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PCR and FISH Detection Extends the Range of *Pfiesteria piscicida* in Estuarine Waters.

P. A. Rublee[1], J. Kempton[1], E. Schaefer[1], J. M. Burkholder[2], H. B. Glasgow, Jr.[2], and David Oldach[3]

ABSTRACT

PCR and fluorescent in situ hybridization probes were used to assay for the presence of the dinoflagellate *Pfiesteria piscicida* in 170 estuarine water samples collected from New York to nothern Florida. 20% of samples tested positive for the presence of *P. piscicida*, including sites where fish kills due to *Pfiesteria* have occurred and sites where there was no historical evidence of such events. The results extend the known range of *P. piscicida* northward to Long Island, New York. The results also suggest that *P. piscicida* is a common, and normally benign, inhabitant of estuarine waters of the eastern US.

INTRODUCTION

Phytoplankton are a critical food resource for many commercially important finfish and crustaceans and shellfish. However, proliferation of this food resource to high densities can have adverse effects on organisms as well, due to development of anoxia or the production of algal toxins (cf., Burkholder 1998). The number of reported red tides and toxicity episodes has been increasing globally for the past 20 years (Anderson 1989; Hallegraeff 1993; Anderson 1994). Although this may be partly due to increased scientific awareness, improved analytical capabilities, and the discovery of new toxic species, it is also likely that environmental change and increased dispersal mechanisms contribute (Anderson 1989; Hallegraeff 1993). Dispersal mechanisms include currents and ballast water transport, while changes in the environment may result from global climate change or coastal eutrophication from domestic, agriculture, industry, and aquaculture sources (Anderson 1989; Hallegraeff 1993; Anderson 1994).

The heterotrophic dinoflagellate *Pfiesteria piscicida* Steidinger et Burkholder (Steidinger et al. 1996a) and at least one other toxic *Pfiesteria*-like dinoflagellate (Steidinger et al. 1996b; Burkholder and Glasgow 1997) were first discovered by accidental contamination of established prey cultures in laboratory aquaria (Noga et al. 1993; Landsberg et al. 1995). *P. piscicida* was first identified in a natural setting during a fish kill in the Pamlico River estuary, North Carolina in 1991 (Burkholder et al. 1992). *P. piscicida* has a complex life cycle including zoospore, amoeboid, and cyst stages (Burkholder et al. 1993; Burkholder and Glasgow 1995), and most stages are colorless unless they have ingested algal prey. It is also ephemeral in the water column.

1 Biology Department, University of North Carolina at Greensboro, PO Box 26174, Greensboro, NC 27402-6174

2 Dept. of Botany, North Carolina State University, PO Box 7612, Raleigh, NC 27695-7612

3 Institute of Human Virology, University of Maryland School of Medicine, 725 W. Lombard St., Baltimore, MD 21201

In general, field studies have found *P. piscicida* to be most prevalent in locations influenced by anthropogenic nutrient loading (Burkholder et al. 1995a). Sources of nutrient loading include municipal wastewater facilities, phosphate mining operations, fish processing plants, and other sources associated with the release of nutrient rich by-products into aquatic systems. Nontoxic zoospore abundance was found to be greater near wastewater discharge sites as compared to control sites without wastewater influence (Burkholder and Glasgow 1997). The increase in anthropogenic nutrient loading into aquatic systems and the subsequent increase in *P. piscicida* zoospore presence may be strongly correlated to the recent surge in *P. piscicida* associated fish kills. Approximately 75% of *P. piscicida*-related fish kills have taken place in nutrient enriched waters (Burkholder et al. 1995a). During the years 1991-1993, *P. piscicida* was implicated as the causative agent of 52 ± 7% (mean ± S.D.) of the 35 major fish kills in the estuarine and coastal waters of North Carolina (Burkholder et al. 1995a). In August, 1997, 1-1.5 x 10^5 fish turned up dead or dying in the lower Pocomoke River, Maryland (Matuszak et al. 1997). Initial analysis of water samples indicated the likely presence of *Pfiesteria*-like dinoflagellates, which was later confirmed. Subsequently, over a 17-day period in August and September, portions of the Pocomoke River, King's Creek, and Chicamicomico River were placed under a closure order in response to *Pfiesteria*-related fish kills and public health concerns (Glasgow, et al. 1995, Gratten, et al.1999).

Many characteristics make *P. piscicida* difficult to detect. First, *P. piscicida* is capable of transforming rapidly among various stages of its life cycle. With at least 24 different life cycle stages covering a 90-fold range of cell lengths, detection of this organism is difficult. Many of the life cycle stages are cryptic. For example, *P. piscicida* amoebae have often been mistaken for debris (Burkholder and Glasgow 1995). In addition, *P. piscicida* is capable of changing its pigmentation by kleptochloroplastidy, stealing chloroplasts from algal prey (Steidinger et al. 1995). *P. piscicida* is also ephemeral in the water column (Burkholder et al. 1992). This behavior can be described as "hit and run" where *P. piscicida* swarms up from the sediments, kills it's prey, and then settles back to the sediments once feeding is complete. There have been exceptions to this behavior; a *P. piscicida* fish kill in the Neuse River estuary where approximately 1.5 x 10^7 fish were killed in 1991 lasted for approximately 90 days (Burkholder and Glasgow 1997). Also, *P. piscicida* typically comprises < 10% of the total planktonic community even during a kill event (Burkholder et al. 1995a). *P.* piscicida's relatively low abundance during fish kills coupled with it's ephemeral nature requires timely sampling to detect this organism at fish kills.

The current method for determining the presence of toxic *P. piscicida* in an environmental sample includes identification of *Pfiesteria*-like organisms based on morphological identification and swimming behavior using standard light microscopy at 600 x with brightfield, phase-contrast, and differential interference optics (Steidinger et al. 1996a; Burkholder and Glasgow 1997). The sample is then tested to confirm toxicity by fish bioassays. Once toxicity has been confirmed, thecal plate tabulation is used to confirm the identity of *P. piscicida* using scanning electron microscopy (SEM). In this study, we have used molecular probes to assay for the presence of *P. piscicida* in water samples from New York to northern Florida. All samples were tested using PCR probing methods (c.f. Oldach, et al. In review), and in some samples

we also employed fluorescent in situ hybridization (FISH, Kempton, et al. In prep.). Molecular probes are sensitive, rapid, and relatively inexpensive, although a disadvantage of the probes we are using is that they are not able to indicate toxicity, even if *P. piscicida* is detected.

MATERIALS AND METHODS

Selection of sampling locations

The presence of *Pfiesteria piscicida* was tested on two sets of samples collected between June and November 1998. The first set of samples was from a north-south coastal transect, and the second set consisted of samples provided by colleagues and state agencies. The first sample set was collected in September and October 1998 and can be characterized as samples of convenience. These samples were selected based on a number of criteria. The most important criterion was accessibility to sampling locations. This was dependent on roadways leading to sampling sites or boat access to areas of interest. A second criterion was appropriate salinity, when feasible areas were selected that fell in the mid to low salinity range (5 - 18 psu). The second set of water samples was provided by colleagues and representatives of various state agencies and collected during routine monitoring trips.

Sampling protocol for determining geographic distribution.

The first set of water samples was assayed for the presence of *P. piscicida* using both PCR probe and FISH assays. Water samples were collected approximately 0.5 m below the surface and the salinity of each individual water sample was determined using a Full Range Specific Gravity Meter (SeaTesT). For PCR probing, cells from the water sample were concentrated for DNA extraction on 25 mm GF/C glass microfibre filters (Whatman International, Ltd.) by vacuum filtration. The volume passed through the filter was dependent on the turbidity of the water sample (30 -375 mL). The filter was then placed in 1 mL of CTAB buffer in a 2 mL microfuge tube.

Water sample aliquots for FISH detection were placed in 120 mL collection bottles, maintained at ambient temperature, and processed within a 12 hr period. 60 or 90 mL of the water sample were concentrated by centrifugation (IEC Clinical Centrifuge) and fixed with paraformaldehyde as described below. These samples were then stored on ice until further processing, within 7 days. In order to prevent cross contamination of samples, the collection bucket and filtering apparatus were washed in 50% bleach, and rinsed multiple times at each location prior to sample collection.

The second set of water samples was assayed for the presence of *P. piscicida* using PCR probing only. All water samples were collected for extraction with CTAB buffer and assays with the *P. piscicida* primer sets with PCR as previously described. Most of these sample sites were selected by the individual collaborating agencies for analysis with the *P. piscicida*-specific primer sets. Collection for FISH analysis was not performed by the agencies for logistical reasons: the concentration of sample for FISH requires a clinical centrifuge and both fixative and fixed samples must be kept refrigerated.

PCR amplification

DNA extractions for PCR were carried out using a rapid CTAB (cetyltrimethylammonium bromide) buffer DNA isolation technique (Schaefer 1997). Briefly, the

TABLE 1: *P. piscicida*-specific primers for PCR.

Primer Label & Direction	Sequence of Primer (5'-3')
65 For	AGCCTAAGCTTGTTAAACGGCAATGC
110 For	GTTAGATTGTCTTTGGTGGTCAATCC
286 For	CATCCGCTGGCGATATACCATATCAC
301 For	TATACCATATCACTTTCTGACC
Euk Rev18S Rev*	TGATCCTTCTGCAGGTTCACCTAC

* Primer sequence from Medlin et al. 1988; Sogin 1990

filtered field samples are macerated in CTAB followed by chloroform extraction and isopropanol precipitation. The dried pellet is then dissolved in 25 μL TE buffer.

Each sample was tested for the presence of *P. piscicida* by PCR probing using at least two and often four primer sets. Each primer set consisted of a species specific forward primer, and a eukaryotic specific small subunit rDNA reverse primer (Table 1). The reaction mixture and conditions had been determined previously by testing with cultured material and several environmental samples from a fish kill in the Neuse River where the presence of toxic *P. piscicida* was been confirmed (Aquatic Botany Laboratory, NC State Univ.). The reaction mixture for each primer set was 50 μL total volume, containing 5 μL of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 9.0 at 25°C, 1% Triton X-100); 5 μL of 25 mM MgCl2; 1.5 U Taq DNA Polymerase (Promega Corp.); 2.5 μL of 4mM dNTP stock (1mM each of dATP, dGTP, dCTP, dTTP; Stratagene); 5 μL of 100 μM Bovine Serum Albumin (BSA; Sigma); and 1 μL of 10 μM of each forward and reverse primer for a final concentration of 0.2 μM per primer. Approximately 100 ng of extracted DNA was used per reaction. Reaction conditions included denaturing at 94°C for 2 min; followed by 39 cycles of 94°C for 1 min, 60°C for 1.5 min, 72°C for 2 min. All PCR reactions were overlaid with 50 μL of sterile mineral oil. PCR reactions were carried out in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc.). Eukaryotic SSU rDNA primers (Sogin 1990) were used to confirm successful extraction of amplifiable DNA from the field sample, and DNA isolated from a known culture of *P. piscicida* was always included as a positive control. DNA isolated from an additional dinoflagellate culture (usually Peridinium foliaceum) was used as a negative control. All PCR amplifications were verified by agarose gel electrophoresis and ethidium bromide staining.

Fluorescent in situ hybridization

The procedure for FISH is described in detail elsewhere (Kempton, 1999.). Briefly, after concentration of the water sample by centrifugation, 300 μL of resuspended cell pellet was transferred into a 1.5 mL microcentrifuge tube and fixed in paraformaldehyde buffer (Hawes 1988; Lin and Carpenter 1996). Upon completion of fixation, cells were resuspended in cold methanol and stored at -20°C until hybridization. For each FISH reaction, fixed cells were resuspended in a hybridization buffer (755 mM NaCl, 70 mM sodium citrate at pH 7.0, 5 mM EDTA, 0.01% SDS, 0.1% CTAB, and 5% formamide) with the fluorescent probes (modified from Adachi et al. 1996). We

utilized two of our oligonucleotide primers (65 For and 286 For) with fluorescein attached at the 5' end as the fluorescent probes. Hybridization reactions took place in a PTC-100TM Programmable Thermal Controller (MJ Research, Inc.) using the following protocol: denaturation at 95°C for 3 minutes; incubation at 4°C for 3 minutes; and hybridization for 1.5 hr at 62°C. After hybridization, the cells were washed with 5X SSC (750 mM NaCl, 75 mM sodium citrate, pH 7.0) at the temperature of hybridization for 15 minutes (modified from Adachi et al. 1996). Cells were then collected on plain black polycarbonate membrane filters (1.0 μm pore size, 25 mm), washed with 15 mL distilled water to remove any unbound or loosely bound probe and to reduce background fluorescence from the membrane filter. The fluorescently labeled cells were then observed using an Olympus BX60 Microscope System with BX-FLA reflected light fluorescence attachment. A DAPI/FITC/Texas Red filter set (Chroma Technologies Corp.) was used for viewing the fluorescein labeled cells. The DAPI/FITC/Texas Red filter set was selected for the FISH assays because it allowed detection of green fluorescence from the fluorescein marker and red fluorescence from chlorophyll autofluorescence. An aliquot of fixed cells from a known *P. piscicida* culture and including non-*Pfiesteria* dinoflagellates (usually *Amphidinium carterae* and *Heterocapsa triquetra*) was always carried through the FISH procedure to serve as positive and negative controls, respectively.

RESULTS AND DISCUSSION

The known distribution of *P. piscicida* has been accumulated primarily from information gathered by conducting water samples where sudden death fish kills and fish lesion events have been reported. The geographic range extends from the Indian River in Delaware to Mobile Bay, Alabama. The epicenter of fish kill activity is the extensive estuarine system of North Carolina, where most documented *Pfiesteria* caused fish kills have occurred, and where the only known *Pfiesteria* related fish kill during 1998 occurred. The presence of *P. piscicida* at these sites was confirmed by SEM and toxicity confirmed in fish bioassays (Table 2). Our goal was to test additional sites to assay for the presence of *P. piscicida*.

Our combined sample sets included 170 water samples from 11 east coast states (Table 3). The samples represented 57 different water bodies, as multiple samples were taken from some water bodies (although at different locations in them). Overall, > 20% of the samples tested positive for the presence of *P. piscicida*, 5 of 38 water samples were positive for either the PCR or FISH assay in the first set, and 30 of 132 water samples were positive in the second set. We found *P. piscicida* present in all states we sampled except New Jersey and Florida (Fig. 1).

Several of the sampling sites are of particular interest. In both sample sets, *P. piscicida* was detected in New York State. This is the first report of the presence of *P. piscicida* north of Delaware. Also in both data sets, *P. piscicida* was detected in the Chicamacomico River, Maryland. The Chicamacomico River is a site of past *Pfiesteria*-related fish kills and experienced fish lesion events during the summer of 1998. In the second sample set, there was also a positive for a Pocomoke River, Maryland sample, another site that has a history of *Pfiesteria*-related fish kills. For the first time, *P. piscicida* was detected in the Rhode River, MD, Mosquito Creek, VA, and the Little Satilla River, GA. Water samples taken from a fish lesion event in the Cooper River, SC, tested positive for *P. piscicida* using both PCR and FISH. In

TABLE 2. Sites of current known distribution of *P. piscicida* and *Pfiesteria* spp. Information compiled based on SEM confirmation and fish bioassays.

Location	Reference
Indian River, DE	Burkholder et al. 1995a, Burkholder and Glasgow 1997
Chicamicomico River, MD	Matuszak et al. 1997
Shiles Creek, MD	Matuszak et al. 1997
Pocomoke River, MD	Burkholder and Glasgow 1997, Matuszak et al. 1997
Kings Creek, MD	Matuszak et al. 1997
Roanoke River, NC	Burkholder and Glasgow 1997
Pamlico River, NC	Burkholder et al. 1995a, Burkholder and Glasgow 1997
Neuse River, NC	Burkholder et al. 1995a, Burkholder and Glasgow 1997
Newport River, NC	Burkholder et al. 1995a
New River, NC	Burkholder and Glasgow 1997
Topsail Beach, NC	Burkholder et al. 1995a
Wrightsville Beach, NC	Burkholder et al. 1995a
Cape Fear River, NC	Burkholder and Glasgow 1997
Clambank Creek, SC	Burkholder et al. 1995a, Burkholder and Glasgow 1997
St. Johns River, FL	Burkholder et al. 1995a, Burkholder et al. 1995a
Indian River, FL	Burkholder and Glasgow 1997
Flamingo Bay, FL	Burkholder and Glasgow 1997
Pensacola, FL	Burkholder et al. 1995a, Burkholder and Glasgow 1997
Mobile Bay, AL	Burkholder et al. 1995a

TABLE 3. Sample locations and results for combined data set

State	Total sites	Total samples	# positive samples
New York	17	26	8
New Jersey	4	4	0
Delaware	4	16	1
Maryland	8	49	14
Virginia	9	15	1
North Carolina	6	43	8
South Carolina	7	12	2
Georgia	4	4	1
Florida	1	1	0

addition, both assays detected *P. piscicida* from the Pamlico River and Neuse River, NC. A water sample taken from an area of fish by-catch in the New River, NC (initially reported to authorities as a fish kill) also tested positive for *P. piscicida* using PCR.

Our PCR primers and fluorescent probes were designed and initially tested on cultured isolates of *P. piscicida*. Assaying field samples presents several potential problems of specificity and sensitivity. Specifically, while the probes may be exten-

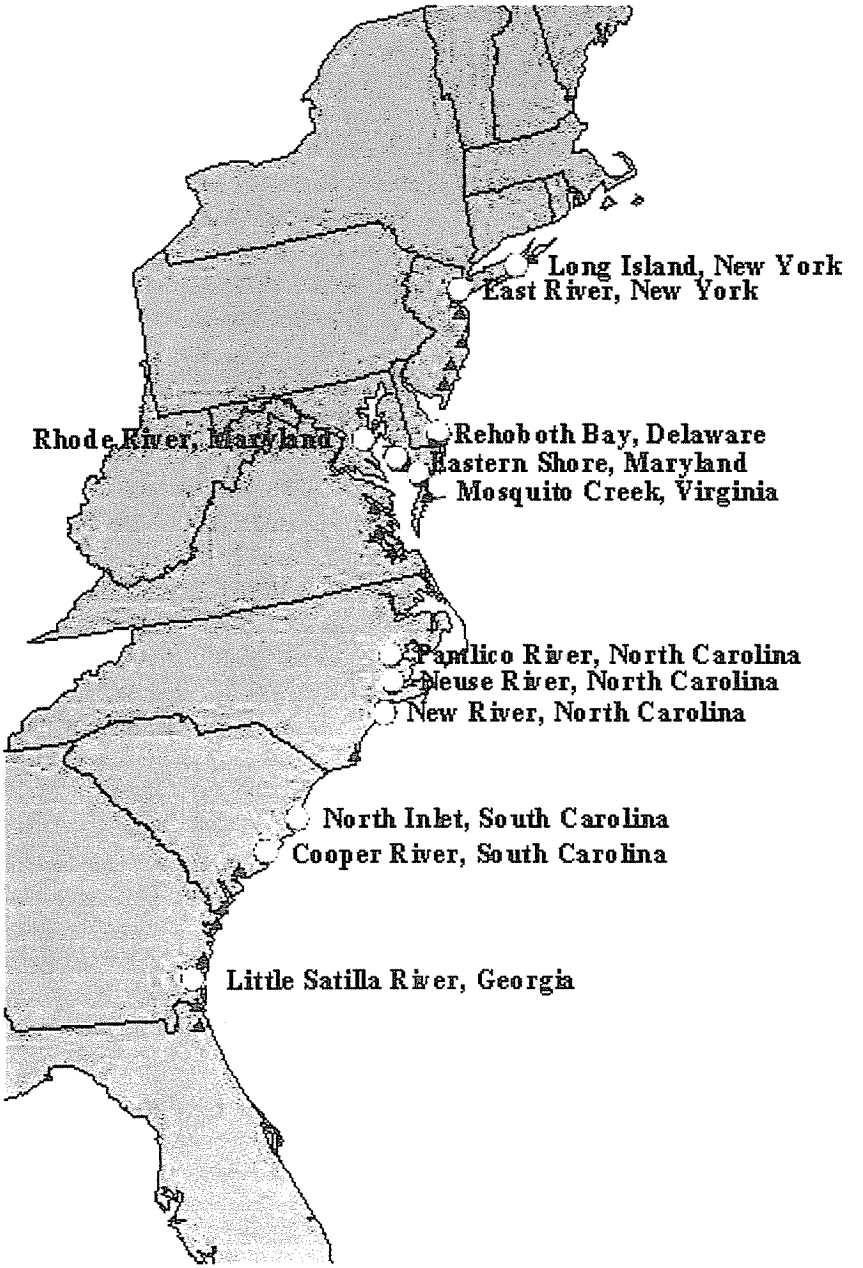

FIGURE 1. Detection of *P. piscicida* in environmental samples using PCR and FISH assays. Open circles = positive sites, closed triangles = not detected.

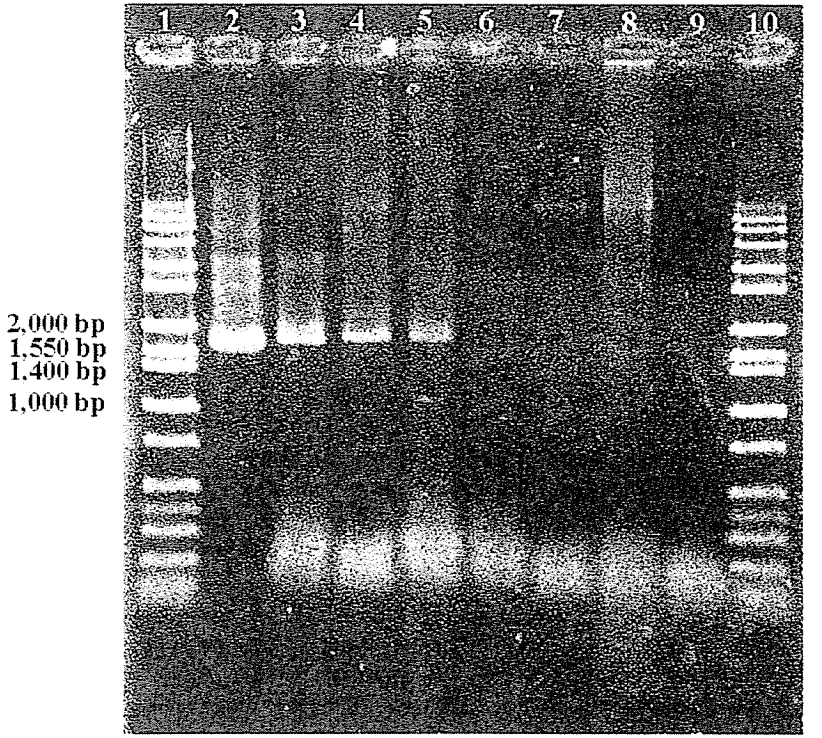

FIGURE 2. Gel electrophoresis of PCR products for selected environmental samples using the *P. piscicida*-specific primer Set 65 For - 18S Rev. Lane 1, Hi LoTM DNA Marker. Lane 2, Positive control (102-1). Lane 3, Neuse River, NC (fish kill 7/98). Lane 4, Chicamacomico River, MD (20% lesion fish). Lane 5, Beaverdam Creek, NY (no known history of *Pfiesteria*). Lane 6, Cape Fear River, NC. Lane 7, Nanticoke River, MD. Lane 8, Carmans River, NY. Lane 9, Negative control (no template). Lane 10, Hi LoTM DNA Marker.

sively tested against other cultured organisms, it is possible that field samples contain species, as yet unknown or uncultured, that may also contain the target sequence. Further, field samples contain considerable amounts of non-target DNA that can interfere with proper hybridization of the probe to the target sequence. This becomes particularly problematic when the number of targeted individuals is low relative to other sources of DNA. Finally, the range of dissolved compounds and particulates in natural water samples may reduce the sensitivity of the PCR assay either by inhibition of the chemical reactions or by adsorption of extracted DNA to particle surfaces. We did note that in some samples only 1 or 2 of the *P. piscicida* primer sets actually produced positive results. Such results are not uncommon in field samples. However, our results overall, as discussed below, suggest that while there are challenges in field applications of oligonucleotide probing, for the most part our assays are robust.

Two water samples taken during this study suggest that the specificity of our probes is good. One of these samples was a July 28, 1998 sample taken during a fish kill in the Neuse River, North Carolina. The sample was confirmed as having toxic *P. piscicida* by fish bioassays and plate tabulation by SEM. Both the fluorescent probe

and all four PCR primer sets gave a strong positive signal for this sample. The fluorescent probe effectively labeled the *P. piscicida* cells in this water sample with a strong fluorescent signal and no apparent cross reactivity to other organisms in the water sample. A second sample, also taken during a fish kill, was from the Indian River, Delaware. This fish kill appeared to be the result from a bloom of Gyrodinium instriatum (E. Humphries, DE DNREC, personal communication), and it tested negative with all our probes as well in fish bioassays.

Additional verification of the specificity of the PCR probes in environmental samples was performed by sequencing the PCR product. Two PCR positives from field samples (Fig. 2) were gel extracted (Qiagen Gel Extraction Kit) and sequenced using the 65 For primer. The sequenced PCR products were then aligned with the *P. piscicida* consensus sequence for verification. This primer was ideal because it enabled us to sequence most of the first 400 bases in the region of the SSU rDNA of *P. piscicida* that has the highest degree of nucleotide variability. Using the RDP database, the rRNA sequence data from the PCR positives for the Neuse River, NC fish kill (7/98) and the Beaverdam Creek, NY river sample were compared to the GenBank *P. piscicida* sequence (AF077055). Comparison of these sequences showed 100% homology between the environmental samples and our GenBank sequence.

Although questions remain regarding probe sensitivity across varied water sample types, this study demonstrates the effectiveness of our probes in determining the presence of *Pfiesteria piscicida*. We emphasize again, however, that these probes do not indicate whether the organism is producing toxin. In fact, given the large number of samples that tested positive with no indication of fish health problems and no history of fish health problems at the site, we suspect that *P. piscicida* exists in a benign state most of the time. Fish kills or human health threats are likely restricted to those circumstances when the appropriate conditions are met (c.f., Burkholder and Glasgow 1997).

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