Molecular Approaches for Identification And Environmental Detection of *Pfiesteria piscicida* and *Pfiesteria*-like Dinoflagellates

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A. Executive Summary and Recommendations

Currently there are at least seven different research groups that have been funded to develop molecular probes for identifying *Pfiesteria* and *Pfiesteria*-like organisms (PLOs). These include: 1) Senjie Lin, University of Connecticut and Ed Carpenter, SUNY Stony Brook, 2) Katheryn Coyne and Craig Cary, University of Delaware, 3) Dave Oldach, University Of Maryland Medical School, 4. Kimberly Reece and Gene Burreson, College of William and Mary, 5) Parke Rublee, University of North Carolina at Greensboro, 6) Karen Steidinger, Florida Fish & Wildlife Conservation Commission, Don Anderson, Woods Hole Oceanographic Institute, Wayne Litaker, University Of North Carolina School Of Medicine, and Chris Scholin, Monterey Bay Aquarium Research Institute, and 7) Gerardo Vasta, University of Maryland Biotechnology Institute. In an effort to avoid duplication of effort and promote better communication between the different teams, a workshop was convened at the Center of Marine Biotechnology in Baltimore, Maryland, on September 3-4, 1999, and was attended by representatives of each research group as well as observers from CDC, EPA and NOAA.

Discussions were broken down into three major sessions: I) Species identification/molecular characterization; II) Technical aspects of preserving and extracting DNA from environmental samples and establishing cultures, and, III) Molecular, morphological and serological identification, taxonomy and quantitation of dinoflagellates in environmental samples. The specific questions posed in each session and a brief summary of the subsequent discussions and conclusions reached thereafter are presented in Section B. Representatives of each group also provided brief summaries of their research initiatives, compiled in Section C.

Workshop participants reached the following conclusions and recommendations:

1. It is advisable to use "certified reference strains" as a focus of analysis given the extensive and wide range of studies driving a relatively small but diverse group of researchers. Whenever possible these strains should be deposited in a public culture collection along with preserved samples of the cells and their DNA.

2. There is a danger that different groups may develop different criteria for delineating between species and strains. Consideration should be given as to how such boundaries should be set, and a mechanism for establishing a convention for naming strains that reflects both morphological and molecular criteria.

3. The extensive sequencing effort now underway is not well coordinated; many groups appear to be sequencing the same genes from the same cultures (in some instances this has already occurred). Wherever feasible, groups should share information to speed analyses and to advance development of cell detection techniques.

4. Creating a central repository of information about cultures or bloom events would be extremely useful. For example, a list of cultures could be accompanied by light and electron micrographs, links to DNA sequence data, information concerning toxicity,

where the culture is held, whether preserved cells and/or DNA is archived, along with contact names for all the above, etc. Likewise, a posting of bloom events, whether samples were collected, and preliminary results of tests on those samples would help inform researchers about opportunities for inter-lab comparisons of different cell detection techniques. A list of species for which probes exist would also be useful. This repository of information could be password-protected.

B. Workshop Summary

Session I: Species Identification/Molecular Characterization (moderated by Chris Scholin)

1. What diagnostic features should be used when describing species?

   Workshop participants agreed that use of morphological based taxonomy (zoospore plate tabulation) should form the basis of the organisms' description, and that results of molecular characterization be viewed within that context. Ideally, strains should be deposited in public culture collections such as the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP). Indeed, many strains are now available through the CCMP and new isolates will be added in the future. A summary sheet listing these cultures is given in Table 1. Since the list of isolates is becoming large, it is advisable to focus research efforts initially on particular strains, or "certified references," that are considered clonal and that are well characterized morphologically and toxicologically. Records of how the organisms were isolated, what feed they may have been reared on, life cycle stages that were observed using a particular technique, available molecular data, as well as preserved reference material (whole cells, extracted DNA) should ideally accompany a strain's deposition into a collection for future inter and intra-strain comparisons.

   Workshop participants also agreed that results of molecular characterizations be incorporated into the description of species and strains. At present, there are no formal guidelines, for example under the Botanical Code of Nomenclature, for integrating classical morphological-based taxonomy and molecular phyologeny to arrive at a universally accepted "standard" for species and strain identification. Historically, workers have used morphology as the primary means of identifying species and provided molecular characterizations that in turn do or do not support such distinctions. This can lead to a proliferation of strain designations, based on molecular data, which are not necessarily adopted by other researchers working on the same or similar organisms. Analysis of the *Alexandrium tamarense/catenella* "species complex" provides a graphic example of this potential problem and the pitfalls to avoid as morphological and molecular characterization of *Pfiesteria* and *Pfiesteria*-like species proceed.

2. To what extent have Pfiesteria and PLOs been characterized at the molecular level?

   Molecular characterizations of *Pfiesteria* and related organisms that are completed, underway or planned are extensive. Two groups (Lin et al., Anderson et al.) are pursuing development of antibodies against cell surface antigens. DNA sequencing efforts are primarily aimed at the ribosomal RNA (rRNA) genes (rDNA; Figure 1).

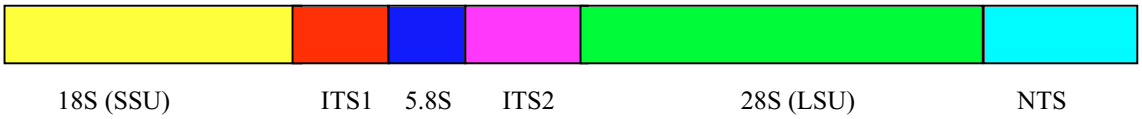

Figure 1. The nuclear encoded ribosomal RNA (rRNA) genes (rDNA), currently the focus of most sequencing efforts. Three genes that are involved in protein synthesis, the 18S or small subunit (SSU) gene, the 5.8S gene, and the 28S or large subunit (LSU) gene, plus two internal transcribed spacer regions abbreviated as ITS1 and ITS2, are arranged in tandem. The rDNA cistrons are separated by a non-transcribed spacer (NTS).

The Oldach and Rublee teams have completed a considerable amount of sequencing of the SSU genes. The VIMS group is concentrating on the ITS regions, 5.8S and 5' portion of the LSU genes. Those workers concentrating on species from the Florida region (Litaker et al.) are sequencing a fragment that includes the SSU through a large portion of the LSU genes, including the ITS's and 5.8S gene. The COMB (Vasta et al) group is focussing its efforts on the non-transcribed spacer (NTS**),** SSU and ITS regions (Table 1).

In addition to rDNA sequences, many groups expressed an interest in or plans to acquire sequences of other "house-keeping" genes (e.g., actin), search for genes that are life stage and cell cycle-specific, and characterize highly repetitive sequences or pseudogenes that may offer markers for fine-scale strain resolution. Given the effort involved in such studies, it is highly desirable to ensure that this effort be spent on a set of isolates examined from multiple perspectives (e.g., morphologically, rDNA sequence, toxin production).

3. Are relationships based on classical morphological-based taxonomy reflected in the organisms' molecular characteristics/molecular phyologeny?

Preliminarily, results of the rDNA and morphological-based analyses appear to cluster species in equivalent groups. A summary of this work is presented in Table 2. For example, Litaker et al. presented a phylogenic tree that demonstrated a high degree of concordance between groups defined by their SSU sequences versus groupings defined by plate tabulations (e.g. apical pore complex, number and shape of apical plates, presence or absence of precingulars, cingular displacement, shape of intercalaries, shape and position of sucal plates, body shape including form of epitheca and hypotheca, and the structure of apical pore complex) of the dinospore after the amphiesma has been removed. Thus a unique SSU sequence in conjunction with a careful examination of plate structure could be used to describe species boundaries with high fidelity.

The SSU phylogeny also indicated that *Pfiesteria piscicida* is in the Blastodiniphycidae along with *Amyloodinium oscellatum*, though probably not in the same order. Placement of *P. piscicida* within the Blastodiniphycidae is consistent with the amoebae life-cycle staged having a typical eukaryotic nucleus. Preliminary work with sequences of recently isolated Cryptoperidiniopsoid species also indicate that other PLOs will fall in this same taxonomic group.

4. How can different research initiatives be coordinated to avoid duplication of effort?

   Given the large degree of overlapping effort revealed in the meeting it was generally agreed that there should be better coordination between laboratories. Some groups have invested considerable time and effort into developing sequence databases and, understandably, are not willing to distribute that information until formally published.

   However, the research community as a whole would benefit considerably if the different groups' initiatives included analysis of a common set of cultures – "standards" – available to all. In this manner, each group could focus on elucidating the relationships of a set of organisms from multiple but distinct perspectives (e.g., morphology, toxicology, antigenicity, and sequences of rDNA, "house-keeping genes," repetitive DNA, mitochondrial DNA, stage-specific-expressed genes, etc.). This would speed characterization of newly identified *Pfiesteria*-like organisms (PLOs), promote a more rapid and thorough understanding of the evolutionary relationships and biogeographic distribution of these species, and speed development of probes and analytical techniques for use on cultured and natural samples.

5. How can information be disseminated between different research groups?

   It would be useful to establish a web site where workers could see a list of isolates, photographs showing distinctive morphological features of representative type species, summaries of what genes have been or are being sequenced and by whom, and a list of associated publications where appropriate. Direct links to abstracts of published papers as well as sequence data or sequence alignments would also be very useful. At a minimum, this web site could serve to keep different groups informed of "who's doing what," so as to avoid duplication of effort to the maximum extent possible.

Session II: Technical Aspects of Preserving and Extracting DNA From Environmental Samples and Establishing Cultures (moderated by Wayne Litaker)

1. What is the best means of extracting DNA from environmental samples?

   Both standard CTAB and Qiagen™ DNAeasy Plant Kit extraction methods produced DNA that amplifies well by polymerase chain reaction (PCR) techniques from 50 to 100 ml water column samples filtered onto GFC glass fiber or Nucleopore filters. Sediment extractions were much more problematic because of the difficulties encountered lysing cells uniformly, as well as the presence of large quantities of phenolic compounds and polysaccharides that co-purify with the DNA or RNA and subsequently inhibit DNA polymerases. The methods used to lyse cells include microwaving, osmotic shock, freeze-thawing, and bead beating in combination with a subsequent proteinase K treatment. Once lysed, extensive extractions in CTAB or phenol/guanadinium (eg. DNAzol ES) were required to remove most of the phenolics

and polysaccharides. Generally a final purification step using DNAeasy (Qiagen™) or some other procedures where the DNA or RNA samples are bound to a glass surface and washed extensively before eluting the DNA or RNA was employed. Even after this involved procedure, there was usually some appreciable inhibition of subsequent PCR reactions. For this reason, quantifying the life stages that are present in sediments using PCR based on extractable nucleic acids is likely to prove challenging.

2. How does one deal with optimization and interference problems?

   Participants agreed that because of the potential for inhibition in both water column and sediment sample extractions, internal amplification controls should be added to a subset of all DNA and RNA extractions to quantify the amount of inhibition. The use of internal controls is particularly important when doing quantitative assays.

   When significant inhibition occurs, then the PCR assays have to be optimized using standard optimization methods to correct for pH, Mg^{++}, and K^{+} imbalances. If optimization fails, extraction protocols have to be modified to produce cleaner DNA or RNA.

3. The collection of the sample before DNA extraction also will be a potential problem. A cell's susceptibly to lysis is likely be influenced by nutritional and growth states, being easy to lyse sometimes and difficult others. Thus, further discussion of how to discern whether or not cells are lysing during filtration or preparation for in situ hybridization would be beneficial.

   Parke Rublee reported on the method developed by David Oldach and Holly Bowers whereby field samples can be successfully stored in Lugol's solution for 40 days with no appreciable degradation of DNA and only slight DNA degradation after 122 days. DNA extracted from these cells worked well in PCR reactions. Given that Lugol's preservation is a recognized method of preserving delicate cells, this represents a major advance that will allow easy preservation of numerous field samples that can be subsequently analyzed in the laboratory.

4. What is the best way to establish new cultures?

   Clonal cultures are best established from single cell isolations. However, many laboratories lack the facilities for making single cell isolations and instead rely on isolation by limiting dilution. Though usually adequate, dilution procedures can sometimes produce cultures with more than one clone or species present. The isolation method used to produce each culture should therefore be listed.

   Other questions were addressed in this session, but ended up carrying over to session III and are therefore addressed below.

Session III: Molecular, Morphological and Serological Identification, Taxonomy and Quantitation of Dinoflagellates in Environmental Samples (moderated by Gerardo R. Vasta)

1. What combinations of sequence determination, specific PCR amplification from known genes, RFLPs or microsatellite data that can be applied to characterizing and quantifying DNA extracted from sediment and water column samples.

   There is great interest in linking molecular features to plate tabulation and other morphological information for the development of comprehensive and rigorous approaches to identification/taxonomy of toxic dinoflagellates. A great deal of discussion was generated on the analysis of various regions of the rRNA gene cluster, namely the SSU, LSU, ITS and NTS for the molecular taxonomy and identification of *Pfiesteria*-like dinoflagellates and other species of interest. It is clear that SSU, ITS, NTS (or ETS) regions have proved very useful for taxonomic/phylogenetic analysis and probe development. These molecular markers , however, are all structurally and evolutionarily "linked". Other genomes (i.e. mitochondrial, plastid), housekeeping genes or other independent genes encoding structural proteins such as actins, should be also included for a more rigorous molecular taxonomy analysis. The application of "long PCR" with "universal" primers for amplification of the mitochondrial genome, followed by restriction analysis (RFLPs) is currently used in fish, and similar approaches could be applied to dinoflagellates. The use of microsatellite analysis, proven as very useful for studies of intra-species variability and population structure, was illustrated with successful application of this approach to *Acanthamoeba* by Bonnie Brown, and proposed as a tool for identification and examining population structure of toxic dinoflagellates in environmental samples. The use of RT-PCR for the identification of transcripts associated to particular life cycle stage would enable the development of stage-specific molecular probes (Coyne and Cary). The application of alternative approaches such as gene complementation in yeast mutants may prove useful for the identification of genes expressed at particular phases of the cell cycle, useful for the molecular characterization of dinoflagellate blooms. Kathryn Coyne and Craig Cary have also developed PCR Fluorescent Fragment Detection assays that can be normalized to chla or 28S RNA and detect down to 1 cell/ml in complex samples using Gene Scan software.

   Pat Gillevet and Bonnie Brown briefly discussed the use of sequence from the SSU of the rRNA gene cluster for the identification of various amoeba obtained from mixed and presumptive clonal cultures. No sequences homologous to *Pfiesteria piscicida* have yet been identified in these amoeba.

   The use of antibodies for genus/species identification, cell cycle status determination, and cell separation was discussed in detail by Sinjie Lin. Conventional antisera developed against whole cells or homogenates show usually various degrees of cross-reactivity, and several procedures have been attempted to make them specific, including dilution and adsorption with cross-reactive cells, with variable results. In many cases, the activity may be directed towards surface carbohydrates, which are

shared by several species. Therefore, most antisera are genus-specific at best, and less frequently, species-specific. Monoclonal antibodies have not shown to be much more advantageous than the conventional antisera. The development of antibodies against gene products specific to certain cell cycle phases would be very useful for understanding how the environmental conditions lead to toxic blooms. The use of detergents and enzymes for increasing cell permeability and access of antibody reagents to intracellular epitopes has been explored to a considerable extent, with DMSO proving the most useful. The use of antibodies conjugated to magnetic beads has enabled the separation of dinoflagellates from mixed populations. The use of flow cytometry with FITC-labeled antibodies has been used to examine the diel cycle of *G. brevis* and may constitute a powerful approach to understand toxic blooms in well-characterized species. The use of lectins has been limited, and so far only the identification of binding patterns with comprehensive panels of plant lectins, such as those tested with *Pseudonitschia* sp and *Alexandrium* sp has shown some degree of success.

2. Quantitative PCR-based assays. Internal competitors and internal standards. Estimation of cell numbers in the environmental samples. Accuracy and sensitivity of quantitative methods.

   The use of internal standards for the assessment of microbes in environmental samples is a well established procedure. Genetically tagged microorganisms can be added to the sample (i.e. sediment) and the performance of the DNA extraction and molecular assays procedures assessed. It was proposed that this approach would be very useful to test extraction and detection methods for dinoflagellate cysts.

   Most of the discussion, however, centered on the development of competitive PCR. The quantitative competitive PCR (QC-PCR) constitutes an established method for the specific and accurate measurement of the concentration of DNA molecules carrying sequences of interest, which can then be used to calculate cell numbers in water, sediment, soil, or body fluids and tissues. This technique is being employed by Dave Oldach's group to assay the abundance of *Pfiesteria piscicida* and other PLOs. The technique is based on the design of a competitor DNA template, with primer recognition sequences identical to those of the target, whose product can be distinguished from the target amplicon on a size basis while exhibiting kinetics of amplification similar to the target DNA. The competitor is added in known quantities to experimental PCR reactions and co-amplified with the target DNA. The EtBr-stained amplicons can be scanned, the densities of the amplicons integrated, and the equivalence point determined. The ratio of competitor product to experimental product can be used to determine the DNA concentration of the experimental template. If the target gene is in single copy and the ploidy of the organism of interest is known, the cell number in the sample can be determined directly. Otherwise, the copy number of the target gene can be determined empirically from the amount of template produced from a standard cell number. The real-time quantitative techniques such as TaqMan had been discussed in the previous session.

Concerns shared by the workshop participants related to the levels of sensitivity and accuracy required in the assessment of dinoflagellates in the environment. This is very important because it appears that most of the detection methods discussed exceed the needs in sensitivity and accuracy that are biologically meaningful when considering toxic outbreaks, in which the cell numbers of the organism of interest is relatively high. However, this has to be examined against the backdrop of the changing environmental conditions that, in a short period of time, can turn low cell densities in a toxic bloom. The participants discussed the possibility of standardizing cell numbers detected in environmental samples, a "cell currency", for example cells/ml extract, in order to make results from different laboratories easily comparable, without having to include sample volume or sampling conditions.

3. Emerging technologies for environmental qualitative and quantitative probing and molecular identification of microbial pathogens and parasites.

   In addition to "real-time" PCR and QC-PCR techniques described above, new technologies are being developed for the assessment of toxic algal blooms, or methods developed in other fields are adapted to this purpose. The participants agreed that when selecting the suitable technology, strong consideration should be given to requirements for sensitivity, assay format (in situ detection, real-time, etc), throughput, human resources, location, cost, and other factors.

   DNA sequencing and fingerprinting technologies continue to improve at a fast pace and multiple instruments are available now, generally at lower prices. Some instruments are now constructed as portable units and their performance has been improved substantially. Such the case of real-time PCR (TaqMan) technologies in which a portable instrument that can identify and quantitate pathogenic bacteria in environmental samples in seven minutes, has recently been introduced in the market. Techniques developed for viral identification such as the heteroduplex DNA mobility assay, have recently been adapted to the detection and preliminary identification of dinoflagellates in environmental samples by Dave Oldach. This very useful method allows the examination of the species diversity in a sample without the need for sequencing all DNA species present.

   More specifically, in the field of harmful algae, devices have been designed and prototypes constructed for sample processing aimed at the application of whole cell assays and homogenate-based sandwich hybridization assays. These two approaches complement each other and some problematic samples (i.e. plankton with large proportion of fecal pellets where the algae are incorporated into a solid matrix) have revealed that the hybridization assays perform well, whereas whole cell assays may fail. Automated units that can both carry out sample processing and the detection assay have also been designed and their performance is currently being tested. These units carry out the sample filtration as the first step, followed by exposure of the particles to fixatives or lysis reagents , and subsequent detection by hybridization in arrays of labeled probes bound to polypropylene filters. Other probing technologies could be applied to the samples (toxins, nucleic acids, proteins, etc). The devices

could be placed in buoys or platforms, and routinely sample the environment for the species of interest. It was the general consensus that these technologies show great promise, both for in situ detection and sample archiving.

4. Processing of the sequence information for taxonomic/phylogenetic analysis of microbes and protista.

   A thorough discussion about the use of algorithms and optimality criteria for phylogenetic analysis of the DNA sequence information took place upon a presentation where the basic concepts were outlined. Whereas the former enable the construction of trees based on the aligned sequences, methods such as maximum parsimony, minimum evolution, maximum likelihood help to determine which tree is better. Among those, the distance methods can be based in models of sequence evolution. It was clear throughout the discussion that the success of the phylogenetic analysis hinges on the quality of the sequence alignments. If the insertions or deletions do not reflect the true evolutionary history of the group analyzed, the tree will not be informative. The use of sequence editors (several currently available and free) may be very helpful in data processing. A number of concerns were addressed, one particularly critical to the success of this type of analysis, which relates to how the low representation of dinoflagellate sequence information in the databases relative to the large number of species in the environment yet to be characterized, introduces a bias in the interpretation of the true evolutionary history of the dinozoa.

C. Research Initiative Summaries

National Center For Culture Of Marine Phytoplankton's (CCMP) Role In Isolating and Maintaining Cultures Of Pfiesteria and Pfiesteria-Like Organisms And The Procedure For Cryopreserving PLO Species

Robert A. Andersen, CCMP, Bigelow Laboratory for Ocean Sciences

The Provasoli-Gulliard National Center for Culture of Marine Phytoplankton (CCMP) maintains culture strains for the scientific community. We maintain and distribute strains isolated and deposited by other scientists and we also isolate organisms ourselves and make these available. We have 45 strains of Pfiesteria or Pfiesteria-like organisms in the collection at this time. Information about these strains can be accessed electronically at our web site (http://ccmp.bigelow.org/) by (1) selecting the "Search Database" option, (2) selecting the "keywords" option, and (3) typing "Pfiesteria" into the "General" category. Typing "Pfiesteria" into the "genus" category will only retrieve those strains that have been identified as Pfiesteria piscicida. The CCMP also has approximately 30 strains that have not been deposited into the CCMP but these are available to all scientists. All strains can be obtained by contacting us at the CCMP.

We can isolate Pfiesteria or Pfiesteria-like organisms from water or mud samples sent to us, with the understanding that (a) the isolates will be deposited in the CCMP and therefore made available to the scientific community, and (b) the isolates will be of value to the scientific community. We are not allowed to isolate strains for a single investigator's research program where the isolates are not deposited in the CCMP. Also, we cannot add to our collection a large number of strains that do not cryopreserve because we lack the space and personnel to maintain them. If we can assist you with isolating strains, please contact us at the CCMP.

Because there are apparently several species of Pfiesteria-like organisms that other scientists are in the process of describing, we list many of our strains as "unidentified species" and we are unable to identify new isolates that originate from you or your water samples. Once these species are described, we should be able to provide more accurate taxonomic identifications for our strains.

Finally, we are very grateful for the assistance from many scientists working on Pfiesteria who have deposited their isolates, have sent us field samples for isolation purposes, and have provided us with identifications of CCMP strains.

The crypreservation procedure used in our laboratory is given below.

Cryopreservation of Pfiesteria and PLO

Pfiesteria piscicida and other PLO cultures are grown in 1.5 L of 12 ppt seawater plus a food supply of Rhodomonas sp. (CCMP768) in a 22 degree C incubator with a 12 hr

light/dark cycle. Cultures are grown to approximately 500-2,500 cell/mL (as dense as possible) before cryopreservation.

1. A few hours before the cells will be cryopreserved, we mix a solution of 20% dimethyl sulfoxide (DMSO) in f/2-si culture medium (autoclaved seawater would probably work just as well). We mix this before hand because the mixture releases heat. We don't actually know if the heat is fatal to Pfiesteria, but we have recorded death in other organisms when we have not allowed sufficient cooling periods for the DMSO stock solution.

2. We concentrate the culture to approximately 10,000-100,000 cells/mL by vacuum filtration (5.0 µm pore-size, 47 mm diameter polycarbonate Nucleopore filters, <250 mmHg vacuum). When filtration rate slows, we transfer the liquid above the filter into a sterile polypropylene centrifuge tube, and we also add the clogged filter to the centrifuge tube. We place a new sterile filter on the filtration apparatus and repeat the process until the 1.5 L of culture has been concentrated down to approximately 15 mL. Naturally, one must practice careful sterile technique during this process.

   Please note: We have found that a gentle vacuum is necessary for success, i.e., gravity concentrated cells do not cryopreserve successfully.

3. We then mix the concentrated cells to ensure homogeneity and to suspend any cells attached to the filters. Using a pipetteman and sterile tips, we dispense 750 µL aliquots of the concentrated cells into sterile, 1.8 mL plastic cryogenic vials. We use Nunc brand vials (Cryo Tube - trademark, part number 377267), however, almost certainly any brand will work.

4. We then add 250-500 µL of the pre-mixed 20% DMSO solution to each cryo-tube, resulting in a final concentration of 5-8% DMSO. We allow the cryo-tubes to sit at room temperature for 15-30 min before initiating the freezing process, so that we achieve good DMSO penetration. The final concentration of DMSO should be between 5-8%; Pfiesteria is killed when higher concentrations are used.

5. We have successfully frozen Pfiesteria using two methods, a simple method (6) and a controlled-rate freezer method (7). Almost all of our efforts have been with the controlled-rate freezer, but the simple method works and may be useful to those who lack a controlled-rate freezer.

6. We use a "Mr. Frosty"-type container (Nalgene cat. No. 5100-0001) for the simple method, but Styrofoam boxes can also be used. The only requirement is that the sample cool at a rate of about 1 degree C per minute. Therefore, after the 15-30-minute "incubation" described in Step 4, we place the cryo-tubes into the freezing container that has been pre-chilled to 10 degrees C. The container is then placed in a -80 degree C freezer for 3 hr. Our freezer is actually set at -75 degrees, but I doubt that this is a meaningful difference. After 3 hours, we remove the vials from the -80

freezer, open the container, and place the cryo-tubes directly into liquid nitrogen. We store our vials on canes in the vapor phase of the liquid nitrogen storage tanks.

7. Our controlled rate freezer system consists of a CryoMed model 1010 programmer, a Cryo-Med 2700C freezing chamber, and a PC computer loaded with CryoMed software. The following is simplified, but if you have the same controlled rate freezer system, we can provide the exact program steps. We place the cryo-tubes into the controlled-rate freezing chamber and activate the cooling program. The program slowly cools the samples to 4 degrees C (~30 min) and then it cools at a constant rate of -1 degree C/min, compensating with a purge of liquid nitrogen for the release of the latent heat of fusion. When samples reach -45 degrees C, the cooling rate increases to -10 degrees C /min until the samples reach -80 degrees C. The samples are then removed immediately and stored in the liquid nitrogen tank (vapor phase).

8. The thawing of cryopreserved samples is as critical as the freezing, i.e., it is easy to kill the cells during the thawing process if not conducted carefully. We thaw samples in a 36 degree C water bath. The water level in the bath is just high enough so that it comes a few millimeters above the level of the frozen sample. We gently transfer the previously frozen cell suspension into a plastic tissue culture flask containing 15 mL of 12 ppt seawater plus one drop of a CCMP768 culture. We place this flask in the same environment that it was previously growing (e.g., 20 degrees C with some light). Depending on the strain, swimming dinoflagellate cells should be visible (using a dissecting scope) within 24-48 hours, although a few strains take a few more days to recover. Depending on the strain, the cells should reach normal density in 7-21 days.

Toward A Mechanistic Understanding Of Outbreaks Of *Pfiesteria piscicida* And Related Dinoflagellates: A Regional Comparative Study.

Kathryn J. Coyne and S. Craig Cary University of Delaware, College of Marine Studies

The principal investigator for this proposal is Patricia Glibert, U of MD, Center of Environmental Studies at Horn Point. Our research includes investigators from NC, SC, MD, and DE. The overall objective of the study is to describe and model the interactions between the environmental conditions and the physiological responses of *P. piscicida*. The specific objectives of the molecular component at the University of Delaware are (i) to develop enumeration capabilities that are independent of microscopic techniques, and (ii) to develop life stage-specific molecular probes to detect, discriminate, and enumerate the key life stages of *P. piscicida*. The integration of quantitative molecular techniques for detection of toxic Pfiesteria with the physical, chemical, and biological characterization of its environment will provide information crucial to the development of monitoring and long-term management strategies for high-risk areas. Two techniques will be used to enumerate *P. piscicida* in field and mesocosm samples. For the first technique, PCR Fluorescent Fragment Detection, the 18S gene is amplified by PCR using a *P. piscicida* -specific primer with a Hex-labeled eukaryotic-specific primer. The fluorescently labeled products are then detected using an ABI Prism 310 Genetic Analyzer and quantified with Genescan Analysis software.

The second technique employs real-time PCR detection of amplification products using primers and probes that are specific to the P. piscicida 18S gene. Again, field samples are compared to a standard curve prepared from spiked samples to determine cells/mL of P. piscicida.

Our second objective is to develop life stage-specific probes. These probes will be used to rapidly assess both the presence and relative abundance of the key toxic and nontoxic life forms of P. piscicida in field and mesocosm samples. There are three steps to our approach:

1. Transcript frequency analysis by Serial Analysis of Gene Expression (SAGE): Transcript tags that are unique to each life stage of interest will be identified for probe development.

2. Transcript retrieval: Full-length sequences of uniquely expressed genes will be obtained from a cDNA library and used to design primers and probes specific to each life stage.

3. Real-time quantitative PCR: Enumeration of targeted life stages of *P. piscicida* will be accomplished by quantitative real-time detection of DNA amplification products using primers and probes developed in step

Characterization Of Pfiesteria Like Dinoflagellates, Co-Isolated Stellate Amoeba And The Total Microbial Consortia Isolated From Sediments Associated With Epizootic Events.

P. M. Gillevet, T. Nerad, and M. Peglar

We have examined sediments from Maryland rivers with (Pocomoke river) and without (Choptank and Patuxent River) reported *Pfiesteria* induced epizootic events and identified both *Pfiesteria*-like dinoflagellates and stellate amoebae at all sites examined. Based on SSU rRNA sequencing of clonal cultures, the stellate amoeba were phylogenetically distinct from any dinoflagellate species (Sawyer, Peglar et al. 1999; Sawyer, Peglar et al. 1999). Our results indicate that all stellate amoebae thus far co-isolated with *Pfiesteria*-like dinoflagellates in sediments associated with epizootic events are not dinoflagellates and that both *Pfiesteria*-like dinoflagellates and stellate amoebae are also found in sediments from sites that have never had a recorded epizootic event.

We have also cultured and continually observed clonal strains of *Pfiesteria*-like dinoflagellates and *Pfiesteria piscicida* reference strains (CCMP 1830,1831,1834) for over two years. None of these cultures has ever yielded an amoeboid form. However, planozygotes have been regularly observed. These results indicate that *P.piscicida* are capable of completing both the haploid and diploid phases of its life cycle in the absence of fish or fish derived materials without converting to amoebae.

Recently, we have used the Amplicon Length Heterogeneity fingerprinting method to monitor cultures of Pfiesteria and fish tank culture systems to correlate the presence of specific genera with fish death. The method interrogates the variable domains of the small subunit rRNA and separates these variable domains on high-resolution sequencing gels (Suzucki et al 1998). These domains tend to vary in length at the genus level so the peaks observed on the fingerprint are correlated to the genus present in the consortia. Amplicon length profiles from the first variable region of the Bacterial SSU rRNA, Fungal SSU rRNA, and Protist SSU rRNA from sediment samples have been completed. Differences in the position of the peaks indicate the presence of genera that differ in the length of the amplified variable region and the height of the peak reflects their relative abundance in the sample. The ALH technique is therefore being used to detect bacterial, fungal, PLO-specific genera in the sediments and in culture. Being able profile which suite of organisms is present will prove useful in resolving the fundamental issues of whether *Pfiesteria* has stellate amoeboid life stages and whether fish death using fish tank culture systems is mediated by a microbial consortia or by a single genera/species.

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Immunocytochemical And Molecular Biological Approaches For Species Identification And Estimation Of In Situ Growth Rate Of *Pfiesteria piscicida* And *Pfiesteria*-like Dinoflagellates

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Immunocytochemical and molecular biological approaches can complement one another for different applications. Immunocytochemical methods such as immunofluorescence have been used widely for identifying phytoplankton species as well as other microoganisms. It has the advantage of simplicity in handling the samples and implementing the detection procedure and less susceptibility to reaction conditions. Molecular biology, on the other hand, is better-defined in terms of the marker used, such as primers for PCR or RT-PCR and oligoprobes for hybridization, although being more sensitive to variation in reaction conditions.

Our project mainly is to establish immunoprobes (antibodies) and gene probes to 1) identify *Pfiesteria* species (flagellated cells and other life stages); 2) perform cell cycle analysis through cell cycle related genes and their products and estimate growth rate (i.e. cell division rate) in situ. Isolation of these genes constitutes a significant portion of the project. Environmental factors will then be correlated with changes in growth rate.

Underway is development of a suite of polyclonal and monoclonal antibodies against *P. piscicida* (Dr. Burkholder's strain), CCMP 1830, 1831, and 1834. The goal is to establish a set of antibodies that can distinguish *P. piscicida* from other similar species and can identify amoeboid and cyst stages of this species.

We also have been cloning and sequencing three cell cycle-related genes (cDNA), proliferating cell nuclear antigen (PCNA), cyclin B, p34cdc2, and MAPK for *P. piscicida*. The aim for this research is to identify unique regions of these genes for primer design for RT-PCR. In addition, after characterization of these genes as to their association with the cell cycle, cDNAs with great promise will be over-expressed in a prokaryote expression system and the proteins will be purified. Polyclonal antibodies will then be produced. In testing the approach, we have cloned a cyclin B conserved region (cyclin box) for a *Pfiesteria* and the related species *Cryptoperidinopsis* (CCMP 1828). Experiments with other *Pfiesteria* strains are currently underway in our laboratory.

Once the probes are ready, we will perform quantitative RT-PCR and immunofluorescence for cultures at different growth phases and cell cycle phases and study how the cell cycle of *Pfiesteria* progress in relation to the natural light dark cycles. Next, field investigation will be conducted.

Molecular Diagnostics For PCOS In Chesapeake Bay

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We are currently funded by the ECOHAB program to develop molecular diagnostics for *Pfiesteria*–complex organisms (PCOs) found in the Chesapeake Bay. During this first year of funding 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 PCO clonal cultures. Clonal isolates of *Cryptoperidiniopsis* spp., *Pfiesteria piscicida*, "Shepherd's crook", "Lucy" and another unidentified PCO were obtained for molecular analysis. Cultures were obtained from Drs. Ed Noga and Pat Tester, the Provasoli–Guillard National Center for Culture of Marine Phytoplankton (CCMP) and the VIMS PCO culturing facility (see table for cultures in the VIMS collection). DNA was isolated from a total of 17 PCO cultures and 3 food source cultures. DNA sequences were obtained from the food sources to assure that PCO rather than food source DNA clones were selected for analysis. Cultures were first identified by scanning electron microscopy (SEM) at VIMS before selection for molecular analysis.

Initial PCR amplifications of the ITS region using "universal" primers resulted in preferential amplification of food source DNA. To circumvent this problem a "dinoflagellate–specific" PCR primer was designed. The primer sequence selected allows amplification of the 3' end of the SSU gene and the entire ITS region when coupled with a "universal" primer that binds to the 5' end of the LSU gene. This primer pair allows exclusive amplification of PCO DNA from the cultures that contain both PCO and food source cells. In addition, we have identified "diagnostic" restriction enzyme sites that distinguish PCO and food source LSU amplification products.

SSU–ITS and LSU sequences from PCOs have been completed and are now being aligned using the Clustal–W algorithm in the MacVector software package (Oxford Molecular Group). Sequence comparisons among the PCO cultures and to other dinoflagellates and protozoans will be used to develop species–specific and genus–specific PCR primers and DNA probes. Phylogenetic analyses will also be competed using PAUP 4.0 for the 3 different regions sequenced.

To facilitate molecular screening of the PCO cultures we are developing the technique of amplified fragment length polymorphism (AFLP) analysis. AFLP profiles will be developed for the available PCO taxa as well as the food sources. Following SEM identification, culture DNAs will be subjected to AFLP analysis to identify cultures that represent the range of genetic variation within each taxon. This technique should allow us to more effectively target a subset of the available PCO clonal cultures for the more intensive sequence analysis. As taxon–specific PCR primers and probes are developed we will test them on the available cultures and begin analyses of environmental water and sediment samples.

Molecular Probes To *Pfiesteria piscicida*.

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First, we are using and refining field methods for handling aqueous environmental samples provided to us by various state agencies and researchers. One key aspects of this work has been to compare sample extraction methodologies to determine optimal methods for field sampling, particularly under the constraints of timely sampling by personnel with little or no training in sampling procedures, and sample storage, handling, and shipment when optimal conditions (e.g. refrigeration) may not be available. To date, we find that fresh samples taken on site are best handled by immediate filtration onto a glass fiber filter followed by immersion in a CTAB buffer, or preservation with acid Lugol's solution. In either case, shipment of samples at room temperature to analytical laboratories appears acceptable. Once received in the analytical laboratory, extraction of the samples in CTAB progresses with a chloroform-alcohol extraction, while preserved samples are processed by passage through a membrane filter followed by extraction with a Qiagen DNeasy plant kit. We are actively pursuing questions of sensitivity of our probes and concerns about internal and external standards, particularly in light of variable ratios of target to non-target DNA likely to be encountered in field samples.

We are also pursuing optimal methods for detecting Pfiesteria DNA in sediments. Tests of several extraction methods (CTAB lysis buffer [Doyle and Doyle 1987] and a method specifically designed for sediments [Tsai and Olsen 1991]) have demonstrated limited success. Both methods appeared to effectively extract DNA from clean sand that had been seeded with Pfiesteria culture material, but increasing proportions of silty organic sediments from the Neuse River reduced the quality of the extracted DNA as determined by A260/A280 ratios. Primers to eukaryotic rDNA (Sogin 1990) were unable to amplify the crude PCR extract. However, after 100-fold dilution, passage through a Sephadex G-200 column, or use of a commercial column (Elutip-D, Schleicher and Schuell, Keene, NH)) and the addition of BSA, we were able to amplify with eukaryotic primers. The problems of low target abundance and hi levels of inhibitor compounds remain challenges to analysis of sediment samples for the presence of *P. piscicida*.

Another aspect of our work has been to map the distribution of *P. piscicida* on the US eastern seaboard. A number of states are providing us with samples from estuarine waters. In some cases these are spot surveys, while in other cases they represent samples from routine monitoring of sites. When prudent (e.g. suspected kill or lesion event) we always recommend submission and coordinate sampling with the Aquatic Botany Lab at NCSU to test for toxicity. In addition to *P. piscicida*, we are probing the samples for a second *Pfeisteria* species and for a cryptoperidiniopsoid. Our primary method is PCR probing, but we are also using fluorescent in situ hybridization to a limited degree. A long term goal is to identify environmental parameters that show high degrees of correlation to positive samples locations, particularly if the also demonstrate toxicity and/or fish kill or lesion events.

In conjunction with both of these efforts, we are looking for signatures of genetic variability and potential new species among geographic locations and from culture isolates provided by our colleague JoAnn Burkholder at NCSU. Of particular interest is whether there are identifiable differences among toxic and non-toxic isolates of *Pfiesteria*.

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Development Of Molecular Probes For The Rapid Identification Of *Pfiesteria*-Like Organisms Known In Florida's Coastal

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The overall objective of this project (funded by the Florida Fish and Wildlife Conservation Commission) is to explore the feasibility of using antibody and nucleic acid probes to discriminate among *Pfiesteria*-like organisms from Florida and other Gulf States. Specific project activities are as follows:

Antibody Probes

1. Obtain and maintain cultures of target *Pfiesteria*-like organisms from the Gulf of Mexico region for probe development. Isolates will likely include those nick-named 'Lucy' and 'Shepherd's Crook'. Probes will also be developed for *Cryptoperidiniopsis,* and *Gyrodinium galatheanum.*
2. Develop methods to prepare some or all of the isolates for antibody production, minimizing the quantity of food algae present as contaminants.
3. Work with private contractors to develop polyclonal and monoclonal antibodies to target isolates.
4. Purify and test probes for cross-reactivity and specificity.
5. If suitable antisera are obtained, explore the use of ELISA assay procedures as a means of rapid cell enumeration.
6. Test the antibody probes on field samples collected by Florida Marine Research Institute personnel from the Gulf states region.
7. Refine or improve the probes as necessary to increase specificity.

Nucleic Acid Probes

1. Obtain sequence information from the target isolates from the Florida region. Also obtain sequence information for *Cryptoperidiniopsis*, *Pfiesteria piscicida*, and other dinoflagellates of interest from other regions, such as North Carolina.
2. Obtain sequence information for food algae.
3. Identify possible sites for oligonucleotide probe development and synthesize probes.
4. Modify probe design to maximize specificity and sensitivity using cross-reactivity tests.
5. Explore the use of these probes in both whole cell and sandwich hybridization formats.
6. Develop a diagnostic PCR assay based on small subunit rRNA sequences.
7. Test nucleic acid probes and PCR assays on field samples collected by Florida Marine Research Institute personnel from the Gulf States region.

Life Cycle, Cell Cycle and Growth Regulatory Mechanisms in *G. breve*

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The work presented is part of the ECOHAB Florida regional field study on oceanographic and biological mechanisms controlling *Gymnodinium breve* red tides in the gulf of Mexico. My laboratory's role in this project is: 1. characterization of probes for *G. breve* to assist in elucidating life cycle stages, 2. determination of *in situ* growth rates in *G. breve* blooms, and 3. identification of cell cycle regulatory mechanisms in *G. breve*.

Probes Much of the life cycle of *G. breve* has been worked out by Walker (1982) and consists of both a vegetative growth stage and sexual cycle. Although resting cysts are hypothesized to be the source for bloom initiation off the west coast of Florida, cysts of *G. breve* have not yet been found in field samples. Recently a benthic palmelloid stage has been identified in culture (Steidinger et al., 1987), which evolve into swimming cells with pointed epithecae atypical of the classical *G. breve* vegetative cells. This opens the question as to its role in the life cycle as well as in bloom formation. To assist in identifying *G. breve* life stages, two polyclonal antibodies (rabbit and sheep) to whole cell lysates of *G. breve* (P. McGuire, Univ. Florida) are currently being tested for specificity. The rabbit anti-*G. breve* IgG is suitable for immunofluorescent labelling of *G. breve*. The antibody shows specificity for *Gymnodinium,* with no cross-reactivity with non-*Gymnidinium* species tested. We do see some cross-reactivity with *G. mikimotoi* and *G. sanguinium*. However, the antibody was able to identify *G. breve* in dinoflagellate mixtures. Therefore, it may be useful in its present form at least for preliminary identification of unusual cell morphologies as *G. breve* in field samples. Current efforts include pre-adsorption of the IgG fraction against other *Gymnodinium* species in order to increase specificity toward *G. breve*, changes in antigen expression during growth cycle, antibody characterization by western blotting, and flow cytometry applications.

In situ Growth Rates A fundamental unresolved question for most HAB species is whether blooms result from events which trigger "explosive" growth rates, or whether cells replicate at a relatively constant rate, with blooms resulting from the advection and subsequent maintenance of dense populations by physical or biological mechanisms. Division rates of 0.2-0.5 div/day observed in lab and field populations of *G. breve* are not sufficiently high to account for its dominance in the water column. The objective of this project is to determine specific growth rates of *G. breve* during bloom initiation, accumulation and decline phases, using flow cytometry.

Cell Cycle Regulation Our interests are in mechanisms that regulate the cell cycle in *G. breve* and signaling pathways involved in diel phasing of the cell cycle (Van Dolah and Leighfield, in press). We have characterized diel phasing of the cell cycle in *G. breve*, in both laboratory cultures and natural blooms: cells are in G1 phase during the light, with S-phase beginning 6-8 h into the light phase, and mitosis following 12-14 h later. The *G. breve* cell cycle is entrained to the diel cycle by the dark:light transition We have evidence for the presence of the eukaryotic cell cycle regulator, cyclin dependent kinase (CDK) in using two approaches: (1) identification of a 34 kD protein immunoreactive to an antibody against a conserved amino acid sequence (_ -PSTAIR) unique to the CDK

protein family and (2) inhibition of the cell cycle by olomoucine, a selective CDK inhibitor. We are currently interested in identifying cyclin (and other cell cycle-dependent genes). For this work we are using two approaches: RNA differential display and yeast complementation. Differential display is useful for identifying transcriptionally regulated *genes;* we have thus far identified four cDNAs of potential interest. The yeast expression system has the advantage of identifying genes that are not necessarily regulated at the transcriptional level.

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Development of Genetic, Lectin and Antibody Probes For *P. piscicida* And Pfiesteria-like Dinoflagellates

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We are applying a multi-targeted approach to establish molecular markers for *P. piscicida* and related species, that will be evaluated in the context of morphotype, physiology, toxicity, and life cycle and will include the following: (1) DNA/RNA probes or PCR primers, (2) lectins of well characterized carbohydrate specificity, in combination with mild proteolytic, glycolytic or glycooxidative cell surface pre-treatments, and (3) species- or stage-specific conventional and monoclonal antibodies. Comparison of divergent ribosomal DNA sequences is rapidly becoming standard methodology for the delineation of taxonomic relationships and development of species-specific probes. We are using a PCR-based approach to develop species- and strain-specific probes that are assessed for sensitivity and specificity in environmental samples, and adapted to quantitative methods (Competitive and "real time" PCR) for evaluation of conditions that influence growth or toxicity. We have developed a PCR-based assay for the specific detection of *Pfiesteria piscicida*, and most recently a competitive quantitative format. In the near future, subtractive cDNA genetics will also be employed to identify differentially expressed genes for the development of stage- or toxin-specific molecular probes. In addition to rRNA, we will apply molecular approaches for identification and classification of these organisms based on a number of gene sequences, including mtDNA, microsatellite DNA, and conserved protein-coding sequences such as actin. Similarly, in order to identify species-, strain- and life stage-specific gene products of Pfiesteria-like dinoflagellates and other related organisms, we are characterizing cell surface carbohydrate moieties and protein epitopes that show potential as targets for molecular probes. Because of the ubiquity of surface oligosaccharide and protein structures, potential cross-reactivity is one of the major hurdles to clear in the application of antibodies and lectins for specific and sensitive detection of cells, including toxic dinoflagellates. In order to overcome this potential problem, we will make use of proteolytic and glycolytic/glycooxidative mild enzyme treatments to modify the cell surface structures of interest and amplify putative surface differences between HAB species, strains and life stages. A lectin "library", together with a collection of proteases, glycosidases and glycooxidases, all available in our laboratory, is used for screening and characterization of dinoflagellate surface glycans and exposing masked epitopes. Biochemical analysis of the carbohydrate composition of these surface structures will be followed by development of species- and/or stage-specific conventional and monoclonal antibodies. The selected lectin and antibody reagents will be conjugated to enzyme or fluorescent tags in order to develop useful tools for the specific and sensitive identification and quantification of the dinoflagellates of interest. Their conjugation to chromatography resins and magnetic beads may enable us to develop cell separation technologies.

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