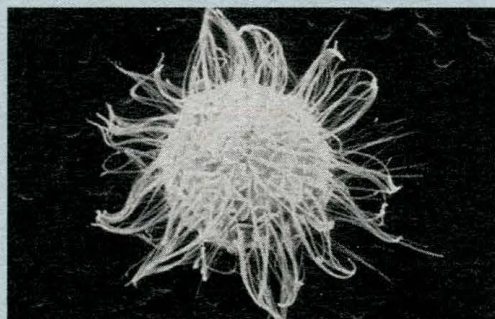
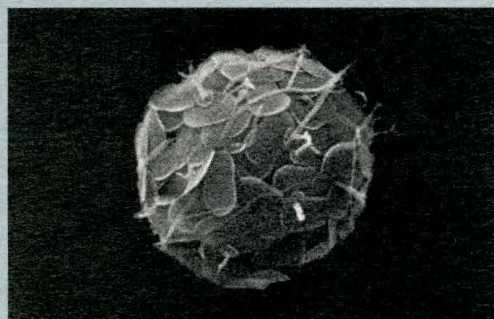
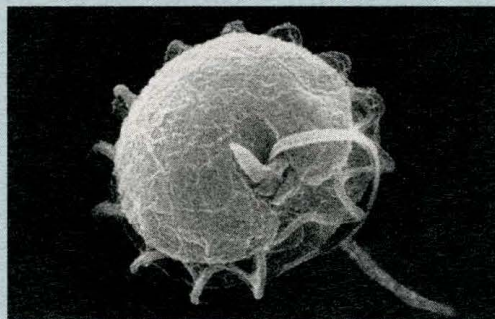
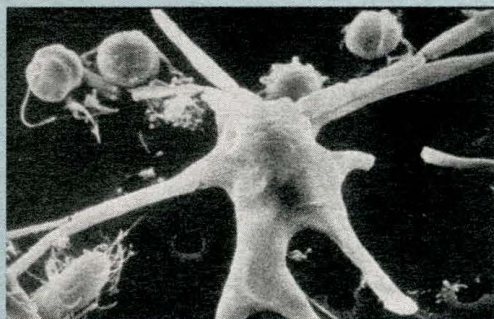


A WORKSHOP REPORT

Molecular Technologies and *Pfiesteria* Research

A Scientific Synthesis



*Prepared by the Center of Marine Biotechnology
and the Maryland Sea Grant College
in cooperation with the
USDA Agricultural Research Service*



A Maryland Sea Grant Publication
College Park, Maryland

Molecular Technologies and *Pfiesteria* Research

A Scientific Synthesis

This report results from a workshop held
October 28-30, 1997
at the Center of Marine Biotechnology
University of Maryland Biotechnology Institute

Organizing Committee
Yonathan Zohar, Robert Belas, Gerardo Vasta,
Allen Place and Jonathan Kramer

Prepared for the
Maryland Sea Grant College and the
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by
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Jonathan Kramer and Allen Place



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REPORT HIGHLIGHTS

Pfiesteria piscicida has emerged as the most recent threat to environmental and human health posed by harmful algae such as dinoflagellates and diatoms. Unlike most of those algae, which reveal themselves in red or brown or mahogany tides, *Pfiesteria*-like species show their presence only by the appearance of dead fish or fish with severe lesions. Preliminary evidence suggests that human health effects may occur even before large fish kills are present. To protect both coastal fisheries and the public health, we need to detect potential *Pfiesteria* outbreaks before they occur. Towards these ends, there are critical needs that scientific research must address if we are to minimize the impact of *Pfiesteria* and, in the long term, eliminate its reoccurrence. Those needs include:

- ▶ Developing certified “pure” cultures of *Pfiesteria*-like organisms so that research among different laboratories is comparable and transferable. Pure cultures can be characterized as “monocultural,” “unialgal,” or “axenic,” depending on the precise nature of the culture in question (that is, whether it is composed of only one species of alga — “unialgal” — or whether certified as bacteria-free — “axenic”).
- ▶ Distinguishing species that make up the complex of *Pfiesteria*-like organisms so that determination of toxin-producing species and stages can be clarified.
- ▶ Developing sophisticated molecular probes that can rapidly detect the presence of *Pfiesteria* and *Pfiesteria*-like organisms and their toxins. These probes must be able to distinguish between toxin- and nontoxin-producing stages; such probes must be able to determine the fate of these toxins, for example, whether fish and shellfish concentrate them, whether they break down and, if so, how long it takes.
- ▶ Characterizing the chemical composition of *Pfiesteria* toxins so that researchers can detail the biochemical mechanisms of how *Pfiesteria*-like species affect fish and human health.
- ▶ Continuing studies on the impact of *Pfiesteria*-like toxins on human health, for instance, respiratory problems and memory loss.
- ▶ Encouraging cooperation among experts and laboratories to achieve the best results in the shortest amount of time.

EXECUTIVE SUMMARY

This report synthesizes the findings of a diverse group of scientists, health care professionals and science managers who assembled at the Center of Marine Biotechnology in Baltimore, Maryland in October, 1997 to address the emerging problem of *Pfiesteria* and *Pfiesteria*-like organisms. The purpose of this two-day meeting was to develop a consensus of important research strategies for understanding and managing this group of organisms. Within this framework, the program concentrated on four major topics: *Pfiesteria* biology, taxonomy, toxins and human health concerns.

The workshop focused in particular on contributions that the application of molecular biology and biotechnology could make to research. These applications have the potential to provide a rapid, highly sensitive means to determine the prevalence and toxicity of *Pfiesteria*-like organisms in the natural environment. Workshop participants agreed, however, that to understand these organisms — in particular their ecological role and potential impact on human health — will require engaging a suite of scientific disciplines. This means cooperation and collaboration not only across traditional fields, but also among universities, public agencies and research laboratories throughout the region and beyond.

Listed below are priority concerns that emerged from the workshop, goals essential to understanding the nature of *Pfiesteria*-related human disease and the ecological impacts — especially fish kills and skin wounds — attributed to *Pfiesteria*-like dinoflagellates.

Establish and make available unialgal and ultimately axenic¹ type culture(s) of *Pfiesteria piscicida* and related dinoflagellates. This will require:

- ▶ Standardization of culture procedures and taxonomic identification.
- ▶ Standard operating protocols for culture growth, maintenance and preservation.
- ▶ Standard operating protocols for diagnostic procedures related to human health.
- ▶ Standard operating protocols for the detoxification of toxin-contaminated material.
- ▶ Determination of the diversity of *Pfiesteria*-like dinoflagellates.

Determine the chemical nature of the toxin(s). This will require:

¹ Cultures can be described as monocultural, unialgal and axenic. Monoculture signifies one species, in this case *Pfiesteria piscicida*. Unialgal signifies a single alga, even though *Pfiesteria* is strictly a protist. Axenic means free from other organisms, specifically bacteria, and therefore describes a certain type of pure culture. In every case the focus is on a culture not contaminated by other organisms or species.

² A “gold standard” refers to an agreed-upon reference point used in all analyzes; in the case of *Pfiesteria* toxin this means a highly purified and certified reference chemical, and the protocol related to the isolation of that pure chemical.

- ▶ Establishment of certified “gold standard”² tests for toxin determination.
- ▶ Development of molecular probes and bioassays for the rapid detection of toxin(s) and the life stages of *Pfiesteria*-like dinoflagellates.
- ▶ Characterization of both the environmental and prey-derived elicitors that induce toxin production.
- ▶ Determination of the role of microbial consortia in toxin production, modification and degradation.

Clarify the molecular and cellular mechanisms of toxicity. This will require:

- ▶ Determination of the molecular target(s) of the toxin(s).
- ▶ Determination of molecular causes for fish kills and ulceration.
- ▶ Understanding the effects of acute and chronic exposure to the toxins.
- ▶ Understanding the role of aerosols in dispersing toxins.

Develop probe technologies for the rapid identification and enumeration of toxic *Pfiesteria* and *Pfiesteria*-like species. This includes:

- ▶ Identification of appropriate nucleic acid or cell surface-based (antigenic or lectin-based) targets for probes.
- ▶ Development of probes for different life history stages of *P. piscicida* and other *Pfiesteria*-like organisms in the water column and sediments (zoospores, amoebae and cysts).
- ▶ Development of standard probing protocols that can be readily applied by management agencies.

Determine the environmental conditions under which *Pfiesteria* and *Pfiesteria*-like organisms can grow and survive.

- ▶ Identify chemical and physical conditions, such as temperature, salinity and nutrient regime, that enable and/or encourage the growth of *Pfiesteria*.
- ▶ Analyze the potential for *Pfiesteria* to spread (e.g., through ballast water, transplanted species, dredging/spoil dispersal) to susceptible areas either along the coast or to other continents and marine areas.

Achieving the aforementioned goals will lead to better ways of determining safe levels for human exposure to *Pfiesteria* toxins, and to improved models for accurate prediction of when and where blooms of *Pfiesteria* are likely to occur. Combined with active programs designed to monitor important ecological conditions that may influence potential *Pfiesteria*-related fish kills, such predictive models will ultimately provide the best means for reducing risks to human health.

INTRODUCTION

In 1988, researchers in North Carolina identified a newly discovered dinoflagellate, *Pfiesteria piscicida*, as the cause of extensive fish deaths in Pamlico Sound. Since that time, it is estimated that more than a billion fish, mostly menhaden, have died in North Carolina waters as a result of *P. piscicida* or other *Pfiesteria*-like organisms.

JoAnn Burkholder and her colleagues at N.C. State University first reported their findings in *Nature* (Burkholder et al. 1992) and speculated that *Pfiesteria* was not confined to North Carolina estuaries. They were right. In 1992, a team of scientists led by Allen Lewitus, then at the University of Maryland's Center for Environmental Science, sampled sediments in Jenkins Creek, near the mouth of the Choptank River on Maryland's Eastern Shore, and found *Pfiesteria* in the Chesapeake Bay watershed (Lewitus et al. 1995).

In studies of *Pfiesteria*'s life cycle, Burkholder has uncovered more than 20 forms that include cysts, flagellated zoospores and amoeboid stages (Burkholder and Glasgow 1997). In its flagellated form, *Pfiesteria* can release powerful toxins that either directly cause or lead to deep ulcerated lesions (and skin sloughing) in fish, part of an attack that can also lead to large fish kills. They can also have serious implications for human health — the first evidence for these came in North Carolina where researchers were themselves sickened while studying the behavior of *Pfiesteria* and its release of toxins in the presence of fish. North Carolina fishermen working in waters where *Pfiesteria* was present also reported health symptoms that ranged from skin sores to dizziness to memory loss, though for some time the link between *Pfiesteria* and health effects was not established.

In Maryland, *Pfiesteria* was first identified as the cause of fish deaths in aquarium tanks in 1994 at the Academy of Natural Science Benedict Estuarine Research Laboratory on the Patuxent River (Breitburg, Academy of Natural Sciences Estuarine Research Center, Pers. Comm. 1996-1997). There were no reported kills or fish with lesions in the river at that time, nor in 1996, when a similar kill occurred in the Academy's new lab on St. Leonard's Creek. These and other observations suggested a possible system-wide distribution in the Bay. While *Pfiesteria* was present in the death of several thousand striped bass in aquaculture ponds on the Manokin River in 1996 (Terlizzi 1996, 1997), so were other potentially harmful algae; thus, scientists have not been able to link conclusively those deaths to *Pfiesteria*. There were no observations of fish with lesions or deaths in either the Manokin or the Patuxent rivers.

When fish with lesions began appearing from Shelltown to Pocomoke Sound in the lower Pocomoke River in the fall of 1997, resource managers were not overly concerned; while lesions and fish kills are not everyday occurrences, neither are they unusual in some regions of the Chesapeake. Fungi, amoeba and other diseases can cause lesions, and when episodes of oxygen depletion occur during hot summer weather, fish unable to escape to safer waters may become stressed, leading to secondary infection and disease or death. Events in the Pocomoke, however, resembled those in North Carolina, especially the tell-tale circular sores, often near the anal area of affected fish. Of greater concern were reports by commercial watermen of maladies including physical reactions and memory loss, ailments also under investigation in North Carolina. *Pfiesteria* soon became a prime suspect.

More extensive evidence of fish lesions and then a large fish kill in the Pocomoke River during August, 1997, as well as assays conducted by Burkholder's laboratory, confirmed that *Pfiesteria*-like organisms were active there. After conferring with his advisors and a team of physicians, Maryland Governor Parris Glendening closed the Pocomoke to fishing and recreational use in order to minimize further effects, particularly over the concern for safeguarding human health. At the same time, Maryland took new actions that included intensive monitoring for *Pfiesteria*, nutrients and man-made chemicals in the river; medical studies of individuals who may have been exposed to the organism during fish kills; and the development of a plan on how best to reduce its immediate impacts on natural resources and human health (Boesch 1997b).

In August, the governor appointed former governor Harry Hughes to head a Blue Ribbon Commission charged with investigating conditions that led to the fish kills and evaluating prospects for eliminating their recurrence. The report reflects just how much uncertainty there is about *Pfiesteria* and closely related dinoflagellates, in particular, their biology, their behavior, their processes of toxin production, the chemistry of the toxins and their means of delivery (Hughes et al. 1997).

The ability to shape management actions that will minimize the effects of *Pfiesteria* on the ecosystem and human health will depend on a much improved understanding of these newly described dinoflagellates. What exactly will it take to improve that understanding, in terms of laboratory and field technologies? There is widespread agreement among researchers that the complex nature of the *Pfiesteria* problem requires a multi-disciplinary approach that employs novel methodologies and techniques. For example, monitoring for different stages of *Pfiesteria* and detecting toxins will require (and, in fact, demand the use of) molecular probes and other tools of biotechnology. What capabilities are available now? What is needed? Can institutions with different capabilities collaborate to overcome obstacles more effectively?

To address these and related questions, the University of Maryland Biotechnology Institute's Center of Marine Biotechnology hosted a meeting of researchers on October 28-30, 1997 at the Columbus Center in Baltimore, Maryland. The meeting aimed at (1) clarifying the state of our scientific knowledge on the biology of *Pfiesteria* and its health impacts and (2) prioritizing the most effective directions for employing the powerful tools of molecular biology.

To meet these objectives, attendees at the two-day meeting participated in presentations covering four major areas of *Pfiesteria* research: biology, taxonomy, toxin production and human health impacts (see Appendix I). On the first meeting day, researchers addressed current scientific knowledge in each of these areas, and on the second day participants divided into four work groups to identify gaps in our knowledge, priorities for research and the means for trying to meet those priorities. The means in many instances will require a toolkit of molecular technologies — molecular probes, bioassays and other techniques for use in laboratory studies but also in the field for monitoring. The need for rapidly distinguishing among the life stages of *Pfiesteria*-like species and for detecting toxins will be critical in order to protect human health.

WORKSHOP SUMMARY

The discussion that follows derives from the workshop's scientific presentations and breakout sessions. Focusing on the workshop's four major topics — biology, taxonomy, toxins and human health — this report sets a context for the kinds of questions about *Pfiesteria*-like organisms that molecular biology can help address. Common to all these discussions were the need for safe operating procedures in conducting research on *Pfiesteria*, the need for training and technology transfer in working with *Pfiesteria*-like organisms, and the need for well-defined stock cultures of these dinoflagellates. The discussions in each work group also emphasized the urgent need that health and resource management agencies have for tools that would enable them to determine the presence of toxin-producing *Pfiesteria*-like species before these organisms can affect the health of workers and others. In the best of situations, monitoring agencies should be able to routinely and rapidly detect or anticipate the potential outbreak of toxins. To reach this goal, researchers must better understand the biology of these dinoflagellates and their mode of toxin production and delivery.

Pfiesteria Biology

The life cycle of *Pfiesteria piscicida* makes it a “versatile predator.” JoAnn Burkholder has identified 24 life stages — at least nine of which are benthic amoeboid and cyst stages (Figure 1). Amoeboid stages inhabit both sediments and

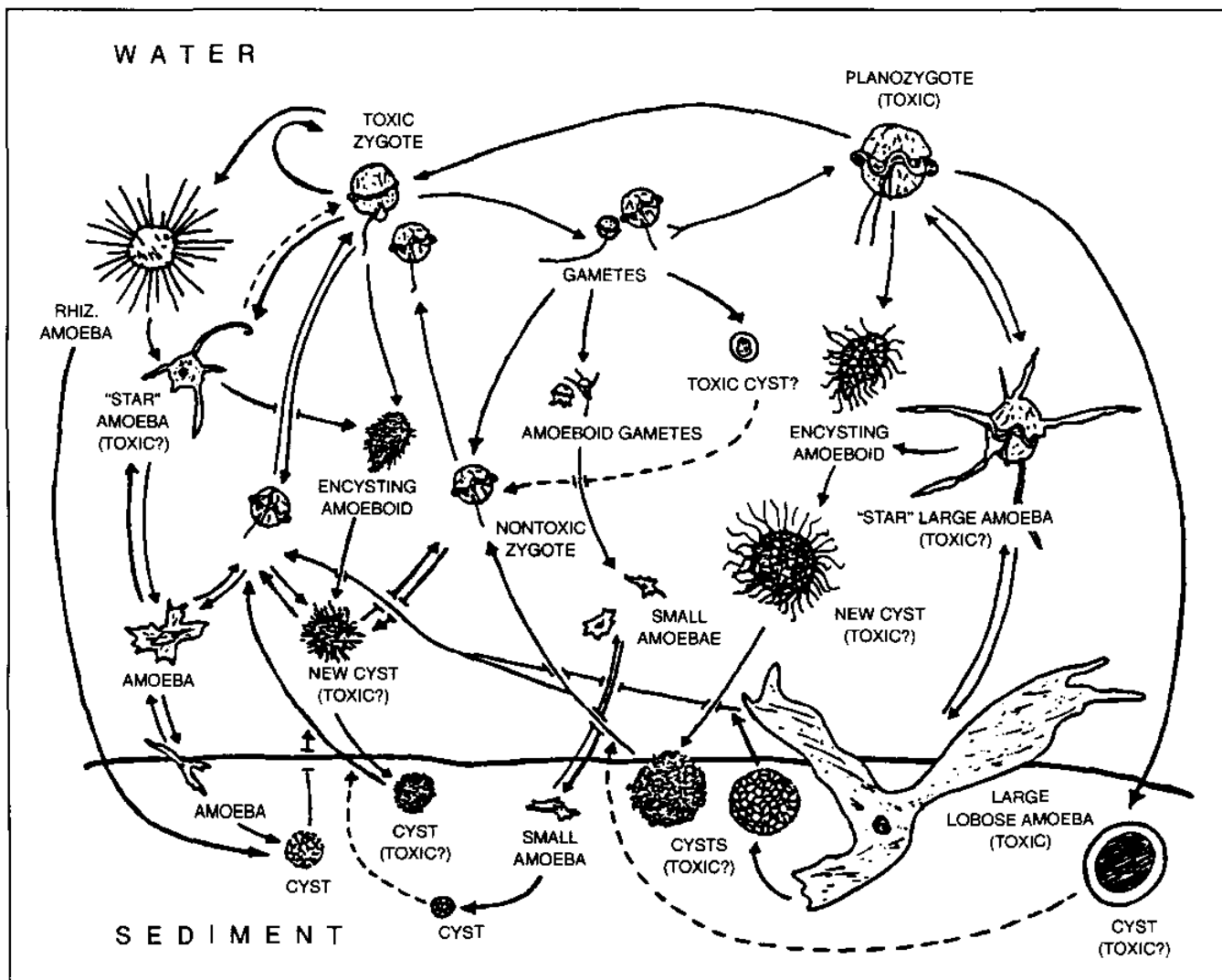


Figure 1. *Pfiesteria piscicida* appears to have an extremely complex life cycle, manifesting more than twenty different forms. Most of those forms come under several broad headings, such as cyst, amoeba, or flagellated cell. Many forms are apparently nontoxic; the presence of live fish reportedly stimulates the presence and activity of toxic forms, especially the flagellated zygote stages. Schematic provided by JoAnn Burkholder and Howard Glasgow.

the water column, while encysted forms are predominantly in the sediment. While nontoxic amoeboid forms feed primarily on bacteria, algae and other microscopic organisms, toxic flagellated stages (zoospores) feed by extending a peduncle, attaching to the phytoplankton prey and sucking in tissues (Figure 2).

Pfiesteria appears to be common in estuarine and coastal waters and has been found as far south and west as Alabama (Mobile Bay) and Florida (Pensacola) and as far north as the inland bays of Delaware. In general, it has been responsible for

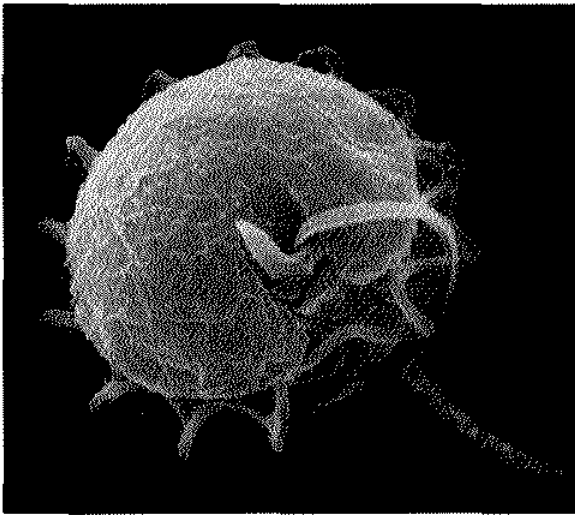


Figure 2. The toxic planozygote stage of *Pfiesteria* extends a peduncle, shown here alongside the whip-like flagellum, which it apparently uses to draw fluids and tissues from its prey. Figure from JoAnn Burkholder.

attacking fish in poorly flushed areas — Burkholder and others speculate that in more heavily flushed waters, *Pfiesteria* may not be able to accumulate in large enough numbers to cause significant problems.

According to some researchers, the abundance of *Pfiesteria* in any given area appears to correlate with fish density and with the abundance of phytoplankton.

Phytoplankton, in turn, are dependent on nutrient availability (often the result of rainfall runoff from nearby terrestrial habitats); therefore, Burkholder and other researchers argue, the abundance

of *Pfiesteria* is likely to be affected indirectly by nutrient runoff. They also point out that other environmental factors, such as standing nutrient loads and physical circulation, play important roles (Cambridge Consensus, Boesch 1997a).

Menhaden appear very susceptible to *Pfiesteria* with lesions induced in both the wild and in the laboratory, though striped bass and other species such as catfish appear to be highly vulnerable as well. For example, tilapia are used in laboratory experiments because they are highly susceptible to attack, while guppies, Burkholder reports, are not, although these fish in time also succumb to *Pfiesteria*'s toxin. Several reasons have been advanced for the large number of menhaden deaths. Menhaden are schooling fish and often, in efforts to forage phytoplankton food sources, get caught in poorly oxygenated areas of bays and estuaries, where they may become stressed and weakened (due to the lack of oxygen), and therefore are more susceptible to attack. Gathering as they do in large numbers, menhaden also release large amounts of excrement that may cue *Pfiesteria* to transform from cysts into one of the toxin-producing stages. Menhaden are also extremely oily fish, which may — or may not — be a factor in their vulnerability.

Pfiesteria appear to require live fish to trigger or stimulate production of toxin and attack behavior. That stimulus seems to be derived from chemical cues in fish excreta, though these cues may work in concert with other conditions. According to Burkholder, "active amoeboid and flagellated cells which are present also

become toxic in the presence of fish excreta. The small cells swim toward the fish prey and, in turn, excrete potent toxins.” The toxins paralyze fish and, reports Burkholder, injure their ability to maintain an internal salt balance. Lesions and sores often occur near the cloacal region (although lesions may be found on any part of the body) — fish show erratic behavior, disorientation, and a gasping at the water’s surface. The open wounds also leave fish vulnerable to opportunistic infections from other microorganisms, a factor which complicates cause and effect issues regarding *Pfiesteria*-induced lesions.

Burkholder has observed *Pfiesteria* feeding on the tissue, blood and other substances that come from the sores. As the skin is destroyed, open bleeding sores and hemorrhaging often occur. Once the fish are incapacitated, Burkholder reports, *Pfiesteria* feed on the sloughed epidermal tissue, blood, and other substances that leak from the sores. When the fish are dead, flagellated stages transform to amoeboid stages and feed on the fish remains or, alternatively, if conditions become unfavorable, *Pfiesteria* cells make protective outer coverings and sink out of the water column as dormant cyst stages. All of these changes can occur in a matter of hours. The ephemeral, transient nature of this life cycle is a major complication for monitoring *Pfiesteria*-related fish kills because, soon after a fish kill is sighted, *Pfiesteria* population density in the water diminishes to undetectable levels.

Current collaborative research projects have been investigating the impacts of *Pfiesteria* on fish and shellfish (The Raleigh Report 1998). While shellfish consume *Pfiesteria* and other algae through normal filtering processes, no lethal effects on adult shellfish have yet been observed. On the other hand, oyster pediveligers that ingest toxic forms of *Pfiesteria* have been observed to die within 24 hours, which suggests that *Pfiesteria* toxins may in some instances be released after cells are eaten or could accumulate. Research in this area has been limited. Jeffrey Springer, JoAnn Burkholder and Sandra Shumway are conducting experiments on the effects of *Pfiesteria* zoospores on commercially important shellfish. In a short-term study of grazing on zoospores by the bay scallop *Argopecten irradians*, they found a significant decrease in clearance rate, with a “narcotizing” effect on exposed scallops; scallops ceased feeding after 15 minutes of continuous exposure to zoospores (Springer et al. 1996). According to Shumway, there is no information that documents filter-feeding vectors for the toxin (Pers. Comm. 1998). This work is preliminary and considerably more needs to be done on the effects of long-term exposure to repeated toxic outbreaks of *Pfiesteria*-like organisms.

Major Research Needs

While researchers at North Carolina State University have uncovered fundamental attributes of *Pfiesteria piscicida*, funding support for research has been limited. For

example, much of the research to date has focused on water column stages of *Pfiesteria*, not the benthic (bottom-dwelling) stages, especially the encysted forms of this dinoflagellate. With coast-wide concern over the impacts of *Pfiesteria*-like organisms on human health and natural resources, opportunities for expanding research are more promising. Basic questions on the biology, ecology and behavior of *Pfiesteria* will need to be answered if there is to be any chance of minimizing toxic outbreaks in the future. For instance:

How does the organism behave when it is in non-outbreak situations?

- Are *Pfiesteria*-like organisms likely to be found in some habitats more than others, i.e., what is the relation between biogeochemical conditions and cyst or amoeboid distribution?
- What percentage of time does *Pfiesteria* spend as a cyst and what is its composition and physiology in that state?
- Are cysts metabolically active? Are they toxic?
- To what extent do endosymbionts and other bacteria contribute to *Pfiesteria* toxin production?
- Do consumed bacteria remain viable within the cell?

The general supposition is that *Pfiesteria* has been with us a long time, though just how long (on the order of hundreds or thousands of years?) is unknown. A number of questions remain:

- If *Pfiesteria* has been in east coast waters for a long time, why is it only within the last decade that outbreaks and, more significantly, human health effects have been observed?
- Have earlier outbreaks gone unnoticed?
- Are there sublethal effects that researchers have not even thought to look for?
- What environmental conditions, natural and/or manmade, have proven favorable for toxin-producing stages?

Pfiesteria and *Pfiesteria*-like organisms are believed to be widely distributed, though there has been little corroboration of this, in part because of the difficulty in easily distinguishing *Pfiesteria* and its cysts from the cysts of other microorganisms. Current methods of identification require highly specialized skills, are labor-intensive and time-consuming (see discussion on Taxonomy). The ability to map accurately the distribution of *Pfiesteria* and to determine the presence of toxins (see discussion on Toxins) will be critical for identifying waters that are at risk to *Pfiesteria* so that they can be managed as potential toxic areas.

The ecology of *Pfiesteria*-like organisms has barely begun to be investigated. For example, fundamental knowledge is lacking on how food web dynamics may

affect *Pfiesteria*'s growth, reproduction and abundance, or how manipulating the food web (by changing nutrient inputs, for example) could affect *Pfiesteria*'s life cycle, including its toxicity and conditions necessary for encystment. What are the natural controls on *Pfiesteria*-like organisms, and what do *Pfiesteria*-like organisms feed on? Burkholder reported that *P. piscicida* favored *Procoentrum minimum* in laboratory experiments while Lewitus observed highest growth when fed cryptophytes — is *Pfiesteria* a selective feeder under natural field conditions? What is the linkage between *Pfiesteria* and fish behavior, e.g., what chemical signals actually cue attack behavior?

Major unknowns remain about how *Pfiesteria* interacts with finfish and shellfish. Burkholder's laboratory has been collaborating with others on the impact of toxin-producing *Pfiesteria* stages on the reproduction, recruitment and disease resistance of commercially important finfish and shellfish. Little study has been done on sublethal effects, for instance on the response of vertebrate and invertebrate immune systems to *Pfiesteria*, or on reproduction and early life history survival. There is a pressing need for definitive pathology of ulcers in fish and shellfish.

Based on the current knowledge of *Pfiesteria* biology and the research needs that would best serve the aims of management, the biology work group identified the following issues as priority needs:

- ▶ Map the distribution of *Pfiesteria* cysts.
- ▶ Characterize the natural history of *Pfiesteria*-like organisms, e.g., trophic controls on stage transformation and toxicity.
- ▶ Identify environmental factors (e.g., temperature and other physico-chemical bases and thresholds) for *Pfiesteria* growth and toxin production.
- ▶ Uncover the interactions between *Pfiesteria* and finfish/shellfish, e.g., cues for triggering toxin production, pathogenesis of ulcers.
- ▶ Determine the mechanisms for toxicity, including bacterial interactions affecting both toxin production and breakdown.

The means for meeting these needs include molecular capabilities that in some instances are under development; in others, they do not exist. (See "A Primer on Molecular Probes," p. 12.)

***Pfiesteria* Taxonomy**

Pfiesteria piscicida, first identified by JoAnn Burkholder and Karen Steidinger (Steidinger et al. 1996), is now known to be one of at least three *Pfiesteria* look-alike species that Steidinger and J. Landsberg at the Florida Marine Research

Institute first discovered and are now classifying. These *Pfiesteria*-like dinoflagellates were isolated from estuarine waters in Maryland, North Carolina and Florida where fish kills have occurred.

While *Pfiesteria*'s aggressive attack behavior distinguishes it from other dinoflagellates, in most other respects it shares the same characteristics in terms of morphology and physiology. Clearly identifying individual *Pfiesteria* species and potentially different strains is labor intensive. It first requires "bringing up" or culturing *Pfiesteria* cells in laboratory aquaria containing mixed populations of algae, dinoflagellates and other microorganisms or, alternatively, fish. Culturing from such mixed populations can take days or weeks.

Two types of culturing procedures are currently being used. The first uses algal food sources (primarily cryptomonads) to increase *Pfiesteria* population density — this produces nontoxic forms of *Pfiesteria*. *Pfiesteria* cultured in aquaria containing fish, on the other hand, produce toxic forms of the dinoflagellate, a process that requires a Biohazard Safety Level 3 (BSL3) facility. These methodologies rely on finding appropriate culture conditions (i.e., food resources, water chemistry, specific cues) that will favor the growth of the target organism over all others. Because the conditions tend to be of a more general nature, it is often impossible to select for a single organism; rather the procedure yields increased populations of more than one species that are capable of responding to the stimulus imposed.

Once a high density population of *Pfiesteria* is obtained, Steidinger concentrates the cells and removes their outer membranes in order to examine the plates (theca), which are the armor surrounding many dinoflagellates. *Pfiesteria*, like many dinoflagellates, has very distinctive plates, which Steidinger has used to characterize the species. This requires a scanning electron microscope for positive identification — a light microscope is not adequate for the task,

Such time-consuming methods of species identification are not sufficient for management needs, which require rapid answers concerning the presence of *Pfiesteria* in the ecosystem, the species or strain(s), and whether or not they are toxic. It is for this reason that molecular probes are needed; as Steidinger says, "this is not a taxonomic issue but a resource issue: we need to know if a species produces a toxin and kills fish or not."

Parke Rublee and a team of researchers at the University of North Carolina at Greensboro have been developing a gene probe that identifies a DNA sequence associated with *Pfiesteria piscicida*. The ultimate goal, says Rublee, is to take water samples suspected of containing *Pfiesteria* cells, treat them chemically to make the cells permeable and then allow these small DNA fragments that are specific to

A PRIMER ON MOLECULAR PROBES

Traditionally, organisms have been identified by a particular set of morphological (or structural) characteristics, referred to as the organism's phenotype — for example, size, shape, coloration — as well as its functional characteristics, such as the ability to produce a certain enzyme. In the case of dinoflagellates, the particular arrangement and number of thecal plates — the armor covering most dinoflagellates — are employed to differentiate among species.

Over the past decade, an increased understanding of the cell biology of microscopic organisms has enabled researchers to identify specific traits that are found at the molecular rather than at the morphological level. This capability has been especially valuable in detecting differences among species that even under the microscope appear to be structurally similar. Each species, even individuals within a species, has a number of unique molecules, or markers, that can be used to identify them. These markers may be found within the cell or they may be located on the cell surface.

When marine scientists speak of using a molecular probe to determine whether an organism is present in a water sample, they are referring to a type of biochemical tool that can detect and visualize the presence of the molecular traits specific to that organism. A considerable amount of effort has already been expended to develop and apply probe technologies to the study of harmful algal species other than *Pfiesteria*, and the strengths of this approach — and obstacles both real and potential — have been described for more well characterized organisms such as *Aureococcus*, *Gymnodinium*, *Alexandrium* and *Pseudonitzschia* (Anderson 1995). These studies should provide essential benchmarks for the development of methodologies to examine *Pfiesteria*.

Target Molecules

There are many potential molecular "targets" for probes. Particularly well defined targets can be found within the genes of the organism, referred to as the genotype. While each organism possesses many thousands of genes, scientists have focused their attention on two targets: those genes encoding ribosomal RNA (rDNA) and the rRNA molecules themselves (the products of the rDNA genes).

Sequences of the rDNA genes among a wide range of organisms have been cataloged and compared. Common and, in some cases, universally conserved regions have been found, as have regions that are unique to a given species. This is why rDNA genes are such a good target for probes: they can be used to determine the relatedness of one organism with another.

A large number of proteins may also serve as specific targets for probes, although the exact nature of the unique qualities of these proteins is often undefined. Especially useful are specific proteins found on cell surfaces, as are surface-bound carbohydrates.

Each of the targets, whether gene or protein, requires having a suitable molecular probe for detection. Gene probes are often either double-stranded or single-stranded DNA, and occasionally RNA, molecules (see below). Protein probes to detect surface components may use antibody molecules or carbohydrate lectin proteins. In each case, detection is provided by means of a "label" attached to the probe. The type of label used depends on many factors, but it may employ either a radioactive or fluorescent tracer molecule, a linked enzyme, or other easily detected molecules.

Types of Probes

Very different types of probes are needed to identify each class of target molecule; however, in general terms, a probe must meet two requirements. First, it must selectively bind to the target, and second, it must carry some molecular determinant that lends itself to easy detection by the scientist.

Figure 3 provides a graphical representation of several different types of probes: antibody and lectin probes for surface cell detection, gene probes for detecting gene sequences and biosensors for detecting toxin molecules. Each exploits particular biochemical characteristics of the target molecules.

Gene Probes. Probes for ribosomal genes or rRNA itself are themselves short nucleic acid strands designed to bind or hybridize to the complementing sequence in the target. These oligonucleotides can be tagged with fluorescent or radioactive labels that are amenable to a range of detection technologies. In this example, they are hybridized directly in whole cells, in situ, that have been made permeable to these small molecules. When fluorescently labeled and excited by the proper wavelength, these probes will cause the cell to fluoresce or glow with a characteristic color, thus making it easy to detect with instruments designed for this purpose, such as a fluorescence microscope or a fluorescence activated cell sorter (FACS).

Antibody Probes. Probes for surface proteins (antigens) are based on antibodies (polyclonal or monoclonal) that preferentially bind a particular region within the three dimensional shape of the target. Antibodies can be labeled in a manner analogous to oligonucleotides; however, a more common methodology links specific enzymatic reactions that produce a distinct response in the presence of the antibody itself.

Lectin Probes. Similar to antibodies, lectin probes are proteins that bind to various cell surface sugars and do so with high specificity for a specific type of sugar molecule. Lectin probes can also incorporate either radioactive or non-radioactive molecules for detectability.

Biosensors. Somewhat different from molecular probes, biosensors often employ living cells that have been genetically engineered to detect certain molecules, for example, an extracellular toxin produced by a dinoflagellate such as *Pfiesteria*. Whole cell biosensors molecularly fuse a gene which produces bioluminescence to a second cellular gene that can sense a toxin, providing a "molecular stethoscope" that can accurately measure the presence of even dilute amounts of target toxins. The ultimate biosensor involves taking these technologies one step further and incorporates the bioluminescent detection system with silicon chip technology to make a kind of integrated living circuit. Such "chip" biosensors are just becoming practical — the technology offers substantial benefits because of its extremely high sensitivity.

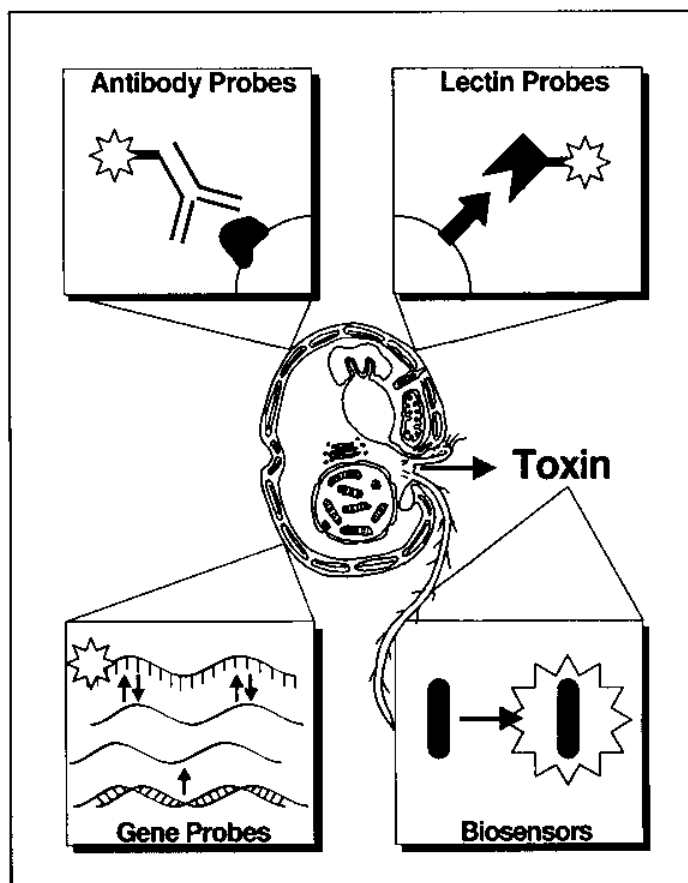


Figure 3. Molecular probes.

Pfiesteria (oligonucleotide probes) to diffuse in and bind (hybridize) to target molecules (ribosomal RNA, or rRNA). The oligonucleotide probes would carry a highly fluorescent tag or label. Therefore, if cells are in the water sample, fluorescent microscopy would reveal them as very bright objects against an otherwise dark background. It would then be possible to identify *Pfiesteria* and count the number of cells.

To date, Rublee has eight oligonucleotide probes directed to rRNA sequences, each working to varying degrees on *Pfiesteria* in the laboratory. While his team has made a great deal of progress in developing the probes, there are a number of limitations. For example, there is no certainty that the fluorescence they see in water samples is *Pfiesteria*; this is because the cultures Rublee is working with are not "pure" cultures (monospecific), and it may be that other microorganisms are present that have similar ribosomal sequences that the oligonucleotide probes are binding to. "There are so many microscopic organisms," Rublee says, "and we know so little about their DNA." While preliminary results suggested that these probes do not detect toxic and nontoxic forms equally, recent adjustments to procedures have resulted in amplifications that show a strong consistency between toxic and nontoxic forms. More work will be required in probe development and the use of these technologies under field conditions.

Major Research Needs

According to Karen Steidinger, discussions are underway with the Bigelow Laboratory for Ocean Sciences (Boothbay Harbor, Maine) to make cultures of isolates available, once Steidinger and Burkholder furnish them. This could take some time, since both investigators stress the need to confirm and corroborate their data, especially the differences observed between *Pfiesteria* reared on algae versus those reared on live fish. While this objective is certainly valid, some researchers argue that we should define a type strain or strains for research first, and then test the strain(s) for toxicity after the type culture is established. This approach may warrant further consideration, including testing by other laboratories, since it has been observed that laboratory-reared cultures lose toxicity over time.

While *Pfiesteria* can be "brought up" from sediments through algal and fish exposures, it is important to clarify and establish consistent use of terms, such as "algal-raised" instead of "nontoxic," when speaking of strains. Often, because of concerns for the health of laboratory workers, *Pfiesteria* cultures are raised on algae and never express toxicity; it has been unclear whether these strains would show toxicity if switched to a fish tank. There is also a question of whether raising cultures of field-caught *Pfiesteria* on algae selects for certain species or strains of the dinoflagellate and eliminates others.

Critical to determining species and strains of the *Pfiesteria* complex of organisms and their toxicity will be the use of a variety of molecular probes combined with certified "gold standard" tests as confirmation of probe identification. To date there are eight candidate oligonucleotide probes to the variable region of the *Pfiesteria piscicida* 18S rRNA gene that have shown promise for detecting this dinoflagellate. The need exists for a battery of probes, not just nucleotide-based, but antibody- and lectin-based, using radioactive and nonradioactive detection methods, i.e., fluorescent, bioluminescent and enzyme-linked techniques. Ideally, in addition to generalized probes for *Pfiesteria*, stage-specific antibody probes would be a tremendously powerful tool to help understand the *Pfiesteria* life cycle and toxin production, although the target antigens to which these probes would be developed remain unknown.

Significant effort has already been expended to develop and apply probe technologies to the study of harmful algal species other than *Pfiesteria*, including more well characterized organisms such as *Aureococcus*, *Gymnodinium*, *Alexandrium* and *Pseudonitzschia* (Anderson 1995). These studies should provide essential benchmarks for the development of methodologies to examine *Pfiesteria*.

There is a crucial need for unialgal (monocultural) and axenic (uncontaminated) cultures of *Pfiesteria*-complex dinoflagellates, since current culture methods start with mixed populations of microorganisms that are then manipulated by the addition of algal prey or live fish to sustain or enhance growth of the *Pfiesteria* complex. The result is an inherent complexity and difficulty in distinguishing which organisms are responsible for effects observed. The development of *Pfiesteria* cultures devoid of other dinoflagellates and algal prey should be the highest priority, as is the development of cultures completely lacking all other microorganisms (especially bacterial epiphytes and endosymbionts). Separating *Pfiesteria*-complex cultures from other organisms will help enable researchers understand the degree to which other elements (bacteria, etc.) may play a role in *Pfiesteria*'s life cycle and toxicity. This is made difficult, of course, because of the need to use bacteria, algae or fish for food to sustain the culture.

There is also a very real need to develop a culture collection of cryopreserved (frozen) stocks of *Pfiesteria piscicida* and other related toxic dinoflagellates. Such storage would ensure strain fidelity for the research community and prevent against the loss of toxicity that results after long-term laboratory culturing of these organisms. The national culture collection at the Bigelow Laboratory or the American Type Culture Collection (ATCC) should ultimately be charged with responsibility for the maintenance of these cryopreserved stocks.

Based on current knowledge of *Pfiesteria* toxins, the taxonomy work group identified the following priorities:

- ▶ Determine what classifies *Pfiesteria* as a dinoflagellate.
- ▶ Determine which are *Pfiesteria*'s nearest evolutionary neighbors.
- ▶ Define which molecular probes are available for enumeration and detection.
- ▶ Determine whether phenotypic variability is genetically based.

***Pfiesteria* Toxins**

Currently, live fish are necessary to stimulate the production of toxin-producing *Pfiesteria* stages in laboratory aquaria. The presence of fish causes excystment, transition to the toxic form, and production of the toxin. One of the long-term goals of *Pfiesteria* research is to avoid the use of live fish as a requirement for producing toxins, substituting instead appropriate, purified molecules that elicit toxin production in the dinoflagellate.

While *Pfiesteria* employs chemical toxin(s) that causes distinctive lesions on many fish prey, the dinoflagellate is itself not infectious, that is, the organism does not invade and propagate within the host and, therefore, is unlike disease-causing viruses and bacteria. Researchers also believe that the toxins break down very quickly and that in the natural environment they are flushed through the system very quickly, especially the toxin which is water-soluble (hydrophilic).

Though the toxins have not been fully characterized, researchers have begun to describe their chemical properties. Techniques for toxin analyses currently involve selective organic extraction, reverse-phase high performance liquid chromatography (HPLC) and electrospray mass spectrometry (MS) and APCL. According to some researchers, we should speak of "toxic activities," rather than of "a toxin," since many known dinoflagellate "toxins" are actually congeners, or families of many similar molecular species.

So far, researchers have reported two *Pfiesteria* toxins from two *Pfiesteria* strains, one that is lipophilic, or fat soluble, and the other hydrophilic, or water soluble. The water-soluble toxin is thought to be a neurotoxin, though it is too early to say, and may be the more toxic of the two. Molecular models are not yet available, though the molecular weight of the water-soluble toxin is estimated by some at 500 daltons and the smaller fat-soluble toxin at about 390 daltons.

Researchers predict that the chemical backbone of both water- and fat-soluble toxins will prove to be very similar. One hypothesis is that a phosphate and/or

sulfate molecule falls off the chemical backbone to create variability in the toxin. The diversity of the different chemical congeners and their abundance in the pool of “toxins” produced by *Pfiesteria* could, some scientists think, depend on the type of prey that *Pfiesteria* cells have been ingesting prior to toxin production.

To date, there is no evidence for long-term persistence in fish or other organisms, including shellfish, an important observation for commercial seafood. This is important for menhaden, the most widespread *Pfiesteria* target, which is used in poultry feed and fish oils; however, investigators acknowledge the need to examine these products once a reliable assay for the toxin is available.

Major Research Needs

Fundamental questions remain about the physico-chemical basis of toxin production, and about the toxin-host and toxin-environment interactions. For example, is it possible to separate “fish” toxin forms from “human” toxin forms? Can the chemistry of the host organism (e.g., the fish being attacked) affect the chemistry of the toxin? Gills are the normal pathway for pathogens and parasites — why are gills not affected by *Pfiesteria*? Why, in fact, do *Pfiesteria* produce toxins and precisely what conditions cause their expression?

Teasing out pathways of expression and delivery is made more difficult by the complex assemblage of microorganisms that exist both in the open environment and in experimental tanks. It is possible that microbes co-existing with *Pfiesteria* (e.g., endosymbionts or epiphytic bacteria) could be involved in toxin production. For now, the role of bacteria in toxin production, modification and breakdown remains unknown. Researchers have also pointed out that transducer molecules may exist in any microbial community associated with fish. This is a complicating factor, since the presence of fish is required in culture to stimulate toxin production. Researchers have observed that high densities of algae in the experimental system appear to inhibit toxin production, though it is not yet known why.

Major unknowns center on the “delivery” mechanism of toxin to the host organism. For example, is actual contact between *Pfiesteria* and fish prey epithelial cells necessary or can toxicity result from general exposure of cells to the toxin(s)? Research shows that the *Pfiesteria* toxins are not volatile, but that aerosols are important, especially in human illness. (Volatilization occurs when molecules assume a gaseous state, as when gasoline vaporizes; aerosols are not gases, but rather tiny droplets of water containing water-soluble materials, as with mist and spray from a breaking wave.) Although not volatile, toxins could end up in the air through aerosol formation and could potentially be absorbed through the skin or inhaled.

Thus far, researchers have not witnessed the presence of living *Pfiesteria* cells in or on a lesion, in either fish or humans. To determine how the toxins function, pure cultures of *Pfiesteria* are required. It is anticipated that ongoing analysis will determine the chemical structure of each of the toxins, and assays now under development will allow testing for the presence of the toxins. Ultimately, there is a need to quantify toxic activity (toxicity measurements) in contrast to simply naming constituent chemical components of the toxins.

Based on current knowledge of *Pfiesteria* toxins, the toxin work group identified the following issues as priority needs:

- ▶ Standard methods for the production, structural characterization and quantification of each of the toxins.
- ▶ Validated detection methodology, “gold standard” methods for assays and analyses for toxins, as well as the development of rapid means to detect the presence of toxins, i.e., luciferase biosensors and PCR (polymerase chain reaction) analysis.
- ▶ Identification of trigger molecules (and stimuli) for toxin production and understanding behind the physico-chemical basis for stimulating toxin production.
- ▶ Characterization of the mechanisms of exposure, toxicity, biological effects, adverse effects and clinical symptoms.

***Pfiesteria* and Human Health**

Concerns over the health impacts of *Pfiesteria* toxins have been mounting ever since researchers working with *Pfiesteria* cultures in laboratories at North Carolina State University reported a range of disturbing symptoms. Symptoms included development of sores by those in direct contact with toxin-containing water as well as more insidious effects from inhaling toxic aerosols, among them, headaches, blurred vision, nausea and vomiting, breathing difficulties, short-term memory loss and difficulty in reading. While most symptoms appeared to be reversible after exposure to the toxins stopped, some of these effects have reportedly recurred (relapsed) in people following strenuous exercise, thus far up to six years after exposure to these toxic fish-killing cultures.

Recent animal research in North Carolina has added evidence of health effects: the injection of small samples from toxic *Pfiesteria* cultures in laboratory rats induced serious learning impairment and memory loss (Glasgow et al. 1995)

In Maryland, outbreaks of fish kills associated with *Pfiesteria* coincided with complaints of various illnesses by watermen and a water skier who were in the affected areas of the Pocomoke River during the summer, 1997 fish kills. A medical team composed of physicians from the medical schools of the University of Maryland and Johns Hopkins University traveled to the Pocomoke River area in August and September, 1997, to examine individuals who had moderate to heavy exposure to the Pocomoke River and reported symptoms that included confusion and memory problems, headaches, skin lesions, and burning of skin on contact with water.

After an initial screening that included a battery of standardized tests — for instance, pulmonary function studies, dermatological exams and blood tests — the physicians found little evidence of immunological dysfunction. They were surprised, however, when cognitive tests revealed problems with learning and memory; the medical team called the problem of confusion “striking” and determined that symptoms correlated highly with levels of exposure. Daily exposure over a period of months, as might be experienced by a waterman working the river, was considered “high” exposure; “low” exposure was far less prolonged. Physicians noted that symptoms in humans appeared when fish lesions were being reported, but *before* fish kills occurred. They therefore surmised that such “kills” are not a “requirement” for symptoms.

The examination included 28 individuals with connections to the Pocomoke and a control of 8 commercial ocean fishermen from the Delmarva Peninsula, who it was assumed had no contact with *Pfiesteria*. Some evidence suggests that people closer to the area of a fish kill or fish lesion event experienced more symptoms than a similar group not exposed to the area. Neurological tests were especially revealing, though it should be emphasized that these findings are preliminary — they have not yet been peer-reviewed, and follow-up examinations of others exposed to Pocomoke waters is underway. As noted earlier, physicians have evidence of health effects when fish had lesions but before a fish kill, which, if true, could suggest that low-level toxins may become aerosolized.

It should not be surprising that a toxic dinoflagellate can impact human health. Other marine dinoflagellates and diatoms produce toxins, including neurotoxins, that can be concentrated in seafood. These include ciguatera toxin, domoic acid and the causative agent of paralytic shellfish poisoning (PSP). Harmful algal blooms such as *Gymnodinium breve*, for example, can produce toxins that become airborne (e.g., in the active surf zone) and cause eye and respiratory irritation (Pierce 1989). Further, amnesic shellfish poisoning (ASP), blamed on the toxin domoic acid, has been reported to cause short-term memory loss and death after ingestion of the toxin.

Major Research Needs

While the medical team is continuing its rigorous examination of suspected cases, many questions still remain. For example, are dermal reactions and memory loss due to the same toxin, or even the same life stage or species? Some researchers suggest that there may be several organisms involved. If this is the case, are these organisms related? Do they have the same, or similar, toxins? Were the causative agents, and the effects, the same in Maryland as in North Carolina? Although *Pfiesteria* has now been confirmed as present in fish kills in the Pocomoke River and Kings Creek, were the causative agents of disease the same in each case?

There are numerous other questions that could be important for protecting human health or for treating those inadvertently exposed. For example, since it is known that some stages are generally more toxic than others, can analysis of life stage provide a means for predicting toxic effects, or are these changes too rapid and dynamic?

Other unknowns center on the basic pathogenic mechanisms, for instance, what is happening in the skin initially. Ed Noga of North Carolina State University has reported pathogenic progression of fish skin lesions — can these findings be related at all to effects on human skin?

Based on experiences in North Carolina, it appears that aerosols were involved. Many questions remain about the nature of the exposures on Maryland's Eastern Shore. It appears that exposure levels for those reporting symptoms varied widely.

Participants in the work group agreed on the necessity for an accepted definition of what constitutes *Pfiesteria*-related illness. Specifically, we need to understand:

- ▶ The potential effects of fish kills on human health.
- ▶ The environmental persistence of the active toxins.
- ▶ The route of exposure to the toxins.
- ▶ A diagnosis for exposure to the toxins.

There are two tracks for concern: environmental and laboratory hazards. The National Center for Environmental Health, part of the Centers for Disease Control and Prevention (CDCP), sponsored a workshop on September 29-30, 1997 for coordinating a multistate response to public health concerns surrounding *Pfiesteria*-like species. The attendees agreed to a set of clinical signs and symptoms that represent adverse consequences of exposure. These include: (1) memory loss, (2) confusion, (3) acute skin burning (on direct contact with water), or (4) three

or more of an additional set of conditions (e.g., headaches, upper respiratory irritation and gastrointestinal complaints).

In addressing environmental conditions that represent adverse consequences from *Pfiesteria*-like organisms, the CDCP workshop attendees agreed that exposure to estuarine water could be characterized by any of the following: (1) fish with lesions consistent with *P. piscicida* or morphologically related organisms (MRO) toxicity (20% of a sample of at least 50 fish of one species having lesions); (2) a fish kill involving fish with lesions consistent with *P. piscicida* or MRO toxicity; or (3) a fish kill involving fish with lesions, if *P. piscicida* or MROs are present and there is no alternative reason for the fish kill (CDCP 1997).

Until probes are developed for rapid detection of toxins — before fish begin to die or during a fish kill — the development of other methods must be accelerated in order to anticipate a potential outbreak as waters warm seasonally.

An animal model is needed that mimics human exposure and pathology resulting from contact with toxin-containing water and aerosols. Less useful are models in which toxicity is assessed after injection of an animal with the toxin, a route of transmission that is unlikely to be the primary means of intoxication by *Pfiesteria*. Also needed are better markers of this disease in animals and, specifically, in humans. At present, the most revealing test appears to be the cognitive one, but this is best administered by a trained expert, and can be time consuming (e.g., a five to six hour test). Physicians noted that there could be a confounding factor between physical and cognitive tests, in that those who feel quite ill are not likely to perform well physically or mentally.

While capabilities are under development to monitor more effectively for the presence of toxins and to determine whether *Pfiesteria* activity (e.g., with regard to fish kills) is a threat to human health, it is critical to develop outreach plans for those who work the water and for consumers of seafood. We clearly need to determine levels of risk — for example at what predetermined level (e.g., cell count or toxin concentration) should people not be allowed near the water? This will require some systematic sampling protocols that take the CDCP recommendations into account.

A major concern is the safety of seafood in the Chesapeake Bay. For example, do shellfish accumulate the *Pfiesteria* toxin(s), as they do with toxins associated with paralytic shellfish poisoning (PSP) or amnesic shellfish poisoning (ASP)? At present, there is no evidence of seafood contamination by *Pfiesteria*, and no reported cases of human illness ascribed to such contamination. While there is no evidence that shellfish or fish without *Pfiesteria*-like lesions present a problem, further study on health effects is critical if the public is to have confidence in recommendations about seafood safety.

SUMMARY

Current scientific methods that draw on the potential of molecular biology, have the potential to significantly improve our understanding of *Pfiesteria*-like organisms and other toxin-producing dinoflagellates. These methods include a variety of molecular probes that could help provide rapid, highly sensitive means for determining the prevalence and toxicity of *Pfiesteria*-like organisms in the natural environment. Workshop participants agreed, however, that understanding these organisms — and their ecological role and potential impact on human health — will require multidisciplinary collaborations that involve universities, agencies and research laboratories throughout the region and beyond.

The workshop participants identified a number of major action items as priority goals, most importantly:

- ▶ Establish and make available unialgal and axenic type culture(s) of *Pfiesteria piscicida* and related dinoflagellates.
- ▶ Determine of the chemical nature of the toxin(s).
- ▶ Describe the molecular and cellular mechanisms of toxicity.
- ▶ Develop probe technologies for the rapid identification and enumeration of toxic and nontoxic stages of *Pfiesteria* species.

Throughout the workshop, several cross-cutting issues arose, among them the following.

Safe Operating Procedures in Conducting Research

The experience of researchers who sustained serious impacts to their health while studying *Pfiesteria* and its toxins has dramatized the need for standards in working with cultures. Because of experiences in North Carolina which showed that *Pfiesteria* has the ability to release toxic aerosols, federal guidelines require all work with fish-killing *Pfiesteria* cultures to be conducted in Biohazard Safety Level 3 (BSL3) facilities. Researchers must follow rigorous safety guidelines and requirements for protective clothing and equipment (e.g., respirators) and must monitor the number of continuous hours they work with toxic cultures. Such concerns are not limited to laboratory research but extend to field monitoring and collection. There is a need for clear guidelines that are available to all research laboratories, resource management agencies and others engaged in field work.

Training and Technology Transfer

There is a need for a greater number of research laboratories to be engaged in studies of *Pfiesteria* biology, the ecological processes involved in the production of toxins and the chemical characterization of the toxins themselves.

Correspondingly, there is a need to train researchers in these laboratories so that they can benefit from the hard-won experience of the researchers who have been instrumental in developing the knowledge of *Pfiesteria* we now have. This is especially important, since the prospects for advancing our understanding of *Pfiesteria*-like organisms and related harmful algal blooms will undoubtedly depend on wide-ranging collaborations and sharing of expertise across institutions, within the United States and elsewhere.

Informed Management

There is an urgent need to develop capabilities that will enable public agencies to act quickly in protecting human health and coastal resources. Towards these ends, management agencies need to know what causes outbreaks, whether some *Pfiesteria*-like populations are more toxic than others, how outbreaks can be identified and whether there are actions that can prevent their further occurrence. Trying to answer such questions will both aid management and improve our general understanding of ecosystem processes that could have profound effects on the prevalence and toxicity of *Pfiesteria*-like organisms.

The scientific challenges are significant. Nevertheless, achieving the goals described above will lead to better ways of determining safe levels for human exposure to *Pfiesteria* toxins, and to improved models for accurate prediction of when and where blooms of *Pfiesteria* are likely to occur. Combined with active programs designed to monitor important ecological conditions that may influence potential *Pfiesteria*-related fish kills, such predictive models will ultimately provide the best means for reducing risks to human health.

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Selected World Wide Web Sites

The following sites are particularly relevant to Maryland:

Maryland Sea Grant/University of Maryland Medical School
Fish Health and *Pfiesteria* Site. (This site contains links to all the following sites.)
www.mdsg.umd.edu/fish-health/pfiesteria

Blue Ribbon Citizens *Pfiesteria* Action Commission Final Report
<http://www.dnr.state.md.ud/Hot/contents.html>

The Cambridge Consensus: Forum on Land-Based Pollution and Toxic
Dinoflagellates in Chesapeake Bay
<http://www.mdsg.umd.edu/fish-health/pfiesteria>

The Agricultural Perspective: Agriculture and Its Relationship to Toxic
Dinoflagellates in the Chesapeake Bay
College of Agriculture Report
<http://www.agnr.umd.edu/pfiesteria/agpros.html>

Maryland DNR Fish Health Facts
<http://www.dnr.state.md.us/fishhealth.html>

The following site is especially relevant for Virginia:

Virginia *Pfiesteria* web page
<http://www.vims.edu/welcome/news/pfiesteria>

The following site is especially relevant for North Carolina but also contains basic
information on *Pfiesteria* generated by Dr. JoAnn Burkholder:

NCSU Aquatic Botony *Pfiesteria* Homepage
http://www2.ncsu.edu/unity/lockers/project/aquatic_botany/pfiest.html

The following sites are national in scope:

National Harmful Algal Bloom Research and Monitoring Strategy: An
Initial Focus on *Pfiesteria*, Fish Lesions, Fish Kills and Public Health
<http://www.redtide.whoi.edu/hab/announcements/pfiesteria/pfiesteriastrategy.html>

The Ecology and Oceanography of Harmful Algal Blooms (ECOHAB):
An Interagency Research Program. Announcement of Opportunity
<http://es.epa.gov/ncerqa/rfa/ecohab.html>

Harmful Algal Blooms in Coastal Waters
NOAA Coastal Ocean Program
<http://hpcc.noaa.gov/cop/#New>

APPENDIX I. WORKSHOP AGENDA

Development and Application of Molecular Technologies to *Pfiesteria* Research: Research Workshop

October 28-30, 1997
Center of Marine Biotechnology
University of Maryland Biotechnology Institute
Baltimore, Maryland 21202

Tuesday, October 28th

- 7:00 Welcome — Hall of Exploration
Edward B. Knipling, Acting Administrator of Agricultural Research Services, USDA
Yonathan Zohar, Director, Center of Marine Biotechnology
Rita R. Colwell, President, University of Maryland Biotechnology Institute

Wednesday, October 29th

- 8:15-9:00 Plenary Talk
JoAnn Burkholder, North Carolina State University
- 9:00-12:00 *Pfiesteria* and Other Toxic Dinoflagellates
Donald Anderson, Woods Hole Oceanographic Institute
“Molecular Probes for Harmful Algal Species”
- Alan Lewitus, University of South Carolina
“Grazing on Behavior and Photosynthetic Potential in *Pfiesteria*”
- Parke Rublee, University of North Carolina
“Development of Ribosomal DNA Probes to *Pfiesteria*”
- Stephen Bates, Fisheries and Oceans Canada, Gulf Fisheries
Centre, New Brunswick
“Pseudo-nitzschia spp. and Other Toxic Algae in Canadian Waters”
- Brian Dougherty, TIGR
“Genome Sequencing and Analysis: Applications to *Pfiesteria* Research”
- 1:30-4:30 Factors Affecting Population Dynamics, Virulence, and Toxic Production
Donald Boesch, University of Maryland Center for Environmental Science
“The Cambridge Consensus: Land Based Pollutants and Toxic Dinoflagellates in the
Chesapeake Bay”
- Karen Steidinger, DEP
“Morphology of *Pfiesteria* and *Pfiesteria*-like Species”

Diane Stoecker, Horn Point Laboratory, University of Maryland Center for Environmental Science
"Mixotrophy Among Bloom-Forming Dinoflagellates"

3:30-4:00 Edward Noga, North Carolina State University
"Ecotoxicology of *Pfiesteria*"

4:00-4:30 John Ramsdell, National Marine Fisheries Service
"Toxin Inducible Genes: Linking Exposure to Effect"

4:30-6:00 Cellular and human health effects

Medical Panel Report

Glenn Morris, M.D.
University of Maryland School of Medicine
"Overview of the Medical Team Report"

Mark Lowitt, M.D.
University of Maryland School of Medicine
"Dermatological Manifestations of *Pfiesteria*-related Toxin Exposure"

Lynn Grattan, Ph.D.
University of Maryland School of Medicine
"Neurobehavioral Sequelae of *Pfiesteria*-related Toxin Exposure"

Patricia Charache, M.D.
University of Maryland School of Medicine
"Report from Atlanta, The CDC Case Definition Criteria"

David Oldach, M.D.
University of Maryland School of Medicine and UMBI
"New Evidence for Aerosol Transmission"

Trish Perl, M.D.
The Johns Hopkins School of Medicine
"The Domoic Acid Study: Echoes in the Chesapeake"

Thursday, October 30th

8:15-1:30 Break-out groups to develop white paper:
Biology Taxonomy
Toxins Human Health

1:30-2:00 Presentation of study group recommendations

2:00-3:00 State, Federal and Corporate Concerns
Representatives from federal and state agencies

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APPENDIX III.



STATE OF MARYLAND
OFFICE OF THE GOVERNOR

PARRIS N. GLENDENING
GOVERNOR

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(410) 974-3901

October 30, 1997

WASHINGTON OFFICE
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Dear Friends:

Thank you for invitation to participate in this three-day research workshop on *Pfiesteria* hosted by Dr. Rita Colwell and the Center of Marine Biotechnology of the University of Maryland Biotechnology Institute. I regret that I was unable to attend the workshop due to prior commitments.

Now that the weather has turned colder, all of us will have time to reflect on how we handled the *Pfiesteria* outbreak this past year and to prepare ourselves for the upcoming warm weather next spring. Throughout this challenge, insuring that the State protects the health of its citizens has been absolutely critical. I remain committed to this end.

As nationally-known experts in the fields of human health and biological sciences, you have been willing to share your expertise to protect the health of the citizens of our State and our many valuable natural resources. With your help, the most accurate and up to date information is being provided to our citizens, scientists, and decision makers at all levels. For this I am very grateful.

Together, we have taken many important steps to address the problem, and Maryland is being recognized as a leader in dealing with *Pfiesteria*. And, together, we need to continue this effort to insure that our seafood and agricultural industries remain economically solvent and environmentally sound and that our Bay and its resources will continue to be admired and enjoyed by all.

Sincerely,

A handwritten signature in black ink that reads 'Parris N. Glendening'.

Parris N. Glendening
Governor

