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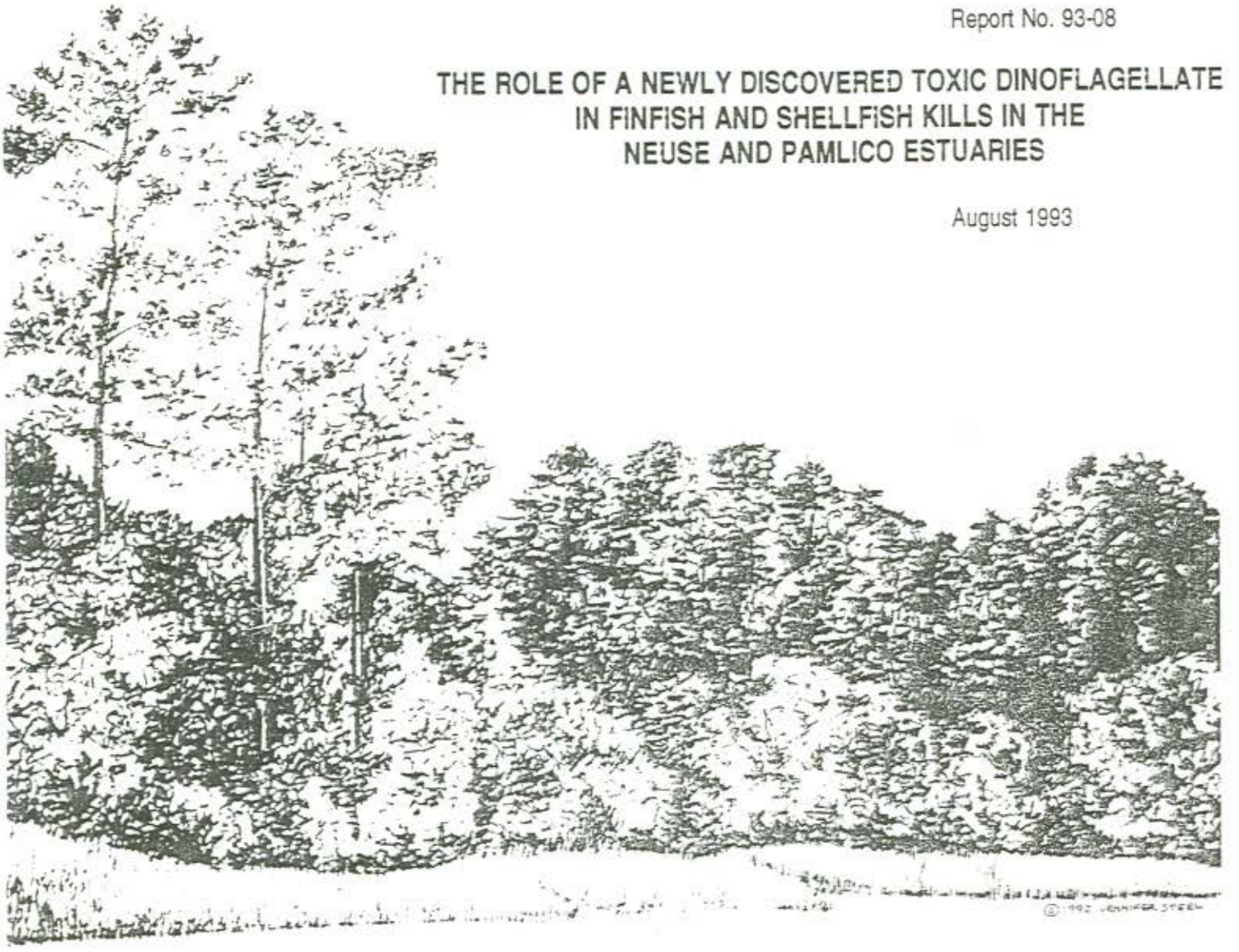
August 1993



ALBEMARLE-PAMLICO ESTUARINE STUDY

NC Department of Environment, Health, and Natural Resources

Environmental Protection Agency National Estuary Program

THE ROLE OF A NEWLY DISCOVERED TOXIC DINOFLAGELLATE IN FINFISH AND SHELLFISH KILLS IN THE NEUSE AND PAMLICO ESTUARIES

by

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This report is dedicated to Dr. Lois Pfiester. Her fascinating research on dinoflagellates opened our eyes to the possibilities inherent in their amazing versatility. Dr. Pfiester's earlier descriptions of freshwater "vampyrelloid" dinoflagellates that transform among amoeboid, saccate and flagellated stages provided the insights needed by PI JMB to approach the complex biology of *Pfiesteria piscimorte* (nov.gen., nov.sp.). Her recent death is a great loss ... we owe her much.

EXECUTIVE SUMMARY

The objectives of this study were to obtain field and experimental information needed to formally speciate and characterize the general ecology of a newly discovered toxic estuarine dinoflagellate, and to examine its significance in causing fish kills in the Neuse and Pamlico Estuaries. Removal of the outer covering membranes on the cells revealed the configuration of an "armor" of protective cellulose plates, and verified that this dinoflagellate represents a new family, genus and species. This information, together with descriptions of 15 flagellated, amoeboid and encysted stages in the complex life cycle, was used to formally name the dinoflagellate to the genus *Pfiesteria* (nov.gen., pronounced "feast-ér-i-a"); it will be formally named within a new family as *Pfiesteria piscimorte* (Latin species name meaning "fish killer;" following publication of Steidinger *et al.* [in prep.]). The predominance of ubiquitous amoeboid stages in the life cycle of this alga will result in its placement within the order Dinamoebales (Division Pyrrhophyta, Class Dinophyceae; Burkholder *et al.* 1992, Steidinger *et al.* in prep.).

In 1991 - 1992 *Pfiesteria piscimorte* (nov.gen., nov.sp.) was the causative agent of at least one-third of the major fish kills in the Neuse and Pamlico Estuaries. The alga is stimulated by fresh fish excreta, and it is lethal to all 18 species of native and exotic finfish and shellfish tested. Preliminary observations suggest that sublethal toxin exposure may also cause long-term damage to epidermal, neural, immune and reproductive systems of affected fish. The toxic flagellated vegetative stage (or dinospore) of *P. piscimorte* (nov.gen., nov.sp.) is lethal across broad temperature, salinity and light gradients. Field data from our monitoring program and State records document toxicity at temperatures ranging from 4-30°C, with most outbreaks occurring at 26°C or higher. Based on field and laboratory data, this dinoflagellate is capable of killing fish at salinities ranging from 2-35‰, with an optimum salinity for toxic outbreaks at 15‰. *P. piscimorte* (nov.gen., nov.sp.) is also lethal to fish in freshwater (~0‰) with high divalent cation content (alkalinity $\geq$ ~20 mg/L). Moreover, it is toxic to fish at available light ranging from 0.2 μEinst m^{-2} sec^{-1} (darkness for all but several minutes per 24-hr period) to 200 μEinst m^{-2} sec^{-1} (12 hr of light and 12 hr of darkness per 24-hr period), with no apparent preference in light availability.

The requirement of an unknown substance in fresh fish excreta by toxic stages of *Pfiesteria piscimorte* (nov.gen., nov.sp.) confounded experiments to test stimulation by absolute supplies and supply ratios of inorganic nutrients phosphate, nitrate and ammonium, since the excreta contains high nutrient concentrations as well as an unidentified stimulatory compound believed to be a form of organic carbon or nitrogen. In the absence of live finfish or their fresh excreta over a 4-day period, however, gamete production increased slightly at 25 μg/L nitrate (as NO_3-N) or 25 μg/L phosphate (as PO_4^{-3}P), and increased significantly at $\geq$ 100 μg/L phosphate. These data indicate that phosphate can play an important role in maintenance of an inoculum of *P. piscimorte* (nov.gen., nov.sp.) in quiet waters as gametes that fuse to form planozygotes which, in turn, produce toxic vegetative cells when finfish enter the area. The gametes in the water column complement the dormant cyst "bank" that serves as an inoculum from the sediments. The laboratory data for gamete response to phosphate, considered along with the fact that nearly 2/3 of the field kills related to this toxic alga have occurred in phosphate-enriched waters of the Pamlico Estuary, provide compelling evidence that phosphate enrichment can act as a major factor in stimulating growth and toxic activity of *P. piscimorte* (nov.gen., nov.sp.).

The naturally occurring predators *Stylonichia* cf. *putricina*, a protozoan ciliate, and *Brachionus* sp., a rotifer, were found to be capable of significantly reducing test populations of *Pfiesteria piscimorte*'s (nov.gen., nov.sp.) toxic flagellated cells and cysts. While the rotifer may eventually offer hope as an agent of bio-control, the ciliate's consumption of flagellated dinospores apparently results in its excretion of a waste product that induces conversion of planozygotes to "giant" toxic amoebae which, in turn, attack and engulf the protozoan as predator becomes prey.

In summer 1991 we confirmed that the toxic flagellated cells (dinospores, planozygotes) and amoebae of *Pfiesteria piscimorte* (nov.gen., nov.sp.) are present in the Indian River, a tributary to the Delaware Bay. Following our guidance, in February 1993 Dr. A. Lewitus (Horn Point Environmental Laboratory, Maryland) confirmed the presence of *P. piscimorte* (nov.gen., nov.sp.) at a known fish kill site in Jenkins Creek, a tributary of the Choptank River in the Chesapeake Bay. Fish from South Carolina estuarine waters carried the dinoflagellate into aquaculture facilities in the Department of Zoology at North Carolina State University, where it subsequently bloomed and caused a major

fish kill during May 1993. These data point toward one emerging theme, brought to light through the discovery of *P. piscimorte* (nov.gen., nov.sp.) at a fish kill in a North Carolina estuary:

> *Given the broad temperature, salinity and light tolerance of this toxic fish ambush predator, its stimulation by eutrophic conditions, and the ephemeral behavior of toxic stages in response to live finfish, we predict that* Pfiesteria piscimorte *(nov.gen., nov.sp.) and its close relatives will be increasingly recognized as a significant source of fish mortality and ulcerative disease in shallow, turbid nutrient-enriched estuaries extending to warm temperate, subtropical, and tropical regions throughout the world.*

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I. INTRODUCTION

A. Coastal "Red Tides" and Toxic Estuarine Dinoflagellates

The rogue algae known as toxic dinoflagellates are considered to be photosynthetic flagellates that appear seasonally in coastal marine waters at bloom concentrations that discolor the water in hues of yellow, brown and, most commonly, red (Steidinger & Vargo 1988). A recent cosmopolitan increase in the frequency and spatial extent of red tide dinoflagellates and other toxic phytoplankton (White 1982, Steidinger & Baden 1984, Anderson *et al.* 1985, Hallegraeff 1993, Smayda 1989) suggests that these organisms may be increasingly significant in determining year class strength of estuarine and marine finfish and shellfish (Shumway 1990, Robineau *et al.* 1991). The frequency of fish ulcerative diseases and kills of unknown cause also has been on the increase worldwide (Steidinger & Baden 1984, Ryther 1989, Noga 1993).

Toxic coastal red tides generally are monospecific; these toxic dinoflagellate blooms remain in surface waters for days to months, causing finfish kills and shellfish poisoning to humans (Steidinger & Baden 1984, Anderson *et al.* 1985, White 1988, Culotta 1992). Only about 20 among more than 3,000 dinoflagellate species produce toxins (Steidinger & Baden 1984, Steidinger & Vargo 1988). The life cycles of these algae have been regarded as simplistic, alternating between flagellated cells and dormant cysts or other resting forms (Lee 1980, Steidinger & Cox 1986). The previously known toxic dinoflagellate species have all been reported in marine coastal waters at relatively high salinities ($\geq$ 20‰) and are most active during warm seasons (Steidinger & Vargo 1988). Although cultural eutrophication has been implicated in stimulating some toxic outbreaks (Paerl 1988, Smayda 1989, Hallegraeff 1993), controlled experiments to resolve the role of nutrient enrichment in promoting toxic dinoflagellate blooms have met with poor success or interpretative difficulties (Smayda 1992), partly because it has not been possible to induce excystment or lethal activity for most toxic dinoflagellates in culture (Steidinger & Baden 1984, Shimizu 1991).

Recent information has begun to alter these general concepts about toxic dinoflagellates. Firstly, a diverse assemblage of small, pigmented to colorless, cryptic flagellated and amoeboid dinoflagellates has been discovered in turbid estuaries and river systems (Mallin *et al.* 1991, Burkholder 1992). Available data suggest that these organisms, most of which have been missed or overlooked, can actually dominate the phytoplankton

community. Secondly, increasing numbers of dinoflagellates with typical biflagellate morphology are now known to have complex life cycles with stages including various cyst morphologies and colorless amoebae (Popovsky & Pfiester 1990, Burkholder *et al.* 1992). Thirdly, dinoflagellates have been known to be capable of digesting and then creating chloroplasts "de novo," implying well-developed ability to utilize both autotrophic and heterotrophic modes of nutrition (Lessard & Swift 1985, Gaines & Elbrachter 1987, Hansen 1991, Lessard 1991). Among the heterotrophic dinoflagellates, some estuarine and coastal species -- with widespread occurrence in areas such as the Mediterranean Sea, the Gulf of Mexico, and the western Atlantic -- additionally have demonstrated "ambush predator" behavior (Spero 1982, Simon *et al.* 1991, Burkholder *et al.* 1992, Burkholder 1993). In each case the animal-like predatory activity has been strikingly similar: The dinoflagellates swarm up from benthic, dormant cysts when they chemically detect the presence of microalgal, protozoan, or fish prey. They devour the prey, described in one instance as being ripped apart in a "feeding frenzy" (Spero 1982) -- and then rapidly re-encyst. These "ambush" dinoflagellates include toxic representatives. Not surprisingly, all of them have been discovered following accidental contamination of established prey cultures. **We now suspect that multiple species with ambush behavior, including toxic forms, are endemic and widespread in estuarine habitat** (Burkholder *et al.* 1992, Burkholder *et al.* 1993, Steidinger *et al.* in prep.), but have been overlooked because their *detection* is extremely difficult under field conditions -- possible, in fact, only with well-timed sampling.

B. A Newly Discovered Toxic Dinoflagellate in the Pamlico Estuary

Among the many estuaries on the Atlantic Coast which increasingly have been associated with unexplained fish kills and ulcerative disease are the Pamlico and Neuse Estuaries in North Carolina (Noga *et al.* 1993). These systems receive high nutrient loading from sewage, agricultural drainage and, in the Pamlico, phosphate mining (Stanley 1987, 1988; Paerl 1990; Rudek *et al.* 1991). In May 1991 a "new" toxic dinoflagellate, to be formally named as *Pfiesteria piscimorte* (nov.gen., nov. sp.; Steidinger *et al.* in prep.), was discovered in the Pamlico and Neuse Estuaries (Figs. 1, 2; Burkholder *et al.* 1992). The biflagellate, toxic vegetative cells ("dinospores") emerge from benthic cysts when a substance is excreted by schools of finfish that linger to feed in the area. The dinoflagellates swim up from the sediment, excrete a potent ichthyotoxin, and consume

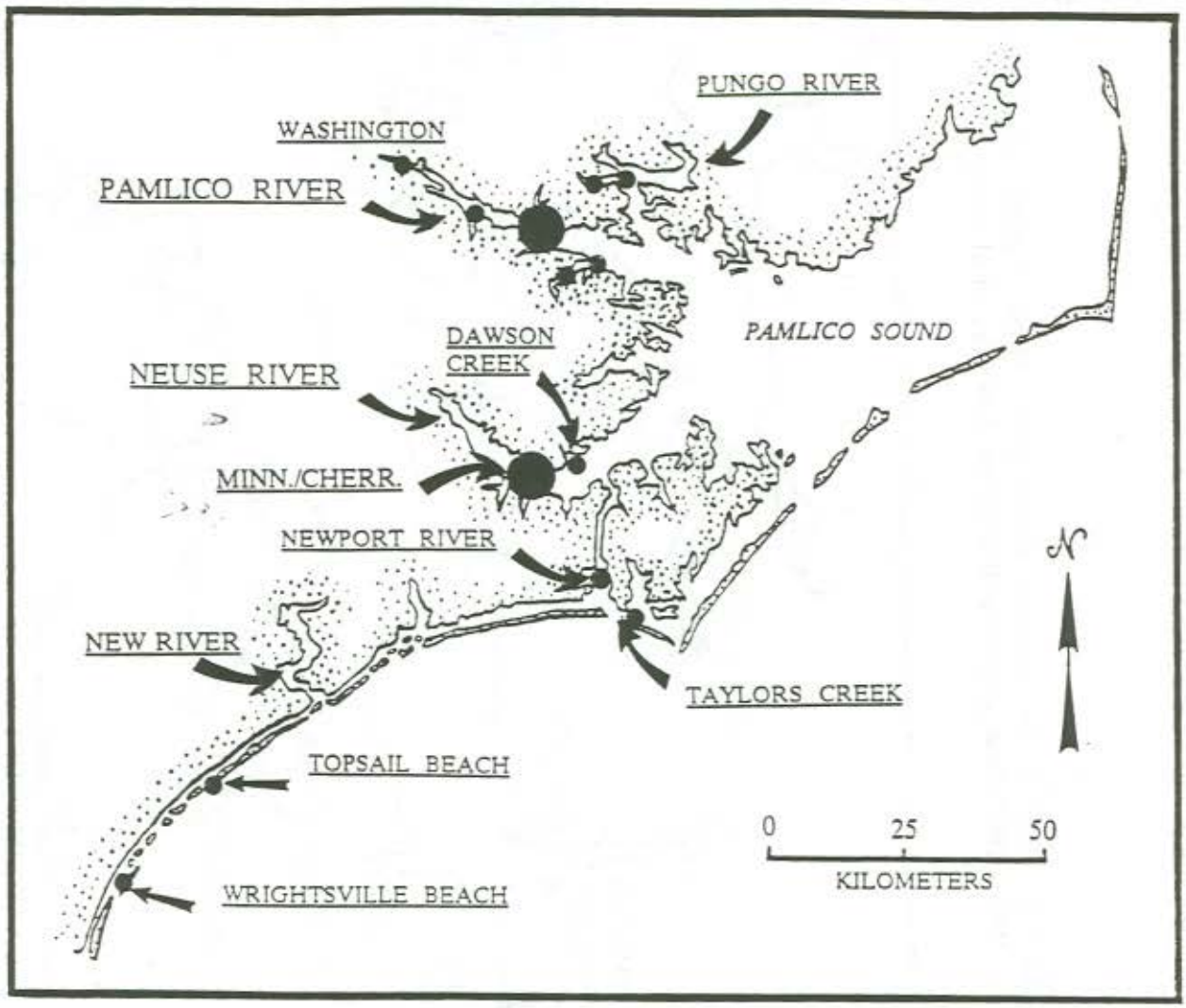
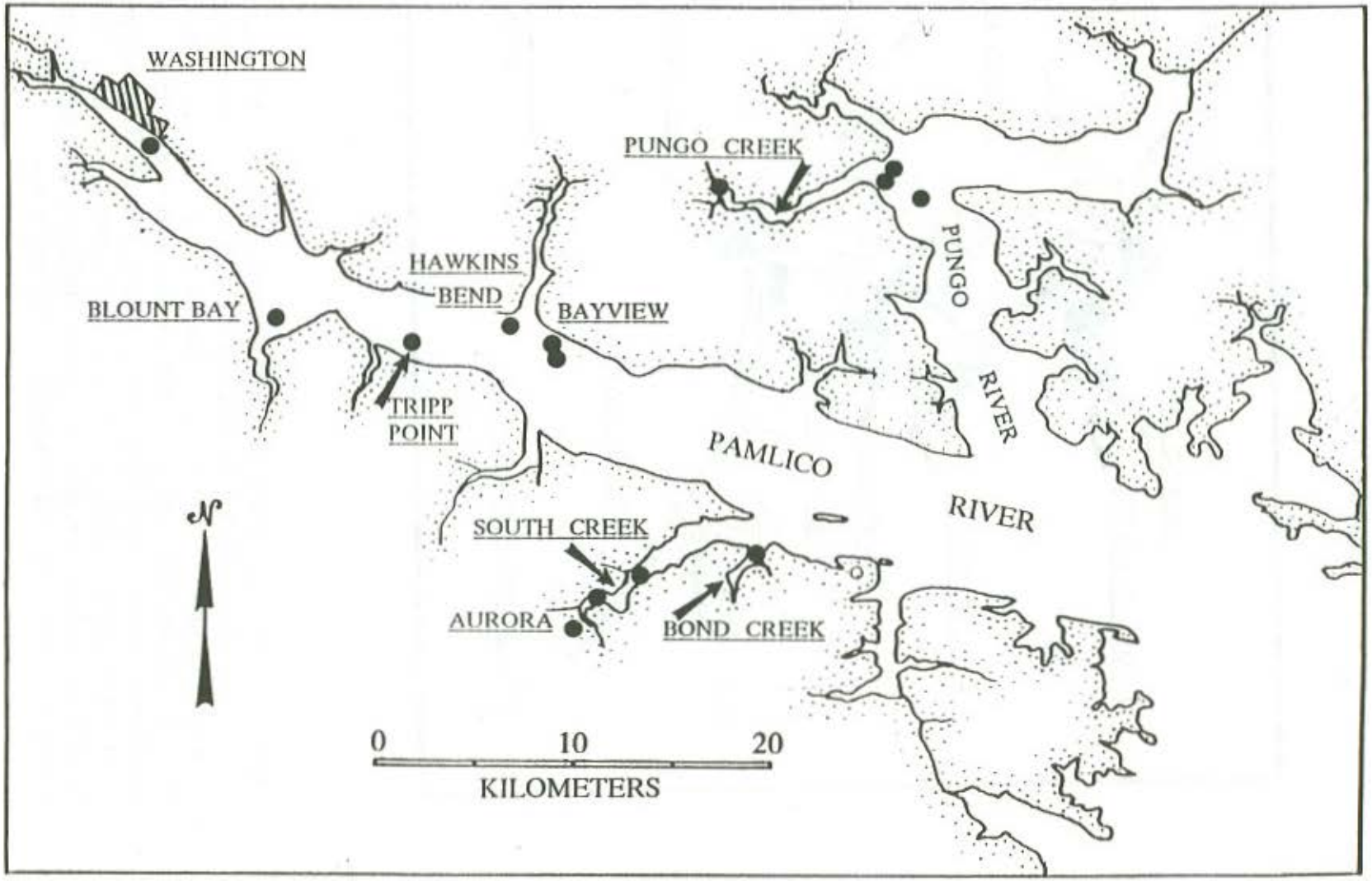


Figure 1. Locations where the toxic dinospore stage of *Pfiesteria piscimorte* (nov.gen., nov.sp.) has been verified in North Carolina. Kill sites (documented in association with this toxic dinoflagellate in all labeled locations except the New River) are designated by blackened circles, with large circles representing sites where fish kills are most frequent with considerable area affected. The large circle on the Neuse River at Minnesott Beach/Cherry Point (MINN./CHERR.) designates the site with highest known loss of fish; more than 1 billion Atlantic menhaden were killed, requiring bulldozers to clear the beaches over a six-week period when menhaden schools were moving out to sea.

Figure 2. Locations of fish kills associated with the new toxic dinoflagellate, *Pfiesteria piscimorte*, in the Pamlico River Estuary from 1986 - 1992 (base graph modified from Kuenzler et al. 1979).



bits of epidermal tissue sloughed from affected fish. They produce gametes that complete sexual fusion when the fish begin to die. Upon fish death, flagellated toxic cells (dinospores, planozygotes) form mostly nontoxic amoeboid stages or encyst and descend back to the sediments (Burkholder 1993).

The newly discovered estuarine toxic dinoflagellate does not form red tides; instead, its most toxic stage is ephemeral in the water column and usually contributes less than 10% of the total cells in the phytoplankton community (Burkholder *et al.* 1992). We first suspected that this type of toxic estuarine dinoflagellate existed only because it was accidentally introduced into fish cultures from local estuarine water (Smith *et al.* 1988, Noga *et al.* in press). Kills related to this dinoflagellate have been most frequent in the phosphate-rich Pamlico Estuary, and in the Neuse River near a recreational beach (Minnesott Beach) and a military air base (Cherry Point; Figs. 1, 2).

C. Objectives of This Research

This study was completed to obtain field and experimental information about the ecology of *Pfiesteria piscimorte* (nov.gen., nov.sp.), and its significance as a causative agent of major fish kills in the Neuse and Pamlico Estuaries. Specific objectives were to (1) obtain required information on the biology and life cycle of this toxic dinoflagellate to enable formal speciation; (2) establish a monitoring network comprised of state staff from the North Carolina Division of Environmental Management (NC DEM) and the North Carolina Division of Marine Fisheries (NC DMF), divisions of the North Carolina Department of Environment, Health & Natural Resources [NC DEHNR]), as well as volunteer citizens, aquaculturists and other scientists to help sample fish kills while in progress so that the dinoflagellate's presence could be accurately quantified; (3) determine optimal physical conditions for its toxic activity; (4) examine potential stimulatory effects of inorganic nitrogen and phosphorus enrichments (N_i and P_i, respectively) on the alga's growth and lethal behavior; (5) determine the dinoflagellate's effects on the skin and gill tissue of representative finfish; and (6) explore the potential for bio-control of the dinoflagellate by a protozoan ciliate, *Stylonichia*, and other natural predators if found. This information is contributed toward the ultimate goal of predicting toxic outbreaks by the dinoflagellate and mitigating its effects on our coastal fisheries.

II. PROJECT PROCEDURES

A. Formal Speciation and Characterization of the Life Cycle

The systematics of armored dinoflagellates requires knowledge of the arrangement and ornamentation of cellulose deposits called armored plates that are obscured by several layers of membrane coverings on the cell exterior (Bold & Wynne 1985). Only when the membranes are carefully stripped from the cell to clarify apical, ventral, dorsal and posterior views, can the plate arrangement or "formula" be obtained. Following procedures recommended by Dr. K. Steidinger (Florida Department of Environmental Protection - Florida Marine Research Institute, St. Petersburg; pers. comm.), we attempted to remove the outer membranes by preserving dinospores in 2% buffered formalin or acidic Lugol's solution (Vollenweider 1974), followed by immersion in various concentrations of Triton X detergent or ethanol for variable exposure periods.

To describe the life cycle of *Pfiesteria piscimorte* (nov.gen., nov.sp.), we isolated individual toxic dinospores and planozygotes, and viewed them over time (hours) under phase optics with a Olympus BH-2 research microscope or an Olympus IMT inverted microscope at 600x. We observed conversions among all described stages using an Olympus PM-10AD automated photomicrographic camera system. Cultures were also subjected to varying light, temperature, salinity and nutrient enrichment to induce transformations among stages. Video photography enabled us to document some transformations (e.g., from flagellated cells to "star-shaped" filipod amoebae, and then to colorless lobate amoebae).

B. Field Monitoring Effort

Numerous meetings and presentations to regulatory agencies, concerned citizens groups, scientists, the state legislature, and students at the elementary, secondary, high school and college level provided information and garnished support for a "network" of assistance in sampling for the new toxic dinoflagellate during fish kills while in progress. This effort was aided by publication of a suggested sampling protocol in local newsletters such as the *Albemarle-Pamlico Advocate* and the *North Carolina Coastal Federation News* (Fig. 3). In this ongoing effort, we have also designed a poster that will be distributed dockside at fish processing centers to aid in alerting fishermen to the toxic dinoflagellate and obtaining their help in sampling kills they encounter. Further, PI JMB has trained appropriate staff of state regulatory agencies to recognize all stages of the toxic

Figure 3. Protocol for volunteer sampling of *Pfiesteria piscimorte* at fish kills.

PROTOCOL: SAMPLING FOR THE NEW TOXIC DINOFLAGELLATE

1. When a fish kill is in progress (finfish and/or shellfish), please observe whether the water is discolored in the vicinity of the kill. Also, note whether the fish are exhibiting erratic behavior, "sudden death" over a short period, or other symptoms which lead you to suspect that the toxic dinoflagellate may be involved.

2. If you suspect a toxic alga, please contact Dr. JoAnn Burkholder or Dr. Edward Noga at NCSU (telephone (919) 515-2726 or (919) 829-4236). Messages can be left on the answering machines, which will be checked at least twice per day.

3. We would very much appreciate your help in obtaining grab samples from water in the kill area (an "elbow's length" below the water surface), including:

   (a) $\geq$ 250 mL of water (about 2 pints) without preservatives, kept in the shade at field / room temperatures;

   (b) $\geq$ 50 mL of water (about 1 cup) preserved with acidic Lugol's solution (which we will be glad to supply if needed). Just add enough (dropwise) to make a golden-orange color (0.01% solution -- can be judged roughly by eye, from water color).

   Any clean, well-rinsed plastic or glass container can be used. It would be best if the samples are collected in the same manner by all who would like to help. If you are only able to sample in one location, please collect water where the most fish are still dying; or, if the fish are already dead, then sample where there are high numbers of dead fish that are drifting toward shore. If you don't happen to have preservative with you, please collect fresh samples -- they will still be very helpful.

   Alternatively, if possible, these 2 types of samples (1 fresh, 1 preserved) should be taken along a (rough)"transect" proceding from a point well outside the kill area, inward to the center or worst area with dying/dead fish. Since the length of this transect would depend on the size of the affected area (and the direction of the current), for consistency's sake, at kills that appear to affect many fish over a relatively large area, 2 samples (1 fresh, 1 preserved) should be taken at each of these locations (12 samples in total):

   (a) Outside the kill area;
   (b) On the margin of the kill area;
   (c) About 1/4 of the distance (roughly) inward toward the center of the kill area;

Figure 3, cont'd.

(d) About 1/2 of the distance inward;
(e) About 3/4 of the distance inward;
(f) At the center of the affected area.

For smaller kills, collect the 1 fresh and 1 preserved sample at locations (a),(b),(d),(f); that is, samples should be collected outside the kill area, on the outer fringe of it, about halfway into it, and then at the center or worst area of the kill (for a total of 8 samples).

4. We would like to help sample, especially in moderate / large kills, so we'd really appreciate your contacting us as soon as you can when you learn that a substantial kill is occurring (or has just occurred), so that we can mobilize quickly and get to the site to collect many other types of samples that would be helpful in tracking this dinoflagellate.

5. To facilitate testing for potential toxic dinoflagellate activity, it would be best to receive the samples as quickly as possible. Please send them to us by State courier mail (# 536121), or call us so that we can make other arrangements. Include a note that briefly describes the kill (date and time, types of fish affected, how dying fish looked or were acting, kill location, whether birds are eating the dead/dying fish, and other details you notice that might be of interest). Also, mark the bottles so that we can determine where, within the kill area, each sample was collected.

   We will be able to confirm the presence of this toxic dinoflagellate within 1 day after receiving water samples (the procedure involves settling the preserved material overnight). However, it often requires several days to 2-4 weeks to confirm toxic activity, depending on whether the alga has encysted by the time we receive the live (fresh, unpreserved) samples.

   We're grateful for your help.

dinoflagellate. Similar training has been provided for staff at the National Marine Fisheries Service (NMFS), Beaufort, North Carolina, the State of Maryland Department of Natural Resources, the phytoplankton consultant for the State of Virginia, and the State of Delaware Department of Natural Resources. We have enlisted the help of Drs. P. Tester and C. Guo (NMFS - Beaufort) in monitoring the seasonal abundance of amoeboid and flagellated forms in the water column and sediments of selected areas where the dinoflagellate repeatedly has caused "sudden death" fish kills.

C. Bioassays to Screen for Toxic Activity

In checking water samples from fish kills for the presence of *Pfiesteria piscimorte* (nov.gen., nov.sp.), we developed a sensitive detection methodology to discern this small, nondescript alga in its dinospore stage from other co-occurring, nontoxic estuarine dinoflagellates that are similar in appearance. This task is also ongoing. We verify the presence of the most toxic forms of the dinoflagellate by conducting aquarium bioassays in which a standard test species, tilapia (*Oreochromis mossambica* Peters) is exposed to the field water samples. The tilapia selected as our standard assay species is not endemic but, nonetheless, is susceptible to the dinoflagellate's toxin. It offers the advantages of constant availability, wide salinity tolerance, and certainty of no prior contamination by local populations of the alga. We check for increasing abundance of toxic stages using the Utermöhl technique as in Burkholder & Wetzel (1989). Dinoflagellate species identifications are confirmed using scanning electron microscopy.

In earlier work we determined that the aerosol accumulations from concentrated aquarium cultures induce disorientation, mild halucinatory effects, nausea, vomiting, eye irritation/swelling, severe asthmatic signs, and short-term memory loss (e.g., Huyghe 1993; Burkholder & Glasgow unpubl. data). Hence, pending toxin identification, the dinoflagellate has been classified as a high level-2 biohazard (NCSU Biosafety Committee), and all research summarized in this report was completed in well-aerated quarantined facilities. As standard operating procedure, we routinely used respirators, disposable gloves, disposable boots, and clothing that was removed and treated with dilute bleach (0.05%) after use. To prevent contamination of control aquaria without dinoflagellates, each aquarium was isolated from all others using shelving with air-tight compartments constructed of clear plexiglass. Cross-contamination also was prevented by using disposable gloves, tubes and pipets to sample each culture. To clean and de-contaminate

aquaria, all reusable labware was soaked in dilute bleach for 12 hr to destroy the cells and cysts, followed by thoroughly rinsing with deionized water.

D. Effects of Acute Exposure to the Toxic Alga on Fish Skin and Gill Tissues

Previous research on the effects of toxic dinoflagellates on fish populations have focused mainly on determining whether the alga can kill the population at risk. Toxins can also cause serious sublethal damage, however, which may render fish less biologically / ecologically "fit." Therefore, we examined impacts of dinoflagellate exposure on potential target tissues of representative finfish to gain understanding of the sequelae of toxin exposure. Gill and skin tissue were considered in this analysis because these tissues would have been in direct contact with the dinoflagellate's toxin. Cultured 1+ striped bass (*Morone saxatilis* Walbaum) were exposed to a blooming culture of toxic dinospores ($\geq$ 300 cells/mL) in 150-L (40 gal.) aquaria at 15‰ salinity and 19°C. Water quality conditions during two repeat-trial experiments were within acceptable ranges for fish growth (unionized ammonia at < 20 μg/L, nitrate at < 100 μg NO_3-N/L, dissolved oxygen at saturation, and pH at 7.8-8.0). Ten fish were sampled just prior to death, and tissues were fixed for histology in 10% neutral buffered formalin. Tissues were processed routinely for analysis by light microscopy after staining with hematoxylin and eosin (Noga *et al.* 1993). Control fish were maintained under similar conditions but without exposure to the dinoflagellate, and their tissues were compared to tissues of the toxin-exposed fish.

In a separate experiment under similar conditions (also repeated once), we also examined effects of sublethal exposure to the toxic dinoflagellate by sampling fish at time intervals that were determined to be non-lethal to the fish. We first exposed 10 striped bass to toxic dinospores (> 300 cells/mL), then sacrificed 5 fish and placed the remaining 5 fish into a "recovery" aquarium without dinoflagellate blooms. None of the fish in the recovery aquarium died; hence, we could assume that the fish had been exposed to sublethal levels of toxin.

E. Optimal Physical Conditions for Toxic Activity

Preliminary experiments and observations established that this dinoflagellate is eurythermal, with demonstrated ability to kill across a wide temperature gradient. Nearly 2 yr of monitoring information (May 1991 - December 1992, encompassing two growing seasons in warm and unusually cold years, respectively -- NOAA 1993) indicated that maximal activity of the most lethal stage of this dinoflagellate -- namely, the flagellated

vegetative cells or dinospores -- occurs during warm seasons (26-30°C). Minimal toxic activity is associated with colder temperatures (4-12°C), wherein the primary lethal stage is a large lobate amoeba (length ca. 50-220 μm) with secondary involvement by the dinospores. In contrast to temperature, the influence of salinity (e.g., from the presence of bottom-water salt wedges) and light (given water- or storm-related stochastic variations) was not clear from field data on toxic outbreaks. Hence, the effects of these two variables on toxic behavior were experimentally examined.

Culture isolates were collected on 23 May 1991 from the Pamlico River Estuary near channel marker no. 9 at the mouth of Blount Bay in Beaufort County during an active bloom of toxic dinospores while approximately 1 million Atlantic menhaden (*Brevoortia tyrannus* Latrobe), southern flounder (*Paralichthys lethostigma* Jordan & Gilbert), hogchokers (*Trinectes maculatus* Block & Schneider) and spot (*Leiostomus xanthuris* Lacepede) were dying. The cultures were maintained in a walk-in environmental chamber under 50 μEinst m^{-2} sec^{-1} illumination (cool white fluorescent lamps) at 18°C with a 12:12-hr light:dark (L:D) cycle in 40-L aquaria filled with artificial seawater at 15‰ (seawater derived by adding Instant Ocean salts to water from a well-water source on the grounds of the North Carolina State University (NCSU) College of Veterinary Medicine. Aquaria were aerated with Harbor Junior box filters containing 50% freshly autoclaved, crushed white coral and 50% filter floss.

Since the toxic dinospore stage requires an unidentified substance in fresh fish excreta (Burkholder *et al.* 1992), it was necessary to maintain cultures using live fish. We routinely used tilapia at a density of 10 fish, each 5 - 7 cm in total length, per culture tank. Fish were fed daily with several flakes of Tetra Marin food. To obtain unialgal cultures without protozoan contamination, the fish were physically separated from dinoflagellate cells using 0.45 or 0.80 μm-pore filters that allowed for passage of the toxin and substances in finfish excreta. In daily checks dead fish were removed and replaced with live fish from uncontaminated stock cultures.

The salinity experiment was conducted for 16 days as batch cultures of dinoflagellates that were maintained under similar conditions as stock cultures in the environmental chamber (50 μEinst m^{-2} sec^{-1} at 18°C with a 12:12-hr L:D cycle in 7.5-L aquaria). Artificial seawater was adjusted to treatment salinities of 0, 5, 10, 15, 25, and 35‰ by altering the quantity of Instant Ocean salts added to the groundwater source, which was

low in divalent cations (approximately 4 mg/L alkalinity). Each treatment was maintained in triplicate 7.5-L aquaria that were randomly distributed on tables. The aquaria were filled, aerated and filtered, with filters acclimated for 14 days before introducing 3 small tilapia (total length 5 - 7 cm). After 7 days with daily feeding of fish, the aquaria were each inoculated with 200 mL of dinoflagellate culture containing approximately 60 cysts or amoebae/mL, from stock aquaria with *Pfiesteria piscimorte* (nov.gen., nov.sp.) that had been inactive (i.e., without finfish) for about 1 month. During the 16-day experiment, fish in triplicate controls (without the dinoflagellate) and treatments were not fed, and expired fish were replaced within 8 hr of death. One 50-mL sample was taken from each aquarium at 2-day intervals, and was preserved immediately in acidic Lugol's solution (Vollenweider 1974) for analysis using the Utermohl method (Lund *et al.* 1958) following the procedure of Burkholder & Wetzel (1989). Samples were settled for 20 hr in darkness within an enclosure that was protected from vibration and temperature alteration. Dinoflagellate stages were quantified under phase contrast at 600x using an Olympus IMT2 inverted microscope. At least 400 cells were analyzed from each sample.

The illumination source used in testing dinoflagellate response to light intensity consisted of VHO cool white fluorescent lamps (model F48T12/CW/VHO) that maintained constant intensity of 350 μEinst m^{-2} sec^{-1} at a 30-cm distance. Fifteen 10-L culture aquaria were wrapped with neutral-density fiberglass screening to achieve 5 light treatments (intensities of 0.2, 25, 50, 75, and 200 μEinst m^{-2} sec^{-1}), each established in triplicate. These irradiances are within the range encountered by phytoplankton in turbid estuarine waters (Cloern 1987, Mallin & Paerl 1992). Treatment replicates and replicated controls (containing fish under various light regimes but without the dinoflagellate) were positioned at randomly assigned locations on four shelves adjacent to the light banks. Each aquarium was filled with artificial seawater at 15‰ salinity that was continually filtered and aerated, and each was maintained for 14 days before introducing 3 fish (5- to 7-cm tilapia). Fish were acclimated for 7 days to ensure viability. Each aquarium was then inoculated with 200 mL of dinoflagellate stock culture containing approximately 300 cells/mL of the toxic dinospore stage. Light experiments were conducted for 25 days as batch cultures. The aquaria were monitored twice daily to exchange dead with live fish and to add fish food, exposing affected aquaria to approximately 10 μEinst m^{-2} sec^{-1} (i.e., at minimal background lighting in the environmental chamber) for 20-30 sec during each

exchange. One 50-mL sample was also taken (for a 20- to 30- sec period at about 10 μEinst m^{-2} sec^{-1}) from each aquarium at 3-day intervals and was preserved with acidic Lugol's solution for quantification of dinoflagellate stages.

F. Response to Inorganic Nitrogen and Phosphorus Enrichments

Experiments to test the effects of inorganic nutrient enrichments on growth of *Pfiesteria piscimorte* (nov.gen., nov.sp.) were conducted in the walk-in environmental chamber for 4-7 days as batch cultures maintained in gently aerated 500-mL Erlenmeyer flasks (50 μEinst m^{-2} sec^{-1} at 18°C with a 12:12-hr L:D cycle). Artificial seawater was made using deionized water that was adjusted to 15‰ salinity with Instant Ocean salts. We gently concentrated dinoflagellate cells and separated them from enriched water with fish excreta by drop-filtering 200 mL of culture through Whatman GFC glass-fiber filters. The filtering procedure induced most toxic dinospores and planozygotes to form cysts or amoebae, but many gametes retained their flagella. One wet filter with cells was transferred to each flask and, after acclimation overnight, nutrient treatments were initiated in triplicate.

Two experiments were completed to test the response of *Pfiesteria piscimorte* (nov.gen., nov.sp.) to absolute supplies and supply ratios of N_i and P_i enrichments. In the first experiment we established controls (without nutrient additions) and treatments as phosphate ($PO_4^{-3}P$), nitrate (NO_3^-N), or ammonium (NH_4^+N) enrichment at 5, 25, 100, or 1,000 μg/L, with each nutrient added as an initial spike. These concentrations lie within the range reported by Paerl *et al.* (1990) for the lower Neuse River ($PO_4^{-3}P$ typically at ~60 μg/L with maxima at ~250 μg/L; NO_3^-N at 15-20 μg/L except for increases up to ~170 μg/L during precipitation / runoff events; NH_4^+N at ~30 μg/L, with increases up to ~140 μg/L during precipitation / runoff events; similar concentration means for these nutrients were reported by Christian *et al.* 1991). Stanley's (1987) data for the Pamlico Estuary showed phosphate at ~300 to $\geq$ 640 μg/L during summer low-flow periods in 8 of 12 sampling sites from below the city of Washington downstream to the lower reaches, with highest concentrations in the vicinity of the Texasgulf phosphate mining company on South Creek.

The second inorganic nutrient enrichment experiment was similar to the first in design, but tested dinoflagellate response to varying supply ratios of nitrate and phosphate. Treatments were imposed as 5:1, 15:1, 25:1, or 50:1 N_i/P_i, holding phosphate

constant at 50 or 100 μg $PO_4^{-3}P$ L^{-1} and adjusting N_i with nitrate (as 0.5, 1.5, 2.5 or 5.0 mg $NO_3^{-1}N$ L^{-1}, respectively). Phosphate was measured using the ascorbic acid procedure of Parsons *et al.* (1985). Nitrate was determined on a Technicon autoanalyzer (model II), using the copper-cadmium reduction procedure of Parsons *et al.* (1985). Ammonium was measured with the Solorzano method (Parsons *et al.* 1985), following modifications of Burkholder & Sheath (1985) for immediate preservation with phenol. In both experiments a 50-mL sample was taken from each flask after 4-5 days, and was preserved and analyzed for abundance of dinoflagellate stages.

G. Statistical Analyses

Correlation analysis was performed initially by date to examine relationships between the abundance of various stages of *Pfiesteria piscimorte* (nov.gen., nov.sp.) and salinity or light. After testing for homogeneity of variance (Hartley's test; Gill 1978), data were square root-transformed where appropriate (SAS 1987). One-way ANOVAs were used to detect differences among treatments for abundances of dinospores, gametes, planozygotes, cysts, and amoebae. Treatment means were compared using Fisher's protected least significant difference test, wtih a comparison-wise error rate (= 0.05; SAS 1987; Day & Quinn 1989). Repeated measures analysis was used to test for differences among treatments over time in abundances of life cycle stages (SAS 1987, Merideth & Stehman 1990, Potvin & Lechowicz 1990).

H. Interactions with Natural Ciliate and Rotifer Predators

Field samples collected from the water column during fish kills were intensively examined for feeding activity on the toxic vegetative stage of *Pfiesteria piscimorte* (nov.gen., nov.sp.) by naturally occurring microinvertebrate animals. The dinoflagellate commonly coexists with the ciliated protozoans *Saprophilus* and *Microthorax* (Lee et al. 1985). These small estuarine protozoans (Lee *et al.* 1985) are saprotrophs or bacteriovores and apparently do not rely upon *P. piscimorte* (nov.gen., nov.sp.) as a food resource, although interactions with stages other than the toxic dinospores are likely. In samples from both estuarine locations and laboratory aquarium bioassays, we commonly observed the protozoan ciliate *Stylonichia* cf. *putricina*, and the rotifer *Brachionus* sp. feeding upon the dinospores, large gametes, planozygotes, and cysts of *P. piscimorte* (nov.gen., nov.sp.) during and following fish kills. We photographed and videotaped interactions between these predators and various stages of the toxic dinoflagellate.

III. DATA SUMMARY

A. Formal Speciation and Description of the Complex Life Cycle

When attempts to follow standard protocol for outer membrane removal using short-term (15 min) immersion in 15% Triton X detergent proved unsuccessful, we used periods of 30 min, 1 hr, 8 hr, and 24 hr in 15%, 25%, 50% and full-strength (100%) Triton X. We repeated all detergent trials after having preserved the cells in acidic Lugol's solution (Vollenweider 1974), but the sutures between plates were covered with nodule-like deposits, and they did not separate. Further, the plates of cells preserved in acidic Lugol's or in 2% formalin did not separate when immersed in dilute chlorox, and the membranes mostly remained intact. We then immersed live cells in 15%, 50%, and 100% Triton X; but even in full-strength detergent, the cells maintained swimming activity. In separate trials we attempted to remove the outer membranes from formalin-fixed cells, acid Lugol's-fixed cells, and live cells by immersion in 10% and 25% (i.e., up to 50-proof) ethanol. We observed no apparent effect on swimming activity by dinospores even in 50-proof ethanol. Dr. Steidinger's laboratory offered assistance; after sonication and attempts with various fixatives and surfactants, Steidinger and her colleagues immersed live cells in 40% (90-proof) ethanol (Steidinger, pers. comm.). The cells died and partial membrane removal enabled the Florida research team to obtain the plate formula needed to verify that this dinoflagellate is a newly reported "armored" genus and species (Steidinger *et al.* 1989).

The armor of this toxic dinoflagellate resembles that of members of the Order Peridiniales, with thin plates arranged in a Kofoidian series of P_o, cp, x, 4', la, 5", 6c, 4s, 6‴, and 2‴ (Popovsky & Pfiester 1990, Steidinger *et al.* 1993, Steidinger *et al.* in prep.). The thin plates are obscured by several outer covering membranes (Steidinger *et al.* 1993); hence, under light microscopy all flagellated stages (dinospores [10-20 μm-diameter], gametes [5-8 μm- diameter], planozygotes [25-60 μm-diameter; Burkholder *et al.* 1992, 1993) appear as "naked" or unarmored ("gymnodinioid") cells except for the hint of an angular profile, suggestive of the presence of plates, in the region of the transverse groove (cingulum) on the cells.

Surprisingly, through many hours of observations using light microscopy, we learned that the complex life cycle of this toxic estuarine dinoflagellate is dominated by

filipodial and lobate amoebae that can transform from all known flagellated stages (Burkholder *et al.* 1992, Burkholder 1993; Fig. 4). The occurrence of repeated sudden-death kills of cultured hybrid striped bass (*Morone saxatilis x Morone chrysops* Rafinesque) in supposedly uncontaminated cold-water aquaria (~9°C) led to our first discovery of a large toxic amoeboid stage of the toxic dinoflagellate (Burkholder *et al.* 1992). These lobate amoebae typically have rigid tapering extensions as well as flexible pseudopodia, and their external covering (fibrous and reticulate in appearance under scanning electron microscopy) often mimics the coloration of stained starch in acidic Lugol's iodine preparations. Filipodial amoebae can be formed from dinospores, gametes, and planozygotes; lobate amoebae may arise from filipodial amoebae or directly from gametes. The largest amoebae, formed from the sexually produced planozygotes, range from ca. 50-220 μm in length; that is, they can be up to 20-fold longer than the diameter of the toxic dinospores. At the cold temperature in which they were first discovered, the large lobate amoebae act with similar ambush predator behavior as the dinospores - they swim up from the bottom of the aquarium when live finfish are added, kill the fish, and then sink out of the water column. They do not, however, kill fish as rapidly as the dinospores, and probably carry residual toxin that was produced by the flagellated planozygotes.

Transformations from small flagellates to filiform and lobate amoebae have not previously been reported for estuarine dinoflagellates (Bursa 1970; K. Steidinger, pers. comm.). But research in freshwaters by Dr. L. Pfiester and coworkers (collectively summarized in Pfiester & Popovsky 1979, Popovsky & Pfiester 1990) uncovered dinoflagellate species with up to 38 distinct life cycle stages including numerous heterotrophic amoebae as well as [few] photosynthetic and heterotrophic flagellates. This newly discovered toxic estuarine dinoflagellate fits a similar description; the dominant amoeboid stages in its life cycle are ubiquitous in the water column and, especially, in the sediments regardless of fish availability (Burkholder 1993). In fact, the only ephemeral or "phantom" stages are the flagellated forms (dinospores, planozygotes, and sometimes the gametes) that were recognized as dinoflagellates of "typical" biflagellate appearance in the early kills of cultured fish. We have since observed transformations by the known flagellated stages of this alga to amoebae that range in length from 10-220 μm (Burkholder 1993; Fig. 4). After weeks without live fish the amoebae may encyst or,

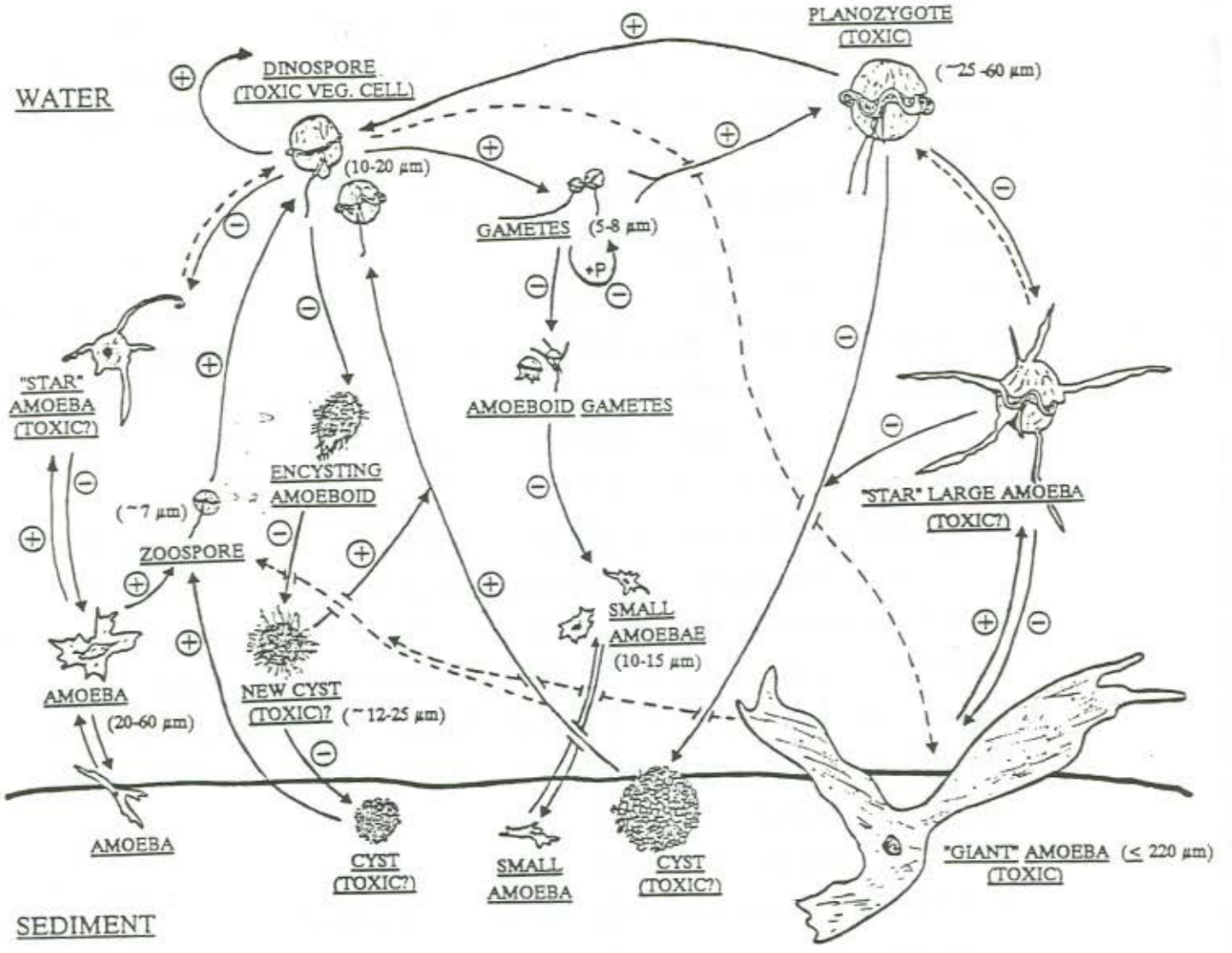


Figure 4. Symbols indicate how the dinoflagellate appears in the presence (+) versus absence (-) of live finfish. Solid arrows represent verified pathways in the complicated life cycle, and dashed arrow lines indicate hypothesized additional pathways. The "P" designates stimulation of gamete production by phosphate enrichment; stages suspected to be toxic are also indicated (toxic?). This figure was compiled from research by Burkholder & Glasgow (Burkholder *et al.* 1993).

alternatively, they may maintain activity as saprotrophs or phagotrophs; we have held live samples in complete darkness for up to 4 months and still have found many active amoebae on the bottom of the containers. Upon addition of live finfish, most amoeboid stages apparently can produce small zoospores that develop into the toxic dinospore stage.

We are completing formal procedures to name the alga to genus and species as *Pfiesteria piscimorte* (nov.gen., nov.sp.) in honor of Dr. L. Pfiester (names selected by Steidinger and Burkholder, to be published with formal description by Steidinger *et al.* in prep.; the genus name is pronounced "feast-er-i-a," with the Latin species name meaning "fish killer"). The predominance of amoebae with few coccoid forms among the 15 known life cycle stages will result in placement of *P. piscimorte* (nov.gen., nov.sp.) within the order Dinamoebales (new family, revised order; Steidinger *et al.* in prep.).

B. Occurrence and Toxic Activity of *Pfiesteria piscimorte* (Nov.Gen., Nov.Sp.)

On the basis of phytoplankton sample analyses by Ms. K. Lynch (NC DEM), we obtained limited historical data relating abundance of the toxic dinospore stage of *Pfiesteria piscimorte* (nov.gen., nov.sp.) to fish kills from 1986 - 1990, prior to confirmation of the alga as a toxic causative agent of estuarine fish kills. During that period *P. piscimorte* (nov.gen., nov.sp.) was tentatively identified as "*Gymnodinium aurantium,*" a name unofficially given by Campbell (1973).

"*G. aurantium*" was assumed to be an incidental species at the kills, and its abundance was recorded in fish kill records if it comprised 8% or more of the total biovolume of the phytoplankton community. Analysis of State DEM records revealed that "*G. aurantium*" generally was low in abundance relative to other phytoplankters, but often maintained higher population densities (10% or more of the total phytoplankton biovolume) at the scene of finfish or shellfish kills (NC Department of Natural Resources & Community Development [NC DNRCD] / NC DEHNR fish kill reports; Table 1). Exceptions to this pattern included (1) a bloom of "*G. aurantium*" that was sufficiently concentrated to cause a reddish-brown discoloration to water in the lower Pamlico Estuary during January 1988 (not linked to a known fish kill or disease outbreak; NC DNRCD 1987 - 1989), and (2) some blooms of "*G. aurantium*" with high cell densities in the New River Estuary in southeastern North Carolina (also with unknown / undetermined linkage to fish

Table 1. "Historical record" of fish kills subsequently linked with the toxic dinospore stage of *Pfiesteria piscimorte* (nov.gen., nov.sp.) from Aug. 1986 - Dec. 1990.

DATE	LOCATION	AFFECTED	*PFIESTERIA* [1]
1986 - AUG.	PAMLICO R., TEXAS GULF	CRAB SPP.	13% OF BV (300 mm^3/m^3).
1987 - JULY	PUNGO CR.[2]	FISH SPP.	6,930/mL (34%).
AUG.	S. SHORE, PAMLICO R.[2]	FISH SPP.	1,310/mL (8%).
1988 - MAY	PUNGO CR.: YEATSVILLE NEAR NC-92 BRIDGE	MULLET, TROUT, MENHADEN	13% OF BV (1,660 mm^3/m^3); 29-53% OF BV.
1989 - JUNE	BOND CR.	FISH, CLAMS	18% OF BV.
JUNE	PAMLICO R., BAYVIEW	FLOUNDER, SPOT, HOGCHOKERS	29% OF BV (1,030 mm^3/m^3).
JULY	DAWSON CR.	FISH SPP.	5,250/mL (11%).
JULY	PAMLICO R., SOUTH CR.[2] NEAR AURORA	FISH, CLAMS	950/mL (9%); 32% OF BV.
JULY	PAMLICO R., SOUTH CR.	CLAMS	6,100/mL (13%).
AUG.	NEUSE R.,[2] MINNESOTT BEACH/ CHERRY PT.	MASSIVE: CRABS, SPOT, CROAKER, PERCH, MENHADEN, FLOUNDER...	1,850-15,000/mL (18-32%); < 60% OF BV.
SEPT.	NEUSE R.,[2] MINNESOTT BEACH	FISH SPP.	3,700/mL (11%).
1990 - JULY	RAGGED PT.-BAYVIEW[2]	MENHADEN, FLOUNDER, HOGCHOKERS, SPOT	N.A.[2]
AUG.	PUNGO CR.	FISH, CRABS	N.A.[2]
AUG.	BATH CR.	CRABS	N.A.[2]

[1] These data were provided by the NC DEHNR with help from K. Lynch, who quantified the toxic dinospore stage of *P. piscimorte* (nov.gen., nov.sp.; then unknown) as the incidental phytoplankter, "*Gymnodinium aurantium.*" Note: BV designates biovolume; percentages indicate relative contribution of the dinospores to the total phytoplankton community.

[2] The kill was also associated with low dissolved oxygen (~ 3.0 mg/L) in the bottom water.

[3] Data were not available.

kills or ulcerative disease; NC DEM unpublished data; Fig. 1). From the historical record for fish kills associated with moderate to high concentrations of "*G. aurantium*," approximately 70% of the incidents (or 10 of 14 kills) occurred in nutrient-enriched areas of the Pamlico (phosphate mining region, Washington to South Creek) and Neuse (Minnesott Beach / Cherry Point) estuaries (Table 1).

Prior to our discovery of *Pfiesteria piscimorte* (nov.gen., nov.sp.) at the Pamlico during a menhaden kill in May 1991, the most thorough monitoring of fish kills in North Carolina occurred in the Pamlico Estuary during 1988. At that time a group of State personnel and volunteer scientists and citizens, the Pamlico Environmental Response Team (PERT), monitored 82 fish kills (Miller *et al.* 1990). Of these, 22 were associated with "sudden-death" neurotoxic signs and apparent suffocation. In 22 additional kills, ulcerative lesions and other signs of fish disease indicated that fish may have been weakened and, therefore, more susceptible to environmental stresses such as low dissolved oxygen in the bottom water. However, two points were of concern to the monitoring staff: (1) in many of the kills, dissolved oxygen was apparently non-limiting at 5 mg/L or higher throughout the water column, nor was any other cause apparent, and (2) the affected fish ranged from having few to many lesions, so that it seemed unlikely that known pathogens such as opportunistic fungi in the lesions could have caused the sudden-death effect. The role of *Pfiesteria piscimorte* (nov.gen., nov.sp.) in the numerous, unexplained sudden-death kills prior to 1991 cannot be determined. It is of interest, nonetheless, that affected fish from kills during 1988, and from kills linked to the toxic dinoflagellate in 1991 - 1992, were similar in appearance to fish from our laboratory bioassays in which ulcerative lesions, subcutaneous hemmorrhaging, and sloughing of large areas of epidermal tissue frequently developed during exposure to toxic flagellated cells (dinospores, planozygotes) of *P. piscimorte* (nov.gen., nov.sp.).

With help from many State fisheries staff and concerned citizens, during 1991 - 1992 we were able to obtain water samples from 23 fish kills in the Neuse and Pamlico Rivers and local aquaculture facilities (Table 2, Figs. 1-2). In about 2/3 of these cases, samples were taken while kills were in progress. In the remainder, samples were collected 2-4 days after fish death, and the presence of common filipodial ("star") and lobate amoebae suggested that transformations from toxic flagellated stages had already

Table 2. Fish kills with documented activity of *Pfiesteria piscimorte* from May 1991 - July 1993.

DATE	LOCATION	SALINITY (‰)	TEMP. (°C)	AFFECTED FISH	*PFIESTERIA* [1] (Cells/mL)
1991 -					
MAY	PAMLICO, BLOUNT BAY	6	28	MENHADEN (NET)	1,300
JUNE	PAMLICO, TRIPP PT.[2]	8	28	MENHADEN, OTHERS	1,100
AUG.	PAMLICO, WASHINGTON	6	30	MENHADEN	600
AUG.	PAMLICO, HAWKINS BEND	12	29	FLOUNDER "WALK"	800
SEP.-OCT.	NEUSE, CHERRY PT.	8 - 10	18 - 27	MENHADEN, BLUE CRABS	1,200
DEC.	WRIGHTSVILLE BEACH	30	17	MENHADEN[2]	40
DEC.	TAYLOR CREEK	30	15	FISH "WALK" (FLOUNDER, EEL ...)	~35,000 [2]
1992 -					
JAN.	AQUACULTURE (PAMLICO R.)	0	6	STRIPED BASS	SUBDOM. [2]
FEB.	NC MARITIME MUSEUM, BEAUFORT (NEWPORT R.)	27	21	FISH SPP.	ALL STAGES [2]
FEB.	NAT. MAR. FISH. SERVICE (NMFS - NOAA), BEAUFORT	24	19	FISH SPP.	ALL STAGES [2]
JULY	PAMLICO, HAWKINS BEND	10	27	MENHADEN, OTHERS	280
JULY	NEUSE, MINNESOTT BEACH/ CHERRY PT.	9	26	MENHADEN, OTHERS	340

Table 2, cont'd.

DATE	LOCATION	SALINITY (‰)	TEMP. (°C)	AFFECTED FISH	*PFIESTERIA* [1] (Cells/mL)
1992 (cont'd.) -					
NOV.	NMFS, BEAUFORT	25	14	FLOUNDER	N.A.[2]
DEC.	TOPSAIL BEACH	29	9	MENHADEN	N.A.[2]
1993 -					
JAN.	NMFS, BEAUFORT	28	20	BAY SCALLOPS, SEA URCHINS	LARGE AMOEBAE [2]
JAN.	DEPT. OF ZOOLOGY, NCSU	15	20	TILAPIA, WHITE PERCH	ALL STAGES [3]
MAY	DEPT. OF ZOOLOGY, NCSU	15	20	TILAPIA	ALL STAGES [3]
JULY	PAMLICO, SOUTH CREEK	11	30	MENHADEN, OTHERS	ALL STAGES [2]

[1] In samples provided by B. Anderson & R. Lewis from the Delaware DNR, *Pfiesteria piscimorte* (nov.gen., nov.sp.) was also confirmed from the Indian River, a tributary to the Delaware Bay (Burkholder & Glasgow, May 1992). Further, this alga was tracked to a known "sudden death" fish kill site in Jenkins Creek, a tributary of the Choptank River in the Chesapeake Bay (A. Lewitus - confirmed by Burkholder & Glasgow, February 1992).

[2] The Tripp Pt. kill was also associated with low dissolved oxygen in the bottom water. During kills in Jan. - Feb. 1992, samples were taken 1-4 days after fish death (~25 cells/mL present). Water samples collected 1-2 days after fish kills at Wrightsville Beach and Topsail Beach, and from the Pamlico (South Creek) in July 1993 still contained ~50 dinospores/mL. The Taylor Creek kill (Dec. 1992) had the highest field abundance of this alga reported (mostly gametes). In other field kills prior to Nov. 1992, only the dinospore stage was quantified. The Jan. 1993 kill at NMFS was confirmed by P. Tester & J. Rivera (pers. comm.).

[3] In the first Dept. of Zoology kill, aquaria were contaminated by two white perch that were caught in the Neuse River Estuary; in the second kill, fish carried the alga from estuarine waters in South Carolina (Dr. C. Sullivan, pers. comm.).

occurred. Concentrations of at least 45 cells/mL of the toxic vegetative stage of *Pfiesteria piscimorte* (nov.gen., nov.sp.) were found in preserved samples from 19 kills. We obtained fresh (unpreserved) samples from 12 of the 19 kills after 2-4 days, and confirmed toxic activity of the dinoflagellate in laboratory bioassays in all 12 instances. In the laboratory this dinoflagellate was often lethal to fish at levels of ~300 cells/mL, also the abundance at which marine red tide dinoflagellates such as *Gymnodinium breve* Davis can be lethal to fish (Tester *et al.* 1991). Cell counts were consistently at 280/mL or higher from samples taken while field kills were in progress and checked for dinospore abundance within 1 day. In contrast, 4 kills with delayed sampling (2-4 days) yielded no evidence of *P. piscimorte* (nov. gen., nov.sp.) in fresh or preserved material, nor in subsequent bioassays of live samples with tilapia (i.e., "negative" tests). Among the 11 field kills listed with documented toxic activity by *P. piscimorte*, 80% (9 of 11) of the incidents occurred in nutrient-enriched areas of the Pamlico, the Neuse, and Taylor Creek (the latter near inputs from a municipal wastewater treatment facility, fish processing plant, etc.). We confirmed that toxic outbreaks of this dinoflagellate were linked to at least 3 of the 8 major fish kills (involving $\geq$ 1,000 fish) that were monitored by NC DEHNR occurred in the Pamlico and Neuse estuaries during 1991 - 1992 (Burkholder 1993, in combination with fish kill records of Mr. K. Miller, Washington regional office, NC DEHNR - DEM).

Thus far, our monitoring network has proven successful for sampling fish kill sites both within North Carolina and in other mid- and south-Atlantic coastal states. In contrast to the relatively warm conditions from spring through fall in 1991 (with water temperatures at 28-30°C by late May; NC DEM 1992), summer 1992 was the third coldest in a 50-yr record with few field kills and more numerous aquaculture kills relative to the previous year. We hope to continue our monitoring effort toward accumulating a long-term data base on toxic outbreaks by *Pfiesteria piscimorte* (nov.gen., nov.sp.), including related environmental conditions and quantification of fishery losses where possible. As part of this effort, during 1992 we completed workshops to train staff from state and federal agencies in North Carolina, Maryland, Delaware, California, and Florida to recognize all stages of this toxic dinoflagellate. In 1993 regulatory agencies in Maryland, Delaware, and Florida initiated surveillance programs to detect *P. piscimorte* (nov.gen., nov.sp.) at known sites of fish ulcerative disease and "sudden death" kills. Researchers

in Maine are involved in a similar monitoring effort to determine whether *P. piscimorte* (nov.gen., nov.sp.) or a closely related species is involved with kills of schooling fish such as pogies. These states have all been experiencing ulcerative disease outbreaks and sudden-death fish kills without apparent cause in their coastal waters, and their regulatory staff suspect that, with appropriate sampling, *Pfiesteria piscimorte* (nov.gen., nov.sp.) or close relatives could be detected in kill / ulcerative disease locations.

Their suspicions seem well-founded. During summer 1992 we confirmed the presence of toxic stages of *Pfiesteria piscimorte* (nov.gen., nov.sp.) in the vicinity of a wastewater treatment facility in the Indian River, a tributary to the Delaware Bay (work in conjunction with Mr. B. Anderson and Dr. R. Miller, DE Dept. of Natural Resources; identification and toxicity confirmed by Burkholder & Glasgow; Table 2). In February 1993, toxic stages of *P. piscimorte* (nov.gen., nov.sp.) were confirmed from a known sudden-death fish kill site in Jenkins Creek, tributary to the Choptank River in the Chesapeake Bay (Dr. A. Lewitus and colleagues, Horn Point Environmental Laboratory; identification confirmed by Glasgow & Burkholder, toxicity confirmed by Lewitus). Descriptions by Maryland state biologists suggest that a second, "conical, green-colored" dinoflagellate species (as opposed to *P. piscimorte* [nov.gen., nov.sp.] with colorless to pale golden cells) with similar toxic ambush behavior toward fish may be active in the Wye River, another tributary to the Chesapeake (Anderson & Hall 1992). In April 1993, fish from an aquaculture facility in South Carolina apparently carried *P. piscimorte* into previously uncontaminated aquaculture tanks in the Department of Zoology at NCSU, where it caused death of more than 1,000 tilapia (fish origin confirmed by Dr. C. Sullivan; algal identification confirmed by Burkholder & Glasgow).

C. Affected Species of Finfish and Shellfish

The toxic estuarine dinoflagellate *Pfiesteria piscimorte* (nov.gen., nov.sp.) was lethal to all fish observed in estuarine habitat or aquarium water when toxic dinoflagellate concentrations were $\geq$ approximately 300 cells/mL (Table 3). The typical response followed this sequence (Burkholder *et al.* 1992, Burkholder 1993): Upon initial exposure to water with high activity by the toxic dinospore stage, the fish quickly darkened in color, became lethargic, and settled on the bottom of the aquarium. In increasingly frequent episodes, the fish suddenly struggled to the water surface and gulped air, but was not able to maintain its position and slowly sank back down where it landed on its side or on its tail and,

Table 3. Species of finfish and shellfish that are known to be killed by this dinoflagellate.*

NATIVE ESTUARINE / MARINE SPECIES

American eel (*Anguilla rostrata* Lesueur)
American menhaden (*Brevoortia tyrannus* Latrobe)
Bay scallop (*Aequipecten irradians* Lamarck)
Blue crab (*Callinectes sapidus* Rathbun)
Littleneck clam (*Mercenaria mercenaria* L.)
Atlantic croaker (*Micropogonias undulatus* L.)
Red drum (*Sciaenops ocellatus* L.)
Hogchoker (*Trinectes maculatus* Block & Schneider)
Black grouper (*Mycteroperca bonaci* Poey)
Largemouth bass (*Micropterus salmoides* Lacepede)
Striped mullet (*Mugil cephalus* L.)
White perch (*Morone americana* Gmelin)
Pinfish (*Lagodon rhomboides* L.)
Spotted sea trout (*Cynoscion nebulosus* Cuvier)
Spot (*Leiostomus xanthuris* Lacepede)
Striped bass (*Morone saxatilis* Walbaum)
Southern flounder (*Paralichthys lethostigma* Jordan & Gilbert)

EXOTIC (INTRODUCED) SPECIES

Clownfish (clown anemonefish) (*Amphiprion percula* Lacepede)
Goldfish (*Carrasius auratus* L.)
Guppie (*Poecilia reticulata* Peters)
Hybrid striped bass (*Morone saxatilis x Morone chrysops* Rafinesque)
Mosquitofish (*Gambusia affinis* Baird & Girard)
Tilapia (*Oreochromis aureus* Steindachner, *Oreochromis mossambica* Peters, *Tilapia nolotica* L.)

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* Note: All finfish and shellfish species tested thus far have been susceptible to the lethal effects of this toxic dinoflagellate.

having lost its ability to maintain balance, leaned against the aquarium wall. This behavior seemed somewhat analogous to fish "walks" reported by State biologists during dinoflagellate-related kills in natural habitat, in which affected finfish and shellfish attempt to leave the water and beach before they die (Table 2). Lesions with sloughing of flecks or patches of epidermal tissue, and/or subcutaneous hemorrhaging developed as the exposure period lengthened and the fish began to expire. Subcutaneous bleeding was most noticeable for hybrid striped bass and striped bass in the region under the dorsal fin, and for southern flounder in sores that formed on the ventral surface by the mouth. Many of the tested fish attempted to reach the well-aerated area by the aquarium filter and, if successful, remained there until death.

When we exposed fish to water with aging dinoflagellate cysts (dormant for ~2 yr), lethal effects often required about 6-8 wk. Cysts that had been inactive for 1-2 months resulted in fish death within approximately 2 wk. Recently formed cysts from highly active, toxic populations, however, were capable of producing flagellated cells that killed fish within 15-20 min. Cultures maintained to provide high levels of toxin for biochemical characterization require 15 or more fish daily. In replicated bioassay tests with exotic fish species, hybrid striped bass were most susceptible whereas some guppies and mosquitofish survived several days of exposure to toxic dinospores at concentrations of > 300 cells/mL. The lethal ichthyotoxic agent from *Pfiesteria piscimorte* (nov.gen., nov.sp.) is an unidentified neurotoxin (Dr. D. Baden, University of Miami, pers. comm.) that is actively released when the dinoflagellate is stimulated by a substance in fresh fish excreta (Smith *et al.* 1988, Burkholder *et al.* 1992). We completed toxicity bioassays on blue crabs (carapace width 8-10 cm) and bay scallops (shell width 4-5 cm) as representative shellfish. These animals did not stimulate excystment or toxic activity; they remained viable for a 9-day test period while filtering low concentrations of the dinoflagellate (~50 cells/mL), although the scallop closing reflex slowed perceptibly. Such impairment of reflex response likely would result in greater susceptibility of scallops to predators in natural habitat. When placed in aquaria with dying finfish, however, blue crabs were killed within hours to several days after numerous frenzied attempts to leave the water, and scallops died within minutes (note: an interactive role of other pathogens such as *Vibrio* in these shellfish deaths is also possible [Dr. G. Krantz, Cooperative Oxford Laboratory, Maryland Department of Natural Resources, pers. comm.], and remains to

be determined). Observations from a scallop/sea urchin kill at NMFS-Beaufort in December 1992 (with nearly 200 scallops affected) suggested that these invertebrates alone (i.e., without live finfish) may stimulate the dinoflagellate to high toxicity for lethal effects (P. Tester & J. Rivera, NMFS-Beaufort, pers. comm.).

D. Acute and Chronic Damage to Fish Skin and Gill Tissues

All control fish (without exposure to *Pfiesteria piscimorte* [nov.gen., nov.sp.]) remained unaffected in all experiments that tested the dinoflagellate's effects on fish tissue. In contrast, all moribund striped bass that had been exposed to lethal concentrations of toxic dinospores developed severe damage to the epidermis. These fish commonly showed mild to severe reddening on the flanks and fins due to hyperemia and hemorrhage, and many specimens lost nearly all of their epidermal epithelium. Fish that received sublethal exposure to the dinoflagellate exhibited mild to severe erosions of the epithelium that were associated with vacuolation of epithelial cells. None of the toxin-exposed fish from any of the experiments showed histologically detectable gill lesions.

E. Dinoflagellate Response to Salinity, Light and Nutrient Gradients

The toxic dinospore stage of *Pfiesteria piscimorte* (nov.gen., nov.sp.) did not emerge from cysts that were placed in approximately 0‰ salinity from the wellwater source, although we documented a hybrid striped bass kill in an aquaculture facility at ca. 0‰ (Table 2, Fig. 5). The water used in the aquaculture ponds originated from a calcium-rich coastal aquifer; the dinoflagellate was believed either to have been transported to the ponds by waterfowl, or to have entered the water from cysts deposited on the pond sediments from previous use of Pamlico River water. Among the salinities tested, 15‰ salinity was optimal for growth and toxic activity of *P. piscimorte*'s dinospore stage (Hobbs *et al.* 1991, Burkholder *et al.* 1992). Fish death occurred at 10‰ or higher during the 16-day experiment (Fig. 6). On day 17 the fish were fed in all tanks, and 8 of the 9 total fish from the 5‰ replicate aquaria died by day 20.

Pfiesteria piscimorte (nov.gen., nov.sp.) also was shown to be capable of lethal activity across a broad gradient of light intensity. The alga showed comparable toxicity to fish across the tested range of available light with no apparent optimum, indicating that light had little effect on toxic activity (Fig. 7). Abundances of the four most common stages (dinospores, gametes, amoebae and cysts) were highly variable among replicates within each treatment (Fig. 8). Considering median abundances, each common stage was

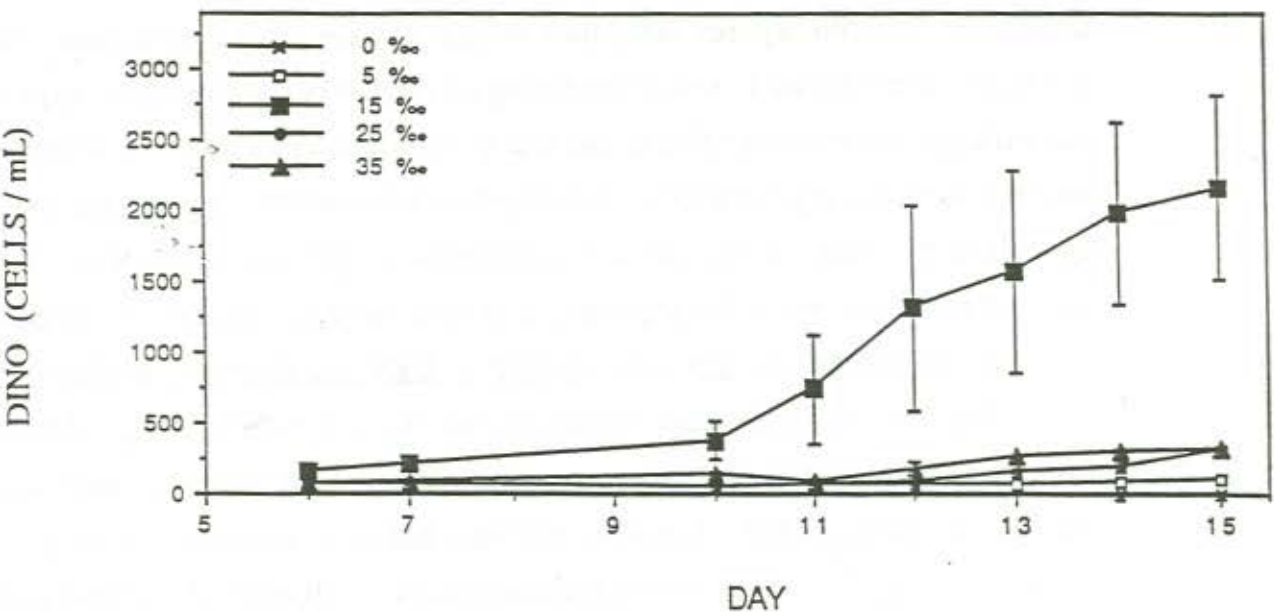

Figure 5. Response of *Pfiesteria piscimorte* to a salinity gradient imposed by mixing Instant Ocean salts with a [soft] wellwater source (alkalinity ~ 4 mg/L). Data are given as the mean $\pm$ 1 standard error (SE; n = 3). Note: The large error bars associated with the 15‰ curve reflect the delayed response of the dinoflagellate in 1 of the 3 replicate aquaria.

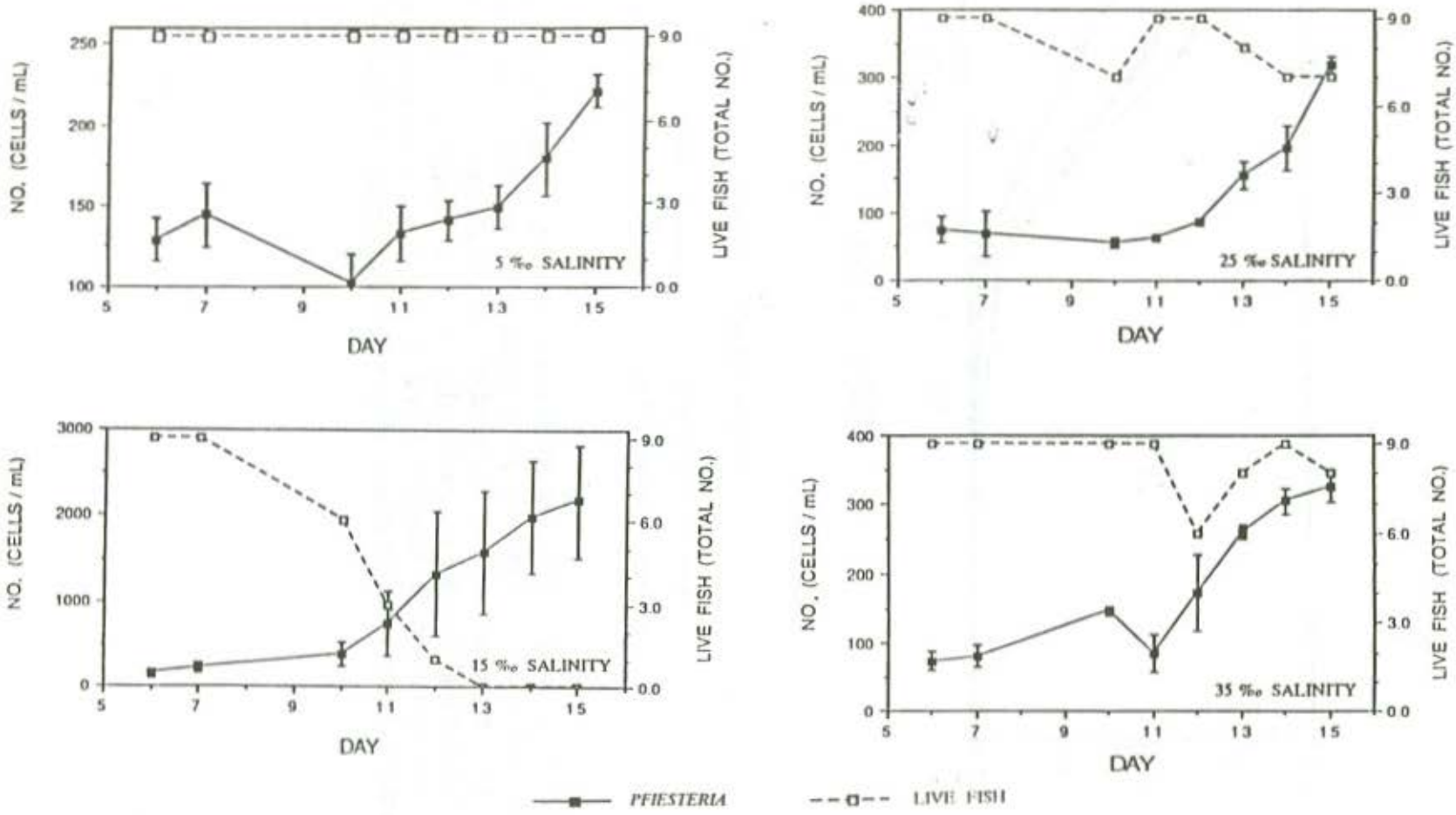

Figure 6. Lethality of the toxic flagellated vegetative cells to test fish across a salinity gradient, considering the total number of live fish in all three replicates daily for each treatment. Fish were not fed during the 15-day test period. Note that abundance of toxic *Pfiesteria piscimorte* cells began to increase at day ~10 among all salinities indicated (data given as the mean ± 1 SE, n = 3), with earliest fish death at 15‰. Fish did not die at 5‰ during the first 15 days, but they were fed at day 16, and 8 of the 9 fish died by day 20 in the 5‰ regime.

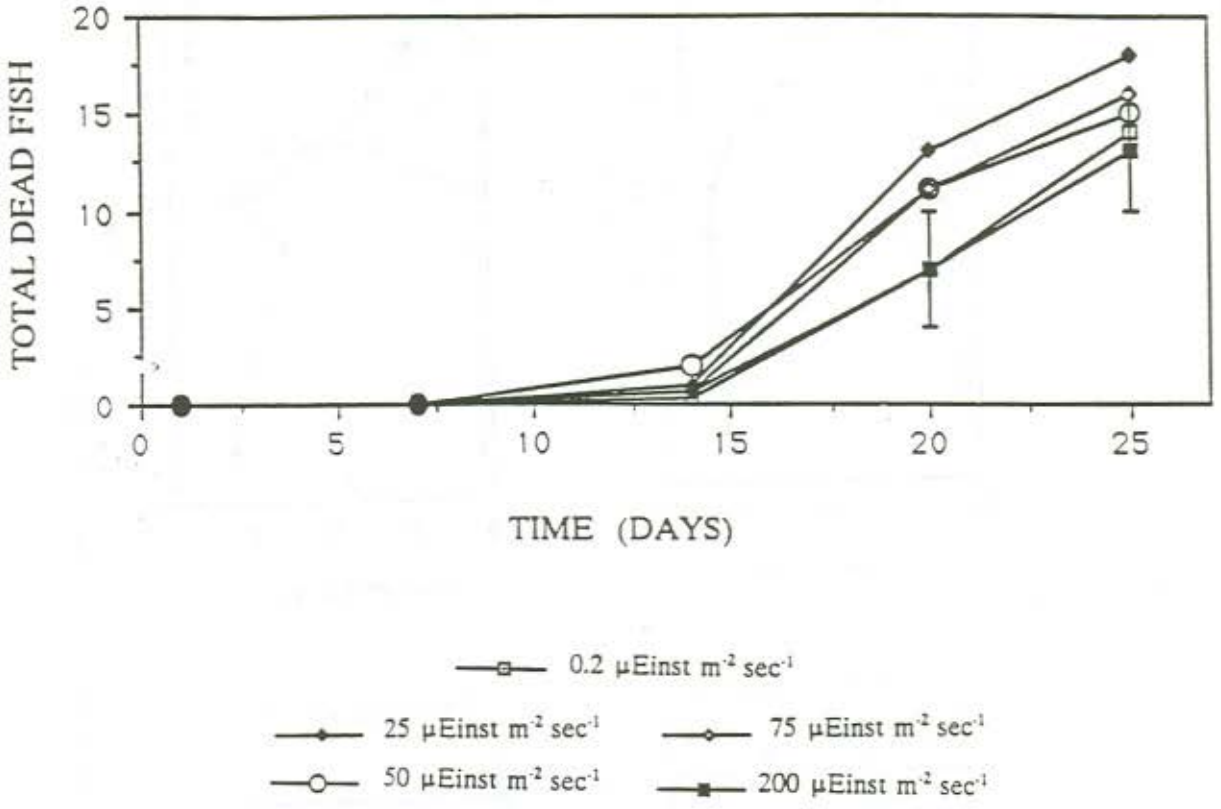

Figure 7. Toxicity of *Pfiesteria piscimorte* to test fish across light gradient from 0.2 - 200 μEinst m^{-2} sec^{-1}. Data are given as the mean $\pm$ 1 SE of the total fish killed in each replicate aquarium after the number of days indicated. Note that the dinoflagellate became slightly more toxic at 25 μEinst m^{-2} sec^{-1} (significantly more lethal than at the other light intensities tested by days 20 and 25; $p < 0.45$ for each of the two dates). In general, however, lethal effects were relatively comparable among treatments.

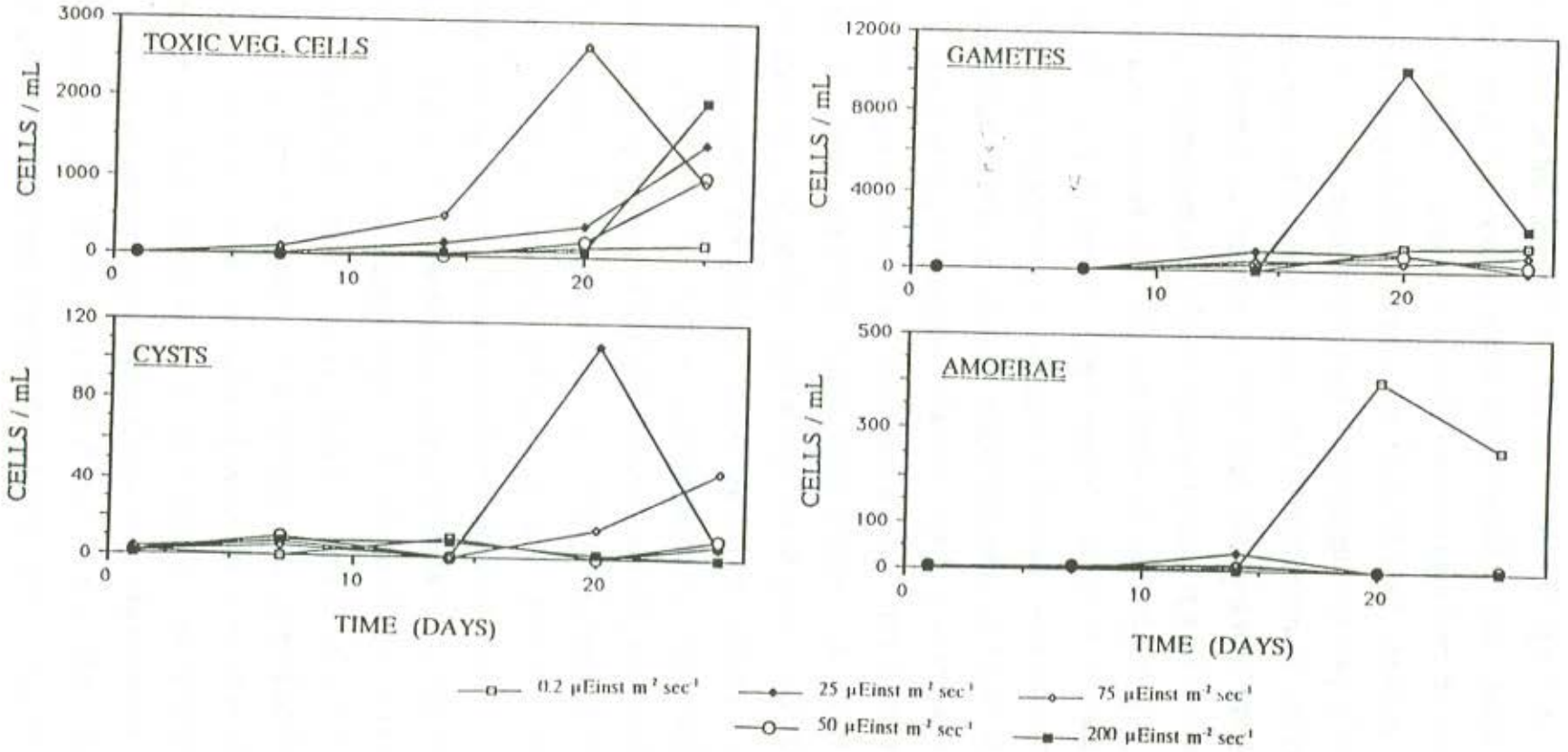

Figure 8. Abundances of toxic flagellated vegetative cells, cysts, gametes and amoebae grown in water with fresh fish excreta across a light gradient of 0.2 - 200 µEinst m^{-2} sec^{-1} (18°C, using cool white fluorescent lights at a 12/12 L:D cycle). Because the data were markedly asymmetrical, median values were used rather than means to indicate average abundance of each stage (Gill 1978).

similar in number at all light intensities for the first 15 days, and each stage reached its maximal concentration at day 20 followed by a decline at day 25. The maximum for each stage occurred at a different light intensity (toxic dinospores, 75 μEinst m^{-2} sec^{-1}; cysts, 25 μEinst m^{-2} sec; gametes, 200 μEinst m^{-2} sec^{-1}, the highest light level tested; amoebae, 0.2 μEinst m^{-2} sec^{-1}), perhaps suggesting a selective tendency for optimal light levels among stages. Recent documentation of "cleptochloroplasts" in the vegetative flagellated stage of *P. piscimorte* (nov.gen., nov.sp.), that is, chloroplasts retained for use after digestion of photosynthetic algal prey (transmission electron micrographs of Steidinger *et al.* 1993), points to a mechanism by which this alga can benefit from a "borrowed" photosynthetic mode of nutrition. Interestingly, cultures of toxic dinospores were successfully maintained for 4-7 days when supplied with microalgae (diatoms or small green flagellates) as a food resource, but vegetative reproduction gradually decreased and transformations to amoebae and cysts increased without live fish or fresh fish excreta.

After 4 days under batch culture conditions, the reproductive gametes of *Pfiesteria piscimorte* (nov.gen., nov.sp.) were strongly stimulated by phosphate enrichment at all levels $\geq$ 100 μg PO_4^{-3}P/L (1-way ANOVA, $p < 0.01$; Burkholder *et al.* 1992; Fig. 9). Low nitrate or phosphate enrichment (25 μg NO_3^-N/L) mildly stimulated growth relative to that in controls without nutrient enrichments ($p < 0.05$), whereas ammonium negligibly affected gamete abundance (Burkholder *et al.* 1993). Tests of dinoflagellate response to nutrient ratios were confounded by difficulties encountered in maintaining toxic stages separately from finfish excreta. The phosphate level used in establishing the treatment ratios (100 μg PO_4^{-3}/L) was within the stimulatory range for gamete growth, based on the previous experiment that tested absolute supply concentrations. In repeated experiments holding phosphate at 50 or 100 μg PO_4^{-3}P/L, clear stimulation by a particular nutrient supply ratio was not detected during 5- to 10-day periods. Active flagellated gametes and small amoebae derived from gametes were observed across the range of nutrient ratios, with gametes consistently most abundant (significantly more numerous than all other stages at each nutrient ratio; $p < 0.05$). However, as for the response to light, abundances of gametes, amoebae and cysts were highly variable among treatment replicates. Gametes were most numerous at the 50:1 N_i/P_i ratio, whereas amoebae were intermediate in abundance and comparable among all N_i/P_i ratios (median values; Fig.10).

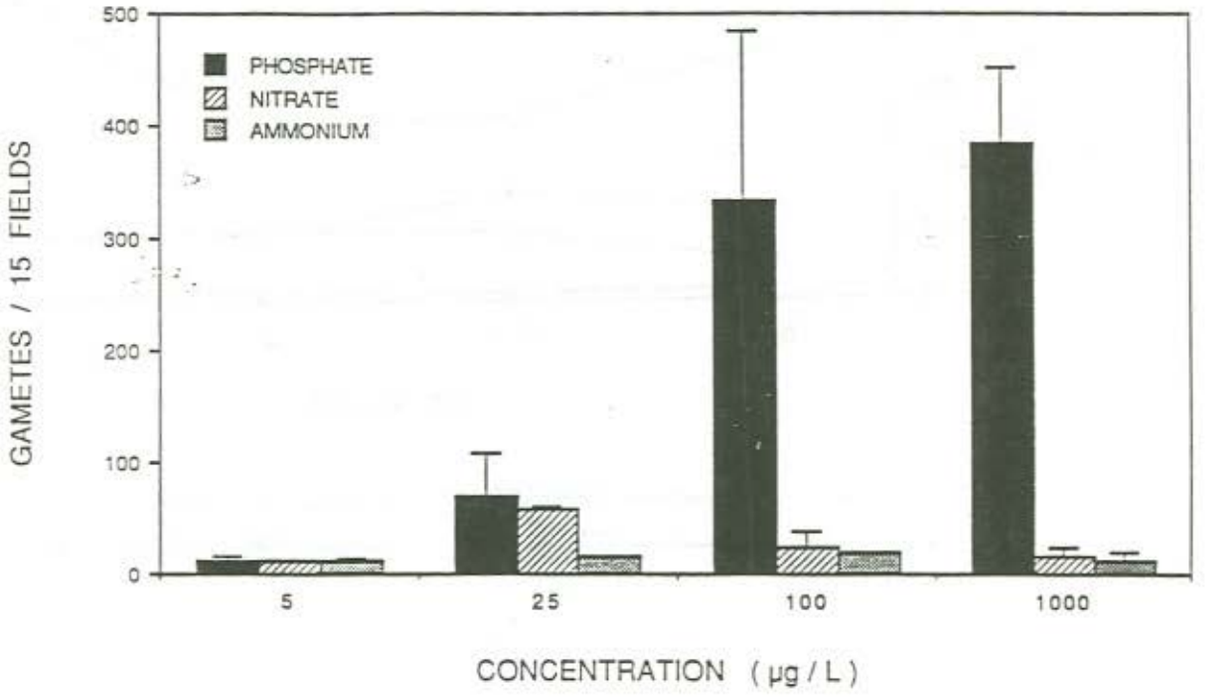

Figure 9. Response of *Pfiesteria piscimorte* gametes to gradients of phosphate, nitrate and ammonium enrichment after 4 days in batch cultures without finfish. By comparison, gamete numbers were negligible in controls without nutrient additions. Data are given as the mean $\pm$ 1 SE (n = 3).

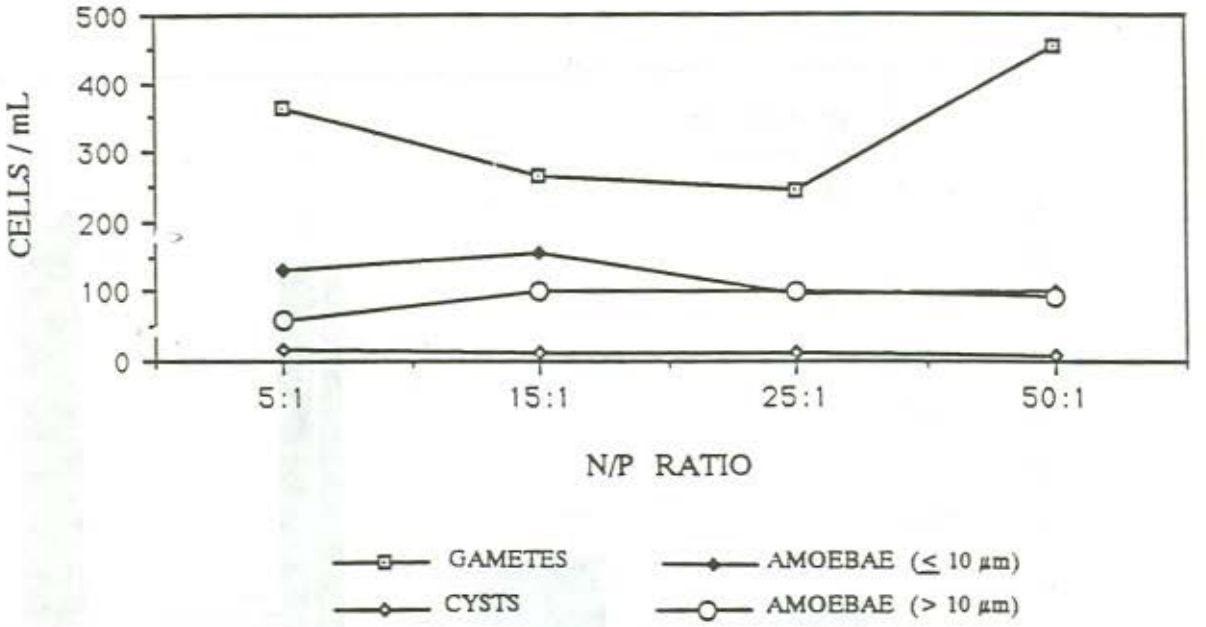

Figure 10. Abundances of gametes, amoebae and cysts at varying N_i/P_i supply ratios after 7 days in batch culture without fish. Culture inocula were obtained from a toxic stock population of *Pfiesteria piscimorte* grown in water with fresh fish excreta. Ratios were obtained by altering nitrate, holding phosphate constant at 100 µg $PO_4^{-3}P/L$. Because the data were markedly asymmetrical, median values were used rather than means to indicate average abundance of each stage (Gill 1978).

F. Exploration of Potential Agents of Bio-Control

Observations on the response of *Pfiesteria piscimorte* (nov.gen., nov.sp.) to feeding activity of *Stylonichia* cf. *putricina* led to abandonment of consideration of this ciliated protozoan as a potential bio-control agent. Based on observations of cultures under light microscopy, the protozoan predator is capable of significantly reducing populations of highly toxic flagellated vegetative cells, and it also consumes toxic cysts; further, it does not appear to be adversely affected by the toxin. However, on several occasions after a short feeding period (~15-30 min), its excreta apparently provided a chemical "cue" to remaining dinospores which began to swarm around individual ciliaties much like the feeding behavior described by Spero (1982) for the small dinoflagellate, *Katodinium fungiforme* (Anisimova) Loeblich (formerly *Gymnodinium fungiforme* Anisimova; now suspected to be armored and closely related to *P. piscimorte* [nov.gen., nov.sp.]) in its ambush predator behavior toward protozoan ciliate prey. The small dinospores bombarded *S. putricina* until the animals attempted to leave the area. During this time, however, remaining planozygotes were induced to transform into the large lobate ("giant") amoeboid stage (Fig. 4). Animals that did not rapidly retreat encountered these large lobate amoebae, which engulfed them as prey became predator. Unlike the protozoan ciliate, the rotifer *Brachionus* sp. apparently does not excrete substances that stimulate dinoflagellate transformations or attacks. This animal is capable of feeding on the toxic dinospores, planozygotes and cysts for extended periods (7-10 days) with no adverse effects observed thus far in preliminary trials (Mallin *et al.* unpublished data).

IV. DISCUSSION / CONCLUSIONS

Within the past two years, an ichthyotoxic dinoflagellate was discovered in the Neuse and Pamlico Estuaries of the Albmemarle-Pamlico system (Burkholder *et al.* 1992). In this research we obtained field and experimental information needed to formally name and characterize the general ecology of this dinoflagellate, and to examine its significance as a causative agent of fish kills in the Neuse and Pamlico.

Disruption and removal of the outer membranes on the toxic dinospore cells revealed the number, shape, patterning and external structure of hardened exterior cellulose deposits referred to as armored plates. The plate formula information, required for formal speciation, confirmed that this organism represents a new genus and species

of armored dinoflagellate. Additional information established that toxic and nontoxic amoeboid stages actually predominate in the life cycle of this dinoflagellate; hence, we have placed it within the order Dinamoebales (Division Pyrrhophyta, Class Dinophyceae; new family in the process of being formally named). We are completing formal procedures to name the alga to genus and species as *Pfiesteria piscimorte* (Steidinger *et al.* in prep.).

Thus far we have determined that this dinoflagellate has at least 15 stages in its life cycle and can transform from flagellated to amoeboid forms with ~2 minutes (Burkholder *et al.* 1992, Burkholder *et al.* 1993). We hypothesize that 5 or more stages still remain to be described (Burkholder 1993). Although this report represents the first description of an estuarine dinoflagellate with a complex life cycle that includes multiple amoeboid and flagellated stages, we predict that other toxic and non-toxic estuarine "amoeboid" dinoflagellates will be discovered using appropriate sampling protocol. Several other dinoflagellate species with complex life cycles, including some with more than twice as many stages, have been described previously from freshwater habitats (Popovský & Pfiester 1990).

Two years of field data, obtained with help from a monitoring network of NC DMF and NC DEM staff, concerned volunteer citizens, aquaculturists and other scientists, indicate that *Pfiesteria piscimorte* (nov.gen., nov.sp.) was the causative agent of more than one-third (38%) of the major fish kills in the Neuse and Pamlico Estuaries during 1991 - 1992. The percentage likely is underestimated because of difficulties in reaching many fish kills to obtain water samples while fish were still dying, which is the optimal period for detection of the most easily recognized, most lethal stage. *P. piscimorte* (nov.gen., nov.sp.) is toxic across broad gradients of temperature, salinity and light, with optimal conditions for toxic outbreaks at > 26°C and 15‰.

When stimulated by an unknown organic substance in fresh finfish excreta/secreta (Smith *et al.* 1988, Burkholder *et al.* 1992), the alga emerges from dormant cysts or converts from amoeboid stages to flagellated vegetative cells (dinospores), and it excretes a potent toxin that is highly lethal to a wide array of finfish and shellfish including all 18 native and exotic species tested thus far. Since toxic activity is triggered by the fresh fish excreta / secreta, with no further stimulation by dead fish carcasses, the compound is believed to be an extremely labile organic substance (e.g., a simple amino acid, methylamine

or methylaminoxide, or calmodulin; Flik *et al.* 1984, van Waarde 1988, Shimizu 1991, Pederson & Anderson 1992) that is a general excretory product or secretion of most finfish. Many dinoflagellates actively consume amino acids as a nitrogen source (Droop 1974, Hauser *et al.* 1975, Gaines & Elbrächter 1987, Schnepf & Elbrächter 1992, Glasgow & Burkholder 1993). The labile substance likely is competitively metabolized by bacteria (e.g., Sieburth & Keller 1989), which would enshroud a fish carcass within a few hours (Lagler *et al.* 1962, Cole 1982) and thus would block further access by toxic cells in the water column to residual leakage of excretory products. This postulated series of events could explain the rapid encystment or conversion of toxic flagellated stages shortly after fish death. Utilization of algal prey as a food resource affords the dinospores and planozygotes to "cleptochloroplasts," or "borrowed" chloroplasts for an assumed photosynthetic mode of nutrition. Algal and bacterial prey sustained active dinospore cultures for several days, but gradually the dinospores formed amoebae or encysted unless offered live fish or fresh fish excreta.

Water from which toxic dinospores were removed by gentle drop-filtration (0.45 μm-pore filters) induced neurotoxic signs by fish including sudden sporadic movement, disorientation, lethargy, and apparent suffocation from muscle paralysis followed by death. Tested shellfish did not stimulate toxin production although their reflexes slowed perceptibly. In bioassay experiments they expired quickly, however, when placed in water with dying finfish. Further, observations from a scallop / sea urchin kill in December 1992 (with nearly 200 scallops affected) suggested that these invertebrates alone (i.e., without live finfish) may stimulate the dinoflagellate to cause lethal effects (P. Tester & J. Rivera, NMFS-Beaufort, pers. comm.).

Aside from neurological signs in fish after acute exposure, we also demonstrated that *Pfiesteria piscimorte* (nov.gen., nov.sp.) causes significant damage to the skin of test striped bass. To our knowledge, this represents the first report of such skin lesions as induced by a dinoflagellate. Similar damage to an array of other fish species was also documented, suggesting that epidermal damage / loss is a characteristic response by finfish to toxic stages of *P. piscimorte* (nov.gen., nov.sp.). Interestingly, in sublethal exposures there was no detectable damage to striped bass gills, even though this tissue is potentially in even greater contact with the toxin from constant water flow. The epidermal damage in finfish may result from (1) direct effects of the toxin, or (2) indirect effects such as

impaired blood supply to the skin. Once the toxin is characterized, the mechanism for epidermal damage can be resolved by exposing purified toxin directly to target tissues both *in vivo* and *in vitro.*

Regardless of the mechanism, this epidermal damage has important implications for the viability of fish that are exposed to its toxins. The skin forms a first line of defense against invasion by pathogenic bacteria and fungi. Breaching this epidermal barrier would facilitate colonization by such opportunistic pathogens, many of which are normally present on the skin or in estuarine water. In recent years the Albemarle-Pamlico Estuary has experienced severe outbreaks of ulcerative skin diseases, (Noga 1993, Noga *et al.* 1993). Most prominent among them is ulcerative mycosis, a mixed fungal / bacterial infection that is associated with deep penetrating ulcers in fish. Virtually all common fish species that inhabit the low to moderate salinity regions of the Albemarle-Pamlico have been afflicted by at least one of these diseases. The opportunistic nature of these pathogens strongly suggests that some type of stressor weakens the fish's resistance and predisposes them to the ulcers (Noga 1988). The ubiquitous nature of *Pfiesteria piscimorte* (nov.gen., nov.sp.) in the Albemarle-Pamlico ecosystem, combined with its specific and highly damaging affects on epidermal epithelium, points to this agent as potentially playing a major role in initiating at least some of these ulcerative diseases.

We examined the effects of two naturally occurring estuarine protozoan predators on *Pfiesteria piscimorte* (nov.gen., nov.sp.). The ciliate *Stylonichia* cf. *putricina* and the rotifer *Brachionus* sp. are capable of consuming both toxic cysts and toxic dinospores while fish are dying. However, an unknown chemical "signal" emitted by *S. putricina* apparently triggers transformation of large dinoflagellate planozygotes (product of sexual fusion by the gametes) into large lobate amoebae with length 15- to 20-fold greater than the diameter of the toxic dinospores. When fully formed (within several minutes), the amoebae sometimes attack and engulf the protozoan ciliate, leaving the rotifer as a more promising potential agent of bio-control. Based on insights from these observations, experiments to more closely examine the relationship between *P. piscimorte* (nov.gen., nov.sp.) and *Stylonichia, Brachionus,* and microcrustaceans such as *Acartia* are now in progress.

The requirement of fresh finfish excreta by toxic flagellated and amoeboid stages confounded experiments to separately test their stimulation by inorganic phosphorus and

nitrogen, since the excreta contains high nutrient concentrations as well as the unidentified organic stimulatory compound(s). Gentle drop-filtration was used in an attempt to separate toxic flagellated and amoeboid forms from the nutrients in fish excreta, but the filtration process induced rapid encystment of dinospores and planozygotes, or transformations to nontoxic amoebae. In the absence of fish or fish excreta, the amoeboid and cyst stages of *Pfiesteria piscimorte* (nov.gen, nov.sp.) did not respond to subsequent water-column enrichments with N_i or P_i. In contrast, gamete production was significantly stimulated by phosphate ($\geq$ 100 μg PO_4^{-3}P/L, mildly stimulated by low concentrations of nitrate (25 μg NO_3^-N/L), and negligibly affected by ammonium enrichments (25-1,000 μg NH_4^+N /L). These data indicate that phosphate can play an important role in maintenance of an inoculum of *P. piscimorte* (nov.gen., nov.sp.) as gametes in the water column. The gametes complement the dormant cyst "bank" that serves as an inoculum from the sediments. Gametes complete sexual reproduction by fusing to produce planozygotes when exposed to live finfish.

Tests of the effects of nutrient supply ratios on the abundance of life cycle stages and toxic activity of *Pfiesteria piscimorte* (nov.gen., nov.sp.) require that one of the two nutrients in question, i.e., either N_i or P_i, becomes limiting during the experimental period (Tilman *et al.* 1982, Kilham & Kilham 1984) - a status that is difficult to accomplish at the relative high phosphate enrichment required to ensure continued gamete activity. In future work we plan to test ratio effects on dinoflagellate populations over longer trials (e.g., 30 - 60 days), using inocula as "aged" cysts (dormant for 6 months) versus nutrient-enriched populations that have been actively killing fish. In the latter case, however, additional time likely will be required to deplete abundant stores of the phosphate and nitrogen that were "luxury-consumed" from previous acclimation to excreta-enriched water (Kuhl 1974, Burkholder 1992).

Preliminary experiments indicate that the toxin from *Pfiesteria piscimorte* (nov.gen., nov.sp.) strips the outer layers of skin from finfish, with striped bass being the most susceptible species tested. This dinoflagellate's toxin creates massive regions of subcutaneous hemhorraging, as well as lesions through muscle tissue that can serve as ports of entry for opportunistic pathogens. Lethal toxin levels apparently act by interfering with neurological signal transmittance, leading to paralysis and suffocation. But chronic sub-lethal exposure may also impair fecundity, egg development, and survival of recruiting

stages (unpublished data from infected fish cultures maintained by B. Hettler and A. Powell, NMFS-Beaufort). These observations suggest that a school of fish which successfully leaves a toxic oubreak area may have only escaped on a short-term basis. Although *P. piscimorte* (nov.gen., nov.sp.) appears to be the causative agent of 30% or more of the major fish kills in the Albemarle-Pamlico Estuarine System, fish sudden death may actually represent a small loss in comparison to a more insidious, chronic influence of this new toxic dinoflagellate on fecundity, recruitment, disease resistance, and survival of fish populations in North Carolina estuaries.

The tracking / surveillance efforts for this dinoflagellate, described for North Carolina as well as other mid- and south-Atlantic coastal states, have involved multiple volunteer concerned citizens and scientific colleagues including State staff who used well-timed or "targeted site" sampling to obtain the required algal material. Collectively, the data point toward one emerging theme, brought to light through the discovery of *P. piscimorte* (nov.gen., nov.sp.) at a fish kill in a North Carolina estuary: Given the broad temperature, salinity and light tolerance of this toxic fish ambush predator, its stimulation by eutrophic conditions, and the ephemeral behavior of toxic stages in response to live finfish, **we predict that *Pfiesteria piscimorte* (nov.gen., nov.sp.) and its close relatives will be increasingly recognized as a significant source of fish mortality and ulcerative disease in shallow, turbid nutrient-enriched estuaries extending to warm temperate, subtropical, and tropical regions throughout the world.**

V. RECOMMENDATIONS

Given this prediction, workshops should continue from New England to Florida, and in other Gulf Coast states, to train regulatory agency staff to identify all stages of this toxic dinoflagellate, and to provide sampling protocol designed to maximize its detection in the water column and sediments from areas with high frequency of ulcerative fish diseases and unexplained sudden-death kills. Such efforts to detect this dinoflagellate in regional fish kill monitoring programs would be greatly aided by development of fluorescent markers or molecular "probes" to enable rapid detection of all stages of *P. piscimorte* (nov.gen., nov.sp.) from water samples.

Staff from the NC DMF and the NC DEM, volunteer citizens, and aquaculturists should be encouraged to continue to act as a "network" to sample estuarine waters and

aquaculture facilities in North Carolina during fish kills, so that we can obtain more quantitative information about fishery losses related to *Pfiesteria piscimorte* (nov.gen., nov.sp.). Similar networks have been established and should be maintained along tributaries to the Delaware and Chesapeake Bays, where we have confirmed the toxic dinoflagellate's presence. Fresh samples should be exposed to fish in aquarium bioassays to confirm toxicity, with algal identifications verified by scanning electron microscopy.

This study represents the first step toward understanding the ecology and adverse effects of *Pfiesteria piscimorte* (nov.gen., nov.sp.) on our estuarine natural and cultured fishery resources. There is a critical need to identify and fully characterize the toxin(s) it produces. This information will enable analysis of toxin accumulation by shellfish such as clams and oysters, and assessment of the potential for shellfish poisoning to humans. Shellfish typically concentrate dinoflagellate toxins in organs such as the hepatopancreas (Shumway 1990). Whereas humans would be expected to encounter little risk from consumption of muscle tissue (e.g., from blue crabs and scallops), the potential for neurotoxic effects from clams and oysters could be higher since the "soft parts" frequently are eaten.

We have shown that phosphate enrichment at 100 μg/L or higher can stimulate growth of this toxic dinoflagellate's gametes, which serve as a water-column inoculum for production of toxic cells when a school of fish enters the area. The linkage between phosphate enrichment and stimulation of this dinoflagellate from our laboratory bioassays is supported by our documented field kill data for *P. piscimorte*, wherein approximately 80% of the kills linked to this organism occurred in nutrient-enriched areas. This research strongly points to a critical need to more fully understand the role of both inorganic and organic nutrients in stimulating toxic outbreaks of *Pfiesteria piscimorte* (nov.gen., nov.sp.). The role of absolute supplies and supply ratios of P_i and N_i enrichments in promoting growth of nontoxic stages aside from gametes, and in transformations from nontoxic to toxic stages, remains to be determined. The available data, nonetheless, point to a clear signal - namely, that this dinoflagellate is most abundant in eutrophic estuaries, and is especially stimulated by phosphate enrichment.

Research to characterize in detail the nutritional optima for flagellated and amoeboid stages of this dinoflagellate should be strongly supported by NC DEHNR, on the basis of more than two years of field data and more than 100 confirming laboratory bioassays that establish (1) the potentially major role of *Pfiesteria piscimorte* in causing fish kills

in our estuaries, (2) the linkage between its toxic activity and fish disease, and (3) the linkage between eutrophic conditions and stimulation of its toxic outbreaks. Based on the available data, we recommend that the potential for stimulation of *P. piscimorte* by phosphate enrichment be seriously considered by NC DEM in its ongoing efforts to develop improved guidelines for protecting North Carolina's estuarine water quality. Point source discharges to receiving waters with salinity $\geq$ 2‰ and ambient phosphate $\leq$ 100 μg $PO_4^{-3}P/L$ (particularly under low-flow, warm summer-fall conditions) should be encouraged to maintain phosphate concentrations below that level outside a necessary [localized] dilution zone in the outfall area. In estuaries with higher ambient phosphate concentrations during the summer-fall period, further reductions by existing point and nonpoint sources should be considered by NC DEM as a long-term strategy in strengthening protection of estuarine waters and fishery resources.

Pfiesteria piscimorte (nov.gen., nov.sp.) is strongly stimulated by at least two substances - phosphate and an unidentified component in fresh finfish excreta. Interactions between these organic and inorganic nutritional sources must be examined before we can hope to predict toxic outbreaks and mitigate their effects. We must first identify the stimulatory compound excreted by finfish; this task should include assessment of the threshold level of the substance required to stimulate dinoflagellate excystment or conversion from nontoxic to toxic stages. Quantitative assessment of differential susceptibility of commercially important finfish species to *P. piscimorte* (nov.gen., nov.sp.) will enhance our ability to predict toxic outbreaks and potential fishery losses. Once the stimulatory compound in finfish excreta is identified, differential species susceptibility can be evaluated, in part, by determining the concentration of this substance in the excreta of larvae, juveniles, and adults of valuable finfish species, given the underlying assumption that species or life stages with higher excretion of this substance may be more vulnerable.

Identification of the stimulatory substance in finfish excreta will also greatly facilitate mass culture of *Pfiesteria piscimorte* (nov.gen., nov.sp.) in the high cell concentrations that are needed for toxin characterization. Addition of synthetic sources of the compound will eliminate the necessity of sacrificing 15 fish per day in order to maintain high toxic activity during experimental trials. The excreted stimulatory compound, once identified, may also be useful as a management tool. It may be possible to add large quantities of the substance to waters with high concentrations of cysts in the

sediment "bank" during periods with relatively low abundance of commercially valuable fauna, resulting in mass excystment of the dinoflagellate and entry into the water column where the toxic cells could be more easily destroyed by short-term, high dosages of pesticides or herbicides and the cyst bank could be substantially reduced.

The discovery that *Pfiesteria piscimorte* (nov.gen., nov.sp.) can cause massive, acute skin damage in finfish poses a serious concern for the health of fish populations that are at risk of exposure to this toxic pathogen. Further studies are needed to determine whether exposure to the dinoflagellate can lead to the specific types of ulcerative diseases that have increasingly plagued fish in the Albemarle-Pamlico Estuary. This research should include sublethal exposure to the toxin, and subsequent monitoring of the effects on recovering fish. Additional research is also needed to examine the impacts of dinoflagellate exposure on other organ systems, since other toxic dinoflagellates have been shown to damage these tissues. Chronic exposures should be included, since the ubiquitous distribution of *P. piscimorte* (nov.gen., nov.sp.) in the Albemarle-Pamlico ecosystem suggests that chronic exposures are common.

Extensive research is needed to unravel the role of the many stages of this toxic dinoflagellate in estuarine food webs. The amoeboid stages in the life cycle of *Pfiesteria piscimorte* (nov.gen., nov.sp.) likely have been identified in the protozoan literature as at least three amoeboid genera unrelated to dinoflagellates (e.g., see Sawyer 1975, Sawyer *et al.* 1977), indicating a need for re-evaluation of the role of dinoflagellates in benthic microbial food webs of shallow, turbid eutrophic estuaries. The potential for adverse effects of the flagellate and amoeboid stages of this toxic alga on phytoplankton and zooplankton that serve as indirect or direct food resources of commercially valuable fishes is totally unknown. Thus far, we have documented only that the dinospores, planozygotes, and most amoeboid stages consume diatoms, other small algae, and bacteria; the ubiquitous amoebae have also been observed to feed upon small animal prey. Efforts to understand trophic interactions between *Pfiesteria piscimorte* (nov.gen., nov.sp.) and other food web components should include evaluation of its natural predators (e.g., the rotifer *Brachionus* sp.) as potential agents of "bio-control" that, perhaps, can offer hope as a management strategy to reduce the abundance of both active and encysted populations of this toxic dinoflagellate at known kill sites.

Finally, as a part of the major effort needed to determine this toxic dinoflagellate's overall role in estuarine food webs, there is a critical need to understand its sublethal chronic influences on recruitment, fecundity, and survival of finfish and shellfish. Thus far, we have observed that young hybrid striped bass (< 7 cm length) are more sensitive to the toxin of *P. piscimorte* than adults, and that menhaden eggs will not hatch when toxic dinospores of this alga are abundant (Burkholder, unpublished data; B. Hettler, NMFS-Beaufort, unpublished data). ... But to gain in-depth insights about the subtle, potentially major population-level impacts of this toxic ambush predator on our fisheries, and to become sufficiently skilled to mitigate those impacts, we need to learn much more.

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GLOSSARY

Abbreviations

A. Units of Measurement

°C	Degrees Celsius (or degrees Centigrade)
%	Parts per hundred (percent)
‰	Parts per thousand (salinity units)
µg/L	Micrograms per liter (nutrient concentration units)
mg/L	Milligrams per liter (nutrient concentration units)
μm^3	Cubic micrometers (biovolume units)
mm^3/mL	Cubic millimeters per milliliter (biovolume units)
$\mu Einst\ m^{-2}\ sec^{-1}$	MicroEinsteins per square meter per second (light quantity)
m	Meter(s) (length)
km	Kilometer(s) (length)
km^2	Square kilometers (area)
gal.	Gallons (volume)
L	Liters (volume)
mL	Milliliters (volume)

B. Parameters

pH	-log[H^+], or minus the log of the hydrogen ion concentration; measure of the acidity of the water.
DO	Dissolved oxygen concentration.

Nutrients:

N_i	Inorganic nitrogen (comprised of nitrate and ammonium).
P_i	Inorganic phosphorus (mostly as phosphate).
NH_4^+N	Ammonia, as ammonium-nitrogen concentration (N content of that form), the preferred inorganic nitrogen source for many phytoplankton and submersed macrophytes; generally low in concentration but added by precipitation and high in sewage.
NO_3^-N	Nitrate, as nitrate-nitrogen concentration, the second form of inorganic nitrogen used by plants; also added by precipitation.

SRP — Soluble reactive phosphate concentration, or $PO_4^{-3}P$ (phosphate-phosphorus), the form of phosphorus that is immediately available for uptake by plant cells.

Terms

Aerobic: Referring to an environment or process in which dissolved oxygen is present.

Alkalinity: Refers to the quantity and kinds of compounds present (e.g., ions HCO_3^- [bicarbonate], CO_3^{-2} [carbonate], and OH^- [hydroxyl]) that collectively shift the pH to the alkaline or basic side of neutrality; frequently is used to express the total quantity of base that can be determined by titration with strong acid.

Anaerobic: Referring to an environment or process in which dissolved oxygen is absent.

Anoxia: Status wherein the water (e.g., the lower part of the water column) is depleted of dissolved oxygen. Typically develops in darkness during periods of high respiration by abundant plants and animals.

Algae (plural; alga, singular): Primitive plants that may photosynthesize like higher plants, but mostly lack vascular tissue (and, therefore, have no flowers, roots, stems or leaves). Dinoflagellates are generally considered to be algae, mostly because the most infamous species, the red tide formers, mostly are capable of photosynthesis like higher plants. However, zoologists consider dinoflagellates as animals (protozoans).

Amoeba (plural, amoebae): General term referring to a group of animals (? -- not as clear in the case of *Pfiesteria piscimorte* [nov.gen., nov.sp.]) with temporary body projections called pseudopodia that are used for locomotion and feeding, and which result in a (sometimes constantly) changing body shape. Most amoebae are known to feed on dissolved organic matter or small particles (bacteria, algae, or smaller animals).

Armor (in dinoflagellates): Flattened deposits of cellulose ("plates") of varying thickness beneath surface membranes on the cell.

Autotrophic: Requiring only inorganic compounds for nutrition, along with energy provided by light.

Benthic: Bottom-dwelling; growing on or within the sediment, or growing attached to a substratum that is in contact with the sediment (e.g., on rocks, seagrasses, etc.).

Bio-Control: In management practices, use of one naturally occurring or exotic (introduced) species to control the abundance or distribution of an undesirable species.

Biomass: The total living particulate organic matter (generally reported as fresh or dry weight) present per unit volume of water, or per unit of mud-substratum surface area).

Biovolume: The total volumetric measure of biomass (e.g., phytoplankton biovolume).

Chlorophyll *a*: Green pigment found in all plants that undergo photosynthesis (including, for example, blue-green algae, other algae, and angiosperms). The concentration of this pigment is often used as an indicator of algal biomass.

Chloroplast: Organelle where photosynthesis occurs, wherein plants convert (inorganic) carbon dioxide into organic carbon. This organelle contains the chlorophyll(s) and other pigments that are involved in capturing the light energy needed for the organic carbon production to occur.

Cilia (plural; singular, cilium): A minute, "hairlike" structure ($\leq$ 10 μm in length) present in large numbers on the surface of many aquatic protozoans; synchronous movement enables motility.

Community: A group of interacting populations within a given habitat; e.g., the phytoplankton community refers to all microscopic suspended plants (algae) that inhabit the water column of a given estuary.

Cultural Eutrophication: Enriching of aquatic systems by anthropogenic nutrient sources; somtimes referred to as "accelerated" eutrophication.

Cyst: Protective structure produced by some algae as part of their life cycle (usually after sexual reproduction) or in response to adverse conditions (sometimes known as "temporary cysts" in the dinoflagellates). The cyst usually has a thick protective outer wall, and the cell within may contain a high quantity of stored food reserves.

Dinoflagellate: Solitary or colonial organism, usually 1-celled, with a true nucleus ("eukaryote" nucleus in amoeboid stages, versus a "mesokaryote" permanently condensed chromosomes in flagellated stages), and with both plant and animal affinities. Many species are free-living motile cells, but parasitism and mutualistic endosymbiont habits are also common. The most easily recognizable or "typical" dinoflagellates (Class Dinophyceae) generally have a transverse groove or canal (cingulum) that gives the cell the appearance of being formed of upper and lower "halves;" the anterior or front side of the cell also has a longitudinal groove (sulcus) in the lower "half" of the cell below the transverse canal. In each of these grooves or canals is a flagellum. The transverse flagellum engirdles the cell within the cingulum, and is helically shaped; its beating causes the cell to whirl or spin as it moves forward. The longitudinal flagellum trails lies in the sulcus and trails out from the posterior end of the cell; it is used for steering. Photosynthetic dinoflagellates (which are outnumbered by heterotrophic members) have pigments chlorophyll *a* and *c*, and form the same type of starch (amylose / amylopectin) as higher plants. Dinoflagellates represent the second oldest group of eukaryote algae

(Division Pyrrhophyta, Greek for "fire plant," named from red tides), with a fossil record dating at least to 450 million years (Bold & Wynne 1985; Steidinger & Cox 1986). The correct pronunciation for dino is to use a short "i", from the Greek word for "whirling," but the term is often pronounced with a long "i," indicating the Latin word for "terrible" or "terrifying."

Dinospore (in dinoflagellates): Asexually produced motile (flagellated) cell; in *Pfiesteria piscimorte* (nov.gen., nov.sp.), it is believed to undergo asexual reproduction to form either additional dinospores or, alternatively, gametes.

Divalent Cations: Ions with a +2 charge, such as calcium (Ca^{+2}) or magnesium (Mg^{+2}).

Estuary: An inlet of the sea reaching into a river valley as far as the upper limit of tidal rise, usually being divisible into three sectors -- (a) a marine or lower estuary, in free connection with the open sea; (b) a middle estuary subject to strong salt- and freshwater mixing, and (c) an upper estuary with more freshwater influence, but subject to daily tidal action. Sectors (c) and (to a lesser extent) (b) are sometimes referred to as brackish waters. The limits between these sectors are variable, and subject to constant changes in the river discharge (Fairbridge 1980).

Eutrophic: High in nutrients and high in organic (biological) production (original meaning -- nutrient-rich). Eutrophic estuarine and marine coastal waters typically are shallow with limited light transparency from algal blooms and suspended sediments, and abundant plant nutrients in both the water column and the sediment. Late summer algal blooms by phytoplankton or macro-algae may be common.

Extinction Coefficient: An expression of the exponential attenuation of irradiance at depth in relation to that at the surface. The total extinction of natural waters is the sum of absorption by the water itself, dissolved compounds, and particles suspended in water.

Flagellum (plural, flagella): A long "hair-like" motile organelle (commonly ~20 - 150 μm or greater in length) used by certain algae and other microorganisms for swimming. Its internal construction includes 9 pairs of peripheral microtubules (proteinaceous fibers) surrounding 1 pair of central microtubules. Flagella occur in small numbers, most commonly in pairs.

Gamete: Sexually reproductive cells that act as "males" and "females" in sexual fusion.

Heterotrophy: The mode of nutrition in which an organism utilizes organic compounds provided by other organisms for nutrition, either in particulate (phagotrophy) or dissolved (saprotrophy) form.

Ichthyotoxin: A toxic substance that is lethal to fish.

"Light" (Photosynthetic Photon Flux Density): The number of photons (quanta) in the 400- to 700-nanometer (10^{-9} meters) waveband incident per unit time on a known amount (unit) of surface area (i.e., the photon flux density of photosynthetically active radiation in μEinsteins m^{-2} sec^{-1}) (Wetzel 1983).

Light Absorption: The diminution of light energy with depth by transformation to heat (Wetzel 1983).

Marine: Coastal and offshore habitats at ~ full-strength salinity (usually at 30-35‰).

Mesotrophic: Moderate in production; intermediate between eutrophic and oligotrophic.

Mixotrophic: Having both autotrophic and heterotrophic modes of nutrition.

Monospecific: Referring to an algal bloom that consists mostly of 1 species. Most red tide dinoflagellate blooms are monospecific, but *Pfiesteria piscimorte* (nov.gen., nov.sp.) typically occurs as a minor or subdominant component of mixed phytoplankton communities.

Naked (in dinoflagellates): Species without a protective outer "armor" of cellulose plates; sometimes these species are referred to as "gymnodinioid."

Neurotoxin: A toxic substance that interferes with functioning of the neurological system. Dinoflagellate neurotoxins typically block transmission of nerve impulses, so that death occurs from muscle paralysis and resulting suffocation (Bold & Wynne 1985).

Neutral Density Screen: A screen that removes all light qualities (colors) or wavelengths equally.

Oligotrophic: Low in nutrients and low in plankton production. Oligotrophic waters generally are clear with high light penetration, low in plant nutrients especially in the water column, abundant in water-column DO at all depths at all times, and high in species diversity. In estuarine or coastal habitat, the term usually has a more restricted meaning and refers to waters that are low in nutrients.

Photosynthetically Active Radiation:
Radiant energy (from the sun) in the 400- (blue) to 700- (far-red) nanometer waveband of the visible spectrum (Wetzel 1983).

Phytoplankton: Microscopic algae that are suspended in the water column. Most phytoplankton have only limited ability to control their location, and tend to be distributed by water currents and wind mixing. Note: A given species of phytoplankton is sometimes referred to as a phytoplankter.

Planozygote (in dinoflagellates): Motile zygote; product of sexual fusion by gametes, having 4 flagella (= 2 from the "male" cell + 2 from the "female" cell).

Plate Formula: The numbered series of armored plates that is used for formal identification of armored dinoflagellates. The plate formula gives the number of each plate type by location on the cell (refer to Bold & Wynne 1985, Steidinger *et al.* 1989 for detailed information on each component of the plate formula).

Population: A defined assemblage of individuals of one species.

Production: The increase in biomass (weight, volume) formed over a known period of time (an accumulation over relatively long periods, usually on an annual basis).

Protozoan: Unicellular or acellular (plasmodial or slim mold-forming) animals, usually microscopic. For example, amoebae and ciliated microfauna are types of protozoans. Note: Many [heterotrophic] dinoflagellates fit this definition more closely than that of the [photosynthetic] "algae," although heterotrophy is a well-developed mode of nutrition by many photosynthetic algae ("mixotrophy"). Dinoflagellates typically are regarded as animals by zoologists. They are also widely considered as plants, however, because some of the most famous species (e.g., the symbiotic dinoflagellates that provide food for reef-forming corals) and some of the most infamous (e.g., the red tide formers) are photosynthetic.

Red Tide: A "bloom" or high concentration of toxic dinoflagellates in coastal marine waters. The cell abundance (and contained pigments) results in discoloration of the water, which actually may appear yellowish, brown, orange, greenish, or red.

Rotifer: Member of Phylum Rotifera; a microscopic animal having a rotating anterior ring or "wheel" of cilia, and a posterior extension called a "foot;" seasonally abundant animals in shallow waters and sediments of rivers, riverine impoundments, and estuaries.

Salinity: The saltiness of water, usually expressed as grams of dissolved salts per kilogram of seawater or as parts per thousand (‰ or ppt). Salinity is defined as the weight of solids obtained by drying 1 kg of water under standard conditions (Dawes 1981).

Salt Wedge: A lower stratum of water that forms as saline ocean water moves up-river. The more saline water is heavier than fresh water, so it sinks to the bottom. (Also see "stratification.")

Shellfish Poisoning: Concentration of toxic phytoplankton (or other toxic microorganisms) by filter-feeding shellfish. The shellfish may or may not be adversely affected; shellfish poisoning usually refers to situations in which the shellfish appear healthy and unaffected, but other animals in the food chain as well as unsuspecting humans may consume the contaminated shellfish and be adversely affected. The risk for shellfish poisoning is often highest with consumption of the entire animal, including the hepatopancreas and other organs that concentrate the toxin.

Stratification: State in which distinct layers of water form in an estuary, often as a result of salinty- or thermally-related density differences (e.g., warm water at the surface is lighter than cold water at depth; fresh water is lighter than salt-water). In eutrophic estuaries during calm weather and high temperatures in summer, the lower depth of the water column often develops a "salt wedge" or layer of more saline water at the bottom, as the ocean waters penetrate up-river. The saline lower stratum of water often becomes low or depleted in dissolved oxygen, since it is isolated from the surface and receives dead, decomposing plant and animal remains as they settle out. This condition is often referred to as "anoxia in the bottom-water salt wedge."

Trophic Level: Functionally similar organisms within a biological community. E.g., all primary producers generally are considered to comprise the lowest or primary trophic level that supports the remainder of the food web.

Trophic Structure (of a community):
Refers to the pathways by which energy is transferred and nutrients are cycled through the community trophic levels. Primary producers include phytoplankton, benthic microalgae, and submersed or floating plants; they are eaten by primary consumers or herbivores (second trophic level), which are successively consumed by secondary, tertiary, etc. consumers (third, fourth trophic levels, etc.), up to the "top" carnivores of the food web. But note: This "classic" definition fails to consider organisms that can function in both plant-like and animal-like (mixotrophic) roles.

Vegetative (as in vegetative reproduction or vegetative cell): Asexually produced.

Zooplankton: Microscopic animals (ciliated protozoans, rotifers, microcrustaceans, etc.) that are suspended in the water column. Most have only limited ability to control their location, and tend to be distributed by water currents and wind mixing.

Zygote: Product of sexual reproduction, arising from fusion of "male" and "female" cells.