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Pfiesteria piscicida and other *Pfiesteria*-like dinoflagellates: Behavior, impacts, and environmental controls

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Abstract

Toxic *Pfiesteria*-like dinoflagellates have been implicated as causative agents of major fish kills (affecting 10^3–10^9 fish) in estuaries and coastal waters of the mid-Atlantic and southeastern U.S. Transformations among an array of flagellated, amoeboid, and encysted stages in the complex life cycle of the representative species, *Pfiesteria piscicida,* are controlled by the availability of fresh secretions, blood, or other tissues of fish prey. *P. piscicida* also is a voracious predator on other estuarine microorganisms. *Pfiesteria*-like dinoflagellates require an unidentified substance(s) commonly found in fresh fish excreta-secreta to initiate toxin production. *P. piscicida* is lethal to fish at low cell densities (>250–300 cells ml^{-1}), and at sublethal levels (~100–250 cells ml^{-1}) it has been shown to cause ulcerative fish diseases. *P. piscicida* also has been linked to serious human health impacts. This dinoflagellate is eurythermal and euryhaline, with optima for toxic activity by the most lethal stage (toxic zoospores, TZs) at ≥26°C and 15 psu, respectively. Thus far it has shown no light optimum and is capable of killing fish at any time during a 24-h cycle. In warmer waters (≥15°C) flagellated stages predominate while fish are dying, whereas amoebae predominate in colder conditions and when fish are dead. Nutritional stimuli influencing *P. piscicida* are complex; inorganic phosphate apparently can directly stimulate TZs, whereas inorganic phosphate and nitrate indirectly promote increased production of nontoxic zoospores (NTZs, maintained in the absence of live fish, as potential precursors to lethal TZs) by stimulating their algal prey. Organic phosphate (P_o) and nitrogen are taken up by *P. piscicida* osmotrophically, and P_o is stimulatory to both TZs and NTZs. The available data point to a critical need to characterize the chronic and acute impacts of toxic *Pfiesteria*-like dinoflagellates on fish and other targeted prey in estuarine and coastal waters that are adversely affected by cultural eutrophication.

The diverse heterotrophic dinoflagellates (Pyrrhophyta) include free-living estuarine species that demonstrate pronounced chemosensory "ambush-predator" behavior toward algal, protozoan, or fish prey (Spero and Moree 1981; Spero 1982; Ucko et al. 1989; Burkholder et al. 1995*a,b,* 1997*a*; Landsberg et al. 1995). This behavioral pattern apparently is widespread; thus far, it has been reported from the Mediterranean Sea, the Gulf of Mexico, and the western Atlantic. In each case the feeding activity has been strikingly similar: the dinoflagellates swarm up from benthic dormant cysts when they chemically detect the prey's presence. They devour the prey—described in one case as being ripped apart in a "feeding frenzy" (Spero and Moree 1981)—and then rapidly re-encyst.

Known dinoflagellate species with this behavior include the toxic representative *Pfiesteria piscicida* Steidinger & Burkholder (nomen nudem *P. piscimorte, P. piscimortuis*; Steidinger et al. 1996*a*) and at least one other toxic *Pfiesteria*-like species (Steidinger et al. 1996*b*; K. Steidinger pers. comm.; J.M.B and H.B.G. unpubl. data confirming toxicity). Not surprisingly, *Pfiesteria* spp. were first detected following accidental contamination of established prey cultures (Spero and Moree 1981; Smith et al. unpubl.; Noga et al. 1993; Landsberg et al. 1995). Other similar and apparently co-occurring dinoflagellate species, including toxic forms, likely have been overlooked because they are small and closely resemble many common nontoxic estuarine dinoflagellates without diagnostic characters that readily enable identification by light microscopy (Spero and Moree 1981; Burkholder et al. 1992).

Pfiesteria-like species are prey generalists that attack a wide array of finfish and shellfish after detecting an unknown substance(s) in the fresh prey secreta (Burkholder et al. 1995*b*). When live fish are not available, the nontoxic stages consume bacteria, algae, and microfauna; they also have been observed to scavenge fish remains (Burkholder and Glasgow 1995). A second major distinction that separates *Pfiesteria*-like species from other toxic dinoflagellates is their highly complex life cycles, which include rapid transformations among flagellated, amoeboid, and encysted forms with remarkable size range (e.g. *P. piscicida,* with cell length 5–450 μm depending on the stage and diet; Burkholder and Glasgow 1995; Figs. 1–3). The life cycles of *Pfiesteria*-like

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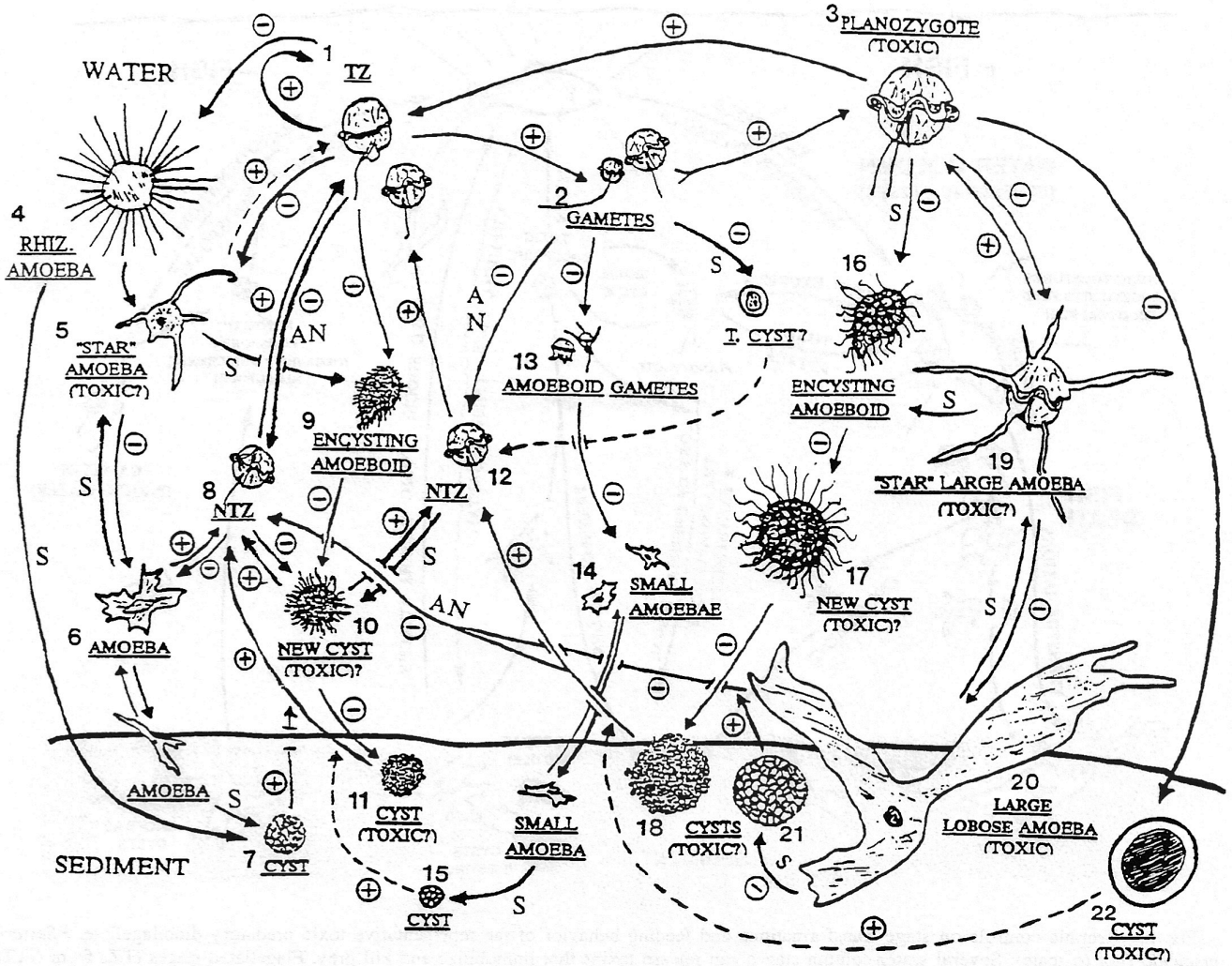

Fig. 1. Schematic of the complex life cycle of *Pfiesteria piscicida* with (+) and without (−) live finish (A—presence of certain flagellated algal prey such as *Cryptomonas* sp., *Dunaliella tertiolecta,* or *Isochrysis galbana*; N—nutrient enrichment as organic or inorganic N and P; S—environmental stress such as sudden shift in temperature or salinity, or physical disturbance). Solid lines—verified transformations; dashed lines—hypothesized pathways. NTZs (stages numbered 8, 12) are produced by lobose amoeboid and cyst stages, by gametes (stage 2) through "reversion" or loss of sexual activity, and by TZs (stage 1—toxic flagellated vegetative cells, TFVCs) through cessation of toxin production. NTZs are similar to "-" anisogamous gametes and TZs in appearance, except for slightly smaller size; these stages are considered immediate precursors to the most lethal form (TZs). Cysts include stages with reticulate covering (stages 7, 19—from amoebae), scaled covering (± bracts; stages 10, 11, 17, and 18—from TZs, NTZs, and planozygotes), and hyaline covering (stage 22)—(presumed hypnocyst) with interior large, swollen, darkened cell. Gametes also have been observed to rapidly form presumed temporary cysts (T.CYST) with thick mucus covering, which may settle out of the water column (arrow not shown); if these cysts yield viable cells, they likely produce zoospores (modified from Burkholder and Glasgow 1995 to include recent observations about rhizopodial amoebae—stage 4, planozygote transformations, and other pathways in the life cycle). Other stages are suspected with uncertain position or role in the life cycle (e.g. saccate stages and a small ephemeral flagellated stage with tear-drop shape that has appeared in abundance on several occasions immediately after fish death).

dinoflagellates also mark them as distinct from all other known free-living estuarine and marine dinoflagellates (Taylor 1987*a,b*; Fensome et al. 1993). The cryptic amoebae, which predominate, previously were not recognized as dinoflagellates (Sawyer 1975; Sawyer et al. 1977; Fensome et al. 1993) and often resemble debris (Burkholder and Glasgow 1995). Some of these amoeboid forms are toxic; all are active in both the water column and, more frequently, the sediments (Burkholder et al. 1992; Burkholder and Glasgow 1995).

The representative species, *P. piscicida,* has been examined in most detail and has been implicated as a causative agent of major finfish and shellfish kills in representative estuarine ecosystems and aquaculture facilities of the mid-Atlantic and southeastern U.S. (Burkholder et al. 1995*a*). Accumulating evidence indicates that toxic *Pfiesteria*-like

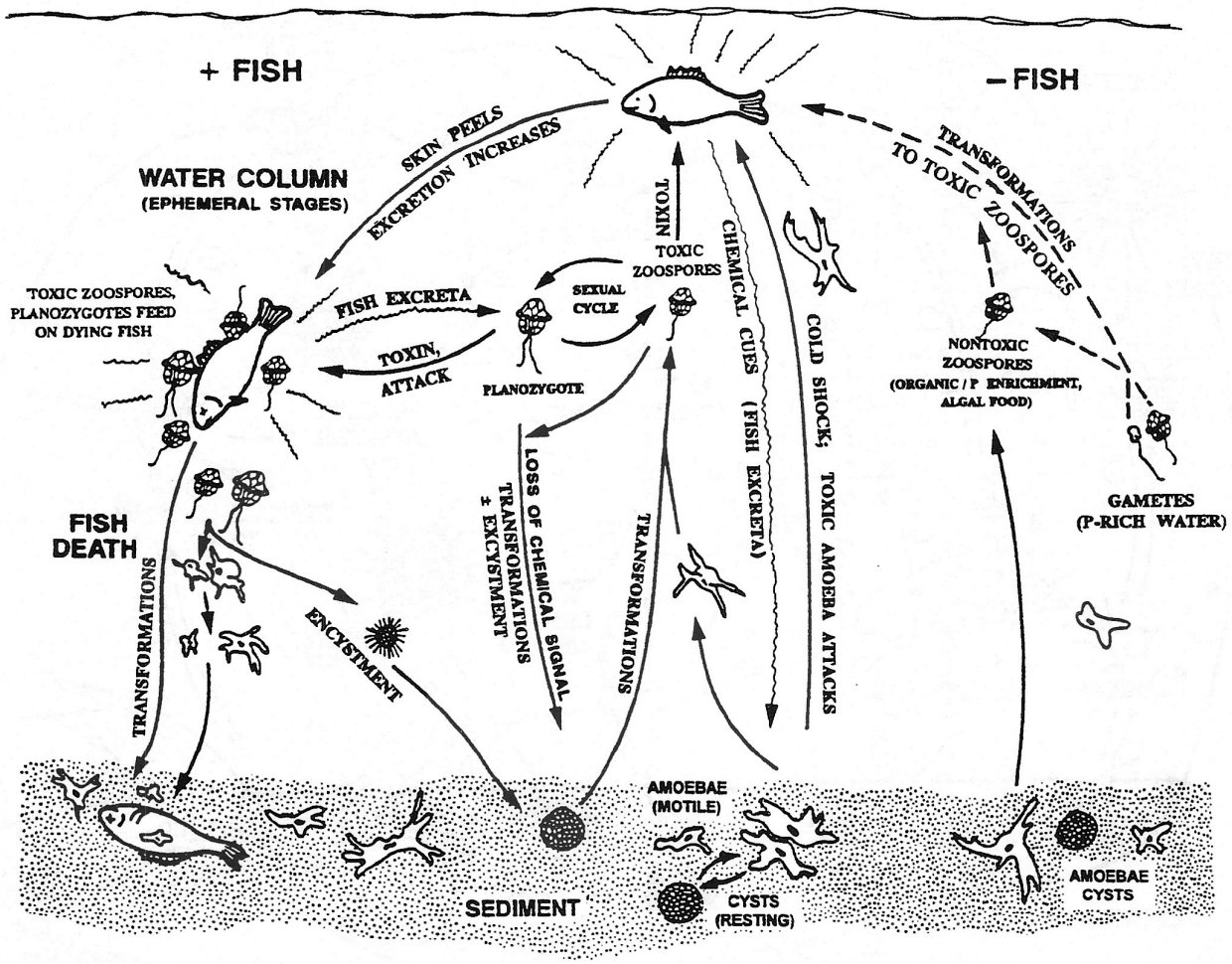

Fig. 2. Trophic controls on stage transformations and feeding behavior of the representative toxic predatory dinoflagellate, *Pfiesteria piscicida* (not to scale). Several water-column stages can release toxins that immobilize and kill prey. Flagellated stages (TZs from NTZs, as well as planozygotes and gametes) attack and feed on fish excreta, secreta, and tissues. After fish death, these flagellated forms either encyst or transform into amoebae and continue to feed. In cold temperatures, large lobose amoebae attack fish with similar behavior as TZs under warmer conditions (≥15°C). When fish are absent, active amoebae persist in the sediments and, less commonly, in the water column as long as alternate microbial prey are available. Gametes (produced from TZs when stressed fish were present) generally remain active as amoeboid forms in phosphate-rich water without fish. Alternatively, upon fish death, gametes and TZs revert to asexual NTZs (also produced by amoebae) when flagellated algal prey are abundant, especially under nutrient-enriched conditions. This schematic was based on data from more than 100 trials (hours to weeks in duration) in which fish access was varied (from Burkholder and Glasgow 1996).

dinoflagellates such as *P. piscicida* likely are widely distributed in temperate-subtropical regions, acting as significant but often undetected sources of fish mortality and disease. Here we review the current status of knowledge about the biology and ecology of *Pfiesteria*-like dinoflagellates, including environmental and trophic controls on their behavior and life cycles, their geographic distributions, their role in fish kills and disease epidemics, and their recent linkage to serious adverse impacts on human health. This work also presents new information about the activity of *P. piscicida* when presented with live fish over diurnal periods; the nutritional ecology of *P. piscicida* when live fish are not accessible, with supporting evidence from field distributions of *Pfiesteria*-like dinoflagellates in relation to sewage outfall sites; and recent fish kills and fish epizootics linked to toxic *Pfiesteria* species.

Considerations for culturing *Pfiesteria* and *Pfiesteria*-like species

Culture isolates of *P. piscicida* for experimentation have been collected from various known sudden-death fish kill sites, during in-progress kills with dying finfish and shellfish, and active blooms of toxic zoospores (TZs; Burkholder et al. 1995*a*). The cultures are maintained in an isolated, quarantined modular biohazard III facility under 3.0×10^{19} quanta m^{-2} s^{-1} (cool-white fluorescent lamps) at 18°C with a 12 : 12 L/D cycle

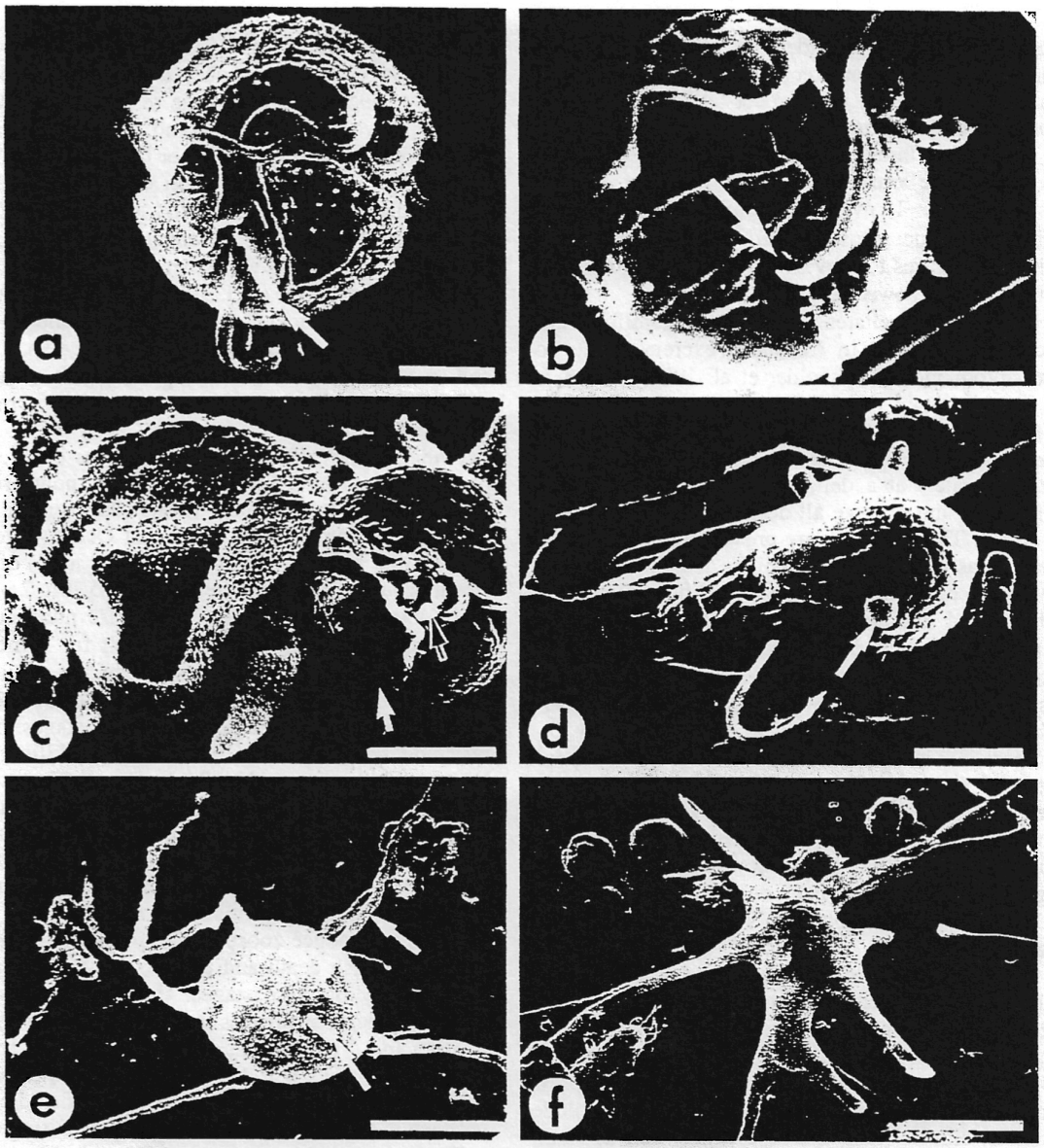

Fig. 3. Scanning electron micrographs of representative flagellated and encysted stages of *Pfiesteria piscicida* from fish-killing cultures. [a.] TZ, the most lethal stage, with retracted peduncle (arrow) (scale bar = 3 μm). [b.] Planozygote formed by gamete fusion, with two longitudinal flagella (upper arrow) and retracted peduncle (lower arrow) (scale bar = 3 μm). [c.] Detailed view of a large lobose amoeba with reticulate organic covering, adjacent to a swollen TZ that was beginning to transform (note the retracted peduncle, white arrow, and the small emerging pseudopodia, black arrow) (scale bar = 5 μm). [d.] TZ in transformation to a filipodial amoeba, with longitudinal flagellum still attached but with amorphous hypotheca or lower cell portion (to left), and with knoblike emerging pseudopodia (arrow) (scale bar = 3 μm). [e.] Filose amoeba in apical view showing emerging and extended, tapering pseudopodia (lower and upper arrows, respectively). When a filose amoeba is disturbed (as observed under light microscopy after tapping and slightly moving the culture plate), it attaches to the bottom of the culture vessel by the tip of one pseudopodium, and undulates the other pseudopodia in the water (scale bar = 3 μm). [f.] Large lobose amoeba that transformed from a planozygote, shown for comparison with TZs which generally are 15–20-fold smaller in maximum cell dimension (scale bar = 20 μm).

(fluorescent bulbs) in 40-liter aerated, covered aquaria filled with artificial seawater at 15 psu salinity (derived by adding Instant Ocean salts to deionized water). This facility has 12 air exchanges per hour and specially designed air flow that is checked weekly by NCSU environmental safety personnel. Contact with culture water and aerosols has been associated with serious human health effects (Glasgow et al. 1995). Hence, work with toxic cultures in the presence of live or freshly dead fish (dead <24 h) is completed using full face respirators with organic acid filters; disposable gloves, boots, and hair covers; and protective clothing that is bleached after use to kill all stages of the dinoflagellates (≥30% bleach). Further,

the aquaria used to culture high densities of toxic stages of *Pfiesteria*-like dinoflagellates are kept in a specially engineered, fully contained isolation unit with proper air flow to prevent exposure of laboratory personnel to toxic fumes from the cultures. Air is expelled through carbon, molecular sieve, and high-efficiency particle arrester filters, and then is exhausted through a stack that is ~10 m high, in compliance with OHSA standards for neurotoxic aerosols. The isolation unit system also includes mechanisms to prevent outward aerosol contamination in the event of power failure.

Pfiesteria-like dinoflagellates require an unidentified substance(s) commonly found in fresh fish excreta-secreta to initiate toxin production (Burkholder et al. 1992); hence, it is necessary to maintain toxic cultures using live fish. We routinely feed these dinoflagellates tilapia (*Oreochromica mossambica* Peters, length 5–7 cm, cleaned by rinsing gently with deionized water) at a density of 70 fish per 25-liter aquarium per day, and remove all dead fish as live replacements are added. These aquaria are preconditioned, aerated, and maintained as described in detail by Burkholder et al. (1995*a*), with fish controls consisting of test fish without the dinoflagellates and dinoflagellate controls consisting of *Pfiesteria* additions to aquarium water without fish. The tilapia selected as the standard test species is not endemic but, nonetheless, is susceptible to the dinoflagellate toxins. It offers the advantages of constant availability, wide salinity tolerance, and certainty of no prior contamination by local populations of toxic *Pfiesteria*-like dinoflagellates. Stock tilapia are cultured in a separate building; to avoid contamination, fish cultures are not visited after personnel have been in the dinoflagellate facility without mandatory shower and change of clothing. These precautions have enabled success in preventing dinoflagellate contamination of stock fish (Burkholder et al. 1995*a*).

Axenic cultures of *P. piscicida* and other *Pfiesteria*-like dinoflagellates have not yet been attained; moreover, transmission electron micrographs have revealed the presence of apparent endosymbiont bacteria in flagellated and amoeboid stages. When grown with direct access to live fish prey, co-occurring microorganisms in the aquarium water include small protozoan ciliates (length ≤20 μm), as well as small populations of the grazers *Stylonychia* cf. *putrina* (larger protozoan ciliate, length ~80 μm) and *Brachionus* sp. (rotifer) which are known to consume *P. piscicida* gametes, nontoxic zoospores (NTZs), and TZs. These animals are uncommon and do not increase in abundance provided that fish are removed within several hours following death. Aquarium water with fish also inevitably develops small populations of blue-greens (Cyanophyceae; *Lyngbya* sp., *Gloeothece* spp., ~2.4×10^5 cells ml^{-1}, quantified following procedures described by Burkholder et al. 1995*a*; nontoxic as inferred by the occurrence of similar populations in control fish cultures without the dinoflagellate, where healthy fish have been maintained for months to years).

The complex life cycle

P. piscicida represents the first known free-living estuarine or marine dinoflagellate with a complex life cycle that involves rapid transformations among at least 24 stages that include flagellates, amoebae, and cysts (Burkholder and Glasgow 1995; Figs. 1–3). The relationship of three stages, including two saccate forms and a small ephemeral flagellated form (~6×4 μm), to other stages remains to be determined. The life cycle has been examined by documenting transformations of isolated cells and hand-picked, isolated populations in response to changing temperature, salinity, and availability of fish and other prey (Burkholder and Glasgow 1995; Steidinger et al. 1996*b*). Contaminants in the medium with hand-picked populations included only bacteria and blue-greens (*Lyngbya* sp., *Gloeothece* sp.; $2.1–2.5\times10^2$ cells ml^{-1}), based on microscopic analysis of acid-Lugol's-preserved samples after *P. piscicida* transformations were documented.

Lethal activity of dominant stages has been compared by placing tilapia (n = 4 per treatment) without prior exposure to *P. piscicida* into aerated filtrate (0.22-μm-porosity Millipore filters) from cultures in Instant Ocean water at 15 psu, with similar biovolumes ($3–5\times10^4$ μm^3 ml^{-1}) of small, mid-sized, or lobose amoebae (length 10–25 μm, 25–50 μm, and >60–400 μm, respectively; in 1% f/2 nutrients and cryptomonad algal prey, and without fish for 2 months); large lobose amoebae (from cultures that had been killing fish 3–4 times daily for 6 months, without live fish for 2 d to induce transformation from larger flagellated stages; note that *P. piscicida* toxins(s) from filtrate of fish-killing cultures is known to denature within 24 h, Burkholder et al. 1995*a*); TZs (>95%, from cultures that had been killing fish 3–4 times daily for 6 months); NTZs (given cryptomonad prey and 1% f/2 medium, without fish for 2 months); and cysts (in f/2 or 1% f/2 medium, without fish for 6 months; note that cysts which are dormant for months usually require 6–8 weeks to produce zoospores, Burkholder et al. 1992). Occurrence of fish death and time to death were used to compare toxicity of these stages. Controls consisted of tilapia placed into similar filtrates that previously had not contained *P. piscicida.* Time to fish death was <4 h in trials with TZ cultures (all fish) and 16–24 h in trials with large lobose amoebae (three of four fish), whereas no fish died in controls without *P. piscicida* or in tests with other stages.

The most lethal known stage, TZs (10–14 μm×8–10 μm, n = 25 cells), consumes microflora and microfauna prey as well as fish substances while multiplying in the presence of live fish. TZs also produce anisogamous gametes which fuse to form planozygotes, thereby completing sexual reproduction, when live fish are available. Depending on environmental conditions and prey availability, TZs may revert to NTZs when denied access to live fish or their fresh tissues [inferred as nontoxic from observations of survival by test tilapia in 24-h trials in ultrafiltered control water (0.22-μm-porosity Millipore filters) with fish but without *P. piscicida,* or in ultrafiltered water from NTZ cultures, as opposed to death of all fish in ultrafiltered water from cultures with >90% of the population as TZs wherein fish had died <1 h before the medium was filtered; n = 4 fish per treatment]. Alternatively, TZs may form cysts with an outer covering of organic, chrysophytelike scales and bracts.

The underlying thick cyst wall is highly resistant to various perturbations. Cysts have been immersed in concen-

trated sulfuric acid (30 min), concentrated ammonium hydroxide (30 min), and bleach (Clorox; 5% hypochlorite, 60 min); they also have been desiccated for 35 d and maintained dormant without fish for 2.2 yr. In each case, there has been ~20% survival when the cysts are added to 15 psu Instant Ocean water with live fish, based on documented excystment under light microscopy (n = 50 cysts per replicated treatment). As other documented options, TZs may form a stage with a thick mucous covering, believed to be temporary cysts (Taylor 1987*a*; Burkholder 1992). Or, they may transform to amoebae following one of two paths as TZs to filose (filipodial) and then lobose (lobopodial) amoebae or TZs to rhizopodial, then filose, then lobose amoebae.

The filose stage seems to be ephemeral and, with long thin pseudopodia that would facilitate suspension in the water column, it likely is involved in dispersal (e.g. Sawyer 1975). Rhizopodial amoebae have been observed to feed on bacteria and small algae for several days and then to transform to filose and then lobose amoebae; alternatively, rhizopodial amoebae may form cysts with reticulate outer covering. The lobose form has been documented to persist indefinitely when given a similar diet of microbial prey such as small flagellated algae and bacteria. Each lobose amoeba can produce two–four NTZs when stimulated by the addition of abundant flagellated algal prey. The amoeba dies after zoospore production, based on shriveling with no further activity for 48 h. Like the NTZs that revert from TZs, the NTZs produced by amoebae are capable of developing toxic activity when they detect live fish [inferred from tests in which tilapia exposed to water from NTZ cultures survived with no signs of stress over 48-h trials; by contrast, 50% of the fish placed directly into water with (initially) NTZs died within 24 h; n = 6]. Without live fish, zoospores consume various microbial prey (bacteria and live or dead small algae and microfauna; Burkholder and Glasgow 1995). Alternatively, the lobose amoeboid stage may form a cyst with a hardened reticulate covering (Burkholder et al. 1995*b*; Steidinger et al. 1995, 1996*b*). Little is yet known about the role of rhizopodial amoebae in the life cycle. Active cells (flagellates or amoebae) can be produced from amoeboid cysts following introduction of high concentrations of mixed algal-bacterial food supplies (e.g. 10^4 *Synechococcus* or *Thalassiosira* spp. with co-occurring bacteria; Glasgow in prep.). Other controls on *Pfiesteria* amoeboid cyst germination have not been determined.

Each planozygote may produce four TZs after a short period of swimming activity; alternatively, it may form a cyst that is larger than, but otherwise similar to, cysts formed by TZs. In transformations that mirror those of the smaller TZs, planozygotes may additionally form larger ephemeral filose and then lobose amoebae. We have not yet observed transformations of planozygotes to rhizopodial amoebae. The lobose amoebae produce reticulate cysts that are larger than, but otherwise similar to the reticulate cysts from amoebae that transformed from TZs (Burkholder and Glasgow 1995). Alternatively, lobose amoebae may produce four NTZs which develop toxin capability as TZs when live fish become available. Planozygotes also have been observed to darken and form cysts with thick mucus covering (Fig. 1, stage 20), presumed to be hypnocysts since a period of dormancy

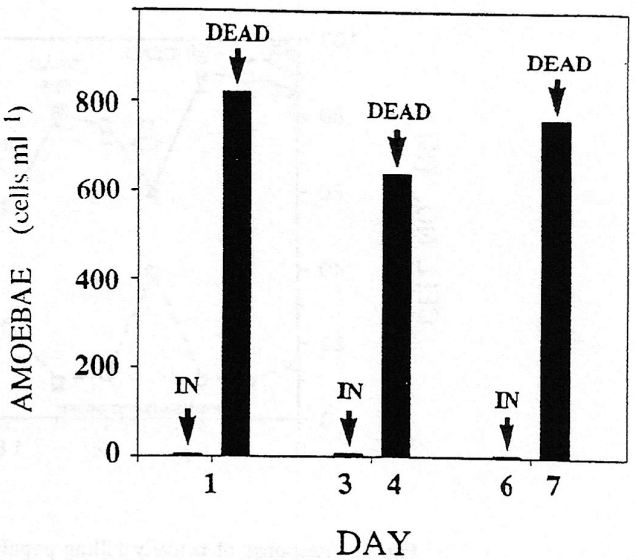

Fig. 4. Apparent lethal activity of lobose amoebae toward juvenile striped bass (day 1) and tilapia (days 3–7) under cold conditions (water temp., 12°C) in repeat-trial laboratory bioassays (38-liter aquaria filled with Instant Ocean seawater at 15 psu; light from fluorescent bulbs, 1.5×10^{19} quanta m^{-2} s^{-1}, 12:12 L/D cycle; four striped bass in trial 1, total length of each fish ~30 cm; 15 tilapia per trial in trials 2–3, total length of each fish ~12–15 cm). Zoospore abundance was negligible (<10 cells ml^{-1}) throughout the 1-d period. Control aquaria with juvenile striped bass or tilapia were similarly maintained but did not contain *P. piscicida*.

(months) has been required before excystment of four TZs in the presence of fresh fish excreta. Gametes without live fish can revert to NTZs that are similar to NTZs produced by other stages in appearance, behavior, and diet. The NTZs, whether from flagellated or amoeboid stages, have been observed to form both scaled cysts similar to those produced by TZs and cysts with thick mucus covering (suspected as temporary cysts).

Environmental influences: Temperature, salinity, light, and physical disturbance

Under field and culture conditions, toxic outbreaks of *P. piscicida* and co-occurring toxic *Pfiesteria*-like dinoflagellates have occurred across a broad temperature gradient from 12 to 33°C, with maximal activity of TZs during warmer seasons when the water temperature is ≥26°C (Burkholder et al. 1995*a*). The most active toxic stage during colder periods is a large lobose amoeba (length ~60–450 μm). This observation was based on dominance by lobose amoebae in aquaria kept under cold conditions with live fish (10–15°C, wherein >90% of the *P. piscicida* population existed as large lobose amoebae that exhibited similar behavior as TZs during warmer seasons, with apparent attraction to fish followed by prey mortality; Fig. 4). We similarly have found >90% of *P. piscicida* cells as large lobose amoebae in research aquaculture facilities along the North Carolina coast during winter (10–14°C; lesions in flounder, and both lesions and death to flounder and scallops; Burkholder et al. 1995*a*).

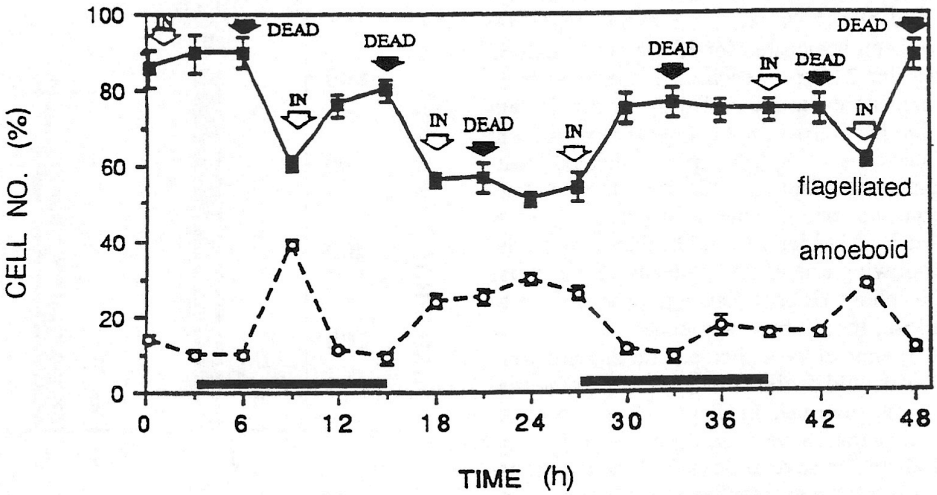

Fig. 5. Response of actively killing populations of *Pfiesteria piscicida* in duplicate cultures to fish availability during two 24-h cycles (IN—introduction of seven live tilapia, each with total length 5–7 cm, into duplicate 27-liter cultures that had been fed fresh fish at least twice daily for the previous 60-d duration; DEAD—point at which all fish were dead and carcasses were removed). The dinoflagellate was capable of lethal activity during dark or light periods, with total abundance of flagellated (TZs, gametes, planozygotes) and amoeboid stages that varied inversely. Flagellated stages reached maximal cell densities when fish were moribund or freshly dead, whereas amoeboid stages seemed to respond more favorably to fish remains (means ±1 SE).

The influences of salinity and light on toxic activity were examined experimentally in aquarium bioassays with fish prey. The optimum salinity for lethal activity by TZs was found to be 15 psu [batch cultures with fish, replicated in triplicate: salinity treatments at 0, 5, 15, 25, and 35 psu as Instant Ocean salts added to a groundwater (softwater) well source at NCSU; Burkholder et al. 1995*a*]. *P. piscicida* was capable of responding more slowly and killing fish at higher or lower salinities ranging from 0 to 35 psu, but at 0 psu it required divalent Ca^{2+} + Mg^{2+} content at >4 mg liter^{-1} for activity and toxin production (Burkholder et al. 1995*a*).

Earlier work testing the influence of light gradients ranging from ~0 to 1.51×10^{20} quanta m^{-2} s^{-1} as photosynthetically active radiation (typical range of available light at depth ≥0.5 m in turbid North Carolina estuaries; Mallin and Paerl 1992) indicated no apparent light optimum for TZs (Burkholder et al. 1995*a*). This observation from bioassays has been supported by field research in which the dinoflagellate was documented to be active in fish kills at night or at full sunlight (~$1{,}446\times10^{21}$ quanta m^{-2} s^{-1}; Burkholder et al. 1995*a*). Laboratory bioassays with fish also were used to examine toxic activity throughout diurnal cycles (two 12 : 12 L/D periods, with light from fluorescent tubes at ~3.0×10^{19} quanta m^{-2} s^{-1}). Under these conditions, TZs were active following introduction of live fish prey, regardless of the time or light availability (Fig. 5).

P. piscicida is sensitive to many types of enclosure effects. For example, flagellated stages (TZs, NTZs, and planozygotes) should be drop-pipetted; if higher pressure is used, the cells encyst within a short time (minutes to several hours; Glasgow et al. 1995). The population must also be gently poured to avoid encystment (e.g. experiments by Mallin et al. 1995), and all methods of mixing solutions with TZs except very gentle swirling (1–2 times at most) have been found to induce encystment. Other toxic dinoflagellates, similarly, have been found to be highly sensitive to mixing of cultures (Heil et al. 1993; Flynn and Flynn 1995; D. Baden pers. comm.). Moreover, if *P. piscicida* TZs are enclosed in standard containers (e.g. carboys, Cubitainers, mesocosms with improper mixing) that are placed in estuarine waters out in the field for more than a few hours, they tend to perform poorly and most of the population (>95%) transforms to amoebae or encysts.

Such sensitivity to mixing, discerned from laboratory research, has been supported by field observations about distribution patterns of *Pfiesteria*-like dinoflagellate activity in natural habitats. During calm weather conditions, the populations may be distributed fairly evenly throughout the water column, but TZs apparently track schooling surface fish such as Atlantic menhaden to the upper 0.5-m-depth (zoospore densities 100–1,000-fold higher among menhaden in distress, located near the water surface). For example, for several days during a *P. piscicida*-related kill of menhaden and other fish in September 1995 (Highway 17 bridge near New Bern, North Carolina, with samples collected from 1000 to 1100 hours), we found 2.52×10^{3} TZs ml^{-1} at depth 0.0 (surface foam) to 0.5 m and <325 cells ml^{-1} at depths ≥2.5 m (cell densities quantified as by Burkholder et al. 1995*a*). But during a storm with substantial wind and rain (1100 hours), we found only $1.5\text{–}3.2\times10^{2}$ zoospores ml^{-1} in the upper 0.5 m of the water column and $1.32\text{–}1.58\times10^{3}$ zoospores ml^{-1} at depths ≥2.5 m. Thus, although we have observed *Pfiesteria*-related kills in rough waters with moderate winds, the more typical situation under such conditions is formation of a "lens" or concentrated layer of TZs on or near the bottom sediments in shallow estuaries including wide expanses of

the Neuse and Pamlico with depth ≤3 m (Glasgow et al. 1995). *Pfiesteria*'s flagellated stages (5–60 μm diam) are capable of traveling at least 3 m d^{-1}, based on laboratory data for swimming velocity together with field observations of population movement. This dinoflagellate thus can easily change vertical location in calm shallow estuarine waters in the course of a 24-h period. Such movement over vertical distance would be less than the documented behavior of various pelagic marine dinoflagellates, which can traverse up to 16 m vertical distance d^{-1} in ocean waters (e.g. Eppley et al. 1968).

Trophic controls on stage transformations: From vertebrates to algae

Interactions with higher trophic levels—Detection of unknown substances secreted and (or) excreted by fish stimulate zoospores of *P. piscicida* to emerge from benthic cysts or transform from amoeboid stages (Burkholder et al. 1995*b*; Fig. 2). We have documented this chemosensory stimulation by fish in several ways. In one approach, 200 TZs were hand-isolated (Olympus BH2 light microscope with micromanipulator, 200×) from fish-killing cultures and placed into ultrafiltered Instant Ocean water (0.22-μm-porosity Millipore filters, 15 psu) within 0.8-μm-porosity dialysis membrane. The dialysis tubing with TZs was placed into new aquaria (15 psu-Instant Ocean water) with live fish that previously had not been exposed to the dinoflagellate. When separated in this manner from direct contact with fish but afforded chemosensory communication, the populations formed small numbers of planozygotes (<10 ml^{-1}), and TZs attained densities of ~$5.2\pm0.3\times10^3$ cells ml^{-1} (mean ±1 SE, $n = 3$) in repeat trials during 15-d periods with frequent replacement of dead with live fish. By comparison, direct access to live fish stimulated more rapid reproduction with ~$9.18\pm1.22\times10^4$ TZs ml^{-1} and common occurrence of planozygotes (>50 ml^{-1}) under otherwise similar conditions.

Lack of access to live fish leads to cessation of sexual reproduction in *Pfiesteria* and loss of toxicity. Within 48 h after live fish were not available, both populations of *P. piscicida* failed to complete the sexual cycle as evidenced by the absence of gamete pairs and planozygotes. In replicated experiments, we tested toxicity by gently gravity-filtering water (without suction pressure, through 0.22-μm porosity Nuclepore filters) from actively killing cultures with daily access to live fish (7–10 tilapia added 2–3 times daily; time to fish death <2 h), and then adding one test tilapia to 100 ml of filtrate. We also experimentally tested fish with filtrate from "inactive" cultures which previously had been lethal to fish, but which had not been given live fish for 14 d. Controls consisted of fish added to filtrate from tilapia cultures without dinoflagellates. Fish in all controls and in filtrate from inactive cultures remained active over the 4-h test period, whereas fish in water from the actively killing cultures died within 3 h. Similar results were obtained when water from both active and inactive cultures was filtered (0.22-μm-porosity Millipore filters) prior to assays with fish.

Using an alternate approach, we hand-isolated small populations of TZs (10–15 cells) and placed them into Petri dishes containing 15 psu ultrafiltered Instant Ocean water. TZ behavior was examined with motion analysis over 120-s trials (Kamykowski et al. 1992) in the presence vs. the absence of fresh fish epidermis. Swimming speed was 655 ± 133 μm s^{-1} (mean ±1 SE; median 670 μm s^{-1}) and 300 ± 190 μm s^{-1} (mean ±1 SE; median 325 μm s^{-1}), respectively, with and without fish tissue. Moreover, swimming direction was noticeably altered from a random pattern without fish detection to a highly directed movement toward the fish tissue (Glasgow and Burkholder unpubl.; Fig. 6).

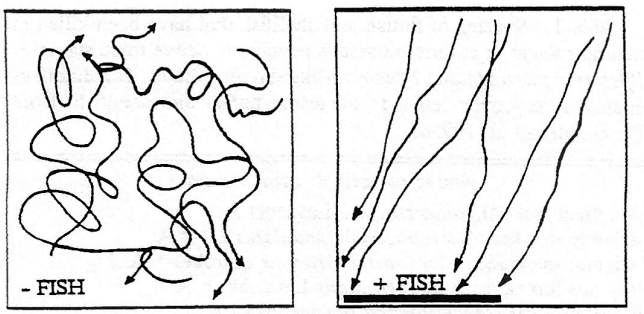

Fig. 6. Chemosensory stimulation of *Pfiesteria piscicida* by fresh fish tissue indicated by motion analysis of six groups of TZs without fish (left panel) vs. with a fresh piece of fish epidermis (right panel; tissue position shown by black bar). Each arrow represents the mean of 10 cells per group. Directed movement is shown as net-to-gross displacement (NGD), a relative measure of path twistedness or convolution, in the absence vs. the presence of fresh fish tissue (each trial = 120 s; a value of 1 describes a straight-line path of travel, whereas a value of 0.50 describes a random path at a 45° angle; Kamykowski et al. 1992). These data were obtained with an Expertvision computer-video system customized for motion analysis following published procedure (Kamykowski et al. 1992), after selecting every sixth frame from a recording made at 30 frames s^{-1} (Dage-MTI CCD 72 camera, black-and-white monitor, and studio-grade Panasonic video cassette recorder VG6300 coupled with a Wild dissecting microscope). Swimming velocity was determined from the same recordings, wherein the overall population was considered irrespective of the swimming direction.

In bioassays with *P. piscicida*, all tested finfish and shellfish species and individuals have died (Burkholder et al. 1995*a*; Table 1). There is an apparent range in prey response to the toxin(s), although narcosis is a common initial sign among all species when exposed to sublethal or lethal TZ densities (100–250 and >250–300 cells ml^{-1}, respectively). Juvenile striped bass are extremely sensitive to the toxin(s) and exhibit signs of distress (darkening, lethargia followed by apparent difficulty in obtaining sufficient oxygen) within a short time (minutes to several hours) after being introduced to toxic *P. piscicida* cultures. As exposure continues, the prominent lymph canal below the dorsal fin can fill with blood as the fish hemorrhage and begin to die. In contrast, species within the guppy family (Poeciliidae) seem less sensitive, requiring days to weeks for lethal effects (Burkholder et al. 1995*a*). Field observations have linked *P. piscicida* kills with flounder "walks," another observed behavioral pattern in which fish with bleeding ventral lesions attempt

Table 1. Species of finfish and shellfish that have been killed in natural habitat or culture bioassays containing active toxic stages of *Pfiesteria piscicida* and *Pfiesteria*-like dinoflagellates. The dinoflagellates have proven lethal to all tested finfish and shellfish (from Burkholder et al. 1995*a*).

Native estuarine/marine species
American eel (*Anguilla rostrata* Lesueur) A
Atlantic croaker (*Micropogonias undulatus* L.) * A
Atlantic menhaden (*Brevoortia tyrannus* Latrobe) * A, J
Bay scallop (*Argopecten irradians* Lamarck) * A
Black grouper (*Mycteroperca bonaci* Poey) A
Blue crab (*Callinectes sapidus* Rathbun) * A
Channel catfish (*Icatalurus punctatus* L.) A
Eastern oyster (*Crassostrea virginica* Gmelin) * P†
Hogchoker (*Trinectes maculatus* Block & Schneider) A
Killifish (mummichog) (*Fundulus heteroclitus* L.) * A
Largemouth bass (*Micropterus salmoides* Lacepede) A‡
Mosquitofish (*Gambusia affinis* Baird & Girard) * A
Naked bogy (*Gobiosoma bosc* Lacepede) A
Northern quahog (littleneck clam; *Mercenaria mercenaria* Linne) L, A
Pinfish (*Lagodon rhomboides* L.) A
Red drum (*Sciaenops ocellatus* L.) * A
Redear sunfish (*Lepomis microlophys* L.) A‡
Sheepshead (*Archosargus probatocephalus* Walbaum) A
Southern flounder (*Paralichthys lethostigma* Jordan & Gilbert) * A
Spot (*Leiostomus xanthuris* Lacepede) * A
Spotted sea trout (*Cynoscion nebulosus* Cuvier) A
Striped bass (*Morone saxatilis* Walbaum) * A
Striped mullet (*Mugil cephalus* L.) A
White perch (*Morone americana* Gmelin) * A
Exotic (introduced) species
Clownfish (clown anemonefish) (*Amphiprion percula* Lacepede) * A
Goldfish (*Carrasius auratus* L.) * A
Guppie (*Poecilia reticulata* Peters) * A
Hybrid striped bass (*Morone saxatilis* × *Morone chrysops* Rafinesque) * A, J
Tilapia (*Oreochromis aureus* Steindachner, *Oreochromis mossambica* Peters, *Tilapia nolotica* L.) * A

* Confirming aquarium bioassays on adults (A), pediveligers (P) or larvae (L), or juveniles (J); mortality of remaining species occurred during field or aquaculture kills in which *P. piscicida* was implicated as the causative agent (based on quantification of toxic stages at ≥250 cells ml^{-1} in water samples from the kills and scanning electron microscopy to verify identification).

† Confirming bioassays on eastern oyster pediveliger larvae were completed by G. Krantz with J.M.B. and H.B.G. (Krantz et al. unpubl.).

‡ Largemouth bass and redear sunfish are considered freshwater species, but they were found in slightly brackish waters.

to leave the water and beach before they die (Burkholder et al. 1995*a*).

In both laboratory bioassays and field kills, representatives from all fish species have developed open bleeding lesions, although lesions are most common on Atlantic menhaden (Burkholder et al. 1995*a*). During lesion formation the epidermal tissue and osmoregulatory functions are destroyed or severely impaired by the dinoflagellate's toxin(s) (Noga et al. 1996). Absence of lesions does not a priori rule out the presence of *P. piscicida,* however, because fish sometimes die too quickly for lesions to develop. Extensive outbreaks of the disease known as ulcerative mycosis have been reported for Atlantic menhaden in the Pamlico estuary since the mid-1980s (Miller et al. 1992; Noga 1993). Up to 98% of all fish present in natural habitats, including all species, can develop such ulcerations especially during May–June and September–October (Miller et al. 1992; Noga 1993; Burkholder et al. 1995*a*). This disease formerly was attributed to a fungal assemblage. Detailed examination by Noga et al. (1996) revealed, however, that the fungi are opportunists; their hyphae generally do not penetrate to the lesion base, suggesting that they do not form the lesions; rather, they colonize lesions formed by toxin(s) from *P. piscicida.* Clinical signs have included severe dermatological intra- and extracellular edema and epidermal necrosis, with severe erosion to the basement membrane; multifocal to regionally extensive lesions that often were apparent before neurological signs; diffuse mild to severe erythema on skin and fins giving the body a dull appearance from destruction of the entire epithelium; mild focal acute myonecrosis in 3–5% of the myocytes; diffuse splenic lymphoid depletion; edematous submucosa in the stomach; and severely increased blood osmolality with elevated serum Na^+, Cl^-, and K^+ (Noga et al. 1996).

Some analogous phenomena have been documented with all assayed shellfish (adult blue crabs, bay scallops, littleneck clams; juvenile clams and eastern oyster pediveligers with adults not yet tested; Table 1). Bioassays with adult blue crabs indicated 10-fold lower production of TZs than when finfish (test tilapia) were also present, however, and crab death occurred more slowly than when finfish were added (Burkholder et al. 1992, 1995*a*). The available evidence thus suggests that shellfish excreta-secreta are less stimulatory to toxic stages of *P. piscicida* than finfish, but that stimulatory substance(s) are produced in varying levels by all finfish and shellfish tested. The signs observed in these prey from aquarium cultures are somewhat similar: motile adult shellfish (scallops, blue crabs) frequently have shown a strong escape response, extending to crab "walks" in which animals attempt to leave the toxic water (Burkholder et al. 1992, 1995*a*). Shells of blue crabs have been damaged to the point that large areas of the carapace are absent and the remaining shell is structurally weakened (observations on six adult crabs, carapace width 7 cm, in bioassays with *P. piscicida* TZs). Sublethal and lethal TZ densities have also been demonstrated to narcotize adult blue crabs, clams, and scallops, which show obvious lethargia (blue crabs), depressed ability to close the valves in fright response (scallops; Burkholder et al. 1995*a*), and significantly decreased filtering activity (oyster pediveligers: Krantz et al. unpubl. scallops: Springer et al. unpubl.). Similar effects of narcosis, depressed feeding and other behavioral alteration have been shown for shellfish exposed to other dinoflagellate toxins (*see* Burkholder 1997). Physiological impacts on shellfish from *P. piscicida*'s toxin(s) remain to be examined.

Feeding trials with tilapia red corpuscles have demonstrated that *P. piscicida*'s TZs and NTZs can phagocytize the red blood cells. More frequently the cells are consumed via myzocytosis (Elbrächter 1991), a process in which flagellated stages (zoospores, negative anisogamous gametes, and planozygotes) attach with an extended peduncle (sometimes almost as long as the cell diameter) and suction the interior

Table 2. Known higher prey of *Pfiesteria piscicida* (from Glasgow and Burkholder unpubl.; Burkholder et al. 1995*a*; Glasgow in prep.), with "+" indicating demonstrated consumption by the dinoflagellate.

Food resource	TZ	Gamete	Planozyg	NTZ	Amoebae
Shellfish					
Oyster pediveligers	+	?	+	?	+
Littleneck clams (adults)	+	?	+	?	+
Clam fingerlings	+	?	+	?	+
Blue crabs (adults)	+	?	+	?	+
Bay scallops (adults)	+	?	+	?	+
Finfish*					
Epidermis*	+	+	+	?	+
Muscle, gill, etc.*	+	?	+	?	+
Red corpuscles*	6±2/10 min	?	+	?	+
Mammals					
Red corpuscles* (human)	6±2/10 min	?	+	?	+

* The epidermis, muscle, gill, and red corpuscles of all finfish species tested (*see Table 1*) must be fresh (separated ≤2 h from the live animal) or from live fish for TZs, but can be either fresh or dead for amoebae. Although fish red corpuscles sustained TZs for long periods (weeks), human red corpuscles (analog for fresh marine mammal tissues) supported growth and survival for only 4 d, after which TZs died or encysted.

cytoplasm and organelles. Both NTZs (acclimated to a diet of the flagellated algal prey, *Dunaliella tertiolecta* Butcher and *Isochrysis galbana* Green, for >2 months) and TZs have shown voracious feeding activity on tilapia red corpuscles, consuming 6±2 cells (mean ±1 SE) on average within a 10-min period (Table 2). Little is known about the response of *P. piscicida* to mammalian prey, except for observations of similar feeding rates by both NTZs and TZs on human (as surrogates for marine mammals) as well as fish red corpuscles (Table 2). Fresh fish tissues including blood cells can sustain zoospore populations indefinitely (months); by comparison, zoospores engorge on the mammalian red corpuscles to the point that they no longer can swim, but they die after 3–4 d when given only this food source [100% of all zoospores shriveled with no apparent signs of metabolic activity after 48 h (n = 50; Glasgow and Burkholder unpubl.)]. Culture filtrate (0.22-μm-porosity Millipore filters) from zoospores given mammalian blood have not shown toxicity to fish in bioassays; fish death or apparent distress were not observed after 24 h, comparable to control fish in ultrafiltered culture medium without *P. piscicida* contamination (n = 3). By contrast, test fish placed into ultrafiltered medium from fish-killing cultures with TZs (>90% of the *P. piscicida* population; fish death had occurred <1 h before filtering) died in <4 h.

Finfish and shellfish kills in estuaries and aquaculture facilities—The role of *Pfiesteria*-like dinoflagellates in fish kills in estuarine ecosystems and aquaculture facilities has been examined in the ~5-yr period since *P. piscicida*'s TZs were first discovered swarming in the water during a fish kill in the Pamlico River estuary in May 1991 (Burkholder et al. 1992; Table 3). The most complete investigations are available for a 3-yr period from 1991 through 1993 and for 1995–1996. In the first 3 yr, laboratory bioassays to confirm toxicity of water from the fish kills were completed by adding test tilapia to aquaria without previous *P. piscicida* contamination and then monitoring dinoflagellate response as TZ abundance and behavior. During 1994–1995 our laboratory was redesigned to ensure human health safety while working with toxic cultures; hence, facilities were not available to demonstrate toxicity of *Pfiesteria*-like populations from field kills in laboratory bioassays. During 1991–1993 the presence of lethal densities of TZs (>250–300 cells ml^{-1}) along with dying or freshly dead fish was strongly correlated with toxicity as confirmed by bioassays (r^2 = 0.94) indicating that similar TZ densities at two fish kills in 1995 could be linked with high probability to toxic *P. piscicida* involvement (Table 4). *P. piscicida* and a second toxic *Pfiesteria*-like species were implicated as the major causative agents in three of seven major fish kills during 1996, but hurricane-related anoxia, with background eutrophic conditions (Burkholder 1996), was associated with the most fish death (Table 4). In 1997, however, fish-kill season began early (mid-June); during a 4-week period, ~6×10^5 fish and 4×10^5 fish with clonal lesions died in the mesohaline Pamlico and Neuse estuaries, respectively. This fish death was linked to toxic activity (confirmed in laboratory bioassays with fish) by two *Pfiesteria*-like species (TZ densities: 380–1,200 cell ml^{-1}) and occurred before the onset of bottom-water hypoxia.

Most fish counts for kills in the wild were completed by North Carolina Division of Water Quality and Division of Marine Fisheries personnel (NC DWQ, formerly the Division of Environmental Management, and NC DMF, respectively; central database initiated by K. Miller), with counts also contributed by H.B.G. Dead fish were quantified following established guidelines (shore-to-shore transect method, extrapolated to the estimated total area affected; Am. Fish. Soc. 1982; Meyer and Barclay 1990), with an average taken from estimates made independently by two scientists at each kill. In implicating these dinoflagellates as the major

Table 3. Environmental conditions and fish affected at kills linked to *Pfiesteria*-like dinoflagellates in North Carolina estuaries, coastal waters, and aquaculture facilities during 1991–1993 and 1995. Data are given as the mean ±1 SE. Nutrient data (total P, TP; and total Kjeldahl N, TKN) were not available (NA) for the coastal waters or aquaculture systems (adapted from Glasgow et al. 1995).

Habitat	*n**	Temp. (°C)	Salinity (psu)	Nutrients (μg liter^{-1})	*Pfiesteria*† (cells ml^{-1})	Fish affected†
Estuaries						
Neuse	9	28±1	10±1	TP 210±40 TKN 1,010 ± 305	940±130	Atlantic menhaden, blue crab, catfish, mullet, spot, white perch (>1×10^9)
Pamlico	11	29±1	7±1	TP 290±4 TKN 580±45	4,300±2,510	Atlantic menhaden, American eel, blue crab, croaker, hogchoker, southern flounder, spot (>2×10^6)
Coastal						
Taylor's Creek near WWTP, inner channel	1	15	30	NA	11,960	Southern flounder, American eel, sheepshead (2×10^4)
Open Atlantic	2	13±4	35	NA	1,400*	Atlantic menhaden (>6×10^3)
Aquaculture	11	17±2	22±3	NA	820±340	Atlantic menhaden, bay scallop, hybrid striped bass, littleneck clam, naked goby, sheepshead, southern flounder, tilapia, white perch (>4×10^4; >$140,000 loss)

* Neuse includes seven major kills defined as affecting ≥10^3 fish, and two moderate kills affecting several hundred fish; Pamlico includes 10 major kills; one major kill in nutrient-enriched coastal waters, Taylors Creek; relatively nutrient-poor coastal waters with major kills at Wrightsville Beach and Topsail Beach; aquaculture facilities include both indoor tanks and outdoor ponds.

† Data include both flagellated and amoeboid stages except for 1991, in which amoeboid stages were not recognized as part of the life cycle. The large error term in Pamlico *Pfiesteria*-like dinoflagellate abundance reflects interannual variability as well as other factors, such as the time lag between the kill and sampling. Fish-affected data include the total number of fish known to have been involved in all kills linked to *P. piscicida* and another toxic *Pfiesteria*-like species, and the total cost for all reported aquaculture kills. Note that of the nonenriched kill sites, the Wrightsville Beach area (3 km from shore) was sampled 2 d after the kill and contained mostly amoeboid forms along with ~50 TZs ml^{-1}. This sample was not considered among the quantitative information used to construct this table because of the substantial time lapse between the kill and the sampling effort.

causative agent of a fish kill, we erred on the side of caution. Even when high densities of toxic *Pfiesteria*-like dinoflagellates (e.g. >2,000 cells ml^{-1}) were swarming in the water during a given kill, we considered the dinoflagellates a contributing lethal factor but did not implicate them as the major causative agent if other causative factors could be identified. Only when dissolved oxygen (DO) was adequate to maintain fish life (≥5 mg liter^{-1} except in the lower third of the water column, unless the fish affected were benthic species such as blue crabs or flounder), and all other known factors could be ruled out based on available data, was toxic *Pfiesteria*-like species implicated as the probable major causative factor (Burkholder et al. 1995*a*). When DO was low in the bottom water and primarily benthic species had been affected, the kills were attributed to low DO as the primary causative factor. We ruled out low DO as the probable cause of other fish kills when hypoxia or anoxia occurred only in the lower third of the water column, based on consideration of the fact that the major species of fish affected in *Pfiesteria*-related kills are Atlantic menhaden (90% of the affected animals; Burkholder et al. 1995*a*). Menhaden are surface schooling fish that avoid the lower water column (Manooch 1988; Migdalski and Fichter 1989). Moreover, we sampled many of the kills while menhaden were dying. In these efforts we often found fish in distress with open bleeding lesions and erratic behavior during the mid-to-late afternoon where DO was more than adequate and likely had been for hours due to algal photosynthesis (Miller et al. 1992). Menhaden are sensitive to low DO and die quickly once they begin to suffocate (Noga 1993). If DO had been low throughout the water column during the previous night, we would have expected to find dead, rather than dying, fish by that time of day; and low DO would not have explained the presence of lesions, most of which occurred in the cloacal area where materials known to attract TZs are excreted (Burkholder et al. 1992).

During 1991–1993 *Pfiesteria*-like dinoflagellates were highly active in toxic outbreaks and were implicated as the causative factor of 52±7% of the 35 major fish kills (affecting 10^3–10^9 fish) in North Carolina's estuaries and coastal waters (nine of 17 kills in 1991, four of eight kills in 1992, five of 10 kills in 1993; Burkholder et al. 1995*a*) (Table 3). Most of the remaining kills that did not involve *Pfiesteria*-like dinoflagellates were related to low DO in the bottom waters [NC Department of Environment, Health & Natural Resources (NC DEHNR) unpubl. fish kill records]. All but three *Pfiesteria*-related kills of wild fish occurred in estuaries (salinity 2–18 psu). Most involved Atlantic menhaden as the dominant affected species (Burkholder et al. 1995*a*), which is logical considering that these fish are naturally oily with copious secreta, and they are known to school in large, tightly packed aggregates. Both characteristics would favor detection by the dinoflagellate when the fish entered quiet, poorly flushed estuarine tributaries and lingered to feed. Atlantic menhaden was the major species affected in the remaining three coastal kills, as well as in the estuarine kills.

Table 4. Major fish kills (>1,000 fish affected) in North Carolina's estuaries during summer 1995 and 1996.

Ecosystem	Duration (d)	No. of fish	Implicated cause(s)
1995			
New River estuary	4	>1,000	Massive spike of raw swine effluent (ruptured lagoon); toxic ammonia levels, anoxia*
New River estuary	1?	~10,000	Lethal *Pfiesteria*-like species densities preceding kill; 2nd harmful alga, *Phaeocystis globosa* Scherffel, also known to be stimulated by raw sewage (Hallegraeff 1993); sublethal *Pfiesteria* 2 d after kill*
Neuse estuary	5	100,000s	Hypoxia; sublethal *P. piscicida* 2 d after kill*
Pamlico estuary	11	100,000s	Lethal *Pfiesteria*-like species during kill (low DO in lower third of water column)
Roanoke River	2	100,000s	Discharge from faulty management of water level in upstream reservoir led to hypoxia-anoxia
Goose Creek (Neuse estuary)	2	110,000	Anoxia, heavy noxious H_2S fumes; also lethal *P. piscicida* during kill (up to 4×10^3 TZs ml^{-1})
Neuse estuary	90†	15,000,000	*P. piscicida* swarming at lethal densities (TZs at 4×10^2–2.9×10^3 cells ml^{-1})
1996			
Cape Fear estuary	14?	1,000,000s	Anoxia (after hurricane)‡
Neuse estuary	2–3	1,000s	Lethal *Pfiesteria*-like species during kill, with TZs at ~4×10^2–1.2×10^3 cells ml^{-1} (hypoxia in lower third of water column)
	3	~10,000	Hypoxia (after hurricane)‡
	2–3	1,000s	Lethal *Pfiesteria*-like species during kill, with TZs at ~8×10^2 to 1.1×10^3 cells ml^{-1} (hypoxia in lower third of water column)
	14	1,000,000s	Anoxia (after hurricane)‡
New River estuary	3	1,000,000s	Lethal *Pfiesteria* during kill, also with *Gyrodinium aureolum* Hulburt§ (hypoxia in lower third of water column)

* In the Neuse estuary kill, data were not available for densities of *Pfiesteria*-like species while fish were dying. Several days before the New River estuary kill, the upstream freshwater area of the river had sustained a massive raw swine effluent waste spill from a ruptured holding lagoon (~1×10^8 liters), which also led to a freshwater fish kill (~4,000 fish affected; Burkholder et al. 1997*b*). Note: Many other kills were anecdotally reported by fishermen who maintained, for example, that few adult menhaden were available to die in the Pamlico because the young or "peanuts" had been dying throughout late spring and summer.

† Fish death from 22 July to 20 October; most fish death from 20 September to 20 October.

‡ Summer 1996 was unusually wet and cool and included two hurricanes (Bertha in July; Fran in September). The combination of cool wet conditions and frequent moderate to severe storms kept salinities low in mid–upper estuaries, so that large schools of menhaden stayed in the lower estuaries and the sounds. These factors probably discouraged growth and toxic activity by *Pfiesteria*-like species; 1996 was associated with fewer kills than any other year in which these dinoflagellates were tracked.

§ The New River estuary kill was associated with ~400 TZs ml^{-1} of a *Pfiesteria*-like species and 2.5×10^3 cells ml^{-1} of a second potentially toxic dinoflagellate, *Gyrodinium aureolum* (Steidinger 1993).

The coastal kills occurred up to 6 km offshore and likely resulted from *Pfiesteria* populations being transported down-estuary with the fish in their normal seasonal migratory pattern. Despite the predominance of menhaden in *Pfiesteria*-related kills, death occurred for all observed and sampled finfish and shellfish species in the affected area (Table 3). Similarly, during episodes of sublethal activity, all finfish species present were affected by open bleeding sores and blue crabs frequently were noted with visible signs of shell disease.

P. piscicida TZs, as well as TZs of two other tested *Pfiesteria*-like species, are capable of killing healthy fish within minutes in a culture setting (Burkholder et al. 1995*a*; Landsberg et al. 1995; J.M.B. and H.B.G. unpubl. data), indicating that fish that have not been stressed by long periods of low dissolved oxygen or other factors can easily succumb to *Pfiesteria*-like dinoflagellates in poorly flushed natural habitats. Moreover, kills documented in May 1991 and June–July 1997 happened before fish were stressed by appreciable low DO, and various other kills (e.g. December 1991, September 1992, September–October 1995) occurred 1–3 months after the estuaries were last affected by low DO (Burkholder et al. 1995*a*; Table 4). Nonetheless, fish that have been physiologically stressed or weakened by adverse environmental conditions, bacterial or fungal pathogens, or other factors likely would tend to be more lethargic than healthy populations and, thus, more susceptible to *Pfiesteria*'s predatory activity. This pattern of events describes July 1995, in which hypoxia and anoxia began to develop at depth by late April and affected about two-thirds the area of most of North Carolina's estuaries by July (depths >1.0–1.5 m; NC DEHNR unpubl. records; J.M.B. and H.B.G. with M. Mallin and coworkers—Table 4 and unpubl. data for the Neuse, Pamlico, New River, and Cape Fear estuaries). Most local scientists outside state agencies have reached consensus that extreme, pervasive, and long-term anthropogenic nutrient and organic, oxygen-demanding loading to these estuaries is the underlying cause (North Carolina Sea Grant 1995). The affected areas included a substantial portion of the Albemarle-Pamlico Estuarine System which is the second largest estuary on the U.S. mainland, historically supplying ~50% of the total area used as nursery grounds by fish species on the Atlantic Coast from Maine to Florida (Steel 1991).

Since the early 1980s North Carolina has experienced what is now commonly referred to by coastal citizens as "fish kill season," with many kills annually in estuarine and coastal waters between April and October (Lowe et al. 1991; Burkholder et al. 1995*a*; Leavenworth 1995; Burkholder 1996, 1997; NC DEHNR unpubl. records). North Carolina's state fish kill records have been rated as the worst (i.e. with poorest documentation of kill events or causality) in the southeastern U.S. (Lowe et al. 1991) and, from scrutiny of the database, the worst of any U.S. coastal area. There were at least six major estuarine kills in summer 1995, with toxic *Pfiesteria*-like species at sublethal or lethal densities in five of these events (Table 4). However, considering the above criteria for implicating these dinoflagellates as the probable causative agent, we attributed two of the six kills primarily to low dissolved oxygen with additional stress that likely was imposed by toxic *Pfiesteria*-like species. Overall, *Pfiesteria* was identified as the primary causative agent of ~33% of the major kills affecting the state's estuaries in the 1995 kill season. One *Pfiesteria*-related kill was by far the biggest of the year (~15×10^6 fish affected) and extended from late July through late October, with most fish manifesting open bleeding sores (diam 1–4 cm, sometimes completely penetrating through the body thickness) and dying in a 6-week period that began in mid-September when DO was >5 mg liter^{-1} throughout the water column (Table 4). The kill extended through a 34-km segment of the Neuse estuary and was associated with the autumn migration of menhaden toward Pamlico Sound.

During 1996 North Carolina sustained a moderate-intensity hurricane in July (Bertha) and a severe hurricane in early September (Fran). Heavy precipitation both during the hurricanes and following the first event resulted in freshwater conditions in many areas of the estuaries that were mesohaline in the other sampled years (NC DEHNR state records). The freshwater and high-volume conditions were believed to contribute to the failure of large schools of Atlantic menhaden to travel up-estuary from their documented distribution in the sounds (NC DMF state records). These factors likely promoted low recorded toxic activity of toxic *Pfiesteria*-like dinoflagellates in 1996. Between the hurricanes, small schools of menhaden slowly moved up-estuary, and we implicated *Pfiesteria* as the causative agent of four small menhaden kills in the Neuse estuary affecting 1–4×10^3 fish (Flanners Beach to Minnesott and Slocum Creek; each kill lasted ≤1 d and coincided with TZ concentrations at 4×10^2 to 1.2×10^3 cells ml^{-1}). Most of the dead fish had open bleeding ulcerations, and we measured DO at >5 mg liter^{-1} in the localized kill sites. Moreover, during most of August 1996, *P. piscicida* zoospores were at sublethal densities (~0.8–2×10^2 cells ml^{-1}), and 43±10% of the Atlantic menhaden sampled from cast nets were affected with bleeding sores (4–7-d sampling intervals; 5–8 casts in 10 or more locations on each date; 50–100 fish taken per cast). Moreover, in late summer 1996, potentially lethal densities of TZs from a second toxic *Pfiesteria*-like species co-occurred with a bloom of the harmful dinoflagellate species, *Gyrodinium aureolum* Hulburt, at a kill of ~10,000 fish (mostly menhaden) with bleeding sores in the New River estuary (~4×10^2 TZs ml^{-1} and 2.5×10^3 *G. aureolum* ml^{-1}).

The economic impacts on wild fish stocks from toxic *Pfiesteria*-like dinoflagellates have not been assessed. However, the massive fish kill of September–October 1995, in which *P. piscicida* was implicated as the major causative agent, marked a turning point for more serious consideration of toxic *Pfiesteria*-like dinoflagellates as an economic threat to commercial and recreational fishing (Burkholder 1997). Although >90% of the fish known to have been affected were Atlantic menhaden, individuals from all fish species in the kill area died (e.g. flounder, spot, croaker, eel, mullet, spotted sea trout, blue crabs), and most carcasses were found with the open bleeding lesions that often characterize toxic activity by toxic *Pfiesteria*-like dinoflagellates (Burkholder et al. 1995*a*; Noga et al. 1996). Divers' reports of dead and dying fish floating or lying on the bottom sediments received national media attention and, along with accounts of lesions on some divers, docksmen, and other local citizens with fre-

quent water contact, coincided with growing public concern about continued heavy commercial fishing in the affected area, primarily for flounders, mullets, and blue crabs.

The situation finally resolved in early October 1995 when state officials at NC DEHNR issued a health warning related to *Pfiesteria,* followed by closure of the affected area to all fishing activity (NC Mar. Fish. Comm. emergency meeting, 13 October 1995). These measures were taken because of public pressure for the state to err on the side of human health protection in the absence of data on the potential for fish to accumulate the dinoflagellate's toxin(s), and also because of an identified need to protect North Carolina's marine fisheries in other locations from economic "halo" effects as large fish houses in New Jersey, New York, California, and elsewhere began to refuse to accept fish from any estuary in the state (North Carolina Div. Mar. Fish. 1995). The closure led to cancellation of two popular recreational fishing tournaments for largemouth bass, with losses estimated at $12,000 (North Carolina Div. Mar. Fish. 1995). Economic loss to commercial fishermen and local fish houses was estimated at about $120,000 for the 2-week period in which the closure and health warning were in effect, with temporary loss of employment for 25 people in the fishing industry, permanent loss of four positions, and one fish house owner forced to leave his business (North Carolina Div. Mar. Fish. 1995; Diaby 1996; D. Jones and family pers. comm.).

The importance of toxic *Pfiesteria*-like dinoflagellates on aquaculture are less well known and underreported (e.g. R. Hodson pers. comm.), partly because many commercial aquaculturists fear that reported kills will translate into substantial loss not only from immediate effects, but also from panic or economic halo effects over larger geographic areas and longer time scales (Shumway 1990; Robineau et al. 1991; Hallegraeff 1993). The first 4 yr of data records (1991–1994) included reported impacts on three commercial and eight research aquaculture operations, verified by analysis of water and sediment samples from these facilities that contained lethal TZ densities (Table 3). Species affected included hybrid striped bass (water source—the Pamlico estuary; $90,000 loss from "sudden death" in one large pond, within 4 h after fish showed signs of distress) and littleneck clams (water source—the Intercoastal Waterway near the White Oak estuary; $20,000 loss of juveniles; Burkholder et al. 1995*a*). During 1995 the juvenile clam operation suffered an additional $12,000 loss, with *P. piscicida* again implicated as the causative agent; in 1996, a $50,000 loss of 20,000 hybrid striped bass that may have been related to *P. piscicida* occurred in a facility near Chesapeake Bay (D. Truluzi pers. comm.; presence of >300 zoospores ml^{-1} confirmed by H.B.G. and J.M.B. with *P. piscicida* identifications by K. Steidinger, J.M.B., and H.B.G.).

Interactions with other microfauna—Given the array of flagellated and amoeboid morphs in its complex life cycle with size range spanning 5–450 μm in maximum cell dimension, *P. piscicida* and other *Pfiesteria*-like species are hypothesized to play multiple roles in estuarine microbial food webs (Burkholder and Glasgow 1995). Although TZs have been shown to be lethal to higher trophic levels such as shellfish and finfish, as plankters they are vulnerable to predation by various microfauna including some protozoan ciliates (e.g. *Stylonychia* cf. *putrina*), rotifers (e.g *Brachionus plicatilis* Mueller and *Brachionus* sp.) and microcrustacean copepod zooplankton (e.g. *Acartic tonsa* Dana) (Burkholder and Glasgow 1995; Mallin et al. 1995). Survival was compared for adult *B. plicatilis* and *A. tonsa* in separate trials (10 and 3 d, respectively) when the animals were given *P. piscicida* TZs, nontoxic algal prey, or a 1 : 1 mixture of TZs and nontoxic algal prey. Rotifer fecundity was also examined. There was no apparent effect of TZ consumption on rotifer survival. For the first 7 d, rotifer fecundity with TZs alone or in mixed prey was comparable to or higher than fecundity with nontoxic algae (Mallin et al. 1995; Fig. 7). From days 8 to 10, however, fecundity with nontoxic algal food was significantly higher than in both treatments with TZs. The copepods maintained similar grazing rates on TZs, nontoxic diatoms, and the mixed prey treatment. Grazing on TZs, however, seemed to promote a behavioral change wherein the animals commonly exhibited more rapid and erratic swimming. Such erratic behavior could be a disadvantage in natural conditions if it resulted in higher visibility to predators, reduced effectiveness in escape mechanisms, or impeded success in mating and other aspects of reproduction. These possibilities merit additional focus.

Larger amoeboid stages of *Pfiesteria*-like dinoflagellates have not been observed to be consumed by common microbial predators thus far, either in field samples or in culture. Moreover, identified predators of smaller flagellated states (zoospores, gametes) have shown no feeding activity on transformed filose or lobose amoebae. Instead, the protozoan ciliate *Stylonychia* and the rotifer *B. plicatilis* Mueller have been observed to be attacked by midsized to large lobose amoebae of *P. piscicida* (length >60 μm). In fact, on several occasions *Stylonychia* has been phagocytized by lobose amoebae while the ciliates were actively consuming TZs (Burkholder et al. 1992). The available data suggest that, at least in the water column, amoeboid stages are less vulnerable to predation than smaller flagellated stages. In what might be regarded as a hunter-gatherer life cycle strategy, rapidly swimming TZs target and immobilize fish prey with their toxin(s) (Burkholder and Glasgow 1995). When the prey are dead, TZs transform to amoebae that are less vulnerable to other microfauna. The amoebae then gather on the carcasses and feed upon the fish remains (Fig. 2). Little work has been directed thus far on amoeba consumption by other predators. This question merits future emphasis toward resolving the role of toxic *Pfiesteria*-like dinoflagellates in estuarine microbial food webs, since these amoeboid stages have been identified as dinoflagellates.

In considering the role of *Pfiesteria*-like dinoflagellates as microfaunal consumers rather than prey, both zoospores and lobose amoebae of *P. piscicida* seem to be voracious predators on microorganisms including other animals between fish kills, and on some occasions, even while fish are dying during a toxic outbreak. Like other free-living heterotrophic dinoflagellates, each zoospore has a large food vacuole that can fill the upper half of the cell (Gaines and Elbrächter 1987; Schnepf and Elbrächter 1992). This feature, along with elasticity of the sulcal area, enables *P. piscicida* to phago-

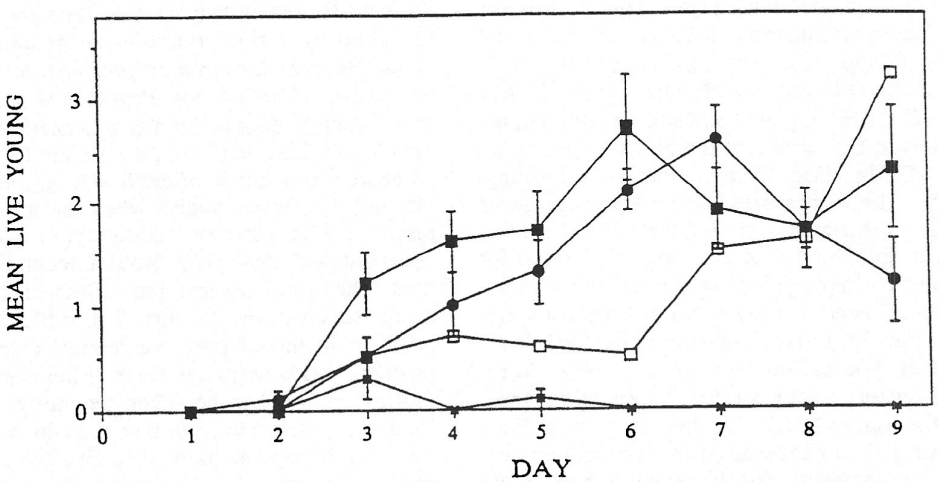

Fig. 7. Live young produced daily by the rotifer *Brachionus plicatilis* in unfed controls (×) and three feeding treatments as nontoxic algal prey: □—mixture of *Nannochloris* sp. (mean diam 3 μm, mean biovol. 9 μm^3; n = 25) and *Tetraselmis* sp. (mean diam 9 μm, mean biovol. 450 μm^3; n = 25) with daily additions to attain initial prey density at 3.38×10^5 μm^3 *Nannochloris* ml^{-1} + 4.92×10 μm^3 *Tetraselmis* ml^{-1} ●—*Pfiesteria piscicida* TZs (2,500 cells ml^{-1}; mean diam 10 μm, mean biovol. 510±18 μm^3, n = 25), culture media gently poured from aquaria with dying fish ■—mixed prey (1,250 TZs ml^{-1} in a 1 : 1 mixture by biovolume with greens). Data given as means ±1 SE (from Mallin et al. 1995).

cytize prey that are two-thirds its size (Gaines and Elbrächter 1987; Burkholder and Glasgow 1995). TZs have also been observed to "spin" a web or velum about *B. plicatilis* and consume it—a mode of prey acquisition that has been reported for certain heterotrophic marine and freshwater dinoflagellates (Jacobson and Anderson 1986; Burkholder 1992). *P. piscicida*'s TZs and NTZs have been found to use myzocytosis to consume small protozoan ciliates (e.g. *Saprophilis* sp.) that are comparable to the dinoflagellates in size and with which they typically co-occur (Burkholder and Glasgow 1995). The trigger that promotes such attacks apparently is given by animals that are either wounded or senescent and visibly weakened with poor feeding activity.

Among the best videotaped observations on consumption of other microfauna by *P. piscicida*'s lobose amoebae are activities recorded by K. Steidinger and coworkers. They filmed a large lobose amoeba as it attracted a small protozoan ciliate so that the prey hovered and moved rapidly back and forth in small motions near the dinoflagellate but did not leave. The attractant likely was a dissolved organic substance that served as a food resource or chemoattractant for the ciliate, which is a method of prey attraction that has been suggested for other predaceous dinoflagellates (Gaines and Elbrächter 1987). Lobose pseudopodia slowly moved to surround the ciliate, which was unable to escape as it became phagocytized into a food vacuole. We have videotaped lobose amoeboe with similar behavior in capturing cryptomonads. As indicated, our knowledge about predation by *P. piscicida* on other microfauna thus far is observational. Experiments are needed to examine grazing rates and bioenergetics of flagellated and amoeboid stages on microfaunal prey species as they are identified.

Pfiesteria, *microflora, and dissolved nutrients*—We have begun to rigorously examine the nutritional ecology of both TZs and NTZs of *P. piscicida* (=the same cells in the presence vs. the absence of fish). Because this dinoflagellate is an animal (protozoan) rather than a photosynthetic alga, its nutritional controls are complex. TZs and gametes, and the NTZs that they revert to in the absence of live fish, all have been observed to phagocytize bacteria, although this activity is rarely observed when flagellated algae are available. These stages are both phagotrophic and myzocytotic on a wide array of algal prey, with demonstrated preference for certain small algal flagellates including cryptomonads, greens, and chrysophytes (Burkholder and Glasgow 1995, 1996; Glasgow et al. unpubl.). Unraveling the nutritional complexities of *P. piscicida* becomes more difficult under conditions when both TZs and NTZs adopt algal- (plant) like nutrition by retaining the chloroplasts that they obtain from algal prey (Steidinger et al 1995)—a phenomenon known as kleptochloroplastidy (Schnepf et al. 1989; Stoecker 1991; Fields and Rhodes 1991; Lewitus et al. unpubl.). That is, utilization of certain dissolved nutrients such as inorganic N and P apparently is dependent in large measure on whether the dinoflagellate has a plantlike response because of retained kleptochloroplasts (Burkholder et al. 1997*a*).

Until the stimulatory substance(s) from fish secreta-excreta is identified, the effects of organic and inorganic nutrient controls on toxic stages of *Pfiesteria*-like dinoflagellates will be difficult to examine because of confounding nutrients (e.g. dissolved phosphorus, nitrogen, and organic carbon) in the fish materials. Flagellated stages have responded poorly to gentle means of separation from culture media with fish; numerous attempts at centrifugation have proven unsatisfac-

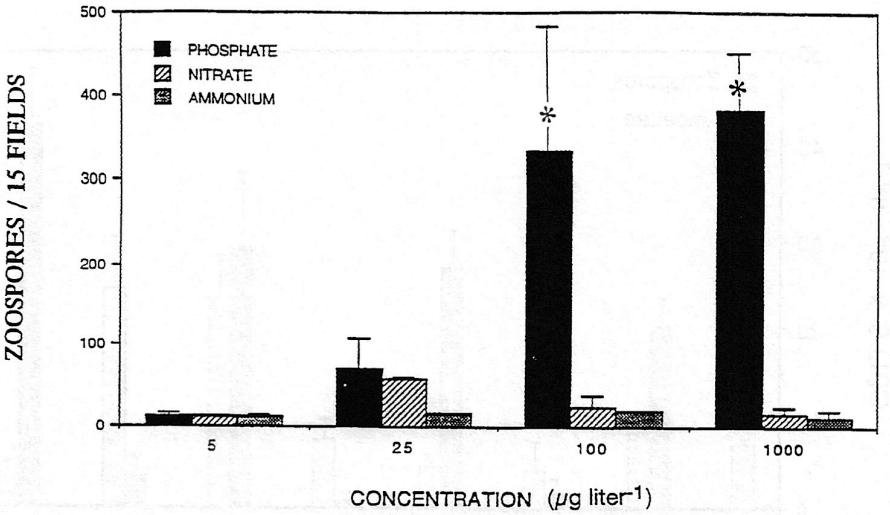

Fig. 8. Response of *Pfiesteria piscicida* zoospores (reverted from gametes, and transformed or transforming into amoebae, that were taken from actively killing cultures) to gradients of phosphate, nitrate, and ammonium enrichment after 4 d in batch cultures without finfish (means ±1 SE, n = 3). The 5 μg liter^{-1} concentration (background nutrient levels in the Instant Ocean media) represented the response in controls without nutrient additions. Asterisks indicate densities significantly higher than control (α = 0.05). In three of four experiments in which we repeated this effort, we obtained similar results. The fourth experiment involved a culture inoculum that had not been exposed to live fish for 5 d before the experiment began, and the culture did not show P stimulation. These results suggest that the response of this heterotrophic-mixotrophic dinoflagellate to nutrient enrichment depends, at least in part, on its recent food supplies and that nutrient controls on growth of various stages are more complex than typically considered for photosynthetic algae.

tory because the toxic cells encyst; by the time they emerge they have been without fish (and, thus, without stimulus for toxin production) typically for several days. Even gentle gravity filtration (without suction pressure) has promoted a similar response by all flagellated stages present (TZs, planozygotes, gametes), with ~75% encystment.

In an attempt to test nutrient controls on TZs apart from fresh fish materials, we gently gravity-filtered TZs, planozygotes, and gametes [1-μm-porosity Millipore filters, using sufficient culture volumes (generally 500 ml) to achieve an initial water-column concentration of 0.8–1.1×10^2 active TZs ml^{-1}]. Filters with remaining active cells were placed in media (15-psu Instant Ocean water) containing gradients of inorganic nutrients as PO_4^{3-}-P, NO_3^--N, or NH_4^+-N [control or background as 15-psu ultrafiltered Instant Ocean water (0.22-μm-porosity Millipore filters) without nutrient additions; concentrations of all N and P inorganic nutrient forms <5 μg liter^{-1}]. At 2 h after filters had been added (=time zero), we sampled the water for TZs and gently removed the filters with remaining cysts and amoebae (presence confirmed in rinse media using light microscopy at 600×). Treatments were established at 25, 100, 500, and 1,000 μg N or P liter^{-1}. The *P. piscicida* population was taken directly from toxic stock cultures that had been killing fish in repeated bioassays for ~1 yr. Samples for quantification of *P. piscicida* stages were coded to prevent bias while counting, with analyses completed separately by both of us (our counts were in agreement to within ±4–7%). After testing for homogeneity of variance (Hartley's test; SAS Inst., Inc. 1987), treatments were analyzed for significant differences with ANOVA. Treatment means were compared with Fisher's protected least-significant-difference (LSD) test with a comparisonwise error rate (α = 0.05; SAS Inst., Inc. 1987).

The TZs in batch cultures (slowly converting to NTZs or transforming to amoebae over 4 d without live fish) in nutrient experiment 1 showed strong stimulation of production (as increase in cell numbers; $P < 0.01$) by P_i at concentrations ≥100 μg PO_4^{3-}-P liter^{-1}, but did not respond to nitrate or ammonium (Glasgow et al. 1995; Fig. 8). Other potential microbial prey (small chrysophyte flagellates, ≤10^2 ml^{-1}; small *Lyngbya* sp., ≤10^{-3} ml^{-1}; bacteria, ≤10^5 ml^{-1}) were also quantified over the 4-d period (techniques for algal cell counts given by Burkholder et al. 1995*a*; for bacteria given by Turley 1993). Changes in algal and bacterial abundances were not significantly correlated with *P. piscicida* densites ($P < 0.2$). In 1-h observations made 4–5 times daily on subsamples during the experiment, we occasionally saw a zoospore consuming an algal flagellate but did not observe zoospores consuming bacteria or blue-greens. More than 90% of the zoospores were translucent or colorless and, in acid-Lugol's-preserved material, did not contain discernible chloroplasts (phase contrast microscopy, 600×). *P. piscicida* zoospores are known to retain kleptochloroplasts for weeks (Lewitus et al. unpubl.) as previously discussed. The predominance of translucent-colorless cells without chloroplasts provided evidence that most of the zoospores had not been consuming algae. The population slowly changed from predominantly zoospores (>95%) to translucent amoebae (>90%) during the 4-d period. The metabolic basis for the

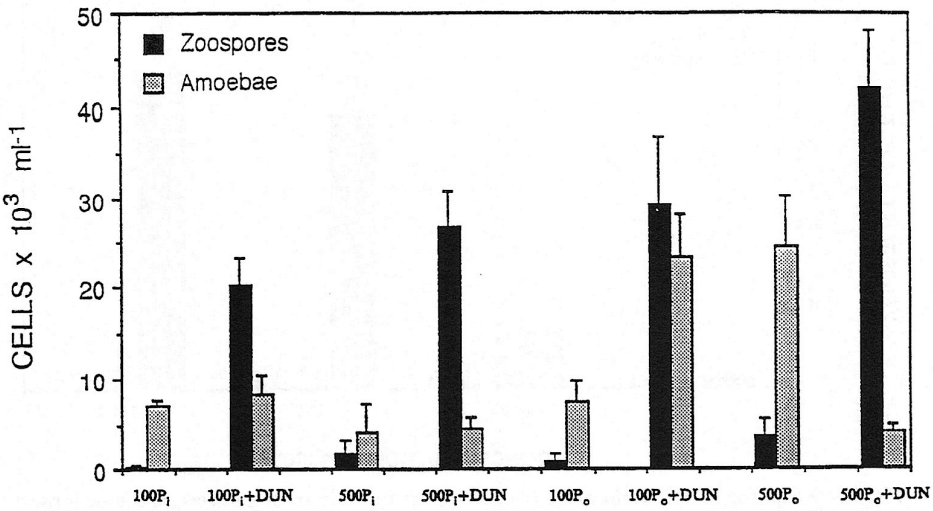

Fig. 9. Abundance of *Pfiesteria piscicida* NTZ precursor stage to TZs, and of amoebae after 7 d in batch cultures with 100 or 500 μg PO_4^{3-}-P liter^{-1} as NaH_2PO_4 (P_i100 and P_i500) or as glycerophosphate (P_o100 and P_o500) ± *Dunaliella tertiolecta* in a 1 : 5 ratio of dinoflagellates to algal prey with prey added daily. The treatments were kept at 21°C, 3.0×10^{19} quanta m^{-2} s^{-1} illumination ("cool-white" fluorescent tubes), and a 12 : 12 L/D cycle. *Pfiesteria* cell production (NTZs or amoebae) was significantly higher in all treatments with P_i or P_o, ± algal prey, than initially or in controls without P ± algal prey ($P < 0.05$; *see text*). NTZ abundance in all treatments with algal prey was significantly higher than in the corresponding treatments without algal prey (ANOVA followed by LSD, $P < 0.01$). Cultures with P_o + algal prey yielded significantly more NTZs than cultures enriched with P_i + algal prey ($P < 0.05$).

observed stimulatory effect of P_i is unknown; the data may reflect a high P requirement for toxin production (e.g. Tomas and Baden 1993, working with other toxic dinoflagellate species).

Toxic *P. piscicida* populations also have been isolated from culture media with fish for a second experiment on nutritional ecology, using a protocol similar to that of experiment 1 with initial densities at $0.7–1.0\times10^2$ active zoospores ml^{-1}. In experiment 2, we examined the response of NTZs with recent toxic prehistory to inorganic vs. organic P enrichment (P_i or P_o, respectively), with and without available algal prey. The response to algal prey was examined under P enrichment because such algae would be expected to coincide with nutrient loading in natural estuarine habitat (Mallin 1994). Treatments were established in triplicate within batch cultures (15-psu ultrafiltered Instant Ocean water) as follows: P_i as sodium monobasic phosphate at 100 or 500 μg PO_4^{3-}-P liter^{-1} (P_i100 and P_i500) and P_o as glycerophosphate at 100 or 500 μg PO_4^{3-}-P liter^{-1} (P_o100 and P_o500). These treatments were imposed alone or with addition of an algal prey species, *D. tertiolecta* Butcher (Pamlico isolate, clone NC9302, length 4 μm; previously grown in f/2 medium with 1% P_i or P_o depending or the treatment and added daily with sufficient prey to maintain a zoospore : prey ratio of 1 : 5 immediately after addition). Cultures were gently mixed after algal prey were added, and 1-ml subsamples were taken daily to check prey densities and ensure that food was still available after 24 h. Prey usually had decreased to a ratio of ~1 : 1 with NTZs at 24 h after the previous prey addition. Controls contained NTZs without P_i or P_o, or without both P and algal prey.

After 10 d (again, with reversion to nontoxic populations, confirmed by testing ultrafiltered culture water with test tilapia), *P. piscicida* in controls without P_i, P_o, or algal prey showed negligible production when compared to initial densities; NTZs (formerly TZs and gametes, with all planozygotes having remained encysted) in controls without P but with algal prey increased to $2.4\pm0.3\times10^3$ cells ml^{-1}; amoeba densities remained low and were comparable to initial densities. NTZs showed stimulation by P_i (ANOVA, $P < 0.05$) and stronger stimulation by P_o ($P < 0.05$), with the higher concentration promoting more population growth (Fig. 9). Both P_i and P_o effects were significantly enhanced in the presence of algal prey ($P < 0.01$). In this experiment, unlike the one previously described, zoospores were still common or abundant in all cultures by the end of the assays, although amoebae predominated in treatments without flagellated algal prey. We also quantified algal contaminants (small chrysophyte flagellates and blue-greens *Lyngbya* sp. and *Gloeothece* sp.) and bacteria at 2-d intervals, using previously indicated methods. As in the first nutritional experiment, none of these potential prey were significantly correlated with *P. piscicida* zoospore densities ($P > 0.2$). However, by the end of the experiment in the P_o treatments without *Dunaliella,* amoeba densities were significantly correlated with abundances of bacteria and of *Gloeothece* ($P < 0.05$).

In a third nutritional experiment, uptake of dissolved amino acids (N_o as ^{14}C-protein hydrolysate; Amersham: L-alanine, 9.5%; L-arginine, 6.9%; L-aspartic acid, 10.0%; L-glutamic acid, 9.0%; glycine, 5.8%; L-histidine, 1.6%; L-leucine, 12.7%; L-isoleucine, 5.8%; L-lysine, 4.8%; L-me-

thionine, 0.6%; L-phenylalanine, 7.4%; L-proline, 5.3%; L-serine, 3.2%; L-threonine, 5.7%; L-tyrosine, 5.8%; L-valine, 5.9%) by a recently toxic population of *P. piscicida* in a cultured fish-killing population was examined by track light microscope-autoradiography (procedure of Burkholder et al. 1990) in 30-min trials. On the date of collection, the culture had killed fish <1 h after exposure and had been given live replacements several times daily for 3 months. Subaliquots of culture medium (15-psu Instant Ocean water containing fish excreta) with dinoflagellates were assayed for uptake of the radiolabeled amino acid mixture. Sufficient substrate was used to effect a target label of ~1 disintegration d^{-1} $cell^{-1}$. Few flagellated algal prey (<10 ml^{-1}) were available for potential consumption by zoospores or amoebae, although these algal cells could have taken up the radiolabeled N_o. Track autoradiography cannot be used to check bacterial uptake of radiolabeled substrates (Rogers 1979). Short incubation times were selected to minimize the potential for incorporation of label by *P. piscicida* as mediated through phagotrophy of labeled bacteria, blue-greens, or small flagellates. Such phagotrophy was observed for only 1 of 30 zoospores (consuming a small chrysophyte flagellate, with low abundance of algal flagellates at ~3×10^2 cells ml^{-1}) in two 30-min observations of culture subsamples.

Assays with radiolabeled N_o were completed in triplicate at 22°C and 3.0×10^{19} quanta m^{-2} s^{-1}, after gently swirling the cultures twice initially to mix the radiolabeled substrate. Controls consisted of cultures with unlabeled protein hydrolysate, using substrate prepared under sterile conditions with the same amino acid mixture as the radiolabeled material. The data were analyzed for significant differences in track counts among controls and radiolabeled stages with ANOVA followed by LSD with a comparisonwise error rate (α = 0.05; SAS Inst., Inc. 1987). *P. piscicida* in controls without radiolabeled substrate was negligibly labeled. In the labeled assays on a per-cell basis, larger lobose amoebae (length ≥40 μm) were associated with significantly higher radiolabeled protein hydrolysate, with TZs moderately labeled and gametes lowest in labeling among active stages ($P < 0.05$; Fig. 10). On a per-volume basis, however, small flagellated stages (considered mostly as gametes, diameter 5–8 μm, also with some recently produced small TZs) took up significantly more ^{14}C-protein hydrolysate.

Other experiments to examine nutritional controls have focused on nontoxic stages of *P. piscicida* that were without live or dead fish for 6–24 months. Working with nontoxic populations has enabled us to gain insights about how this dinoflagellate thrives between fish kills and develops "inocula" for toxic outbreaks when fish are again detected (Burkholder and Glasgow 1995). This approach also has made it possible to avoid the confounding factors inherent in attempts to understand nutrient enrichment effects on populations in media with fish-derived substances. The NTZs (dimensions 9±2 μm×7±2 μm, means ±1 SE, n = 25 cells) used in nutrient experiment 4 had been without live fish for ~1.5 yr. We tested NTZ response [previously fed a diet of *D. tertiolecta* (previously described) and *I. galbana* Green (clone CCMP1323, diam 4 μm); initial NTZ density 300 cells ml^{-1}] across concentration gradients of P_i, P_o (as NaH_2PO_4 or glycerophosphate), N_i (as nitrate), and N_o (as

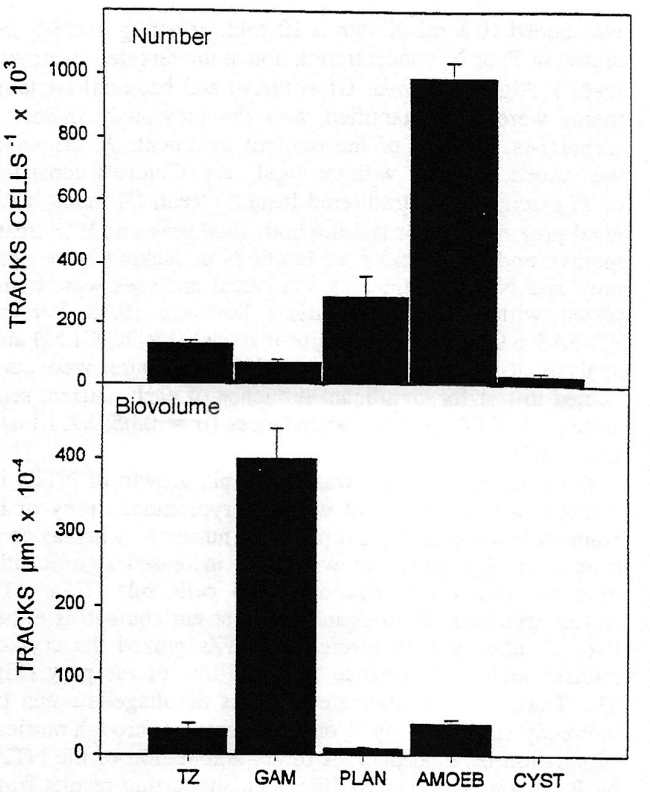

Fig. 10. Uptake of ^{14}C-protein hydrolysate by a *Pfiesteria piscicida* population taken from a culture that was killing tilapia at 20-min intervals (TZs, gametes, planozygotes, lobose amoebae, and cysts; upper panel, tracks per cell number; lower panel, tracks per unit cell biovolume (means ±1 SE; from Glasgow and Burkholder unpubl.)

protein hydrolysate, in similar mixture as the radiolabeled substrate from Amersham described previously) with target levels of 0, 50, 100, 500, or 1,000 μg P or N $liter^{-1}$ (n = 3; ~1,200 samples in total).

Treatments for experiment 4 were established in triplicate batch cultures within 6-ml well plates, using f/2 medium prepared without P (for treatments with P_i, P_o) or N (for treatments with N_i or N_o) in 15-psu ultrafiltered Instant Ocean water. These concentrations encompassed the range of nutrient levels measured in the water column of estuarine areas where the dinoflagellate's zoospores commonly occur (Burkholder et al. 1995*a*), with the exception of the N_o levels which also considered conditions during fish kills in dense prey aggregates or schools (dissolved organic N 1–2 mg $liter^{-1}$; H.B.G. unpubl. data). The P_i, P_o, N_i, and N_o treatments were imposed in 15-psu ultrafiltered Instant Ocean water both with and without addition of nutrient-replete *Cryptomonas* (isolate WH2423, 8±1 μm×5±2 μm, means ±1 SE, n = 25 cells) as prey (from cultures in f/2 medium: McLachlan 1979; initial bloom density of 1.5×10^5 cells ml^{-1}). The prey were added initially to achieve a density of 5×10^{-2} cells ml^{-1} (22°C, 12:12 L/D, 3.0×10^{19} quanta m^{-2} s^{-1}). *Cryptomonas* had been cultured at high density (10^5–10^6 cells ml^{-1}) so that little volume of prey with culture medium

was added (0.3 ml diluted ~10-fold, affecting a <5% increase in P_i or N_i concentration above the targeted treatment levels). Algal (*Lyngbya, Gloeothece*) and bacterial contaminants were also quantified with the previously indicated techniques. To each of the nutrient treatments *P. piscicida* was added, with vs. without algal prey. Controls consisted of *P. piscicida* in ultrafiltered Instant Ocean f/2 media with algal prey minus P or lacking both algal prey and P (P treatments); and with algal prey minus N or lacking both algal prey and N (N treatments). Statistical analyses were completed with Statistical Analysis Software (SAS Version PC-SAS 6.08); and general linear model (PROC-GLM) and analysis of variance (PROC-ANOVA) procedures were conducted to test for significant influence of each nutrient separately on NTZ and prey abundances ($\alpha = 0.05$; SAS Inst., Inc. 1987).

In experiment 4 there was negligible growth of NTZs in controls with P_i or N_i but without cryptomonad prey or in controls lacking both algal prey and nutrients, whereas controls with algal prey but without P increased significantly over the initial densities of 75–95 cells ml^{-1} (Fig. 11). Across gradients of inorganic nutrient enrichment as either P or N, after 8 d *P. piscicida*'s NTZs grazed the cryptomonads and its abundance followed that of the prey (Fig. 11). Thus, the data indicate that this dinoflagellate can be indirectly stimulated by P_i or N_i enrichment through nutrient stimulation of algal prey. A direct stimulation of the NTZs by P_o also was suggested (Fig. 11), supporting results from the experiment with former TZs and glycerophosphate (Fig. 9). We inferred direct stimulation by P_o since zoospore abundances were not significantly correlated with bacterial densities ($P > 0.2$); nor were *Cryptomonas* abundances significantly correlated with bacterial numbers ($P > 0.3$), although in microscopic observations of the cultures we observed cryptomonads and, less commonly, *P. piscicida* consuming bacteria. Other dinoflagellates have been found to be osmotrophic on dissolved N- and P-containing organic substrates (Gaines and Elbrächter 1987; Schnepf and Elbrächter 1992). Results of trials with N_o indicated that under these conditions, *P. piscicida* was outcompeted by bacteria and cryptomonad prey for N_o substrate. *P. piscicida* was mildly stimulated by the lowest addition of N_o ($P < 0.10$), but at higher additions bacteria developed as dense populations, along with increases in the cryptomonad which commonly phagocytized bacteria and which likely also consumed protein hydrolysate (Droop 1974). The response of *P. piscicida*'s NTZs to N_o merits more in-depth examination with a variety of dissolved organic nitrogen substrates and a range of conditions across gradients of microflora and microfaunal prey abundance. Investigations of the effects of organic carbon on flagellated and amoeboid stages of *P. piscicida* are also in progress.

The observed general trend of *P. piscicida* stimulation by inorganic nutrient enrichments in laboratory bioassays, mediated through stimulation of algal prey, has been supported thus far in field observations. About 75% of toxic outbreaks by *Pfiesteria*-like dinoflagellates have been in nutrient-enriched waters (Burkholder et al. 1995*a*). We have begun to examine the influence of nutrient stimulation on these dinoflagellates in natural habitats using a two-pronged approach

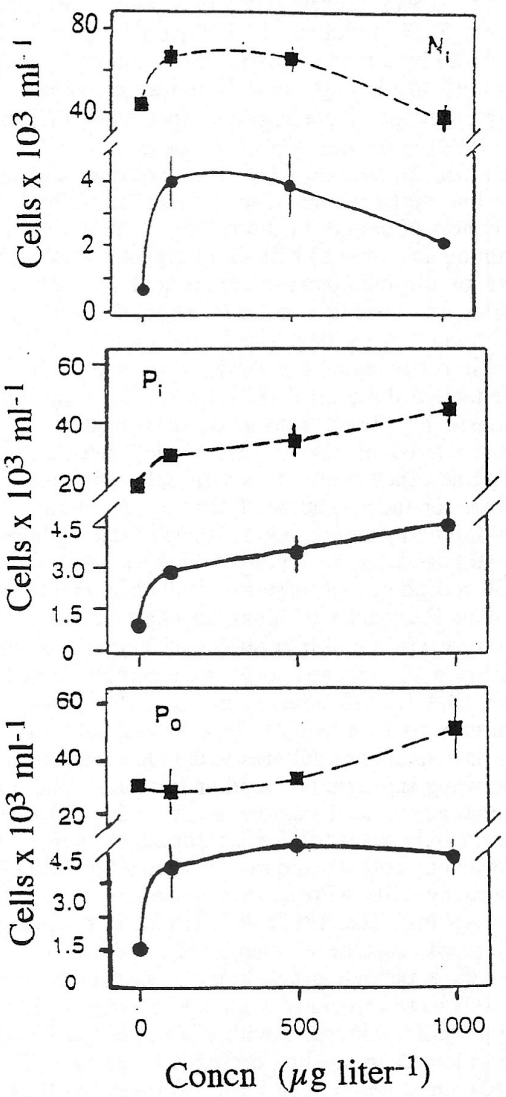

Fig. 11. Response of NTZs of *Pfiesteria piscicida* (●) to nutrient enrichment with N_i (as NO_3^{-1}-N), (as PO_4^{3-}-P), and P_o (as glycerophosphate), when fed *Cryptomonas* sp. (■) in 7-d trials (means ±1 SE).

that includes both correlative field research and mesocosm experiments with NTZs. For the correlative effort we have focused on easily identified areas known to be impacted by anthropogenic nutrient inputs, namely, major point-source discharges of municipal sewage (>1.9×10^6 liters d^{-1}). At the end of two growing seasons (October 1993, 1994), four control sites without wastewater influence (as isolated from anthropogenic nutrient sources as possible) were selected for comparison with four sites near sewage outfalls in the New River estuary, North Carolina. Large tracts of land on either side of this large shallow lagoon are owned by the U.S. Marine Corps—Camp Lejeune Base, which keeps the land mostly as forest for training maneuvers.

Surface-water samples were collected in shallow nearshore areas (depth <1.5 m) during a period of calm, warm

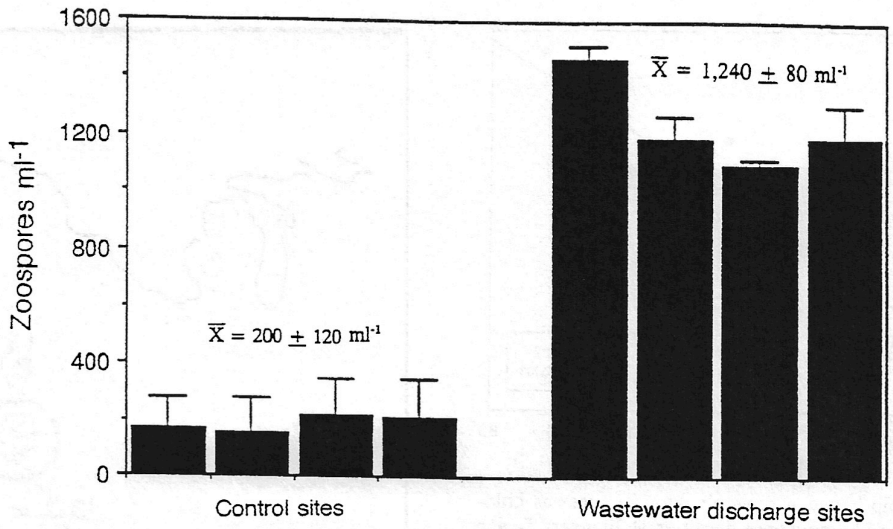

Fig. 12. NTZs of *Pfiesteria*-like dinoflagellates from 0.25-m depth at four control sites (upper Stones Bay, Swan Point, Ellis Cove, and Sneads Ferry Marina, in order of presentation) and four wastewater discharge sites (sampled within ~100 m from the discharge point: lower Northeast Creek, Wallace Creek, Hadnot Point, and Rifle Range–lower Stones Bay) at the end of the growing season in the New River estuary (means ±1 SD; 3 replicates per site).

weather in late October 1993 (water temp. 18–20°C, which is ~6–8°C below the optimal temperature for *P. piscicida* (Burkholder et al. 1995*a*). Dissolved oxygen was >6 mg liter^{-1} in all locations, and salinity ranged from 3 to 15 psu. In sewage-affected areas, surface-water samples were taken ~100 m from the point of entry by discharge from the wastewater treatment plants. Immediate discharge areas were not sampled to avoid negative influences of chlorine and other deleterious substances potentially involved in treatment processes or contained in the effluents, while still remaining in the nutrient-enriched areas of influence (Hynes 1960). Control and sewage sites were selected in the mesohaline estuary (salinities 16–18 psu) and on both western and eastern shores to avoid inherent bias from potential wind distribution of algal populations. Samples were collected at each site in triplicate. They were preserved with acid-Lugol's solution, and cell densities of NTZs of toxic *Pfiesteria*-like dinoflagellates as well as small flagellated algal prey (mostly smaller dinoflagellates, cryptomonads, and chrysophytes) were quantified following the protocols used in previously described laboratory experiments. Small flagellated algal prey with length or diameter ≤8 μm were included in the assessment on the basis of laboratory feeding trials in which *P. piscicida* NTZs were shown to consume prey within and even larger than that size range (Burkholder and Glasgow 1995). Water-column nutrient concentrations (total phosphorus, nitrate, and ammonium) were analyzed from each site in triplicate, using techniques described by Burkholder et al. (1992). Mean densities of *Pfiesteria*-like dinoflagellates at control vs. wastewater discharge sites were compared (Student's *t*-test; SAS Inst., Inc. 1987). The data were also examined for correlations among NTZ abundances, flagellated algal prey densities, and nutrients.

NTZ abundance was significantly greater near wastewater discharge sites than in control areas without wastewater influence (Fig. 12). Ammonium and nitrate concentrations were similar in control and wastewater discharge sites (range 5–38 μg NH_4^+-N liter^{-1} and 25–50 μg NO_3^--N liter^{-1}). Total phosphorus concentrations near wastewater outfalls were significantly higher (60±5 μg TP liter^{-1} in control sites and 115±25 near sewage outfalls; means ±1 SD, $P < 0.01$) and small flagellated algal prey more numerous (cryptomonads, chrysophytes, and dinoflagellates with maximal cell dimension ≤8 μm) than in control sites (Student's *t*-test; $P < 0.01$). NTZ densities were positively correlated with TP ($P < 0.01$, $r = 0.76$). NTZ densities were also positively correlated with abundance of small algal flagellates (2×10^3–4×10^4 cells ml^{-1} in control sites, and 3×10^5–6×10^6 cells ml^{-1} near wastewater discharge sites; 2nd-order polynomial, $P < 0.05$, $r = 0.87$). Moreover, small algal flagellate densities were positively correlated with TP concentration ($P < 0.01$). These data provided field evidence that TP and flagellated algal prey abundance may stimulate growth of NTZs, which represent an immediate precursor stage to TZs.

Although we found a strong positive correlation between NTZ abundance and TP in this field study, organic nutrient sources and other materials in the wastes that were not measured could also have been stimulatory. This field-sampling effort to compare *Pfiesteria* abundance in control vs. sewage-influenced sites has been extended to the general toxic outbreak season for *P. piscicida* and *Pfiesteria*-like dinoflagellates (May–October; work in progress), considering additional control and wastewater discharge sites. In other research, we found a strong stimulation of a toxic *Pfiesteria* by a massive swine effluent spill (Burkholder et al. 1997*b*). Further evidence in support of *Pfiesteria*'s stimulation by nutrient loading, mediated through algal prey, has been obtained by Fensin (1997) with use of a dataset from the laboratory of J.M.B. In that study, two sites in the mesohaline Neuse estuary were sampled weekly during two annual cy-

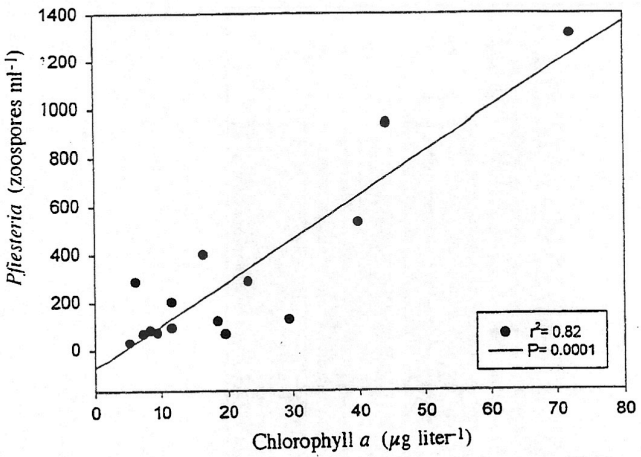

Fig. 13. Relationship between phytoplankton biomass as chlorophyll *a* and *Pfiesteria*-like zoospore densities at Flanners Beach and Minnesott Beach in the mesohaline Neuse estuary (mean salinity 15 psu) based on weekly integrated water-column samples (surface to bottom, with maximum depth 2.0 m; sampler described by Cuker et al. 1990) for two annual cycles from 1994 to 1995 (from Fensin 1997).

cles for physical-chemical variables, phytoplankton biomass as chlorophyll *a*, abundance of dominant phytoplankton taxa, and *Pfiesteria*-like zoospores. Fensin reported a significant positive correlation between zoospore abundance and phytoplankton chlorophyll *a* during the spring season (Fig. 13), which may be a critical period for algal prey (stimulated by nutrients in late winter runoff and rain; Mallin et al. 1993) to support excystment and growth of zoospores after the winter season of low activity. The study also documented positive correlations between zoospore densities and both nitrate and phosphorus concentrations ($P < 0.01$–0.05). These data, like the laboratory bioassays, indicate that *Pfiesteria* is stimulated by P_i (as phosphate) or N_i (as nitrate) especially when loadings effect concentrations ≥ 100 μg liter^{-1}.

P. piscicida is an animal with modes of nutrition that include both mixotrophy using kleptochloroplasts and heterotrophy spanning prey from all trophic levels (bacteria, algae, small animals, fish, and mammalian tissues). Its nutrition is complex and cannot be expected to directly parallel the linear response of algae to nutrient enrichments. Its response to nutrient enrichments depends on several factors including prey type and availability, prehistory of feeding, nutrient form and concentration, and season. Thus far, our extensive field and laboratory information indicates that *Pfiesteria* can be highly stimulated by nutrient enrichment, directly and/(or) indirectly as mediated by the abundance of algae and other prey.

Known geographic range

Toxic *Pfiesteria*-like species have been tracked to shallow nearshore waters and sediments of sudden-death fish kill sites in eutrophic estuaries from the Delaware inland bays to Mobile Bay, Alabama (Fig. 14; Burkholder et al. 1995*a*;

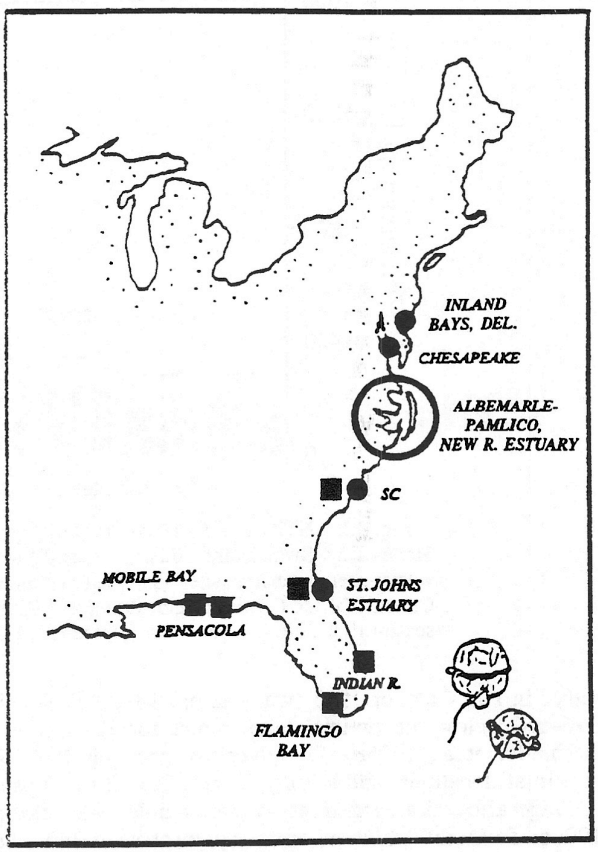

Fig. 14. Known geographic range of toxic *Pfiesteria*-like species. The map indicates locations where TZs have been identified from sites associated with sudden-death fish kills and ulcerative fish disease, and where toxicity has been verified by bioassays with tilapia. Sites where confirming toxicity bioassays have not been completed are not shown, but include the Cape Fear estuary and various tidal creeks in southeastern North Carolina. Apparent species overlap occurs along the midsouthern U.S. Atlantic Coast (modified from Burkholder et al. 1995*a*).

Lewitus et al. 1995). This distribution represents the northernmost boundary of the region surveyed thus far, with presence detected in 70% of the areas examined. Recent research also established that toxic *Pfiesteria* and *Pfiesteria*-like dinoflagellate populations occur in the mouth of the Pocomoke River as it enters Chesapeake Bay. In early May 1997, we found toxic zoospore densities of ~200–250 cells ml^{-1} in water-column samples, coinciding with an epizootic of open bleeding sores which affected most finfish species that were collected from the area.

In all research to establish the occurrence of toxic *Pfiesteria*-like dinoflagellates among regional sites, presence was determined from light microscopy with identifications confirmed by scanning electron microscopy and from aquarium bioassays of water samples and sediments with live fish. Available data indicate that the complex life cycle of the second *Pfiesteria* (Landsberg et al. 1995), and of other known *Pfiesteria*-like dinoflagellates, closely resembles that

of *P. piscicida,* complete with an array of flagellated, amoeboid, and encysted stages (H.B.G. and J.M.B. unpubl.).

Distribution surveys have not yet been conducted outside the mid-Atlantic and southeast regions of the U.S. The evidence to date points to the potential for widespread distribution of toxic *Pfiesteria*-like dinoflagellates in warm temperate to subtropical regions, with high probability for the existance of additional species. Development of DNA probes specific to *Pfiesteria*-like species will facilitate its identification from natural habitats and will provide insights about their genetic diversity and their occurrence through time (by analysis of cyst deposits in sediment profiles). Both 16S and 18S ribosomal rDNAs have been isolated from cultures of *P. piscicida* using the polymerase chain reaction, with eucaryote-specific primers (Toffer 1996). Reaction products have been cloned and sequenced, and two sets of primers based on unique regions of *P. piscicida*'s 18S rDNA have been designed and tested on cultures. The DNA probe has been tested successfully for utility in identifying *P. piscicida* from natural plankton samples (P. Rublee and coworkers unpubl., with J.M.B. and H.B.G.).

Impacts to human health

Strong evidence from a laboratory setting implicates *Pfiesteria*-like species in serious effects on human health (Glasgow et al. 1995). Ten people have been impacted thus far, including three seriously affected, when in close proximity to toxic *P. piscicida* cultures. These scientists worked in five different laboratories. Although the toxins from this dinoflagellate have not yet been identified, the time-locking of the symptomatology convinced these laboratory staff and medical counsel (Duke Medical Center) of the linkage to toxic *P. piscicida* cultures. Effects have occurred after either direct contact with culture water or by inhaling water that was aerosolized by tank aeration systems. Signs have ranged from narcosis, eye irritation, respiratory distress, stomach cramping, and vomiting (of short-term duration, hours) to development of epidermal lesions, cognitive impairment, and Alzheimer's-like short-term memory loss (months). These symptoms reversed in the laboratory workers after contact with toxic cultures ceased. Suppressed T4-cell counts, suggesting the potential for immune system suppression, and peripheral nervous system dysfunction have been associated as more long-term impacts (years). More recently, research has shown that exposure to culture media with TZs (via subcutaneous injection) promotes significant cognitive impairment, short-term memory loss, and learning disability in rats exposed to subcutaneous injection of crude toxin preparation from fish-killing cultures of *P. piscicida* and its co-occurring microbial consortium, relative to control animals without the dinoflagellate (Levin et al. unpubl.). Other impacts of *P. piscicida*'s toxins on small mammal physiology and behavior are being tested (work in progress, E. Levin and D. Schmechel). The possibility of involvement by co-occurring bacteria in toxin production is also under investigation.

Anecdotal information from fishermen, docksmen, and other people who frequent known sites of repeated toxic activity by *P. piscicida* also points to the potential for this dinoflagellate to adversely affect human health in natural habitat (Glasgow et al. 1995; Barker 1997). Such long-term exposure to waters with sublethal TZ populations (manifested, for example, by ulcerative epidemics in fish and by fish kills that last for months) may be a contributing factor in reports by local citizens of epidermal lesions that do not respond to antibiotics, sporadic memory loss, and chronic respiratory infections. Frequently such reports describe a lessening or disappearance of the symptoms following weeks or months without visits to these estuarine areas. Little is yet known about the toxins produced by *Pfiesteria*-like dinoflagellates, except that one major toxin component is water-soluble, and another is highly lipophilic (transported in water as mycelles; Glasgow et al. 1995). These components are known to be denatured after 8–24 h in ultrafiltered estuarine water (Noga et al. 1993, 1996; Burkholder et al. 1995*a*). Until the toxins are identified, and without carefully designed accompanying epidemiology studies, the question of *P. piscicida*'s potential to cause human health impacts for local human populations cannot be resolved.

Pfiesteria-like dinoflagellates are unique from most of their toxic red tide relatives in their complex life cycles, predominance of heterotrophic modes of nutrition along with photosynthesis from kleptochloroplasts (shown for *P. piscicida*), and directed behavior in attacking targeted fish prey (Burkholder and Glasgow 1996). The toxins of these dinoflagellates must be identified to enable determination of the potential for bioaccumulation in food webs and of the full extent of chronic impacts on fish and mammals. Fundamental characteristics about *Pfiesteria*-like dinoflagellates remain to be resolved, such as their overall role as predators across trophic levels from the base to the apex of estuarine food webs. Our laboratory and field data indicate that *P. piscicida* can be strongly stimulated by anthropogenic nutrient enrichment. Further research should continue to examine interactive controls on *Pfiesteria* population survival and success by specific forms of organic and inorganic sources of carbon, nitrogen, phosphorus, and other nutrients. As part of this research, the role of symbiotic and free-living consortium bacteria in *Pfiesteria*'s toxicity and nutrition should be assessed. In the interim, the total available evidence about *Pfiesteria*-like dinoflagellates points to a critical need to characterize their chronic and acute impacts on fish and other targeted prey in estuarine and marine waters that are increasingly affected by high coastal human population growth, intensive agricultural operations, aerial deposition, and other sources of cultural eutrophication (Maiolo and Tschetter 1981; Steel 1991).

References

AMERICAN FISHERIES SOCIETY. 1982. Monetary values of freshwater fish and fish-kill counting guidelines. Am. Fish. Soc. Spec. Publ. 13.

BARKER, R. 1997. And the waters turned to blood. Simon & Schuster.

BURKHOLDER, J. M. 1992. Phytoplankton and episodic suspended sediment loading: Phosphate partitioning and mechanisms for survival. Limnol. Oceanogr. **37:** 974–988.

———. 1996. Surface water issues in North Carolina, p. 11–15.

In Solutions: A technical conference on water quality. Symp. Proc. N.C. State Univ., Raleigh.

———. 1997. Implications of harmful marine microalgae and heterotrophic dinoflagellates in management of sustainable fisheries. Ecol. Appl. **7:** in press.

———, AND H. B. GLASGOW, JR. 1995. Interactions of a toxic estuarine dinoflagellate with microbial predators and prey. Arch. Protistenkd. **145:** 177–188.

———, AND ———. 1996. Trophic controls on stage transformations of a toxic ambush-predator dinoflagellate. J. Eukaryotic Microbiol. **44:** 200–205.

———, ———, AND C. W. HOBBS. 1995*a*. Distribution and environmental conditions for fish kills linked to a toxic ambush-predator dinoflagellate. Mar. Ecol. Prog. Ser. **124:** 43–61.

———, ———, AND A. J. LEWITUS. 1997*a*. The physiological ecology of *Pfiesteria piscicida*, with general comments on ambush-predator dinoflagellates. *In* D. A. Anderson et al. [eds.], The physiological ecology of harmful microalgae. NATO.

———, ———, AND K. A. STEIDINGER. 1995*b*. Stage transformations in the complex life cycle of an ichthyotoxic estuarine dinoflagellate, p. 567–572. *In* Harmful marine phytoplankton blooms. Proc. 6th Int. Conf. on Toxic Marine Phytoplankton. Lavoisier.

———, M. A. MALLIN, AND OTHERS. 1997b. Impacts to a coastal river and estuary from rupture of a large swine waste holding lagoon. J. Environ. Qual. **26:** in press.

———, E. J. NOGA, C. W. HOBBS, H. B. GLASGOW, JR., AND S. A. SMITH. 1992. New "phantom" dinoflagellate is the causative agent of major estuarine fish kills. Nature **358:** 407–410; **360:** 768.

———, R. G. WETZEL, AND K. L. KLOMPARENS. 1990. Direct comparison of phosphate uptake by adnate and loosely attached microalgae within an intact biofilm matrix. Appl. Environ. Microbiol. **56:** 2882–2890.

CUKER, B. E., P. GAMA, AND J. M. BURKHOLDER. 1990. Type of suspended clay influences lake productivity and phytoplankton community response to phosphorus loading. Limnol. Oceanogr. **35:** 830–839.

DIABY, S. 1996. Economic impact of the Neuse River closure on commercial fishing. N.C. Div. Mar. Fish.

DROOP, M. R. 1974. Heterotrophy of carbon, p. 530–559. *In* W. D. P. Stewart [ed.], Algal physiology and biochemistry. Blackwell.

ELBRÄCHTER, M. 1991. Food uptake mechanisms in phagotrophic dinoflagellates and classification, p. 303–312. *In* D. J. Patterson and J. Larsen [eds.], The biology of free-living heterotrophic heteroflagellates. Clarendon.

EPPLEY, R. W., O. HOLM-HANSEN, AND J. D. H. STRICKLAND. 1968. Some observations on the vertical migration of dinoflagellates. J. Phycol. **4:** 333–340.

FENSIN, E. E. 1997. Population dynamics of *Pfiesteria*-like dinoflagellates, and environmental controls in the mesohaline Neuse estuary, 1994–1996. M.S. thesis, North Carolina State Univ., Raleigh.

FENSOME, R. A., AND OTHERS. 1993. A classification of living and fossil dinoflagellates. Micropaleontol. Spec. Publ. 7. Sheridan.

FIELDS, S. D., AND R. G. RHODES. 1991. Ingestion and retention of *Chroomonas* spp. (Cryptophyceae) by *Gymnodinium acidotum* (Dinophyceae). J. Phycol. **27:** 525–529.

FLYNN, K. J., AND K. FLYNN. 1995. Dinoflagellate physiology: nutrient stress and toxicity, p. 541–550. *In* Harmful marine phytoplankton blooms. Proc. 6th Int. Conf. on Toxic Marine Phytoplankton. Lavoisier.

GAINES, G., AND M. ELBRÄCHTER. 1987. Heterotrophic nutrition, p. 224–268. *In* F. J. R. Taylor [ed.], The biology of dinoflagellates. Blackwell.

GLASGOW, H. B., JR., J. M. BURKHOLDER, D. E. SCHMECHEL, P. A. TESTER, AND P. A. RUBLEE. 1995. Insidious effects of a toxic dinoflagellate on fish survival and human health. J. Toxicol. Environ. Health **46:** 101–122.

HALLEGRAEFF, G. M. 1993. A review of harmful algal blooms and their apparent global increase. Phycologia **32:** 79–99.

HEIL, C. A., L. MARANDA, AND Y. SHIMIZA. 1993. Mucus-associated dinoflagellates: Large scale culturing and estimation of growth rate, p. 501–506. *In* Toxic phytoplankton blooms in the sea. Proc. 5th Int. Conf. on Toxic Marine Phytoplankton. Elsevier.

HYNES, H. B. N. 1960. The biology of polluted waters. Liverpool.

JACOBSON, D. M., AND D. M. ANDERSON. 1986. Thecate heterotrophic dinoflagellates: Feeding behavior and mechanisms. J. Phycol. **22:** 249–258.

KAMYKOWSKI, D. M., R. E. REED, AND G. J. KIRKPATRICK. 1992. Comparison of sinking velocity, swimming, rotation and path characteristics among six marine dinoflagellate species. Mar. Biol. **113:** 319–328.

LANDSBERG, J. H., K. A. STEIDINGER, AND B. A. BLAKESLEY. 1995. Fish-killing dinoflagellates in a tropical aquarium, p. 65–70. *In* Harmful marine phytoplankton blooms. Proc. 6th Int. Conf. on Toxic Marine Phytoplankton. Lavoisier.

LEAVENWORTH, S. 1995. Fishing for trouble—can North Carolina save a dying resource? Two-part Ser. 28 and 30 August. Raleigh, News & Observer.

LEWITUS, A. J., AND OTHERS. 1995. Discovery of the "phantom" dinoflagellate in Chesapeake Bay. Estuaries **18:** 373–378.

LOWE, J. A., D. R. G. FARROW, A. S. PAIT, S. J. ARENSTAM, AND E. F. LAVAN. 1991. Fish kills in coastal waters 1980–1989. NOAA, Strategic Environ. Assessments Div.

MCLACHLAN, J. 1979. Growth media—marine, p. 25–51. *In* J. R. Stein [ed.], Handbook of phycological methods—culture methods and growth measurements. Cambridge.

MAIOLO, J. R., AND P. TSCHETTER. 1981. Relating population growth to shellfish bed closures: A case study from North Carolina. Coastal Zone Manag. J. **9:** 1–17.

MALLIN, M. A. 1994. Phytoplankton ecology in North Carolina estuaries. Estuaries **17:** 561–574.

———, J. M. BURKHOLDER, L. M. LARSEN, AND H. B. GLASGOW, JR. 1995. Response of two zooplankton grazers to an ichthyotoxic estuarine dinoflagellate. J. Plankton Res. **17:** 351–363.

———, AND H. W. PAERL. 1992. Effects of variable surface irradiance on phytoplankton productivity in shallow estuaries. Limnol. Oceanogr. **37:** 54–62.

———, J. RUDEK, AND P. W. BATES. 1993. Regulation of estuarine primary production by watershed rainfall and river flow. Mar. Ecol. Prog. Ser. **93:** 199–203.

MANOOCH, C. S., III. 1988. Fisherman's guide—fishes of the southeastern United States. N.C. State Museum Nat. History.

MEYER, F. P., AND L. A. BARCLAY. 1990. Field manual for the investigation of fish kills. U.S. Fish. Wildlife Serv. Resour. Publ. 177.

MIGDALSKI, E. C., AND G. S. FICHTER. 1989. The fresh and salt water fishes of the world. Greenwich House.

MILLER, K. H., AND OTHERS. 1992. Pamlico Environmental Response Team report (June–December 1988). N.C. DEHNR.

NOGA, E. J. 1993. Fungal diseases of marine and estuarine fishes, p. 85–100. *In* J. A. Couch and J. W. Fourrie [eds.], Pathobiology of marine and estuarine organisms. CRC.

———, L. KHOO, J. B. STEVENS, Z. FAN, AND J. M. BURKHOLDER. 1996. Novel toxic dinoflagellate causes epidemic disease in estuarine fish. Mar. Pollut. Bull. **32:** 219–224.

———, S. A. SMITH, J. M. BURKHOLDER, C. W. HOBBS, AND R. A. BULLIS. 1993. A new ichthyotoxic dinoflagellate: Cause of acute mortality in aquarium fishes. Vet. Record **133:** 96–97.

NORTH CAROLINA DIVISION OF MARINE FISHERIES. 1995. Economic impacts of the Neuse River fishing closure related to *Pfiesteria piscicida*. Fact sheets on economic projections.

NORTH CAROLINA SEA GRANT. 1995. Nutrient summit overview.

ROBINEAU, B., J. A. GAGNE, L. FORTIER, AND A. D. CEMBELLA. 1991. Potential impact of a toxic dinoflagellate (*Alexandrium excavatum*) bloom on survival of fish and crustacean larvae. Mar. Biol. **108:** 293–301.

ROGERS, A. W. 1979. Techniques of autoradiography, revised ed. Elsevier/North-Holland.

SAS INSTITUTE, INC. 1987. SAS/STAT guide for personal computers. Version 6.

SAWYER, T. K. 1975. Marine amoebae from surface waters of Chincoteague Bay, Virginia: Two new genera and nine new species within the families Mayorellidae, Flabellulidae, and Stereomyxidae. Trans. Am. Microsc. Soc. **94:** 71–92.

———, G. S. VISVESVARA, AND B. A. HARKE. 1977. Pathogenic amoebas from brackish and ocean sediments, with a description of *Acanthamoeba hatchetti* n.sp. Science **196:** 1324–1325.

SCHNEPF, E., AND M. ELBRÄCHTER. 1992. Nutritional strategies in dinoflagellates. A review with emphasis on cell biological aspects. Eur. J. Protistol. **28:** 3–24.

———, S. WINTER, AND D. MOLLENHAUER. 1989. *Gymnodinium aeruginosum* (Dinophyta): A blue-green dinoflagellate with a vestigial, cryptophycean symbiont. Plant Syst. Evol. **164:** 75–91.

SHUMWAY, S. E. 1990. A review of the effects of algal blooms on shellfish and aquaculture. J. World Aquaculture Soc. **21:** 65–104.

SPERO, H. J. 1982. Phagotrophy in *Gymnodinium fungiforme* (Pyrrhophyta): The peduncle as an organelle of ingestion. J. Phycol. **18:** 356–360.

———, AND M. MOREE. 1981. Phagotrophic feeding and its importance in the life cycle of the holozoic dinoflagellate, *Gymnodinium fungiforme*. J. Phycol. **17:** 43–51.

STEEL, J. [ED.]. 1991. Status and trends report of the Albemarle-Pamlico estuarine study. N.C. DEHNR and U.S. EPA Natl. Estuarine Program.

STEIDINGER, K. A. 1993. Some taxonomic and biologic aspects of toxic dinoflagellates, p. 1–28. *In* I. R. Falconer [ed.], Algal toxins in seafood and drinking water. Academic.

———, J. M. BURKHOLDER, AND OTHERS. 1996*a*. *Pfiesteria piscicida* gen. et sp. nov. (Pfiesteriaccae fam. nov.), a new toxic dinoflagellate with a complex life cycle and behavior. J. Phycol. **32:** 157–164.

———, J. H. Landsberg, E. W. Truby, and B. A. Blakesley. 1996*b*. The use of scanning electron microscopy in identifying small "gymnodinoid" dinoflagellates. Nova Hedwigia **112:** 415–422.

———, E. W. TRUBY, J. K. GARRETT, AND J. M. BURKHOLDER. 1995. The morphology and cytology of a newly discovered toxic dinoflagellate, p. 83–88. *In* Harmful marine phytoplankton blooms. Proc. 6th Int. Conf. on Toxic Marine Phytoplankton. Lavoisier.

STOECKER, D. K. 1991. Mixotrophy in marine planktonic ciliates: Physiological and ecological aspects of plastid retention by oligotrichs, p. 161–180. *In* P. C. Reid et al. [eds.], Protozoa and their role in marine processes. Springer.

TAYLOR, F. J. R. 1987*a*. Dinoflagellate morphology, p. 24–92. *In* F. J. R. Taylor [ed.], The biology of dinoflagellates. Blackwell.

———. 1987*b*. Ecology of dinoflagellates. A. General and marine ecosystems, p. 399–502. *In* F. J. R. Taylor [ed.], The biology of dinoflagellates. Blackwell.

TOFFER, K. L. 1996. Analysis of ribosomal DNA from the toxic dinoflagellate *Pfiesteria piscicida* for the development of a PCR-based probe. M.S. thesis, Univ. North Carolina, Greensboro.

TOMAS, C. R., AND D. G. BADEN. 1993. The influence of phosphorus source on the growth and cellular toxin content of the benthic dinoflagellate *Prorocentrum lima*, p. 565–570. *In* Toxic phytoplankton blooms in the sea. Proc. 5th Int. Conf. on Toxic Marine Phytoplankton. Elsevier.

TURLEY, C. M. 1993. Direct estimates of bacterial numbers in seawater samples without incurring cell loss due to sample storage, p. 143–147. *In* P. F. Kemp et al. [eds.], Handbook of methods in aquatic microbial ecology. Lewis.

UCKO, M., E. COHEN, H. GORDIN, AND S. ARAD. 1989. Relationship between the unicellular alga *Porphyridium* sp. and its predator, the dinoflagellate *Gymnodinium* sp. Appl. Environ. Microbiol. **52:** 2990–2994.