Development of Real-Time PCR Assays for Rapid Detection of *Pfiesteria piscicida* and Related Dinoflagellates

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Abstract:

Pfiesteria complex species are heterotrophic and mixotrophic dinoflagellates that have been recognized as harmful algal bloom species associated with adverse fish and human health effects along the East Coast of North America, particularly in its largest (Chesapeake Bay in Maryland) and second largest (Albermarle-Pamlico Sound in North Carolina) estuaries. In response to impacts on human health and the economy, monitoring programs to detect the organism have been implemented in affected areas. However, until recently, specific identification of the two toxic species known thus far, *Pfiesteria piscicida* and *P. shumwayae* (sp. nov.), required scanning electron microscopy (SEM). SEM is a labor-intensive process in which a small number of cells can be analyzed, posing limitations when the method is applied to environmental estuarine water samples. To overcome these problems, we developed a real-time PCR-based assay that permits rapid and specific identification of these organisms in culture and heterogeneous environmental water samples. Various factors likely to be encountered when assessing environmental samples were addressed, and assay specificity was validated through screening of a comprehensive panel of cultures, including the two recognized *Pfiesteria* species, morphologically similar species, and a wide range of other estuarine dinoflagellates. Assay sensitivity and sample stability were established for both unpreserved and fixative (acidic Lugol's solution)-preserved samples. The effects of background DNA on organism detection and enumeration were also explored, and based on these results, we conclude that the assay may be utilized to derive quantitative data. This real-time PCR-based method will be useful for many other applications, including adaptation for field-based technology.

Article:

Pfiesteria complex species are heterotrophic and mixotrophic dinoflagellates that have been recognized as harmful algal bloom (HAB) species. Many HAB species are believed to be increasing in frequency and worldwide distribution, with negative effects on the economy, human health, and the environment (12, 13, 20). Of the approximately 5,000 recognized species of marine phytoplankton (21), about 300 can occur in sufficient concentration to discolor the water while at least 90 of these are classified as HAB species because they can produce potent toxins that have adverse effects on fish and human health (2, 3, 13). Other species, although harmless to humans, may have direct effects on fish through damage to their gills (13) or by leading to low dissolved-oxygen concentrations (2).

Toxicity-associated *Pfiesteria* species have been identified in both the Chesapeake Bay (Maryland) and Albermarle-Pamlico Sound (North Carolina) estuaries, where adverse fish and human health effects attributed to these organisms have been reported (1, 5, 7, 10, 22). In 1997, detection of *Pfiesteria piscicida* was correlated with three major fish kills affecting the Pocomoke, Chicamacomico, and Manokin Rivers in Maryland. In that same year, five major fish kill-disease events occurred in the Neuse and Pamlico estuaries in North Carolina.

Watermen and other individuals exposed to those affected river systems at these times complained of symptoms including gastrointestinal disturbance, headache, respiratory difficulties, burning skin, eye irritation and, for some, confusion and memory difficulty (8–10). In addition to complaints of these symptoms, reversible deficits in learning efficiency and concentration were observed among individuals who were clinically evaluated in Maryland shortly after exposure to *Pfiesteria*-related fish kills (5, 10, 11, 17). Laboratory staff who worked with toxic, fish-killing *P. piscicida* cultures previously had been reported to have similar symptoms (8). Thus, a tentative linkage between human health effects and exposure to partially characterized toxins present during environmental, as well as laboratory, exposure to *Pfiesteria*-associated fish kill-disease events was established. Although no correlation was or has been made between seafood consumption and illness, public concern led to significant impacts on the seafood industry along the eastern seaboard and consequently affected the livelihoods of many watermen (16).

TABLE 1. Specificity of species-selective primers

Organism or collection site and date (mo/yr)	Source; strain(s)	*P. piscicida* PCR	*P. shumwayae* sp. nov. PCR
Prorocentrum minimum	CCMP; 699	Neg[b]	Neg
Heterocapsa triquetra	CCMP; 449	Neg	Neg
Amphidinium carterae	CCMP; 1314	Neg	Neg
Katodinium rotundatum	CCMP; 1542	Neg	Neg
Pyrocystis lunula	CCMP; 731	Neg	Neg
Ceratium longipes	CCMP; 1770	Neg	Neg
Lingoludinium polyedrum	CCMP; 407	Neg	Neg
Gymnodinium breve	CCMP; 718	Neg	Neg
Chattonella subsalsa (*Heterokontophyta*)	CCMP; 217	Neg	Neg
Gymnodinium galatheanum	CCMP; 415, 416	Neg	Neg
Prorocentrum hoffmannianum	CCMP; 683	Neg	Neg
Prorocentrum triestinum	CCMP; 700	Neg	Neg
Scrippsiella faroensae	CCMP; 771	Neg	Neg
Scrippsiella sp.	CCMP; 772, 735	Neg	Neg
Gambierdiscus toxicus	CCMP; 1600	Neg	Neg
Protoceratium reticulatum	CCMP; 1889	Neg	Neg
Adenoides eludens	CCMP; 1891	Neg	Neg
Coolia monotis	CCMP; 305	Neg	Neg
Gymnodinium varians	CCMP; 421	Neg	Neg
Gymnodinium mikimotoi	CCMP; 429, 430	Neg	Neg
Heterocapsa niei	CCMP; 447	Neg	Neg
Amphidinium operculatum	CCMP; 1342	Neg	Neg
Prorocentrum sp.	CCMP; 1541	Neg	Neg
Prorocentrum compressum	CCMP; 1786	Neg	Neg
Thecadinium inclinatum	CCMP; 1890	Neg	Neg
Alexandrium tamarense	CCMP; 116	Neg	Neg
Oxyrrhis marina (*Alveolata*)	CCMP; 604, 605, 1739	Neg	Neg
Peridinium foliaceum	CCMP; 1326	Neg	Neg
Gonyaulax cochlea	CCMP; 1592	Neg	Neg
Gymnodinium sanguineum	CCMP; 417	Neg	Neg
Prorocentrum balticum	CCMP; 1260	Neg	Neg
Prorocentrum sp.	CCMP; 703	Neg	Neg
Gyrodinium impudicum	CCMP; 1678	Neg	Neg
Gyrodinium instriatum	CCMP; 431	Neg	Neg
Gymnodinium simplex	CCMP; 419	Neg	Neg
Gyrodinium uncatenum	CCMP; 1310	Neg	Neg
Gymnodinium catenatum	CCMP; 414	Neg	Neg
Prorocentrum mexicanum	CCMP; 1370	Neg	Neg
Rhodomonas sp.	CCMP; 767	Neg	Neg
Rhodomonas sp.	CCMP; 768	Neg	Neg
Pfiesteria piscicida	NCSU; 102-1	Pos[c]	Neg
Pfiesteria piscicida	FL DEP; MDFDEPMR23	Pos	Neg
Pfiesteria piscicida	NCSU; 97-1	Pos	Neg
Pfiesteria piscicida	CCMP; 1831	Pos	Neg
Pfiesteria piscicida	FL DEP; MMRCC981020BR01C5	Pos	Neg
Pfiesteria shumwayae sp. nov.	Species 'B' (GenBank AF218805)	Neg	Pos
Pfiesteria shumwayae sp. nov.	NCSU; B-Vandemere	Neg	Pos
Pfiesteria shumwayae sp. nov.	NCSU; 7-28-T	Neg	Pos
Pfiesteria shumwayae sp. nov.	NCSU; BP	Neg	Pos
Cryptoperidiniopsis sp. (gen. nov.)	CCMP; 1827a	Neg	Neg
Cryptoperidiniopsis sp. (gen. nov.)	CCMP; 1827b	Neg	Neg
Gymnodinium galatheanum	HPEL[a]; GE	Neg	Neg
Pfiesteria-like isolate sites			
Neuse River (N.C.) 10/98	CCMP; 1872	Neg	Neg
Wilmington River (Ga.) 11/98	CCMP; 1873	Neg	Neg
Wilmington River (Ga.) 11/98	CCMP; 1874	Neg	Neg
Wilmington River (Ga.) 11/98	CCMP; 1875	Neg	Neg
Wilmington River (Ga.) 11/98	CCMP; 1876	Neg	Neg
Wilmington River (Ga.) 11/98	CCMP; 1877	Neg	Neg
Neuse River Isolate (N.C.) 12/98	CCMP; 1878	Neg	Neg
Wilmington River (Ga.) 11/98	CCMP; 1879	Neg	Neg
Wilmington River (Ga.) 11/98	CCMP; 1880	Neg	Neg
Wilmington River (Ga.) 11/98	CCMP; 1881	Neg	Neg
Wilmington River (Ga.) 11/98	CCMP; 1882	Neg	Neg
Pocomoke River (Md.) 1/98	CCMP; A8925	Pos	Neg
Chicamacomico River (Md.) 1/98	CCMP; A8932	Pos	Neg

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TABLE 1—*Continued*

Organism or collection site and date (mo/yr)	Source; strain(s)	*P. piscicida* PCR	*P. shumwayae* sp. nov. PCR
Pocomoke Sound (Md.) 1/98	CCMP; A8942	Neg	Neg
Pocomoke Sound (Md.) 1/98	CCMP; A8941	Neg	Neg
Kings Creek (Md.) 9/97	CCMP; 1827	Neg	Neg
Kings Creek (Md.) 9/97	CCMP; 1828	Neg	Neg
Rhode River (Md.) 9/97	CCMP; 1829	Neg	Neg
Chicamacomico River (Md.) 1/98	CCMP; 1830	Pos	Neg
Chicamacomico River (Md.) 1/98	CCMP; 1832	Neg	Neg
Chicamacomico River (Md.) 1/98	CCMP; 1833	Neg	Neg
Pocomoke River (Md.) 1/98	CCMP; 1834	Pos	Neg
Pocomoke Sound (Md.) 1/98	CCMP; 1835	Neg	Neg
Pocomoke Sound (Md.) 1/98	CCMP; 1836	Neg	Neg
Neuse River (N.C.) 2/98	CCMP; 1838	Neg	Neg
Neuse River (N.C.) 2/98	CCMP; 1839	Neg	Neg
Neuse River (N.C.) 2/98	CCMP; 1840	Neg	Neg
Neuse River (N.C.) 2/98	CCMP; 1841	Neg	Neg
Neuse River (N.C.) 2/98	CCMP; 1842	Neg	Neg
Neuse River (N.C.) 2/98	CCMP; 1843	Neg	Neg
Neuse River (N.C.) 2/98	CCMP; 1844	Neg	Neg
Neuse River (N.C.) 2/98	CCMP; 1845	Neg	Neg
Ciliophora spp.			
Mesodinium pulex	HPEL	Neg	Neg
Strombium sp.	HPEL	Neg	Neg
Tontonia sp.	HPEL	Neg	Neg

[a] HPEL, Horn Point Environmental Laboratories (University of Maryland Center for Environmental Studies).
[b] Neg, negative.
[c] Pos, positive.

In consideration of the association of toxic *Pfiesteria* species (*P. piscicida* Steidinger and Burkholder and a second species, *P. shumwayae* sp. nov.; 7, 22) with human health and the adverse economic impact of the 1997 events, comprehensive monitoring programs were developed and implemented by several Atlantic coast states (19). In Maryland, Virginia, and North Carolina, monitoring programs are now in place, with weekly to bimonthly collection of biophysical parameter data, including efforts to identify and enumerate *Pfiesteria* spp. Assessment of algal communities and fish health monitoring programs have also been implemented. Furthermore, programs have been established to rapidly assess these same parameters in response to reports of fish health disturbance or of human illness in association with estuarine exposure to toxic *Pfiesteria* outbreaks.

However, detection and quantification of *Pfiesteria* spp. have been problematic. The two known organisms (*P. piscicida* and *P. shumwayae* sp. nov.) are relatively nondescript heterotrophic-mixotrophic dinoflagellates (5, 15). Their life cycles are complex and may include multiple flagellated, amoeboid, and cyst forms with a considerable size range (major cell axis, 5 to 750 µm; 4, 5). These forms or stages cannot be positively identified by light microscopy (LM) alone because they closely resemble various other flagellates and amoebae. Moreover, specific antibodies or lectins for organism labeling are not yet available. *Pfiesteria* spp. (flagellated zoospores) can be identified by scanning electron microscopy (SEM) of membrane-stripped or suture-swollen cells (7, 23); however, this painstaking process requires considerable time and expertise, thus limiting the number of specimens that can be analyzed. Until recently, no genetic sequence data were available to permit development of sequence-based detection methods. This bottleneck was recently overcome (18), permitting development of new assays for these organisms.

We developed and implemented real-time PCR-based assays utilizing the 5'-to-3' exonuclease activity of *Taq* polymerase (Taqman; 14, 26) for detection of *P. piscicida* and *P. shumwayae* sp. nov. in both fixative-preserved and unpreserved environmental estuarine water samples and cultures. In these assays, detection of amplified target DNA requires annealing of fluorescently labeled oligonucleotide probes, resulting in an added level of specificity compared with assays based on traditional PCR methodology. As the reaction proceeds, the 5'- to-3' exonuclease activity of *Taq* polymerase cleaves the probe. This cleavage frees the quencher dye from the emitter dye, which is then able to fluoresce. Amplification was observed via real-time fluorescence monitoring on the Lightcycler.

The specificity of both *Pfiesteria* sp. assays was tested against a panel of dinoflagellate cultures characterized by SEM or LM. After specificity was determined, it was imperative to test the sensitivity of the assays on both fixative (acidic Lugol's solution)-preserved (24) and unpreserved (fresh) culture and environmental samples to aid in designing the optimal protocol for sample collection and storage until the time of processing. In addition, given the availability of archived samples and an interest in investigating prior algal blooms and fish kill events, it was essential to determine the long-term stability of preserved samples. Given the anticipated use of the assay in environmental screening and the marked heterogeneity (species composition and relative abundance) of estuarine water samples, the effect of variable background DNA concentrations on assay performance was investigated.

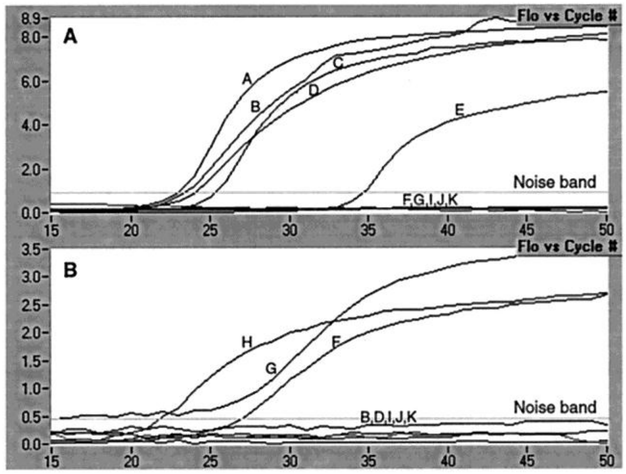

FIG. 1. Specificity of *P. piscicida* (A) and *P. shumwayae* sp. nov. (B) real-time PCR assays. DNA was extracted from five cultures (A, B, C, D, and E) determined to be *P. piscicida* by either SEM or LM (coupled with 18S rDNA sequence analysis) and analyzed with the real-time PCR assay specific for *P. piscicida*. DNA was extracted from three cultures (F, G, and H) determined to be *P. shumwayae* sp. nov. by SEM and analyzed with the real-time PCR assay specific for *P. shumwayae* sp. nov. Negative results in both graphs (below the noise band) represent morphologically close relatives. The negative (no-DNA) controls were negative. The corresponding results obtained are presented in Table 2.

TABLE 2. Specificity of *P. piscicida* and *P. shumwayae* sp. nov. real-time PCR assays (see Fig. 1)

Sample[a]	Species	Method	*P. piscicida* PCR	*P. shumwayae* sp. nov. PCR
A; NCSU; 102-1	*P. piscicida*	SEM	+	−
B; FL DEP; MDFDEPMR23	*P. piscicida*	SEM	+	−
C; NCSU; 97-1	*P. piscicida*	SEM	+	−
D; CCMP; 1831	*P. piscicida*	LM-18S rDNA sequencing	+	−
E; FL DEP; MMRCC981020BR01C5	*P. piscicida*	SEM	+	−
F; NCSU; B-Vandemere	*P. shumwayae* sp. nov.	SEM	−	+
G; NCSU; 7-28-T	*P. shumwayae* sp. nov.	SEM	−	+
H; NCSU; BP	*P. shumwayae* sp. nov.	SEM	−	+
I; CCMP; 1827a	*Cryptoperidiniopsis* sp. (gen. nov.)	SEM	−	−
J; CCMP; 1827b	*Cryptoperidiniopsis* sp. (gen. nov.)	SEM	−	−
K; Horn Point; GE	*G. galatheanum*	LM-18S rDNA sequencing	−	−

[a] The first letter corresponds to a designation in Fig. 1, and the source and the strain designation follow.

MATERIALS AND METHODS

Cultures. For dilution experiments, two *P. piscicida* zoospore cultures were utilized: strain 113-3 (Aquatic Botany Laboratory, North Carolina State University [NCSU], Raleigh) and a strain (MDFDEPMR23, characterized by K. Steidinger, Florida Department of Environmental Protection [FL DEP], St. Petersburg) maintained by Horn Point Environmental Laboratories (University of Maryland Center for Environmental Studies, Cambridge) using previously described methods (5). *P. piscicida* zoospores were quantified from acidic Lugol's solution-preserved samples (24) using a Palmer-Maloney counting chamber (25) and an Olympus IMT-2 inverted microscope (magnification, ×600, phase contrast). Four additional *P. piscicida* cultures were utilized for assay specificity experiments (NCSU cultures 102-1 and 97-1, Provasoli-Guillard National Center for Culture of Marine Phytoplankton [CCMP] culture 1831, and FL DEP culture MMRCC981020BR01C5). *P. shumwayae* sp. nov. cultures (B-Vandemere, 7-28-T, and BP) were provided by NCSU.

Additional cultures were received from the Horn Point Environmental Laboratory, including *Gymnodinium* *galatheanum*, three *Ciliophora* cultures, and *Rhodomonas* sp. *P. piscicida* and *Pfiesteria*-like (morphologically similar to *Pfiesteria* complex species) cultures were provided by CCMP (R. Anderson, West Boothbay Harbor, Maine), and additional *Pfiesteria*-like dinoflagellate cultures were supplied by Old Dominion University (H. Marshall, Norfolk, Va.). Culture material characterization was confirmed by at least two methods and in at least two laboratories in all cases. Table 1 lists the cultures and isolates used in this study.

Acidic Lugol's solution fixation. For fixation of cultures and environmental estuarine water samples, acidic Lugol's solution (hydrated iodine-potassium iodide, acetic acid solution; 24) was used at a final concentration of 1% (Sigma, St. Louis, Mo.).

DNA extraction. For all experiments, sample aliquots were filtered through a 5-µm-pore-size hydrophilic Durapore filter (Millipore, Bedford, Mass.). The filter was then placed into an Eppendorf tube, and DNA extraction was performed by following the protocol supplied with the DNeasy Plant Kit (Qiagen, Valencia, Calif.). DNA was eluted with 100 µl of elution buffer and stored at —20°C.

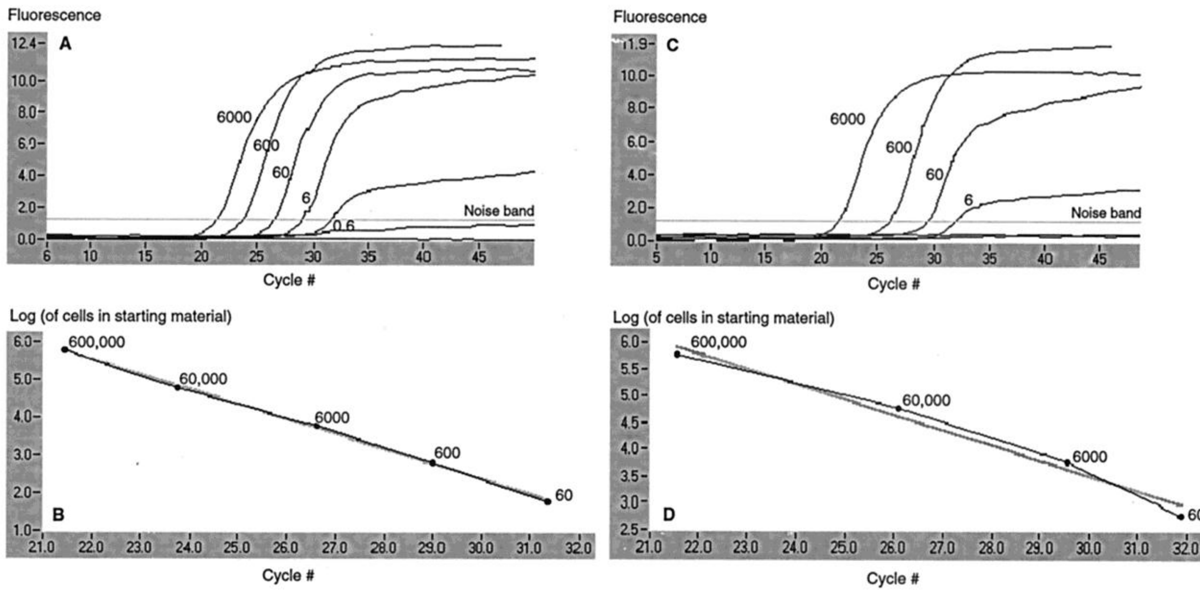

FIG. 2. Real-time *P. piscicida* PCR assay on the Lightcycler to detect the organism in 10-fold serial dilutions of unpreserved and fixative (acidic Lugol's solution)-preserved culture material. A 10-ml volume of each dilution was filtered through a 5-µm-pore-size filter, and DNA was extracted from the retained organism. In graphs A and C (unpreserved and fixative preserved, respectively), fluorescence acquired from dilutions detected with the probe is plotted against the cycle number. The numbers indicate the equivalent numbers of cells (genomes) aliquoted into the PCR (i.e., extracted DNA was eluted in 100 µl, and 1 µl 1/100 was assayed). In graphs B and D (unpreserved and fixative preserved, respectively), the log of the number of cells in the starting material is plotted against the cycle number at which the signal exceeded the threshold (set at 10% of the total fluorescence for the data set). In the unpreserved dilution, fewer than one cell per reaction could be detected, while in the fixative-preserved sample, the lower limit of detection was six cells per reaction.

PCR. The primers and probes were designed utilizing the Primer Express software (Test Version; Perkin-Elmer) and an alignment of >100 dinoflagellate small-subunit ribosomal DNA sequences. The alignment was constructed using the Pileup software (Genetics Computer Group) and sequences downloaded from GenBank (in addition to multiple unpublished dinoflagellate sequences [T. Tengs, University of Maryland, unpublished data]). The alignment included *P. piscicida* (GenBank accession no. AF077055) and *P. shumwayae* sp. nov. (GenBank accession no. AF218805), and primers and probes were designed to target signature sequences unique to these species. PCR assays with these assays were performed on the Lightcycler (Idaho Technology, Idaho Falls, Idaho). The following reagents were added for a 10-µl *P. piscicida*-specific reaction: primers 107 (5'-CAGTTAGATTGTCTTTGGTGGTCAA-3') and 320 (5'-TACCATAT CACTTTCTGACCTATCA-3'), each at a final concentration of 0.2 µM (Operon, Alameda, Calif.); a *P. pisc*. probe labeled with FAM (carboxyfluorescein) and TAMRA (carboxytetramethylrhodamine) (5'-FAM-CATGCACCAAAGCCCG ACTTCTCG-TAMRA-3') at a final concentration of 0.15 µM (Operon); *Taq* polymerase at a final concentration of 0.1 U μl^{-1} (Life Technologies, Rockville, Md.); $MgCl_2$ at a final concentration of 4 mM (Life Technologies); a deoxynucleoside triphosphate mixture with each deoxynucleoside triphosphate at a final concentration of 0.2 mM (Bioline, Reno, Nev.); bovine serum albumin at a final concentration of 0.25 mg ml^{-1} (Idaho Technologies); PCR buffer at a final concentration of 1 × (Life Technologies); approximately 10 ng of template DNA; and PCR grade water to a final volume of 10 µl (Sigma). For a 10-µl *P. shumwayae*-specific reaction, primers Pshumfor (5'-TGCATGTCTCAGTTTAAGTC A-3') and Pshumrev (5'-TCGATCATCAAATACACTAAAACTGTTTT-3') each at a final concentration of 0.2 µM (Operon), were used. The probe used in this assay, at a final concentration of 0.30 µM, was *P. shum* (5'-FAM-TACGG CGAAACTGCGAATGGCTCAT-TAMRA-3'). The same reagents and concentrations were used as described above to obtain a 10-µl reaction mixture. Seven microliters of the reaction mixture was added to a cuvette (Idaho Technologies) and pulse spun on a tabletop centrifuge (Sorvall). Cuvettes were loaded into the Lightcycler, and the following quantification cycling protocol was used: 50 cycles at 94°C for 0 s and 60°C for 20 s, with a temperature transition time of 20°C s^{-1}. Fluorescence acquisition was 100 ms after each incubation at 60°C, and the display mode was CH1 1^{-1} with the gain set at 1.

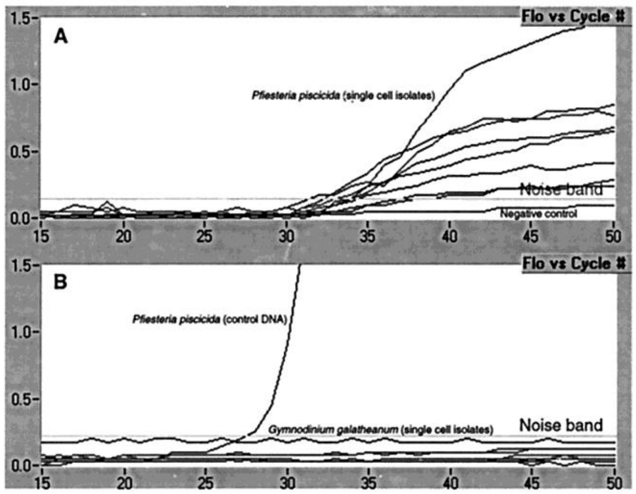

FIG. 3. Single-cell specificity and sensitivity of *P. piscicida* real-time PCR-based assay. (A) Results of PCR performed on eight replicates of single *P. piscicida* cells (all detectable). (B) Results of PCR performed on *G. galatheanum* (seven replicates), a close morphological relative, to test assay specificity. The positive control was total DNA isolated from a *P. piscicida* culture. In both graphs, the values for the negative control are below the noise band.

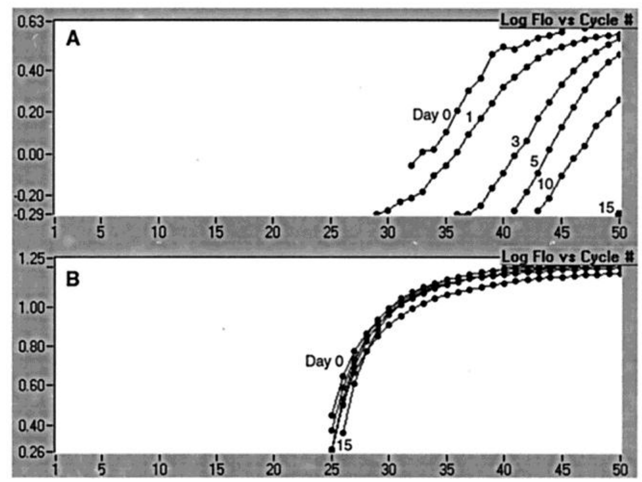

FIG. 4. Detection of *P. piscicida* over time in unpreserved (A) and fixative (acidic Lugol's solution)-preserved (B) environmental water spiked with a known number of organisms. Spiked samples were stored on the benchtop, and DNA was extracted from 40-ml aliquots on the days indicated.

RESULTS

Assay specificity. DNA extraction and PCR were performed utilizing SEM-verified *P. piscicida* and *P. shumwayae* sp. nov. culture DNA and panels of control organism DNA. Extensive specificity testing was performed with a panel of 36 well-characterized dinoflagellate cultures, 2 cryptophyte prey cultures, other protist representatives (*Heterokontophyta* and *Alveolata*), three *Ciliophora* representatives, and a panel of 32 dinoflagellate cultures characterized as *Pfiesteria*-like by the reference laboratory from which they were obtained (CCMP). Of these 32 cultures, 4 were positive by the PCR assay (Table 1) and have been confirmed via SEM and/or 18S rDNA sequencing to be *P. piscicida*. The remaining 28 cultures, all heterotrophic estuarine dinoflagellates, have been demonstrated through either 18S rDNA sequencing or heteroduplex mobility assay (18) to be distinct from *P. piscicida* (data available upon request). Figure 1A and B and Table 2 depict the specificity of the *P. piscicida* and *P. shumwayae* sp. nov. PCR assays against a representative panel of dinoflagellates, including SEM- and small-subunit ribosomal DNA sequence-validated *P. piscicida* (five cultures), *P. shumwayae* sp. nov. (three cultures), and the morphologically similar (*Pfiesteria*-like) dinoflagellates *G. galatheanum* and *Cryptoperidiniopsis* sp. Controls containing no template DNA were negative.

Sensitivity. The sensitivity of the *P. piscicida* assay was assessed by performing PCR on fixative (acidic Lugol's solution)- preserved and unpreserved 10-fold serial dilutions of a pure *P. piscicida* culture (NCSU strain 113-3). Figure 2A reflects the sensitivity limits of the *P. piscicida*-specific assay on an unpreserved culture, with a detection limit of approximately 0.6 cell in a reaction. This value corresponds to DNA extracted from a total of 60 cells, assuming 100% extraction efficiency with the protocol used (under our experimental conditions, 1 µl of extracted DNA from 100 µl of total eluate was used as a template). Sensitivity decreased by 1 log with a fixative-preserved culture (Fig. 2B).

Sensitivity was further assessed by performing a single-cell PCR assay. Single *P. piscicida* strain MDFDEPMR23 cells were isolated with a capillary tube and placed directly into reaction cuvettes, and a PCR assay was performed immediately. Amplification was evident in all eight single-cell trials (Fig. 3).

Stability. The ability to recover and detect P. piscicida DNA over time from fixative (acidic Lugol's solution)-preserved and unpreserved environmental water samples spiked with a known number of organisms was assessed. Environmental water samples collected from the Choptank River (Maryland) tested negative for the presence of *P. piscicida* with our PCR-based assay. Two 950-ml aliquots of this Choptank River water were spiked with 50 ml of a *P. piscicida* culture of 60,000 cells ml^{-1} (NCSU strain 113-3) for a final concentration of 3,000 cells ml-1. One sample was preserved with 1% acidic Lugol's solution, and both samples were maintained at room temperature on the benchtop. DNA was extracted from 40-ml aliquots on days 0, 1, 3, 5, 10, and 15. PCR was performed on all of the samples in the same run.

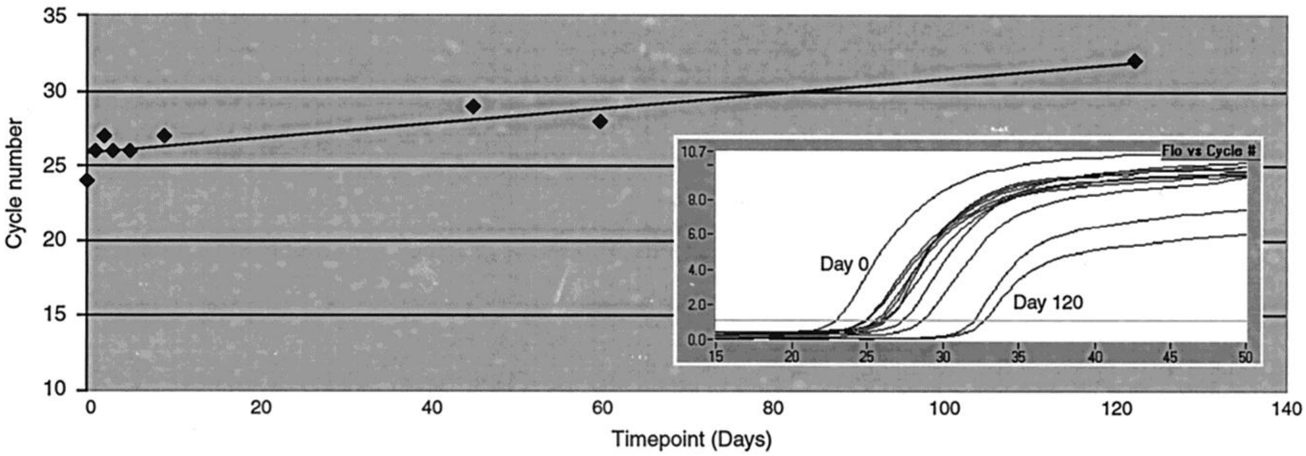

FIG. 5. Detection of *P. piscicida* to 120 days in a fixative (acidic Lugol's solution)-preserved culture. At time point indicated, DNA was extracted from a 2-ml aliquot of the culture. DNA from all time points was assayed with the *P. piscicida* probe assay in the same Lighcycler run. The inset is a graph depicting fluorescence versus cycle number for each time point.

Detection of *P. piscicida* in the unpreserved sample was dramatically reduced over time, with undetectable levels by day 15 (Fig. 4A). In contrast, the fixative-preserved sample was markedly more stable, with *P. piscicida* at detectable levels throughout the experimental period and fluorescence detection consistent for all time points (Fig. 4B).

A further experiment was designed to assess the long-term stability of a fixative-preserved sample. A 22-ml aliquot of a *P. piscicida* culture (NCSU strain 113-3; concentration, 60,000 cells ml^{-1}) was preserved with 1% acidic Lugol's solution and stored at room temperature on the benchtop. DNA was extracted from 2-ml aliquots on days 0, 1, 2, 3, 5, 9, 45, 60, and 120. A PCR assay was performed on all of the samples in the same run, and the cycle number at which fluorescence was detected at each time point was recorded (Fig. 5). Although there was an approximate shift of five cycles over the course of 4 months, long-term stability was apparent.

Effects of background DNA. The performance of the *P. piscicida* assay was assessed in the presence of various background DNA concentrations either present prefiltration as prey organisms in the culture or introduced postfiltration through addition of extraneous organism DNA derived from environmental water. Three 10-fold serial dilution sets were prepared from a pure culture (strain MDFDEPMR23; concentration, 35,000 cells ml^{-1}). One set was filtered, and DNA was extracted. The second set was filtered, DNA was extracted, and aliquots were then spiked with 640 ng of background environmental DNA (for a total of 12.8 ng in the PCR) to represent postfiltration spiking. In the third serial dilution set prepared from the same strain, a total of 1,860,000 *Rhodomois* sp. cells were spiked into each dilution prior to filtration and DNA extraction.

PCR was performed on all three sets of serial dilutions in the same run. A 1-log decrease in the sensitivity of *P. piscicida* detection was observed when high extraneous background DNA concentrations were added to samples postextraction (Fig. 6). However, assay sensitivity was not affected by high background DNA concentrations when they were present as high extraneous organism loads in samples to be filtered, a condition more closely approximating screening of environmental samples. Regardless of the presence or absence of exogenous DNA, correlation of cell cycle number at detection versus concentration of target cells was highly significant (R values for the unspiked, spiked postextraction, and spiked preextraction conditions were 0.98, 0.94, and 0.91, respectively).

DISCUSSION

Based on the testing of available characterized cultures of *P. piscicida* and *P. shumwayae* sp. nov., a wide array of cultures representing morphological and genetically closely related organisms, and representatives of other photosynthetic protist groups, the real-time PCR-based assays described here have proven to be highly specific and sensitive for the detection of *P. piscicida* and *P. shumwayae* sp. nov. In our experience, the use of fluorescein-labeled species-specific probes in conjunction with species-specific primers added additional assay specificity in comparison to detection with SyBr Green or other double-stranded DNA intercalating dyes (data not shown), probably due to the conserved nature of the ribosomal gene targets assayed.

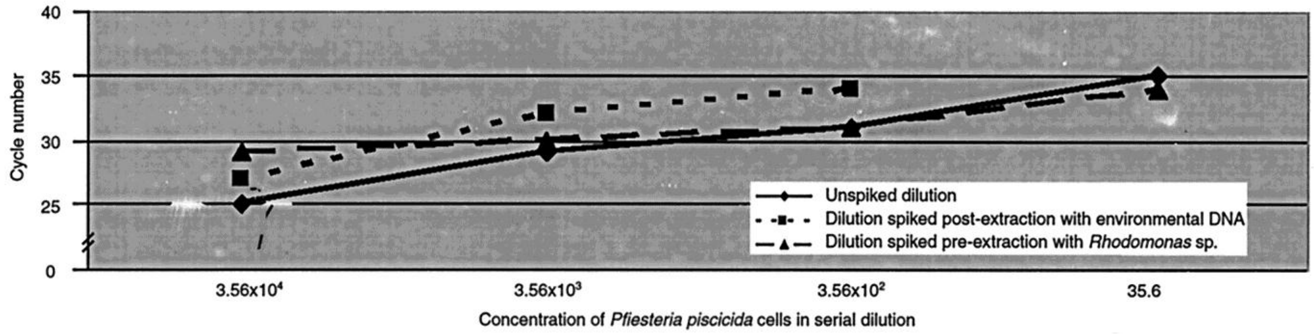

FIG. 6. Effects of background DNA on detection of *P. piscicida*. Three 10-fold serial dilutions were prepared from a pure *P. piscicida* strain MDFDEPMR23 culture. Aliquots from one dilution set were spiked postfiltration with 12.8 ng of organism DNA extracted from a heterogeneous environmental water sample (Choptank River in Maryland). The third dilution set was spiked with 1,860,000 cells of *Rhodomonas* sp. prefiltration.

The demonstration of PCR assay sensitivity utilizing fixed (acidic Lugol's solution) samples over time will prove valuable for ongoing investigations of *Pfiesteria* biology. As demonstrated, the confounding effects of variable time intervals between sample collection and laboratory analysis, an often unavoidable consequence of oceanographic field work, can be addressed with a standard fixation methodology that has minimal (and consistent) impacts on downstream molecular analysis. The fixation method is simple to use, and it provides the means to assay archived samples. Further experiments will include assessment of assay stability over longer time periods (i.e., greater than 1 year) and efficiency of DNA extraction from samples preserved with other fixatives (glutaraldehyde, formalin).

In addition to a high level of specificity and stability of detection over time, the *P. piscicida* PCR assay demonstrated high sensitivity, with a detection limit of 0.6 cell. Further results showing detection of single *P. piscicida* cells in a PCR support the assay's sensitivity. Future efforts will include comparison of single-cell PCR assays of various described life stages (zoospores, cysts, and amoebae). The assay cannot yet be used in an absolutely quantitative manner due to (i) the fact that the number of 18S gene copies per cell is unknown and (ii) the possible variance of 18S gene copy number during the growth cycle. However, it can and currently is being used to determine relative concentrations of *P. piscicida* in environmental field samples, permitting statistical assessment of parameters believed to be associated with Pfiesteria blooms.

SEM methods are regarded by dinoflagellate systematists as the "gold standard" for identification of *Pfiesteria* spp. (e.g., see references 7 and 23). However, these procedures require membrane stripping or suture swelling techniques which are tedious and limit SEM's utility for environmental monitoring (7). Limitations also arise in utilizing SEM methods for detection of *Pfiesteria* spp. in estuarine water samples because these organisms are often minor components of the species composition (10^1 to 10^3 cells ml^{-1} versus 10^5 or more total phytoplankton cells ml^{-1}; 5). In contrast, our real-time PCR assays developed for these organisms may be run rapidly with large sample sets and thus have proven to be useful tools for the detection of these species in both culture and environmental samples.

Molecular methods are rapid and allow phylogenetic analyses based on genetic data, but they also have limitations. For example, molecular techniques are subject to uncertainty in species specificity because various *Pfiesteria*-like estuarine dinoflagellates have not yet been formally described (22). In addition, the assay, which detects nuclear encoded DNA sequences, does not differentiate between *Pfiesteria* cultures in a toxic versus a nontoxic state as assayed in laboratory settings by estimation of toxin detectable in a reporter gene assay (6) or by ichthyotoxicity (4). This limitation can be addressed when the genetics of *Pfiesteria* toxicity are determined, permitting development of assays targeting toxicity-associated mRNA transcripts.

In summary, we have developed a highly sensitive and specific assay for detection of toxicity-associated dinoflagellates (*P. piscicida* and *P. shumwayae* sp. nov.) that can be used to explore *Pfiesteria* biology and the epidemiology of human health impacts of the organisms. The methods developed can be applied to a variety of critically important environmental monitoring initiatives (for instance, water quality screening for the presence of fecal coliforms or cryptosporidia). Fundamental questions about *Pfiesteria* biology, such as characterization of toxins and of mechanisms of toxin production, determinants of population blooms, and the full range of impacts on human health, must be resolved. The assays described here can be used as tools to address these important questions.

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