

Use of Molecular Probes to Assess Geographic Distribution of *Pfiesteria* Species

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We have developed multiple polymerase chain reaction (PCR)-based methods for the detection of *Pfiesteria* sp. in cultures and environmental samples. More than 2,100 water and sediment samples from estuarine sites of the U.S. Atlantic and gulf coasts were assayed for the presence of *Pfiesteria piscicida* Steidinger & Burkholder and *Pfiesteria shumwayae* Glasgow & Burkholder by PCR probing of extracted DNA. Positive results were found in about 3% of samples derived from routine monitoring of coastal waters and about 8% of sediments. The geographic range of both species was the same, ranging from New York to Texas. *Pfiesteria* spp. are likely common and generally benign inhabitants of coastal areas, but their presence maintains a potential for fish and human health problems. **Key words:** molecular probes, PCR, *Pfiesteria*, toxic dinoflagellates. — *Environ Health Perspect* 109(suppl 5):765–767 (2001). <http://ehpnet1.niehs.nih.gov/docs/2001/suppl-5/765-767rublee/abstract.html>

Molecular probes to harmful algae are powerful tools that can be used to positively identify the presence or absence of the organisms in cultures and environmental samples (1). The gene probe approach has the potential for high specificity, lower cost, and quicker turnaround (<48 hr) than methods that rely on bioassays, culturing, and detailed microscopic observation. Disadvantages of the molecular methods are the potentially long lead time needed for development of the specific probes and the limitations that may be imposed by the specific target. For example, probes to structural genes such as SSU rDNA cannot be used to determine whether the populations detected are producing or capable of producing toxins. Thus, in efforts to determine the distribution of actively toxic populations, a combination of molecular probes, followed by toxin assays or fish bioassays, is currently necessary.

In 1998 we determined the sequence of the small subunit ribosomal RNA gene of *Pfiesteria piscicida* Steidinger & Burkholder (2) from cultures (Genbank Accession AF077055) and developed a suite of primers that could be used to identify *P. piscicida*. The approach included three methods. First, primer pairs specific to *P. piscicida* could be used in a polymerase chain reaction (PCR) amplification of purified DNA from culture or field samples to indicate the presence or absence of the target nucleic acid sequence (3,4). Second, primers specific to a broader taxonomic category were used in heteroduplex mobility assays (4), which have proven useful for determining the purity of cultures and for use in the discovery of new variants (strains, species, genera) of *Pfiesteria*-like organisms. Third, combinations of the species-specific primers could be labeled with a fluorescent compound and then used to

visualize whole target cells by fluorescent *in situ* hybridization.

During the summer and fall of 1998, we confirmed the capability of the primers to detect *P. piscicida* during a fish kill event in the Neuse River, North Carolina. We also implemented field collection protocols for DNA extraction and purification from field samples we had developed previously, and began to use the assay methods to test estuarine coastal water samples along the U.S. east coast (3). In 1999, primers were developed to a second *Pfiesteria* species, *Pfiesteria shumwayae* Glasgow & Burkholder (5), which were incorporated into our field-sampling protocols, and we began testing estuarine sediments as well as water.

In this article we summarize our results of field assays for *Pfiesteria* species from 1998 to 2000. Primarily, these results are from samples collected by state agency personnel from New York State to Texas either in response to fish lesion or fish kill events or as additions to routine monitoring of coastal waters.

Materials and Methods

For most field samples, surface water samples were concentrated by vacuum filtration of 30–150 mL onto a 25-mm glass microfiber filter (GF/C; Whatman, Tewksbury, MA, USA). The filter was then placed in 1 mL hexadecyltrimethylammonium bromide buffer in a 2-mL microfuge tube and stored at room temperature until extraction (6). In some cases samples were concentrated on 5- μ m pore-size nylon filters and extracted immediately with a commercial kit (DNeasy plant kit; Qiagen, Valencia, CA, USA) or concentrated by centrifugation and extracted (Invitrogen, Carlsbad, CA, USA). Extracted DNA was resuspended in 25 μ L sterile Tris-ethylenediaminetetraacetate buffer, pH 7.4, and stored at -20°C until use.

The extracted DNA from each sample was first probed by PCR with universal 18S rDNA primers to assure that the sample contained amplifiable DNA. PCR reactions for detection of *P. piscicida* and *P. shumwayae* followed the protocols previously reported (2,3). Briefly, 50- μ L reactions contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂ (4.0 mM for *P. shumwayae*), 200 μ M nucleotides, 1 U *Taq* DNA polymerase (PCR Supermix, Gibco/Life Technologies, Gaithersburg, MD), 0.2 μ M of each primer (0.8 μ M for *P. shumwayae*), and 0.1–1 μ L of the sample DNA. PCR reaction conditions for *P. piscicida* assay were $94^{\circ}\text{C} \times 4$ min, then 39 cycles ($95^{\circ}\text{C} \times 1$ min/ $40^{\circ}\text{C} \times 1.5$ min/ $72^{\circ}\text{C} \times 2.5$ min), then $72^{\circ}\text{C} \times 7$ min followed by cooling to 4°C . *P. shumwayae* reaction conditions were $95^{\circ}\text{C} \times 3$ min, then 49 cycles ($95^{\circ}\text{C} \times 30$ sec/ $60^{\circ}\text{C} \times 30$ sec/ $72^{\circ}\text{C} \times 40$ sec), then $72^{\circ}\text{C} \times 5$ min followed by cooling to 4°C . Reaction products were visualized by electrophoresis and ethidium bromide staining on agarose gels. Both positive controls (DNA derived from scanning electron microscopy-confirmed cultures of each target species) and negative controls (reagent blanks) are run in all assays. In most cases, field samples that test positive are confirmed by a second set of primers and/or corroborated by testing in a second laboratory. In many cases amplicons are also sequenced and compared to Genbank sequences as a further verification.

We also tested sediment samples from three sites in the Neuse River, including two sites that have a history of fish health problems and the presence of *Pfiesteria* sp., and an upstream site that had no history of such problem. Triplicate box cores were taken at each site and the sediments were assayed at

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Table 1. Incidence of *Pfiesteria* sp. in estuarine water samples from the U.S. east and gulf coasts, 1998–2000, as determined by PCR probes.

State	Type	Number of samples	Number of positive samples	
			<i>P. piscicida</i>	<i>P. shumwayae</i>
New York	Water	225	18	6
	Sediments	21	7	4
New Jersey	Water	46	1	0
	Sediments	26	3	0
Delaware	Water	434	8	19
	Sediments	47	4	1
Maryland	Water	220	33	8
Virginia	Water	86	0	5
North Carolina	Water	206	2	17
	Sediments	28	2	0
South Carolina	Water	97	0	2
	Sediments	59	4	0
Georgia	Water	139	1	0
	Sediments	41	0	0
Florida	Water	338	1	2
	Sediments	66	0	0
Texas	Water	73	3	3
	Sediments	8	0	2
Total	Water	1,864	67	64
	Sediments	296	20	7

depths of 0, 1, 2, 4, and 8 cm into the sediment. Overlying water was also sampled at 0.5- or 1-m intervals from the water surface to the sediment–water interface.

Results and Discussion

Prior to 1998 *Pfiesteria* species had been reported to occur from Delaware to Mississippi on the east and gulf coasts of the United States (7,8), primarily as a result of investigating fish kill or fish lesion events. In 1998, a year in which few fish kills were attributed to *Pfiesteria*, 21% of 170 samples probed by PCR tested positive for *P. piscicida* (3). Again, however, most samples were from sites of fish health problems. Our current cumulative data (Table 1) show that approximately 7% of field samples demonstrate the presence of one species of *Pfiesteria*. Further, sediment samples show a higher incidence of positive results (10%) than water samples, and most of these are from routine monitoring samples. The results also demonstrate the first records of *Pfiesteria* sp. in Texas coastal waters, thus extending the known range to the southern limit of the U.S. gulf coast.

We have previously suggested that *Pfiesteria* sp. are probably common and normally benign inhabitants of many estuarine systems (9). Our cumulative results tend to support this point of view. Interestingly, although there have been more fish kill and lesion events sampled in the mid-Atlantic states during 2000 than in the previous 2 years, the identification of *Pfiesteria* in samples from the fish kill sites has been limited to only a few occasions, even when many other potential causes (e.g., low dissolved oxygen)

could be ruled out. Although this observation may be because other pathogens or parasites were the causative factors, it may also be related to the importance and difficulty of sampling while the kill event is in progress, which is critical to establishing *Pfiesteria* sp. as a causative factor (10,11).

Our observations from the cumulative data set led us to several cautions regarding sampling. First, the observations combined with results from sediment assays suggest to us that routine monitoring of water is not the optimal method to detect *Pfiesteria* sp. Because benthic amoeboid stages of *Pfiesteria* may be common forms, except during those conditions that lead to fish lesions or kill events, monitoring of sediments may give a picture of the presence of endemic populations and may also be a means of finding a residual signal from an event. For example, a citizen-reported fish kill during a drought year (1999) in the Tuckahoe River in southern New Jersey was not sampled for PCR analysis during the event. Twenty-three days later, single water and surface sediment samples were taken at each of three sites: 1 mile above the kill site, at the reported site, and 1 mile downstream. Further, between the occurrence of the event and the sampling for PCR assay, the area had been subject to extensive rainfall as a result of Hurricane Floyd. Although the single water sample taken downstream from the fish kill site was positive, all three sediment samples were positive for *P. piscicida* (12). Second, samples taken through the water column and into the sediment at three sites on the Neuse River showed positive signals for *Pfiesteria* only in sediments of most, though not all, replicate cores taken at two

Table 2. Results of *P. piscicida* assays in Neuse River, North Carolina sediment cores. Flanners Beach and Carolina Pines are sites with a history of *Pfiesteria*-caused fish health problems. Mills Branch has no history of the presence of *Pfiesteria*. Assays of overlying water were negative for *P. piscicida*, and all samples were negative for *P. shumwayae*.

Site	Sediment depth (cm)	Positive assays out of three reps (no.)
Flanners Beach	0	2
	1	2
	2	2
	4	1
Carolina Pines	8	0
	0	0
	1	1
	2	1
Mills Branch	4	0
	8	0
	0	0
	1	0
	2	0
	4	0
	8	0
	8	0

sites with a known history of *Pfiesteria* events (Table 2). Although these positive results do not prove linkage of *Pfiesteria* to specific fish kills, they are suggestive.

The sediment samples from the Neuse River also suggest that *Pfiesteria* may be distributed in patches at fine spatial scale in bottom sediments, as replicate cores from the same site do not always give the same result. A previous study conducted in Maryland waters (13) suggested short-term temporal variability in water samples. Given these indications of fine-scale variability, the importance of carefully designed and timed sampling is significant, especially when trying to determine if *Pfiesteria* is present at the site of a fish health event. The development of toxin assays suitable for field use will be a welcome addition in this regard.

A broad range of other approaches have been or are in development in other laboratories (14). These include real-time PCR methods (15) that have great sensitivity and provide rapid results. Additional approaches include PCR-based probing to other regions of the ribosomal DNA or RNA, reverse transcriptase–PCR as a means of developing stage-specific probes, PCR fluorescent fragment detection assays, as well as antibody and lectin probes, and electrochemical detection methods (14,16). Many of these approaches have potential for speed, sensitivity, and simplicity for field sampling, and some may also be able to be automated for unattended field use. As there is potential for both human and fish health problems as a result of *Pfiesteria* toxins, continued development of improved methods and continued study of both broad and fine-scale distribution of *Pfiesteria* sp. is prudent.

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