of Maryland Center for Environmental Science, Maryland Department of Natural Resources, and NOAA, are ongoing for determining environmental conditions leading to the outbreaks of *Pfiesteria* and related HAB species. Our intent is to develop an adaptable, autonomous system measuring a key suite of variables and reporting in real time. Initial efforts have been brute-force, deploying a wide range of new and conventional sensors from both fixed and floating platforms, in an attempt to assemble a tractable and sensitive detection system. In addition to monitoring meteorological and physical variables, sensors are deployed to monitor chlorophyll, zooplankton, optics, oxygen, nutrients and presence of fish. Acoustic telemetry is employed for underwater communications, and spread-spectrum radio relays the data to shore stations where the data are placed on the internet. A modified Chesapeake Bay Observing System (CBOS) buoy and shallow water platforms were employed to mount the various sensors in the lower Pocomoke River, a tributary of Chesapeake Bay, during late summer/early fall 2000.

Nutrient enrichment has been shown to stimulate *Pfiesteria*, but the relationships between nutrient loading and *Pfiesteria* growth are not clear. For nutrient monitoring, WS Ocean System nutrient monitors are employed to autonomously determine nitrate+nitrite and phosphate concentrations approximately every two hours. During instrument deployments, discrete samples are collected at the same location and time every few days for calibration of nutrients and water column chemistry. We are working on developing a system that can autonomously collect ammonium data during future deployments.

A suite of bio-optical instruments (fluorometer, turbidimeter and AC-9) are used for detecting variations on the optical characteristics of the water. Absorption spectra are resolved into the contribution of CDOM, detritus, and plant pigments at 8 wavelengths, using empirically-derived relationships based on fluorescence and back-scatter. The approach may permit the development of species-specific spectral signatures, depending on the robustness of the algorithms for correcting the very high concentrations of CDOM and suspended sediment at the site. Although *Pfiesteria* cannot be detected with this approach directly, various spectral signatures may be correlated with its presence.

Two biological components which have the potential to affect *Pfiesteria* are zooplankton and fish. Zooplankton consume *Pfiesteria* and thus are a biological control of their abundance and potential to bloom. High concentrations of fish can potentially trigger the metamorphosis of toxic stages of *Pfiesteria*. An *in situ* acoustic sensor capable of monitoring zooplankton and fish is being used to detect zooplankton and fish. The instrument is moored on the sediment surface, and includes a side-looking low-frequency acoustic transducer for detection of fish schools, and a high-frequency sensor aimed at the surface for monitoring the zooplankton in the water column above the mooring.

FUNCTIONAL TYPE (TOXICITY STATUS) CONTROLS *PFIESTERIA* RESPONSE TO NUTRIENTS AND ALGAL VERSUS FISH PREY

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Many "toxic algae" (here, including heterotrophic dinoflagellates and blue-green algae or cyanobacteria, as well as photosynthetic taxa such as toxic *Alexandrium* spp.) have naturally oc-curring toxic as well as apparently benign (non-inducible) strains with undetectable toxin produc-tion. About 60% of 470 *Pfiesteria* clones isolated by JB/HG over the past decade from estuaries known for toxic *Pfiesteria* outbreaks have tested as ichthyotoxic. *Pfiesteria* zoospores may be 1of 3 functional types as actively toxic (TOX-A, in fish-killing mode), temporarily nontoxic (TOX-B, capable of toxin production but without recent access to live fish), or non-inducible (NON-IND, apparently unable to cause fish stress, disease, and death). As a separate phenomenon and an apparent artifact of culture conditions, toxic *Pfiesteria* strains (like many other toxic algae) gradually lose toxin-producing capability in culture. About 98% of all cultured toxic *Pfiesteria* strains have become NON-IND after 6-8 months even when maintained with live fish, and after a shorter duration when maintained with other prey).

We tested the hypothesis that the three functional types of *Pfiesteria* differ significantly in response to N and P enrichments, algal prey, fish prey, and other factors. The cultures of *Pfiesteria piscicida* and *P. shumwayae* used in this research were isolated from the Neuse Estuary. One isolate each of each species was confirmed by JB/HG as toxic to fish (toxicity cross-con-firmed by H.Marshall, ODU; uni-dinoflagellate clonal quality – Heteroduplex mobility assay, D.Oldach, U.MD; species identifications from suture-swollen cells with SEM, cross-confirmed by PCR probes from HG/JB and FISH probes of P. Rublee, UNC-G). A second isolate of each species was cloned and tested as NON-IND, with cross-confirmations as above. TOX-A *Pfiesteria* spp. were grown with live fish for 3-4 months prior to the experiments; TOX-B cultures were grown with live fish for 2-3 months, then were switched to a diet of cryptomonad prey for 3 weeks; and NON-IND cultures were similarly grown with cryptomonad prey. In the 1st experi-mental series, we tested *Pfiesteria* zoospore production in response to N_i or P_i enrichment + cryp-tomonad prey (500 µg NO_3^- or PO_4^{-3}/L). A 2nd experiment tested *Pfiesteria* response to crypto-monad prey (batch mode, 6 d, n=4). In a 3rd experiment, we examined *Pfiesteria* response to fresh fish mucus (minutes, microcapillary tube assay).

NON-IND *Pfiesteria* attained highest zoospore production on both N and P enrichments, and on cryptomonad algal prey. This functional type retained kleptochloroplasts for longer peri-ods and exerted the lowest grazing pressure on algal prey, suggesting increased reliance on photo-synthesis in a more 'plant-like' mode. TOX-B *Pfiesteria* was intermediate in stimulation by N and P, kleptochloroplast retention, and cell production with cryptomonad prey. TOX-A zoospores had lowest cell production on N and P enrichment, and on algal prey; and their kleptochloroplast retention was negligible. Although this functional type had the highest algal grazing rates and most tightly coupled Lotka-Volterra fluctuations with cryptomonad prey, the consumed prey supported negligible zoospore production relative to that of unfed controls. Trials with fresh fish mucus indicated strongest attraction by TOX-A *Pfiesteria*, with intermediate to low attraction by TOX-B zoospores and negligible attraction by NON-IND *Pfiesteria.* These data demonstrate that functional types of *Pfiesteria* spp. are strikingly different in response to nutrient enrichment, algal prey, and fish prey. The data raise a 'red flag' in warning against use of NON-IND strains in research to gain insights about environmental controls on toxic *Pfiesteria.*

ASSESSING TEMPORAL AND SPATIAL VARIABILITY IN *PFIESTERIA PISCICIDA* DISTRIBUTIONS USING MOLECULAR PROBING TECHNIQUES

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The toxic dinoflagellate, *Pfiesteria piscicida*, poses a significant threat to natural resources and to public health. Although often linked to increases in pollution and nutrient loading, the environmental factors that contribute to toxic *Pfiesteria* blooms are unclear. An understanding of these relationships is critical to the development of monitoring strategies for high risk areas and requires that the presence of *P. piscicida* be rapidly and accurately assessed. Routine monitoring by light microscopy lacks both the sensitivity and accuracy required for species-specific detection of low levels of *Pfiesteria*. In this study, we used molecular probes to identify and enumerate *Pfiesteria piscicida* and several closely related members of the *Pfiesteria* species complex in the Delaware Inland Bays and the Pocomoke River, Maryland. Low levels of *Pfiesteria* were detected in water and sediment samples confirming the presence of *Pfiesteria* as a minor but prevalent member of the phytoplankton community in mid-Atlantic estuaries. We also describe a novel technique, Polymerase Chain Reaction-Fluorescent Fragment Detection (PCR-FFD), for specific and quantitative detection of low levels of *Pfiesteria* in environmental samples. Using PCR-FFD, we conducted a diel study of *Pfiesteria* in the Broadkill River, Delaware. Preliminary data generated by this study indicates a possible tidal influence on the presence and concentration of *Pfiesteria* as well as possible correlations to biological variables and phosphate.

THE AMBUSH PREDATOR *PFIESTERIA PISCICIDA*: FAD OR FALLACY

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The ambush predator hypothesis is based on three principal tenets: (1) in the presence of fish, *Pfiesteria piscicda* undergoes sexual reproduction and transforms into an amoeba that feeds on lesioned fish, (2) the dinoflagellate releases exotoxins that narcotize and cause lesions in fish, and (3) the toxic ambush predator behavior is induced in aquaria if fish are present during an extended incubation period.

We have isolated and characterized the most prevalent amoebae with stellate floating forms as well as *Pfiesteria* complex (PCOs) dinoflagellates from rivers in Maryland and VA where epizootic events involving large numbers of dead or moribund fish have occurred. Based upon morphological and molecular data the amoebae were shown not to be life stages of the co-isolated dinoflagellates. These findings suggest that the reported amoeboid life stages of *Pfiesteria piscicida* and related dinoflagellates are likely phylogentically distinct taxa. After nearly two years of continual observation, clonal strains of *Pfiesteria*-like dinoflagellates and *P. piscicida* reference strains (CCMP 1830,1831,1834) have never yielded an amoeboid form. Further, clonal cultures of *P. piscicida* have been observed to form planozygotes indicating that *P. piscicida* is capable of undergoing sexual reproduction in the absence of fish or fish derived materials without converting to amoeboid stages.

During a fish survey (Oct 1999) in the lower James River near Richmond, VA, penetrating lesions similar to those reported for *P. piscicida* were found in approximately 75% of Atlantic menhaden sampled. *Kudoa clupeidae* was found in histological sections from fish without evident lesions and from fish with pre-lesions (raised unruptured regions of the epidermis) as well as from fish with open lesions. No evidence of fungal, bacterial, or amoeba involvement were found in fish without lesions or in fish with pre-lesions. This study indicates that *Kudoa clupeidae* may be a significant agent in initiating lesion development in recent outbreaks of fish mortality in eastern Atlantic coastal waters and calls into doubt the role of *P. piscicida* and related dinoflagellates (PCOs) as the causative agents of these epizootics.

We have characterized the complex microbial consortia from fish culture experiments using Amplicon Length Heterogeneity Fingerprinting. We observed the presence of several protist and bacterial taxa that may be correlated with fish mortality. The results indicate the existence of other putative microbial organisms besides *Pfiesteria* that may be the causitive agent of fish mortality in these fish culture experiments.

Thus, the three basic tenets of the ambush-predator hypothesis are called into question by our studies casting further doubt that *Pfiesteria piscicida* plays any role as the causative agent of epizootic events involving penetrating fish lesions in the Chesapeake Bay and its tributaries.

NITROGEN UPTAKE AND NUTRIENT RELATIONSHIPS IN LABORATORY CULTURES AND FIELD ASSEMBLAGES OF *PFIESTERIA*

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Due to the largely heterotrophic nutrition of *Pfiesteria*, the degree to which nutrients are directly or indirectly taken up by this organism is of considerable interest. *Pfiesteria* is typically associated with nutrient rich conditions in the field, but the extent to which eutrophication may stimulate this organism is yet unclear. Laboratory studies have demonstrated the direct uptake of both inorganic and organic nutrients by *Pfiesteria.* Rates of nitrogen uptake, as determined using ^{15}N tracer techniques, compare with rates of nitrogen acquisition by phagotrophy under certain growth conditions. Direct comparisons of patterns in nitrogen uptake were made using actively toxic ("TOX A"), previously toxic ("TOX B") and non-toxic (algal-fed, "NON –IND") *Pfiesteria piscicida*, and rates varied with feeding history and culture incubation conditions.

Direct uptake of nitrogen may not be the only pathway by which this organism obtains dissolved nitrogen. Recently toxic cultures of *Pfiesteria* have been shown to have mechanisms for obtaining nitrogen in the form of amino acids via extracellular oxidation. High rates of extracellular amino acid oxidation have also been observed in the field when *Pfiesteria* has been confirmed to be present. This pathway of nitrogen acquisition has previously been observed for some other phytoplankton species, and in particular, dinoflagellates.

In Maryland's tributaries, outbreaks of toxic *Pfiesteria* have consistently been associated with ratios of organic carbon: organic nitrogen that are elevated above levels that are observed during periods of non-*Pfiesteria* activity. With the ability of *Pfiesteria* to access nutrients by both heterotrophic or autotrophic pathways, it may be able to grow successfully on available particulate or dissolved, inorganic or organic, nutrients when other necessary conditions, such as reduced grazing pressure and appropriate temperatures are present.

PFIESTERIA OR FUNGUS? INDUCTION OF SKIN ULCERS IN MENHADEN WITH ZOOSPORES OF _APHANOMYCES_ SPP.

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Menhaden, *Brevoortia tyrannus*, develop skin ulcers that have been attributed to exposure to *Pfiesteria piscicida* toxins. However, the consistent presence of a fungal agent in the ulcers suggests a different etiology. We have completed three challenge studies to determine the infectivity of *Aphanomyces invadans* in relation to its role as a primary or secondary etiologic agent. (1) Hyphal injection: macerated hyphae of two strains of *A. invadans* (INV, an Asian strain, and WIC, an endemic strain in menhaden from Maryland) were injected subcutaneously into 10-11 menhaden. Fish were kept in static aquaria (6 ‰, 76 L, preconditioned whisper filters) at 23.0°C, and monitored daily for 26 d. Hyphal injections caused characteristic lesions. Small, incipient skin lesions developed after 4-5 d. Large necrotic lesions were present on 60% of fish injected with WIC after 26 d. No lesions were detected in fish injected with INV or the saline control. (2) Zoospore injection: secondary zoospores of WIC and INV and an additional strain, ATCC #62427 (isolated from menhaden from North Carolina) were injected subcutaneously into menhaden. Fish were inoculated with 1.9×10^3 zoospores (WIC-low), 1.9×10^4 (WIC-high), 6.5×10^3 zoospores (INV strain), or 7.5×10^3 zoospores (ATCC). Both low and high doses of WIC caused incipient, mildly granulomatous lesions in fish after 4-5 d. Fish injected with the high dose WIC died within 7 d. After 10 d, all of the fish inoculated with the low dose WIC had died. Fish treated with INV developed lesions after 9 d; i.e., later than those injected with WIC. Fish injected with ATCC or saline did not develop lesions after 21 d. (3) Zoospore bath challenges: fish were exposed in baths containing zoospores of WIC. Treatments consisted of net-stressed fish (Net stress, exposed for 2 h to either 7.0×10^2 and 7.0×10^3 zoospores/ml), fish with a few scales removed (Trauma, exposed for 1 h to 7.0×10^3 zoospores/ml), fish with no handling (No trauma, exposed for 5.5 h to 1.4×10^3 zoospores/ml), and unexposed controls. Mortality and prevalence of the lesions were high (88-100%) for fish in stress treatments (net, trauma), while they were lower (20-23%) for fish in the "No trauma" treatment, a more environmentally realistic exposure.

Lesions from fish inoculated with hyphae or zoospores or bath exposed with zoospores were histologically identical to those observed in wild menhaden collected from several estuaries and rivers along the middle North Atlantic coast. The deeply penetrating ulcers were characterized by dermatitis, myofibrillar degeneration, and a deep necrotizing granulomatous myositis. Experimentally induced lesions, however, exhibited more invasiveness, often involving the liver or kidney. Incipient granulomas were apparent after 5 d with inoculation or bath exposure to zoospores, and became more abundant with time. Infections developed into frank lesions over a relatively short time frame of 7-9 d. We demonstrated that "typical" skin ulcers attributed to exposure to *Pfiesteria piscicida* can be experimentally induced following inoculation or bath exposure with fungal zoospores of an endemic strain of *A. invadans*.

TROPHIC RELATIONSHIPS OF PHYTOPLANKTON AND MICROZOOPLANKTON WITH *PFIESTERIA*-LIKE HETEROTROPHIC DINOFLAGELLATES IN POCOMOKE RIVER AND TRANSQUAKING/CHICAMACOMICO RIVERS, MD, USA.

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In laboratory experiments, reproduction and stage transformations of *P. piscicida* have been linked to abiotic (inorganic nutrients, temperature and salinity) and biotic factors (organic nutrients and algal and fish prey density) (Burkholder and Glasgow 1997). In these experiments, it has been demonstrated that *P. piscicida* has specific algal prey preferences. Highest zoospore production took place in treatments that were fed *Cryptomonas sp.*. Treatments fed flagellates from other taxonomic groups (Chlorophyceae and Prymnesiophyceae) were characterized by significantly lower abundances of *P. piscicda* zoospores. A correlation between non-toxic vegetative cells and chlorophyll *a* and specific algal prey has also been documented in field studies in the Pamlico and Neuse River estuaries, North Carolina (Fensin 1997). In this study, spring chlorophyll *a* and densities of the dinoflagellate *Prorocentrum minimum* were correlated positively with zoospore abundance. This laboratory and field data indicate links between nutrient enrichment, algal prey types and densities and *P. piscicida* zoospore densities.

The population dynamics of *Pfiesteria*-like heterotrophic dinoflagellates are likely controlled by bottom-up factors (food) as well as top-down forces (grazing). Oligotrichous ciliates and tintinnids > 20 µm have been shown to prey on *P. piscicida* zoospores in laboratory experiments (Stoecker et al. in prep.). In contrast, other forms of metazoa and non-ciliate grazers were relatively ineffective in reducing the densities of zoospores. Grazing rates calculated in this laboratory study (0-0.46 zoospores/h) indicate that protistan grazing may be able to regulate the densities of *P. piscicida* zoospores.

In the Pocomoke and Transquaking/Chicamacomico Rivers, the presumptive numbers of *Pfiesteria*-complex organisms closely tracks the density of cryptophytes. The few occasions when there is a disconnect between PCOs and this favorite prey group seem to occur as a result of fluctuating grazing pressure. For instance, during the 9/3/99 sampling in the Chicamacomico River (CCM0069) there was a peak density of cryptophytes but a relatively low number of *Pfiesteria*-like heterotrophic dinoflagellates. This disconnect between food and grazer was likely the result of peak densities of PCO consumers, oligotrichous ciliates and tintinnids > 20 µm. Bi-weekly field data from the Pocomoke River and Transquaking/Chicamacomico Rivers will be analyzed for the summer of 2000 and compared to the results generated from 1998 and 1999 in further attempts to establish trophic linkages between the population dynamics of *P. piscicida* – like heterotrophic dinoflagellates and specific algal prey and microzooplankton predators.

APPROACHES TO THE INVESTIGATION AND INTERPRETATION OF POSSIBLE *PFIESTERIA*-RELATED EVENTS IN MARYLAND

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Pfiesteria was first documented in Maryland in 1992 (Lewitus *et al*, 1995. Estuaries 18: 373-378), but it was not until 1997 that it drew much public attention when several fish kills on Maryland's Lower Eastern Shore associated the dinoflagellate with fish health, fish mortality, and human illness (Gratten *et al*, 1998. Lancet 353: 532-539, Md DNR 1998). In consultation with external scientific panels and numerous local, state and federal agencies, Maryland responded with a conservative public policy that placed a priority on protecting human health, while recognizing that a number of scientific uncertainties still existed. Using multiple lines of evidence, the State temporarily closed several miles of tidal rivers in 1997 until the suspect conditions were not evident for a period of two weeks. This public policy continues and has remained flexible since the beginning to utilize the latest scientific understanding, measurement methods, and decision-making processes to assess the possibility that a toxic outbreak of *Pfiesteria* is occurring.

Since 1998, a system of intensive environmental and fish community monitoring has been established in Maryland. These 'comprehensive assessments' are used as a surveillance system for possible outbreaks and to refine our understanding of the factors that may lead to the occurrences of toxic or non toxic *Pfiesteria* in association with fish health/mortality. Additionally, a 'rapid response' capability has been established to draw upon a pool of trained biologists that can be deployed within hours to investigate suspicious findings. In most cases, these findings involve fish kills or the presence of diseased fish, primarily Atlantic menhaden with ulcerative lesions. These two monitoring programs are being coordinated with a number of *Pfiesteria*-related research studies, including those investigating links to environmental conditions, fish health, and human health, and those pursuing more precise and rapid techniques for the identification of *Pfiesteria* and its toxin(s).

Maryland has employed the presence of unexplained fish lesions as one of the indicators to pursue more targeted investigations of *Pfiesteria* and associated environmental factors. Evidence of active, toxic *Pfiesteria* was documented at the sites of the 1997 fish lesion / mortality events, and the presence of non toxic *Pfiesteria* has been documented at the site of almost every major menhaden lesion outbreak that has been investigated from 1998 to 2000. Although the relationships between lesioned menhaden and *Pfiesteria* remain areas of active research, in the absence of rapid and definitive screening methodologies for potential toxic outbreaks, fish health remains a viable, albeit imperfect, indicator of possible *Pfiesteria* activity. Human health concerns can also be used to trigger an investigation.

The interpretation of data collected during a rapid response investigation is challenging because it is currently impossible to measure *Pfiesteria*-related toxin(s) directly, and river closure decisions must be made quickly (24 -72 hrs.). Thus, multiple lines of evidence are used in a 'weight of evidence' approach using measurements in the field and results from multiple laboratories. One of the most valuable additions to our measurement suite is the rapid (less than 24 hrs) molecular probe technology that has recently been developed (Oldach *et al,* 2000. PNAS 97(8): 4303-4308) and has been incorporated into our monitoring program starting in 1999. If no *Pfiesteria* genetic material is detected with these molecular techniques, we assume that the probability of toxicity due to *Pfiesteria* is very low. An actively toxic event is also ruled out if there are no obvious signs of distressed behavior in resident fish or a significant fish kill in progress. Finally, an actively toxic event is ruled out if the counts of *Pfiesteria*-like cells are below those reported in the literature to cause toxic impacts to fish. Thus, all three conditions described

above - presence of *Pfiesteria* genetic material, fish in distress or fish kill, and high cell counts - are necessary to consider any event as potentially toxic.

RESULTS OF A SERIES OF FISH BIOASSAYS WITH THE TOXIC DINOFLAGELLATE *PFIESTERIA PISCICIDA*

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A series of fish bioassays were conducted to test the ichthyotoxic activity of a toxic strain of the dinoflagellate *Pfiesteria piscicida* using tilapia, with *P. piscicida* producing fish deaths in 9 of 10 of these experimental bioassays. Once toxicity was established, *P. piscicida* maintained this toxicity causing fish deaths in the culture vessels as long as the dead fish were replaced with live fish. In addition, this toxicity was perpetuated following a series of inoculations to other culture vessels. Among these bioassays, there were differences in the period between inoculation of *P. piscicida* cells and the onset of fish deaths. Using *P. piscicida* from a culture that had previously been maintained on algal cells (Cryptomonas sp.) it took 16 days before fish deaths occurred. In contrast, fish deaths occurred within hours when using a culture that had recently (previous day) killed fish. Initial inoculations of live *P. piscicida* into these bioassays was ca. 50-60 zoospores mL^{-1}, with approximately a 10-fold increase in numbers within the culture vessels before the first fish death occurred, with later concentrations at >5,000 zoospores mL^{-1} during periods of highly active toxicity. Throughout these bioassays the control fish remained healthy, with only one death occurring among 90 fish. In contrast, 100's of fish in the culture vessels of the bioassays that were exposed to the toxic *Pfiesteria* died during the study.

There were no discernable differences in the microflora/fauna and bacterial populations in the control versus the bioassay vessels, with protozoan ciliates rarely detected, and autopsies of moribund fish from the bioassay facility indicated a general lack of bacterial infection. Neither oxygen or ammonia levels were determined to be factors in the fish deaths.

Fish bioassays (using tilapia) were also conducted to determine toxicity of the Pfiesteria-like organisms *Cryptoperidiniopsis* sp. and *Gyrodinium galatheanum*. There were no fish deaths associated with these species over periods of 10-14 weeks, but at concentrations that did not exceed 800 zoospores mL^{-1}. These may have been non-toxic strains of these species, or higher cell concentrations may be needed to produce fish toxicity (e.g 115,000 mL^{-1}, Nielsen, 1993).

The toxic *Pfiesteria piscicida* cultures were provided by Dr. J. Burkholder and Dr. H. Glasgow (NCSU) for these studies. Clonal cultures of *Cryptoperidiniopsis* sp. and *G. galatheanum* were developed from sediment samples taken in Virginia estuaries. Prior to and after this series of bioassays took place, cells from these cultures were identified by SEM plate analysis and cross-confirmed by two outside laboratories using gene sequencing protocols.

DEVELOPMENT AND TESTING OF MOLECULAR DIAGNOSTICS FOR *PFIESTERIA*-LIKE ORGANISMS IN LABORATORY AND ENVIRONMENTAL SAMPLES

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Currently the only reliable method to accurately identify and distinguish *Pfiesteria piscicida* and other *Pfiesteria*-like organisms (PLOs) is through 3-D reconstructions of the thecal plate structures by scanning electron microscopy (SEM). We are doing a comprehensive survey of DNA sequences for the internal transcribed spacer (ITS) region and portions of both the small (SSU) and large (LSU) subunit genes of the ribosomal DNA complex for available PLO clonal cultures. Sequence information is being used for phylogenetic studies and to develop DNA-based diagnostics. Sequence comparisons among the PLO cultures and to other dinoflagellates and protozoans allowed design of species–specific and genus–specific PCR primers and DNA probes. Clonal cultures of *Pfiesteria piscicida*, *Pfiesteria* sp. "B", *Cryptoperidiniopsis* spp., "Shepherd's crook" and "Lucy-like" PLOs were first analyzed and identified by SEM and then obtained for molecular analysis. DNA was isolated from a total of 18 clonal PLO cultures and 3 food source cultures. DNA sequences were obtained from the food sources to assure that PLO, rather than food source DNA clones, were selected for analysis. SSU, ITS and LSU DNA sequences from the PLOs were aligned and subjected to phylogenetic analyses. The SSU gene was highly conserved while the ITS region (excluding the 5.8S portion) and portions of the LSU gene fragment demonstrated considerable sequence variation. Comparison of DNA clone sequences obtained from clonal cultures demonstrated degenerate sites in the ITS region and the LSU gene and even a few in the SSU gene. Overall the molecular data strongly supported identifications made by SEM. Phylogenetic analyses grouped together all the cultures identified as *P. piscicida* and placed *Pfiesteria* sp. "B" between the *Pfiesteria piscicida* clade and *Cryptoperidiniopsis* spp. In addition, cultures identified by SEM as "Lucy" or "Lucy-like", grouped together in the DNA-based phylogenies.

Sequence alignments and comparisons were used to design four sets of PCR primers to specifically amplify *P. piscicida*, *Pfiesteria* sp. "B", *Cryptoperidiniopsis* spp. or the "Lucy" group clonal culture DNAs. PCR primers and reaction conditions were optimized for specificity with clonal culture DNAs and then tested for their ability to amplify these DNAs from water and sediment samples. In addition, specific DNA probes were designed for in situ hybridization. Samples from fish bioassay tanks and the environment have been tested with the DNA probes and PCR primers. Results of molecular diagnostic assays agreed with SEM identifications and PCR assays confirmed the presence of PLO species detected by in situ hybridization. For example, the *Pfiesteria* sp. "B" probe hybridized to dinoflagellate cells found in the lateral line canal and alimentary tract of fish from an experimental tank with high cell counts of *Pfiesteria* sp. "B". DNA probe analyses indicated that dinoflagellates found in the alimentary tracts of menhaden collected from two sites in North Carolina were "Lucy-like" cells. PCR amplification of DNA from water samples collected at the same sites as these menhaden indicated the presence of "Lucy-like" DNA.

POTENTIAL GRAZING ON *PFIESTERIA PISCICIDA* BY MICROZOOPLANKTON IN THE POCOMOKE RIVER

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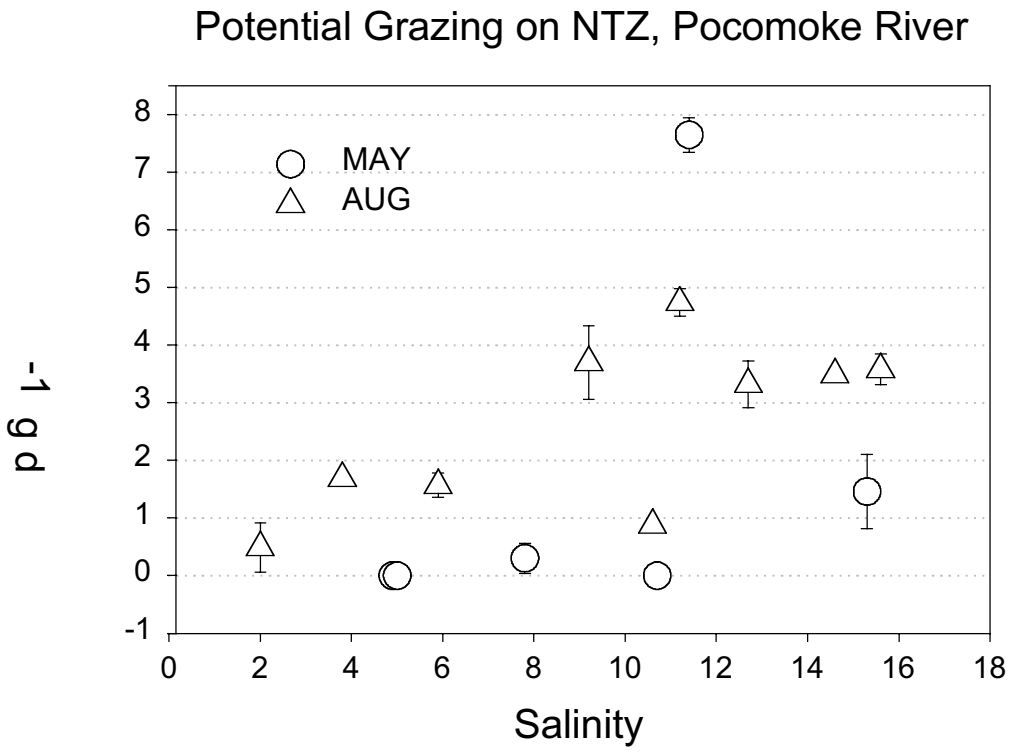
Our objectives were to determine if microzooplankton grazing has the potential to prevent net growth of non-toxic zoopspores (NTZ) and to determine if grazing pressure is related to salinity. In a previous study at a mesohaline site on the Chicamacomico River, the instantaneous rate of grazing mortality, *g*, varied from 0 to 10 d^{-1} and in 6 out of 10 incubations was > 2 d^{-1}. The maximum growth rate of NTZ in culture is < 2 d^{-1}. We wanted to determine if potential grazing pressure in the lower Pocomoke River varied with season or salinity. Although *Pfiesteria* cultures grow best at ~ 15 psu, most fish kills associated with *Pfiesteria*-like dinoflagellates have occurred at salinity <10. We hypothesized that this may be due to less grazing pressure on *Pfiesteria*-sized dinoflagellates at lower salinity sites.

The grazing pressure (*g* d^{-1}) of natural assemblages of microzooplankton on cultured non-toxic zoospores (NTZ) was measured in water samples collected from sites of different salinity on the lower Pocomoke River in May and August 2000. NTZ of a non-inducible strain (FDEPMDR23) of *P. piscicida* were stained with a vital green fluorescent dye, 5-chloromethylfluorescein diacetate, and added to treatments with (<200 µm) and without (<1.2 µm) the natural microzooplankton assemblage. Grazing coefficient, *g*, varied from 0 to 8 d^{-1} in May and from 0 to 5 d^{-1} in August (Fig.). In 6 of 9 incubations with samples of >9 psu, *g* was > 2 d^{-1}. In the 6 incubations with < 9 psu samples, *g* was < 2 d^{-1}, and in 3 cases was zero. Potential grazing pressure on NTZ varied with date and site, but in incubations with > 9 psu water, *g* was usually greater than the maximum potential growth rate of NTZ. Microzooplankton grazing is an important factor that may regulate net growth of *Pfiesteria piscicida* populations in the plankton.



SKIN LESIONS IN ESTUARINE FISHES: A COMPARATIVE PATHOLOGICAL EVALUATION OF WILD AND LABORATORY-EXPOSED FISH

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The toxic dinoflagellate, *Pfiesteria piscicida*, is widely blamed for acute mortalities and skin ulcers in menhaden, *Brevoortia tyrannus*, from Mid-Atlantic U.S. estuaries. However, the underlying causes of these acute fish kills and the so-called "*Pfiesteria*-specific" skin ulcers of menhaden remain poorly understood and controversial. We initiated field and laboratory studies to clarify the role, if any, of *Pfiesteria* spp. and other environmental factors in menhaden lesion development. Histopathologically, skin ulcers in > 250 wild menhaden from Chesapeake Bay and the Pamlico Estuary, NC all harbored a deeply invasive, pathogenic fungus eliciting severe tissue necrosis and intense granulomatous inflammation. One of us (VB) recently determined this fungus to be *Aphanomyces invadans*, the causative agent of epizootic ulcerative syndrome (EUS) in fishes from the Indo-Pacific Region. The consistent presence of this fungus and the granulomatous nature and severity of the resulting inflammatory response suggest these lesions are chronic (age > one week). Their use as an indicator of current, localized *Pfiesteria* activity may therefore not be valid. However, these field data do not allow us to rule out a possible early role for *Pfiesteria* spp. as initiator of lesion development. It is therefore important to better understand the pathology of *Pfiesteria* exposure and to investigate potential environmental factors that might modulate lesion pathogenesis in exposed fishes. To this end, we conducted a series of preliminary laboratory challenges of tilapia (*Oreochromis niloticus*) and mummichog (*Fundulus heteroclitus*) with high concentrations (5000 - 30,000 cells/ml) of *Pfiesteria* sp. "B" (characterization based on SEM and molecular analyses). Results indicate *Pfiesteria*-associated pathology is consistent with acute toxin exposure and differs significantly from pathology observed in wild menhaden with ulcerative mycosis caused by *A. invadans*. Fish mortalities in the assays were directly associated with abundance of *Pfiesteria* "B" cells. Grossly, fish exhibited loss of scales, mucus coat and occasional mild petechial hemorrhage. Histologically, fish exhibited widespread loss of epidermis with bacterial colonization but minimal associated inflammation. Epidermal erosion extended into the oral and branchial cavities. Gills exhibited epithelial lifting, loss of secondary lamellar structure and infiltration by lymphoid cells. Interestingly, dinoflagellates were observed within skin folds, scale pockets and the lateral line canal system of the head region. Epithelial lining of the lateral line canal and associated sensory structures exhibited degeneration and necrosis. However, bacterial colonization of tissues harboring dinoflagellates was a consistent observation. *In situ* hybridization analyses using specific DNA probes to *P. piscicida, Pfiesteria* sp. "B" and the "Lucy" group confirmed that dinoflagellates associated with fish tissues were *Pfiesteria* sp. "B". We consider this association to be an artifact of the very high dinoflagellate concentrations in our exposures. However, *in situ* hybridization analyses of wild menhaden intestine with these probes may serve as a sensitive, specific and quantifyable bioindicator of local *Pfiesteria* activity in east coast estuaries.

ABSTRACTS OF POSTER PRESENTATIONS

***PFIESTERIA* – DE, MD, VA SESSION**

IMPACT OF DINOFLAGELLATE-COEXISTING BACTERIA ON THE PHYSIOLOGY OF *PFIESTERIA*-LIKE DINOFLAGELLATES

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In nature, dinoflagellates exist along side a myriad of other microorganisms. In numbers alone, the largest percentage of this microbial population is made up of the bacteria. Some of these bacteria may directly and indirectly influence the dinoflagellates physiology and metabolism. For this reason, we are interested in understanding how the bacterial community specifically affects the physiology and toxin production of *Pfiesteria*-like dinoflagellates. We have taxonomically classified the bacterial species co-existing in four laboratory cultures of *Pfiesteria* and *Cryptoperidiniopsis* spp originally obtained from sites of Chesapeake Bay fish kills. Our approach was based on the use of oligonucleotide primers and polymerase chain reaction to amplify a portion of the small ribosomal subunit gene (16S rDNA) from both the culturable and nonculturable bacteria in these cultures. The nucleotide sequence of each unique clone was determined and the homology between the dinoflagellate bacteria and known species was used to construct phylogenetic trees of taxonomic relatedness. We then produced axenic dinoflagellate cultures and studied the influence of culturable bacterial isolates on the growth and feeding behavior of these cultures by add-back experiments. Our results indicate that there are substantial differences between axenic dinoflagellate cultures and their bacterized counterparts. These differences include, but may not be limited to, reduction in growth rate and lowered cell density, as well as changes in feeding behavior.

SALINITY TOLERANCE FOR 62 STRAINS OF *PFIESTERIA* AND *PFIESTERIA*-LIKE STRAINS

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Based upon preliminary studies, experimental cultures were prepared at salinities of 0.5, 1, 2, 3, 4, 5 and 6 ppt and 35, 40, 45, 50, 55 and 60 ppt for lower upper tolerance limits, respectively. Cells were grown at 22^{o} C under a 12 hr light/dark cycle. Cultures (at 12 ppt salinity) were fed immediately before beginning the experiment with *Rhodomonas* (CCMP768); no food was added to the cultures during the experiment. For each salinity treatment, the water was prepared as a batch and then dispensed. After 5 days, each test tube was examined with a dissecting microscope to determine the presence of swimming dinoflagellate cells. After this examination, the salinity of each test tube was measured again using a refractometer to verify that the experimental salinity had not changed due to evaporation. If no swimming cells were observed in all three tubes, the contents of two tubes were transferred into a clean Petri dish so that a more thorough examination was possible. If no swimming cells were observed in the Petri dish, then the third tube was adjusted to 12 ppt and the culture was maintained for two additional days. At the end of the two-day period, the test tube was examined again for swimming cells as described above. When swimming cells were observed at the end of the two day period, we assumed that viable cysts were the source of the swimming cells; when no swimming cells were observed at the end of the two day period, we assumed that all cells and cysts died. After five days, most Chesapeake Bay and Neuse River system cells were still swimming at 1 ppt and 40 ppt; one strain had swimming cells at 0.5 ppt and six strains had swimming cells at 50 ppt. After 5 days, most Wilmington River cells were still swimming at 4 ppt and 50 ppt; several strains had swimming cells at 3 ppt and two had swimming cells at 60 ppt. Putative cysts allowed for even greater salinity tolerance because many cultures without swimming cells at extremely low or high salinities produced swimming cells when the salinity was adjusted back to 12 ppt. There was a statistically significant ($p < 0.001$) difference between the mean lower and mean upper salinity tolerances of the Wilmington River system and the Chesapeake Bay system or Neuse River system. The original Wilmington River water samples were more saline, suggesting that salinity tolerance is under genetic control.

DEVELOPMENT OF REAL-TIME PCR ASSAYS FOR RAPID DETECTION OF *PFIESTERIA PISCICIDA* AND RELATED DINOFLAGELLATES

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Pfiesteria complex species are heterotrophic and mixotrophic dinoflagellates that have been recognized as harmful algal bloom species (HAB) associated with adverse fish and human health effects along the East Coast of North America, particularly in its' largest (Chesapeake Bay, Maryland) and second largest (Albemarle-Pamlico, North Carolina) estuaries. In response to impacts on human health and the economy, monitoring programs to detect the organism have been implemented in affected areas. However, until recently, specific identification of the two toxic species known thus far, *Pfiesteria piscicida* and *Pfiesteria shumwayae* (sp. nov.), required scanning electron microscopy (SEM). SEM is a labor-intensive process in which a small number of cells can be analyzed, posing limitations when the method is applied to environmental estuarine water samples. To overcome these problems, we developed a real-time PCR-based assay that permits rapid and specific identification of these organisms in culture and heterogeneous environmental water samples. Various factors likely to be encountered when assessing environmental samples were addressed and assay specificity was validated through screening of a comprehensive panel of cultures, including the two recognized *Pfiesteria* species, morphologically similar species, and a wide range of other estuarine dinoflagellates. Assay sensitivity and sample stability were established for both un-preserved and fixative-preserved (acidic Lugol's solution) samples. The effects of background DNA on organism detection and enumeration were also explored and, based on these results we conclude that the assay may be utilized to derive quantitative data. This real-time PCR-based method will be useful for many other applications, including adaptation for field-based technology.

TEMPORAL AND SPATIAL DISTRIBUTION OF *PFIESTERIA PISCICIDA* IN CHESAPEAKE BAY WATERS, 1999-2000

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Pfiesteria piscicida, a heterotrophic dinoflagellate, has been associated with adverse fish and human health effects in estuarine environments extending from Delaware to Florida. Due to public concern over the risk of exposure to putative toxins and the economic loss related to fish kills in the Chesapeake Bay in Maryland, the Maryland Department of Natural Resources and collaborating laboratories began monitoring various tributaries of the Bay in 1997 and developed those efforts into an extensive and comprehensive program for 1998 - 2000. Targeted rivers include those in which *P. piscicida* has been previously detected (through molecular methods, scanning electron microscopy and fish kill bioassays), rivers having a possible predisposition to *Pfiesteria* activity (chemical and physical characteristics similar to affected systems), and rivers with no history or apparent tendency for *Pfiesteria*-related problems. A real-time PCR-based assay for detection of the organism developed by our laboratory was utilized in the comprehensive monitoring program. In 1999, *P. piscicida* was not detectable in surface waters through the spring and early summer, with the organism being detected at only one station by mid-July. More intensive sampling during 2000 has detected *P. piscicida* in April at one site and at seven other sites by mid-July. In general, nutrient concentrations and phytoplankton biomass were higher at stations where *P. piscicida* was detectable in the water column, in comparison to sites repeatedly testing negative. Detection of *P. piscicida* in the water column has been repeatedly associated with the occurrence of summer/fall populations of Atlantic menhaden with ulcerative lesions that have been found to harbor a number of pathogens (e.g. fungi, bacteria, parasites). Further analysis of biological and physicochemical parameters associated with the detection of *P. piscicida* and assessment of those parameters in waters consistently negative for the organism may enable identification of conditions conducive to *Pfiesteria* blooms. Hypotheses generated with these data will be tested through ongoing longitudinal analyses of samples collected at multiple stations throughout the Chesapeake Bay region.

RELATIONSHIP BETWEEN OCTADECAPENTAENOIC ACID, ALGICIDAL TREATMENTS, AND TOXICITY IN TWO BLOOM FORMING DINOFLAGELLATES, *GYRODINIUM GALATHEANUM* AND *PROROCENTRUM MINIMUM,* FOUND IN THE CHESAPEAKE BAY, MD

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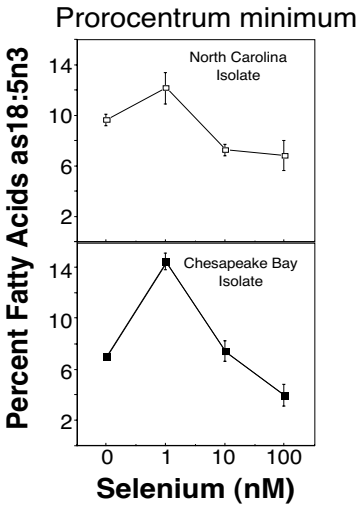
Since 1996, fish kill events associated with blooms of *Gyrodinium galatheanum*, a small (10-15 μm) mixotrophic, non-thecate dinoflagellate, have occurred periodically at an estuarine pond production facility, raising hybrid striped bass on the Chesapeake Bay. It has been observed at this facility that *G. galatheanum* blooms treated with potassium permanganate ($KMnO_4$) result in fewer, or no, fish mortalities, compared with treatments of copper sulfate ($CuSO_4$). One bloom in 1996, treated with $CuSO_4$, resulted in the loss of 20,000 fish. We have shown Chesapeake Bay isolates of both *Gyrodinium galatheanum* and *Prorocentrum minimum* to contain the hemolytic endotoxin octadecapentaenoic acid (18:5n3). The fatty acid 18:5n3 has been suggested as the toxic agent responsible for aquaculture related fish kills due to the dinoflagellates *Gyrodinium aureolum* and *Gymnodinium* cf. *mikimotoi* . This study was undertaken to determine if differences exist between algicidal treatments of $CuSO_4$ and $KMnO_4$ on two bloom forming dinoflagellates, *G. galatheanum* and *P. minimum*, and if these differences influence ichthyotoxicity.

We have shown that, depending upon the addition or deletion of soil extract and/or chicken manure extract to culture media, *G. galatheanum* and *P. minimum* cells can contain from 4.88% - 15.43% and from 5.81% -19.44%, respectively, of their total fatty acids as 18:5n3. The addition of 1 nM selenium to selenium replete culture media was shown to stimulate a similar effect in two different isolates of *P. minimum*. Further, we have shown that for *G. galatheanum* cells, the major pools for 18:5n3 are the monogalactosyl diacylgylcerols (MGDG) and diagalactosyl diacylglycerols (DGDG). This is also in accordance with other toxic dinoflagellates containing 18:5n3. *Pfiesteria piscicida*, another dinoflagellate implicated in MD fish kills, was shown to be present at the facility and its role in the kills cannot be ruled out. Several isolates of *P. piscicida,* cultured on cryptomonads, were found to contain no 18:5n3. This study was part of a continuing research effort to determine if poly-unsaturated fatty acids (PUFAs) contribute to ichthyotoxicity in selected Chesapeake Bay dinoflagellates.

Cell lysis (70-90% max.) occurred in both clonal cultures tested upon exposure to varying levels of $KMnO_4$, while cell lysis due to $CuSO_4$ only occurred in *G. galatheanum* cultures (approx. 75% max.). In a related experiment at the above mentioned facility, samples of a bloom of *Katodinium rotandatum* treated with either $CuSO_4$ or $KMnO_4$, showed increased aqueous inorganic N and P levels in both treatments, suggesting cell lysis or leakage. However, chlorophyll *a* concentrations dropped only in $KMnO_4$ treated samples suggesting oxidation of certain cellular components. The results obtained thus far suggest that: 1. Both *G. galatheanum* and *P. minimum* contain a potentially toxic hemolytic lipid fraction, and 2. Although both $CuSO_4$ and $KMnO_4$ treatments were algicidal, $CuSO_4$ treatments may allow the intact release of cellular contents while $KMnO_4$ treatments may not. Further experimentation will be required to validate these hypotheses and to determine their respective involvement in



ichthyotoxicity. Results will be discussed in relation to algicidal treatment effects on PUFA content and release.

GROWTH CHARACTERISTICS OF CLONAL *PFIESTERIA PISCICIDA* IN CULTURE

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Pfiesteria piscicida is a heterotrophic dinoflagellate that exhibits predatory behavior towards live algae and other marine microorganisms. Due to it's association with fish kills along the mid-Atlantic coast of North America and possible impact on human health it has become the subject of intense research on its biological and molecular aspects. This required the development and optimization of reliable culture methodologies that would yield a reproducible biomass. Published reports on the culture and life cycle of *P. piscicida*, have described multiple life stages present in aquaria containing fish and environmental water or sediments. We examined selected growth characteristics of clonal *P. piscicida* cultured in the presence of cryptomonads, and attempted to produce high density cultures to be used as a source for isolation of DNA, RNA, proteins or other molecules of interest. Cultures of *Rhodomonas* sp. (50 ml; $3x10^5$ cells/ml) in 75 ml flasks were inoculated with 5 ml of a fast-growing culture of clonal *P. piscicida* to a final density of 10^3 cells/ml, and grown under standard conditions (85 $\mu M.m^{-2}sec^{-1}$ light intensity; 14h/10h light/dark; 23° C temp). Samples were collected daily, and relative cell numbers of *P. piscicida* and *Rhodomonas* sp., *P. piscicida* cells containing two nuclei, and cells with ingested *Rhodomonas* sp. were determined by microscopy. *Rhodomonas* sp. actin by assessed by PCR. Size distribution of *P. piscicida* cells was determined microscopically by measuring the cells' long axis (n= 50-100) with a micrometer eyepiece. Reproducible high-density *P. piscicida* cultures ($2x10^5$ cells/ml) were obtained by the optimized protocol described above. One day after inoculation there was a substantial increase in numbers of round-shaped binucleated *P. piscicida* cells. This was followed by increases in *P. piscicida* cell numbers and median cell size, and a sharp decline in *Rhodomonas* sp. numbers, which was followed by a decrease in *P. piscicida* cells with visible ingested *Rhodomonas* sp. This was correlated with the disappearance of the actin signal for *Rhodomonas* sp. After the disappearance of *Rhodomonas* sp.cells from the culture, *P. piscicida* cells with visible ingested *Rhodomonas* sp. were only observed during the following 1 - 2 days. After the culture was depleted of *Rhodomonas* sp., there was a sharp drop in *P. piscicida* cell numbers, with a concomitant decrease in median cell size. An increase in the proportion of *P. piscicida* cells with two or more nuclei was observed at the end of the 10-day culture period. These cells, however, differed morphologically from those observed immediately after inoculation.

CHARACTERIZATION OF ACTINS AND TUBULINS FROM THE HETEROTROPHIC DINOFLAGELLATE *PFIESTERIA PISCICIDA* . SUBCELLULAR LOCALIZATION BY IMMUNOFLUORESCENCE

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The phagotrophic dinoflagellate *Pfiesteria piscicida*, a species associated to fish kills in mid-Atlantic estuaries, requires algal prey as a source of nutrition. *P. piscicida* biflagellated zoospores actively swim towards their prey, attach to it, and feed on its contents in a process known as myzocytosis. When the prey is abundant, and ambient conditions favorable, *P. piscicida* multiplies vegetatively by cell division. Mitosis in dinoflagellates differs from nuclear division in other eukaryotes by the permanent presence of a nuclear envelope and an extranuclearly localized spindle, the apparatus responsible for separation of chromosomes. Swimming, feeding and cell division rely on functional actin and tubulin cytoskeletons, and their characterization is critical for understanding the molecular aspects of these processes. Furthermore, actins and tubulins are well conserved throughout the eukaryotic lineages, and their primary structures have proven useful tools for phylogenetic analysis. Thus, *P. piscicida* actins and tubulins may provide valuable information, in addition to that from rRNA genes, for revealing its taxonomic relationships to other Dinozoa. DNA from *P. piscicida* was isolated and used as a template for PCR amplification with "universal" actin primers (kindly provided by Dr. G.W. Warr, Medical University of SC, USA). Two PCR products, 0.7 kb and 1.9 kb were cloned and sequenced. A new set of primers designed on the 0.7 kb amplicon was used to confirm its origin in *P. piscicida* DNA. This *P. piscicida* actin fragment had no introns and was about 70 % identical to the corresponding actin fragments from the dinoflagellates *Prorocentrum minimum, Gyrodinium galatheanum,* and *Amphidinium carterae.* Three partial _-tubulin sequences from *P. piscicida* were obtained using degenerate primers designed by Keeling *et al.* (J. Euk. Microbiol. 45: 561-570, 1998). Two of the sequences were 97 % identical to each other, 70 % identical to the third one, and all three contained introns. Alpha-tubulin was localized by indirect immunofluorescence to the longitudinal flagellum.

DEVELOPMENT AND APPLICATION OF NEW CELL-SURFACE ANTIBODIES IN THE STUDY OF *PFIESTERIA PISCICIDA*

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We developed cell-surface antibodies against *Pfiesteria piscicida* and a major prey species, *Rhodomonas* sp., in an attempt to develop specific new tools in the study of this toxic dinoflagellate. Well-designed antibodies allow the quantitative measurement of field concentrations and have additional uses when coupled with immunomagnetic beads. We tested the antibodies' affinity and specificity on cultures *Pfiesteria* and related species and field samples. Some nonspecific binding by crude anti-*Pfiesteria* sera was encountered and was significantly mitigated through pre-adsorption of the serum onto other species and through blocking procedures. We also tested Immunomagnetic protocols using mixtures of *Pfiesteria* with *Rhodomonas* as well as field samples, and negative separation. Direct and indirect methods with different sample fixation and blocking protocols were compared. We achieved a good negative separation (use of anti-*Rhodomonas*-coated beads to remove *Rhodomonas* and leave behind *Pfiesteria*) using anti-*Rhodomonas* but efforts continue to improve the specificity of the positive separation procedure (use of anti-*Pfiesteria*-coated beads to isolate *Pfiesteria*) that may be more useful for *in situ* applications.

CANNIBALISM IN *PFIESTERIA PISCICIDA, PFIESTERIA* "B" AND PFIESTERIA-LIKE DINOFLAGELLATES

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Under laboratory culture conditions, the fate of heterotrophic dinoflagellates, including *Pfiesteria piscicida*, *Pfiesteria* "B", "Lucy", *Cryptoperidiniopsis sp*., and "Shepherd's crook" is directly related to the abundance of available cryptophyte prey. We observed that as prey abundance decreased, heterotrophic dinoflagellate cultures cannibalized each other resulting in decreased cell concentrations. All observations were made from clonal, aseptically maintained stock cultures of heterotrophic dinoflagellates. Cannibalism is evident by the appearance of two nuclei visible inside a single dinoflagellate cell using epifluorescent microscopy. Depending on the time elapsed since the prey dinoflagellate was ingested, various-sized remnants of ingested nuclei are visible within a food vacuole inside the cannibalizing dinoflagellate. In the presence of abundant cryptophyte prey, cannibalism was observed at a low rate in all of the heterotrophic dinoflagellate cultures. In cultures where cryptophyte prey availability was greatly reduced, an increased rate of cannibalism was observed (ten-fold increase of dinoflagellates containing two nuclei). Our observations provide direct visual evidence of heterotrophic dinoflagellate cannibalism. These results suggest that in experiments where heterotrophic dinoflagellate concentration plays a role, cannibalism must be considered as a factor affecting experimental population concentrations as well as the overall experimental dynamics.

GYRODINIUM GALATHEANUM GRAZING ON *PFIESTERIA PISCICIDA* AND *PFIESTERIA*-LIKE DINOFLAGELLATES

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Gyrodinium galatheanum is a mixotrophic dinoflagellate commonly observed in mid-Atlantic estuarine waters. Past laboratory and field observations revealed a range of *G. galatheanum* algal prey including flagellates and cyanobacteria (Li et al, 1999,2000). We report here the results of grazing studies of *G. galatheanum* on *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates including *Pfiesteria* "B", "Lucy" and *Cryptoperidiniopsis sp*. All grazing experiments were conducted using clonal cultures. Under laboratory conditions (24 C and 12:12 light:dark cycle), *G. galatheanum* was capable of grazing *P. piscicida* and other similar dinoflagellates to near extinction within several days. The effect of the nutritional status of both predator and prey on grazing dynamics was examined. Pre-conditioning of *G. galatheanum* at various levels of inorganic nutrients appeared to have no effect on its grazing rate. The nutritional status of the prey appears to influence their susceptibility to being grazed, as grazing rates increased as food scarcity induced a reduction in prey cell size presumably related to starvation. Our results suggest that the mixotrophic nature of *G. galatheanum* allows this organism to persist under a wide range of environmental conditions and effectively graze other dinoflagellates as the food supply is reduced.

Li. A., D.K. Stoecker, J.E. Adolf. 1999. Aquat Microb Ecol 19:163-176.

Li, A., D.K. Stoecker, D. W. Coats, 2000. J. Phycol. 36:33-45.

BIOMONITORING FOR TOXICITY CAUSED BY HARMFUL ALGAL BLOOMS AND OTHER WATER QUALITY PERTURBATIONS

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Harmful algal blooms, including those associated with toxicity, hypoxia events and other water quality perturbations, have increased in frequency and severity worldwide. An automated, remote biomonitoring system is being developed to continuously monitor waterways that are susceptible to these water quality perturbations. The system consists of a series of flow-through chambers that expose sentinel fish. Changes in ventilatory and movement patterns, as they relate to altered or toxic water quality, were monitored. Paired electrodes in each chamber non-invasively transmit an amplified electrical signal from each fish to a computer. These signals and water quality data can be transmitted to remote locations for further analysis and potential management response. Freshwater laboratory trials with bluegill indicated that exposure to sublethal hypoxia produced an increase in ventilatory rate with minor associated depressions in ventilatory depth. For the purpose of validating the system with HAB toxins, bluegill were also exposed to brevetoxin (PbTx2) and *Pfiesteria* culture water. One hour exposure to 40 ppb PbTx2 at 25˚C, but not 19˚C, produced minor elevations in ventilatory rate with strong cough and movement responses. Brackish (15 ppt) *Pfiesteria* culture water exposures caused a different response pattern: strong elevations in cough rate and % movement. Initial efforts to link biomonitoring signals with stress mechanisms were initiated with PbTx2-exposed bluegill; these animals showed altered patterns of glucose metabolism in their brains based on incorporation of 2-deoxyglucose (2-DG) *in vivo*. These laboratory biomonitoring studies indicate different response patterns under different stress conditions, and demonstrate utility of the biomonitoring system and the 2-DG method.

PFIESTERIA AND OTHER STRESS FACTORS ASSOCIATED WITH KILLS AND ULCERATIVE LESIONS OF ESTUARINE FINFISH

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Ulcerative lesions in mid-Atlantic estuarine finfish are associated with bacterial, fungal, viral and parasitic infectious agents, as well as suboptimal water quality. Atlantic menhaden are a relatively susceptible species to such stressors. In recent years, ulcerative lesions on field collected menhaden have been associated with exposure to *Pfiesteria*-like dinoflagellates and their toxins. These lesions are typically solitary, focal, deep, often perianal, and granulomatous with oomycete hyphae. Although *Pfiesteria*-like dinoflagellates have not been recovered from fish ulcers, and there is little evidence that supports a single causal relationship between toxic *Pfiesteria* and ulcerative lesions, much emphasis has been placed on *Pfiesteria* and fish lesions in the scientific and news media. A group of fish health experts examined ulcerated fish from estuarine waters in Maryland and North Carolina. Ulcerative menhaden lesions histologically demonstrated a marked chronic inflammatory infiltrate in large areas of exposed necrotic muscle. The ulcers contained granulomas with fungal hyphae in the necrotic tissue. Gram negative rod-shaped bacteria were also observed in the lesions, a common finding in ulcers of aquatic organisms. Other menhaden specimens contained *Kudoa*-like protozoan parasites, but lacked granulomas. Menhaden with solitary ulcers sampled from Delaware also lacked granulomas but were associated with Pennellid copepods. Bluegill, channel catfish, yellow perch, striped bass and carp sampled from Maryland waters with *Pfiesteria* were externally non-remarkable. These findings suggest that "typical" ulcerative lesions observed on menhaden from areas with *Pfiesteria*-like dinoflagellate blooms are reflective of dermatosis which may be related to a variety of individual or combined environmental stressors. Exposure to dinoflagellate toxin(s) potentially represents one such stressor. This presentation reviews the multifactorial nature of ulcerative lesions on finfish, and the role of Atlantic menhaden in monitoring the health of estuarine systems.

AN INTEGRATED FIELD STUDY TO IDENTIFY FACTORS RESPONSIBLE FOR ULCERATIVE LESIONS IN JUVENILE MENHADEN IN THE GREAT WICOMICO RIVER, VIRGINIA

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The occurrence of deeply-penetrating ulcerative lesions in young-of-the-year (YOY) Atlantic menhaden (*Brevoortia tyrannus*) was investigated in the Great Wicomico River (GWR) estuary, VA, USA in 1998-2000. In 1998, 6,874 caught YOY menhaden exhibited 211 ulcerative lesions (3% lesion rate). Lesion prevalence increased substantially in menhaden caught at salinities below 12-14 psu with the highest prevalence (11.5 %, n=823) at 10-12 psu. A similar relationship was observed in 13 separate estuaries throughout Virginia in September-October, 1998, where YOY menhaden captured (n= 5,077, lesion rate 5.6%) exhibited a marked increase in lesion rate at salinities of 12-14 psu and lower. In 1999 (through 9/9/99), a total of 4,005 YOY menhaden caught in the GWR exhibited a total of only 7 lesions (0.2% lesion rate). In 1998 a wet spring resulted in water with salinities associated with high lesion-prevalence (i. e., ≤12 psu) occupying the entire upper half of the estuary. Dry conditions in the remainder of 1998 and in the warmer months of 1999 through August reduced the area of salinities of 6-12 psu to only a short segment (ca. 100 m) in the shallow, narrow headwaters of the estuary. The greatly reduced area of ≤12 psu water in the GWR in 1999 is postulated to be the primary cause of reduced lesion prevalence in this year. Comprehensive data sets collected to examine aspects of the biological, chemical and physical characteristics of the GWR did not reveal obvious differences between 1998 and 1999 that could account for the low lesion prevalence in 1999. Low dissolved oxygen, a predisposing factor to fish disease, occurred in bottom waters of the upper reaches of the estuary in all years. Comparative analyses of heterotrophic dinoflagellates, other components of the microbial community, and chlorophyll *a* for both years were similarly unremarkable. Menhaden with typical ulcerative lesions examined in 1998-9 and 2000 confirmed the presence of an aseptate fungus (water mold) belonging to the genus *Aphanomyces*. Studies in 1999 to detect and quantify water molds as an index of *Aphanomyces* presence, suggested runoff was an important factor controlling its occurrence. Although field work and data analysis in 2000 are not yet complete, ulcerative lesion prevalences in GWR YOY menhaden increased from none in May to a maximum of 14% in late August. July, August and early September experienced periods of intense, episodic thunderstorms which produced large reductions in surface salinities in the upper reaches of the GWR. These observations and other data imply lesion occurrence is related to the presence of freshwater runoff which favors the introduction of infective *Aphanomyces* spp. and/or provides very low salinity (ca. 1 psu) conditions required for zoospore formation and release.

A DESCRIPTIVE ACCOUNT OF POTENTIALLY HARMFUL ALGAE IN INLAND BAYS AND NANTICOKE RIVER, DE

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Beginning in 1998, Delaware Department of Natural Resources and Environmental Control (DNREC) initiated a routine monitoring program for phytoplankton species composition is Rehoboth, Indian River and Little Assawoman Bays and Nanticoke River, DE. This effort was largely precipitated from the discovery of several potentially harmful algae. *Pfiesteria piscicida* and *Aureococcus anophagefferens.* Upon completing two years of taxonomic enumerations of samples from these waters, a list of taxa revealed a number of potentially harmful forms of microalgae. These taxa were compiled in a data base which included information on their physiology and ecology, toxin or alternate harmful mechanism, target species, threshold densities, possible environmental triggers, human health risks and trophic interations. The data base will also list references which will enable the user to access more detailed information on these various topics. The ultimate product will be an interactive spreadsheet-type data base which can facilitate selected queries.

cDNA SCREENING FOR *PFIESTERIA PISCICIDA*: SEARCH FOR SPECIES-SPECIFIC DNA MARKERS AND GROWTH-REGULATING GENES

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Due to the unusual life cycle and growth characteristics of *Pfiesteria piscicida* reported previously and observed recently in our laboratory, it is highly desirable to extensively screen cDNA libraries and identify genes that are related to growth regulation. In addition, because of presence of similar genera and species, genes or other DNA markers that contain species-specific signatures will be useful. To identify these genes or DNA markers, *Pfiesteria* cultures were grown under different conditions and RNA/DNA were extracted. Different behaviors of DNA suggested that *Pfiesteria* chromosomal DNA is probably bound by histone proteins as in typical eukaryotes in contrast to DNA in *Prorocentrum minimum*, another dinoflagellate, which appeared not to contain histones. mRNA was isolated from the crude RNA extract, and cDNA was prepared using a SMART technique. With modified poly (dT) primers, a number of genes were cloned, and sequencing effort is underway. Some of the sequences identified thus far include 3'-end of the mitogen-associated protein kinase (MAPK), tubulins, cytochrom b oxidase I, and some others with no homology to known genes. Search for cell cycle dependent genes are still underway. By using a mRNA differential display technique, we are also trying to isolate genes differentially expressed under different growth conditions. A comparative analysis of some of these genes will be presented.

A RAPID LARVAL FISH BIOASSAY FOR TESTING *PFIESTERIA* TOXICITY

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The cause of fish mortality in large-scale static biotoxicity assays with *Pfiesteria* is often difficult to determine. Suboptimal water quality, microbial enrichment, and prior fish health issues have been major impediments to assay interpretation. We have developed a sensitive and rapid (96 hr) "fractionation assay" using larval mummichogs, *Fundulus heteroclitus,* and toxic cultures of *Pfiesteria* sp. “B”. This assay tested fish mortality in relation to the different constituents found in the complex biotic community of our large-scale *Pfiesteria* bioassay systems. Seven day old lab-reared mummichog and juvenile tilapia (25-35 mm), *Oreochromis niloticus*, were exposed to "dinoflagellate fractions", "bacterial fractions", "supernatant (soluble *Pfiesteria* toxin) fractions" and appropriate water quality controls. The source of the fractionation medium was raw water from "toxic" cultures of *Pfiesteria* sp. “B” causing 20-50% daily mortality in tilapia. Water was fractionated by centrifugation and filtration to obtain media enriched in dinoflagellates, bacterial contaminants, and toxin (supernatant fraction). The dinoflagellate fraction (*Pfiesteria* sp. “B” and other protozoa) was collected on a 5 μm filter and rinsed with sterile-filtered artificial seawater (ASW) under vacuum, floated in ASW, examined for viability, and suspended in an identical volume of ASW as that originally filtered. The bacterial fraction (and small bicoecid flagellates) was pelleted by centrifugation (9000 rpm, 45 min, 10E C, Sorvall RC-5B), resuspended in small aliquots of ASW, passed through a 5 μm filter, and resuspended in ASW in an identical volume as that originally centrifuged. The "soluble toxin" fraction was the supernatant from the centrifugation, that was passed through 5 μm and 0.45 μm filters. Controls consisted of (1) artificial seawater (ASW), (2) ASW with ammonia (as NH_4Cl) and pH adjusted to that of raw toxic culture water, and (3) raw toxic culture water. Mortality and water quality (NH_4, NO_2, DO, pH) were assessed every 24 hrs for 96 hrs. Tilapia were exposed in jars with 500 ml medium (treatment: 3 replicates of 4 fish). Mummichogs were exposed in micro-titer well plates with 15 ml medium/well (treatment: 9 replicates of 5 fish/well). Results indicate that tilapia are marginally sensitive to *Pfiesteria* "B", as extremely high cell densities are required to kill fish. However, larval mummichogs exhibited 100% mortality in raw water and "dinoflagellate fractions” yet negligible mortality in controls. Histopathology confirmed toxicity/mortality findings. The mummichog assay appears effective for rapid verification of dinoflagellate toxigenicity and provides a quantifiable approach to study *Pfiesteria* biology.

VISUALIZATION AND IDENTIFICATION OF ATTACHED AND INTRACELLULAR BACTERIA WITHIN SEVERAL STRAINS OF *PFIESTERIA PISCICIDA*

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Bacteria co-occurring with strains of the toxic dinoflagellate *Pfiesteria piscicida* may affect the physiology and/or toxigenesis of the host dinoflagellate. In efforts to understand the role played by these bacteria, we have identified and localized the bacteria in and on *P. piscicida,* using oligonucleotide probes to regions of the bacterial 16S ribosomal RNA gene (rDNA). Polymerase chain reaction (PCR) amplification of bacterial 16S rDNA from DNA templates derived from the total bacterial population associated with the dinoflagellate was performed. The nucleotide sequence of each unique clone was obtained and this information was used to generate the species-specific probes, while group-specific probes were generated based on published data. The population of bacteria that was unattached or loosely attached to the dinoflagellate was removed by gentle washing of the eukaryotic cells, leaving behind only those bacteria that were firmly attached or internal to the host cell. The washed dinoflagellates and associated bacteria were then hybridized to each fluorescently-labeled oligonucleotide probe and the bacteria on and in the dinoflagellate cells were visualized by confocal scanning laser microscopy. These images were used to establish the cellular location of each bacterial species. Taxonomic identification of the attached and intracellular bacteria was accomplished by determining the relatedness of their 16S rDNA nucleotide sequence to known bacterial sequences using computer-based homology and phylogeny software. From these analyses, phylogenetic trees have been constructed showing the taxonomic inter-relatedness of the dinoflagellate-associated bacteria and the niches on and in the dinoflagellate cells that these bacteria occupy. The possible significance of these bacteria to the physiology and toxigenesis of the dinoflagellate is discussed.

INVESTIGATION OF THE CAUSALITY OF DERMAL LESIONS IN MENHADEN FROM THE JAMES RIVER, VA (OCTOBER 1999)

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The dinoflagellate *Pfiesteria piscicida* has been reported as causing massive episodes of menhaden (*Brevoortia tyrannus*) mortalities in North Carolina and several other locations along eastern coastal watersheds of the United States. Focal ulcers primarily localized to the vent region of the fish have been observed in these episodes. These lesions resemble those also illustrated for *Aphanomyces, Saprolegnia* and various species of bacteria. Our recent observations have shown that the myxosporean *Kudoa clupeidae* is also associated with similar lesions. The myxosporean genus *Kudoa* was described as a parasite of Atlantic menhaden in North Carolina waters as early as 1947. The genus now comprises 45 species and has been of interest to parasitologists ever since. Some species of *Kudoa* liquefy muscle tissue making some fish unsuitable for commercial use and *Kudoa clupeidae* is the only species of the genus that has been linked to fish mortalities.

In the recent James River episode, the Atlantic menhaden sampled had lesions that were primarily localized to the vent region. Among several hundred Atlantic menhaden sampled, 75% had penetrating lesions. Twenty-five (25) percent had either no lesions or sub-dermal lesions (swollen areas on the body without any visible breaks in the epidermis) that quickly erupted when the fish were handled. Histological sections from 14 fish exhibiting lesion development ranging from non-evident to advanced, were hematoxylin and silver stained. Sterile swab samples were also taken from the same areas of the fish and examined for bacteria, fungi, free-living amoebae, and other protists. Molecular assays of sediment water and fish tissues were also conducted to determine whether PCOs (*P. piscicida* and related dinoflagellates) were present. No PCOs were detected. However, in fish without evident lesions and with sub-dermal lesions, only *Kudoa clupeidae* was found. On the other hand, various bacteria, fungi and amphizoic amoebae, as well as *Kudoa* were found in fish with all stages of open dermal lesions.

We developed *Kudoa* specific oligonucleotide probes based on published SSU rRNA gene sequences for four species of *Kudoa*. Using these probes we amplified and sequenced a 1395 bp segment of the SSU rRNA gene of *K. clupeidae.* A phylogenetic analysis of the sequence data indicated that *K. clupeidae* was distinct from the four other sequenced taxa and was most closely related to *K. miniauriculata*.

In nature *Kudoa* may initiate epidermal lesions that allow opportunistic pathogens such as bacteria, fungi and amoebae to further compromise fish health. This study suggests that *Kudoa clupeidae* may have been a significant agent in initiating lesion development in other recent outbreaks of fish mortality in eastern Atlantic coastal waters.

MOLECULAR DIFFERENTIATION OF STELLATE AMOEBAE, *PFIESTERIA PISCICIDA* AND RELATED DINOFLAGELLATES RECOVERED FROM THE CHESAPEAKE BAY REGION OF THE EASTERN UNITED STATES

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Commonly occurring amoebae and *Pfiesteria*-like dinoflagellates from major tributaries of the Chesapeake Bay were established in pure culture. Representative taxa of six genera of amoebae capable of forming temporary stellate or rayed stages were studied. Following two years of observation during continuous cultivation with algal prey, clonal cultures of *Pfiesteria*-like dinoflagellates (Pocomoke river isolates 1-8, Patuxent river isolate A) and clonal reference strains of *Pfiesteria piscicida* (CCMP 1830,1831,1834) and *Pfiesteria*-like dinoflagellates (CCMP 1828, 1835, 1838, 1839, 1845) have never yielded amoeboid life-cycle stages. In addition, monoprotist cultures of amoebae have never produced any dinozoite stages. Small subunit ribosomal RNA (SSU rRNA) genes were sequenced from monoprotist cultures of amoebae and several clonal cultures of dinoflagellates. Parsimony analysis of this data demonstrated that six genera of commonly occurring amoebae including *Korotnevella*, *Neoparamoeba*, *Paraflabellula*, *Platyamoeba*, *Rhizamoeba* and *Vannella* cluster in several independent clades that are all distinct from dinoflagellates. In addition, *P. piscicida* reference strain (CCMP 1834), two *Pfiesteria*-like isolates from the Pocomoke River, MD (isolates 6 and 8) and a tentative *Gyrodinium* sp. from the Patuxent River, MD cluster tightly within the dinoflagellate clade, separate from all groups of amoebae for which SSU rRNA gene data exists. The results of this study support the conclusion that representative taxa from six genera of the most commonly occurring amoebae of the Chesapeake Bay are not related to dinoflagellates.

MODULATION OF VERY-LONG-CHAIN (C_{28}) HIGHLY UNSATURATED FATTY ACIDS BY SELENIUM IN TWO BLOOM FORMING DINOFLAGELLATES, *GYRODINIUM GALATHEANUM* AND *PROROCENTRUM MINIMUM,* FOUND IN THE CHESAPEAKE BAY, MD.

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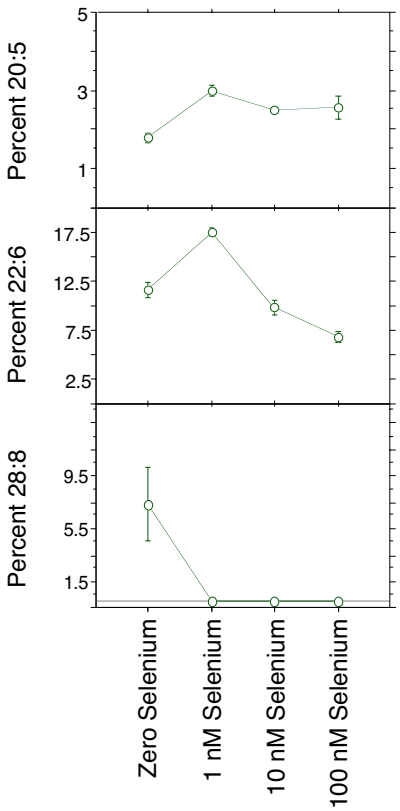
In recent years, harmful algal blooms, in particular those of the ichthyotoxic dinoflagellates *Gyrodinium galatheanum* and *Pfiesteria piscicda*, have been associated with massive mortality of fish in natural riverine systems along the east coast of the United States and in cultured fish ponds. We have shown Chesapeake Bay isolates of *Gyrodinium galatheanum* and *Prorocentrum minimum* both contain the hemolytic endotoxin octadecapentaenoic acid (18:5n3). This unique fatty acid has been suggested to be the toxic agent of *Gyrodinium aureolum* and *Gymnodinium* cf. *mikimotoi* responsible for aquaculture related fish kills. Furthermore, we have shown that for *G. galatheanum* and *P. minimum* cells, the major pools for 18:5n3 are the monogalactosyl diacylgylcerols (MGDG) and diagalactosyl diacylglycerols (DGDG) found in chloroplasts. This finding is also in accordance with other toxic dinoflagellates containing 18:5n3. Several isolates of *Pfiesteria piscicida,* as well as other related heterotrophs, when cultured on cryptomonads, were found to contain no 18:5n3.

As part of our work investigating the modulation of the hemolytic fatty acid 18:5n3 by alteration in culture conditions, cultures of *Prorocentrum mininum* and *Gyrodinium galaltheanum*, were grown in defined media with and without soil extracts and/or chicken manure extracts. Addition of either extract elevated the level of 18:5n3 in both species. When we analyzed the trace elemental composition of the extracts, the level of selenium was found to be high. Accordingly when we grew these species in defined artificial seawater with varying molarities of sodium selenite (0, 1, 10, and 100 nM) the level of 18:5n3 was modulated by the selenium in the culture medium (Deeds et al., 2001), with the highest percentage found at 1 nM for both species. Recently, very-long-chain (C_{28}) highly unsaturated fatty acids (VLC-HUFA) were identified in seven marine dinoflagellate species (Manour *et al.,* Phytochemistry, 50 (1999 541-548). In general, the proportion of these fatty acids accounted for less than 2.3% of the total fatty acids in these species. Unexpectedly the level of VLC-HUFA (28:8n3) increased at 0 nM sodium selenite, while at all other selenite concentrations the VLC-HUFA was less than 1%. The VLC-HUFA (28:8n3) was not found in the MGDG and/or DGDG lipids but instead accumulated in phospholipids. Both 28:8n3 and 22:6n3 (DHA) contain the maximal number of methylene-interrupted double bonds in a straight-chain fatty acid and share a double bond across the 4-5 positions numbered from the carboxyl group. The synthesis of 28:8n3 appears to involve an additional elongation/desaturation cycle proposed for the synthesis of 22:6n3 in mammals from 20:5n3 (EPA). Selenium appears to be involved in restricting this pathway to a 22 carbon length fatty acid. These findings pose new questions as to whether VLC-PUFAs might contribute to ichthyotoxicity and whether environmental selenium levels influence ichthyotoxicity in selected Chesapeake Bay dinoflagellates.



DEVELOPMENT AND VALIDATION OF A PCR-BASED DETECTION ASSAY FOR *PFIESTERIA PISCICIDA* IN WATER AND SEDIMENT ENVIRONMENTAL SAMPLES

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Pfiesteria. piscicida is a heterotrophic dinoflagellate that has been associated to massive fish mortalities, in some cases associated with health problems in water men working along the lower Eastern Shore of the Chesapeake Bay. Similar episodes, although of lesser scale, have occurred in Chesapeake Bay tributaries. Plate tabulation has been the routine method for the identification of dinoflagellate species. Groups of species are characterized by a particular formula, and differences in shape or size of some of the plates can define distinct species within the group. This technique is time consuming and requires special training. We have developed a rapid, sensitive, specific, and easy to implemented in any laboratory PCR-based assay for the diagnostic of *P. piscicida*. Based on genetic information, the PCR assay uses primers from *P. piscicida* NTS and SSU regions yielding an amplicon of 429 bp. We have been routinely applying this assay in the core facility to identify *P. piscicida* in environmental water and sediment samples. Although PCR assay is a very sensitive technique for the detection of specific sequences in complexes DAN mixtures, the amplification reaction can be easily inhibited by compounds present in the sample. Indeed it is fundamental to develop controls that guaranteed the detection of *P. piscicida* in a sample is consequence of the absence and not the result of PCR inhibition. Here we present data on the effect of inhibitors from water and sediment samples in the PCR assay and we developed an internal standard that uses the same priming sites that the PCR-based assay for *P. piscicida*. DNA from environmental samples is spiked with the internal control and an aliquot of the mixture used PCR. If the internal control is detected, samples are subsequently tested for *P. piscicida*. When the internal control is not detected by PCR, serial dilution of the sample are prepared and used to establish the dilution of the sample at which there is no inhibition of the PCR. This sample dilution is then used for detection of *P. piscicida*. The use of internal standards would guaranteed the quality of the sample and avoid false negatives in environmental samples and based on data we suggest it use for routine diagnostic of *P. piscicida*.

PFIESTERIA PISCICIDA IN SEDIMENT SAMPLES FROM FISH-FARMING PONDS: _IN VITRO_ EFFECT(S) OF LIVE FISH ON THE EARLY PCR-BASED DETECTION OF ZOOSPORES IN THE SUPERNATANT WATER

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Pfiesteria piscicida is a heterotrophic dinoflagellate that has been associated to massive fish kills during recent years in the mid-Atlantic estuaries of North America. Thus, the effects of live fish, and their secretions, excretions or metabolic products on the life cycle, proliferation, and toxicity *P. piscicida* has become a research area of great interest. In this preliminary study we examined the possible effect(s) of live fish on the presence and proliferation of *P. piscicida* in water or culture medium that has been incubated with sediments potentially containing putative life stages of this dinoflagellate species. Sediments (10 ml) from 10 active or drained ponds from a fish farming facility were sieved through a 60 _m mesh, and cultured in 750 ml flasks with 500 ml of artificial seawater (7 ppt; pH 8) containing one Sheepshead Minnow (*Cyprinodon variegatus*) (Controls were flasks with water containing no fish), or with culture medium (F/2) containing no fish. Flasks were maintained at 23 ºC and 14 h light/10 h dark cycle. Enumeration of dinoflagellate zoospores in the supernatant and PCR-based specific detection of *P. piscicida* were carried out daily for 21 days, and the health of the fish was monitored throughout the experiment. Numbers of *P. piscicida*-like zoospores increased progressively, and positive PCR results for *P. piscicida* were obtained in flasks corresponding to all ten ponds. In flasks containing fish, however, *P. piscicida* could be detected as early as the second day, and flasks remained PCR-positive throughout the duration of the experiment. No fish deaths were recorded.

DETERMINING THE CAUSES OF MORTALITY IN BIOTOXICITY ASSAYS WITH *PFIESTERIA*: THE DEVELOPMENT OF FRACTIONATION STUDIES

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Aquarium-scale biotoxicity assays with *Pfiesteria* pose inherently difficult problems. Fish health must be carefully balanced with issues such as frequency of water changes, degradation of water quality, the presence of pathogens, and the density of the background community of micro-organisms and protozoa. Add to the equation, long-term exposures of fish to a variably toxic species such as *Pfiesteria piscicida*, or the potential for contamination with related species (*Pfiesteria* sp. B), and the problems quickly appear insurmountable. We undertook long-term (80+ d) bioassays of laboratory and field isolates (Neuse and Pamlico rivers) of several *Pfiesteria*-like dinoflagellates (*P. piscicida, Pfiesteria* sp. B., "Lucy," and "Shepherds Crook"). For field isolates, fish were immediately placed in water collected for biotoxicity assays. Subsamples were processed for counts of heterotrophic dinoflagellates, DNA analysis, and SEM verification. Exposures consisted of 25-50 fish in each aquarium (38 L, 12‰ artificial seawater in de-ionized water, preconditioned Whisper filters, 20° C) inoculated with isolates from cultures, sediments or freshly collected water samples. Fish were fed three times per week and monitored for signs of morbidity, lesions, and mortality. Controls consisted of fish in replicate treatments without dinoflagellates (unexposed). Thirty long-term toxicity bioassays (17 field isolates, 3 subcultures, and 10 clonal cultures, with controls) were conducted. Two aquaria showed moderate mortalities, and four aquaria showed high mortalities in relation to the density of *Pfiesteria* sp. B. Mortality events typically lasted 2-3 weeks with 1-2 sequelae shortly thereafter. In four cultures, heavy bacterial loads (*Vibrio* spp. and *Shewanella putrefasciens*) were present, and water quality was degraded due to low dissolved oxygen (high BOD), high ammonia and nitrite, and low pH in relation to organic wastes. High bacterial loads and low pH (5.8-6.0) occurred in the other two aquaria, but other conditions were ideal to support fish health. All aquaria possessed numerous ciliates (holotrichs, and hymenotrichs), rotifers, amoebae, microflagellates (bicoecids), and other taxa. Thus, even though densities of *Pfiesteria* were high (2.0-20.0 x 10^3 cells ml^{-1}), it was difficult to rule out other causal or contributory factors in fish deaths.

Water quality studies were undertaken with an appraisal of ammonia toxicity in tilapia, *Oreochromis niloticus*, at varying pH. Tilapia were resistant to high levels of reactive ammonia (LC_{50} = 9.72 ppm), and low pH (5.0, 6.5). Thus, ammonia was not a significant factor in the observed mortalities. We then developed a "fractionation" assay where small volumes (1-2 l) of "toxic" aquarium culture were filtered and centrifuged into different components: dinoflagellates (and other protozoa), bacteria (and microflagellates), and the "soluble toxin" fractions. Controls consisted of raw culture water, and sterile artificial seawater. Fractionation assays with tilapia in 500 ml volumes were inconclusive. However, fractionation assays with mummichogs in 15 ml volumes indicated conclusively that the dinoflagellate fraction caused significant mortality compared to different controls. Bacterial and "soluble" fractions did not cause appreciable mortality. Fractionation studies with a sensitive species or sensitive life-history stage, such as larval mummichogs, provide a reliable tool for assessing fish mortality in toxic exposures with *Pfiesteria*.

In conclusion, during our biotoxicity assays, several inherent issues arose regarding culture conditions to support optimal fish health, yet provide suitable conditions for the growth and assessment of *Pfiesteria*. We developed a protocol that accounts for water quality, the possibility of contamination, and an assessment of toxicity, but from our standpoint questions remain regarding the toxigenic nature of *Pfiesteria* sp. B.