Detection of Pfiesteria spp. by PCR in surface sediments collected from Chesapeake Bay tributaries (Maryland)

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Abstract:
In 1997 blooms of Pfiesteria piscicida occurred in association with fish kills and human health problems in tributaries of the Chesapeake Bay (Maryland) and the scientific and media response resulted in large economic losses in seafood sales and tourism. These events prompted the Maryland Department of Natural Resources (MDNR) to begin monitoring for Pfiesteria spp. in water column samples. Real-time PCR assays targeted to the 18S rRNA gene were developed by our laboratories and utilized in conjunction with traditional microscopy and fish kill bioassays for detection of these organisms in estuarine water samples. This monitoring strategy aided in determining temporal and spatial distribution of motile forms of Pfiesteria spp. (i.e. zoospores), but did not assess resting stages of the dinoflagellates' life cycle. To address this area, a 3-year study was designed using real-time PCR assays for analysis of surface sediment samples collected from several Chesapeake Bay tributaries. These samples were tested with the real-time PCR assays previously developed by our laboratories. The data reported herein suggest a strong positive association between presence of Pfiesteria spp. in the sediment and water column, based on long-term water column monitoring data. P. piscicida is detected more commonly in Maryland's estuarine waters than Pfiesteria shumwayae and sediment 'cyst beds' may exist for these organisms.
Keywords: PCR; Pfiesteria; Sediment; Chesapeake Bay

Article:

1. Introduction

Several factors are believed to be associated with the apparent increase in worldwide distribution and frequency of harmful algal bloom (HAB) species (Hallegraeff, 1995). Ballast water transport can introduce non-indigenous species (e.g. Lewis et al., 2003; Joachimsthal et al., 2003), particularly when ballast water exchange procedures are not strictly implemented, leading to expanded distribution. An increase in HAB frequency has also been attributed to nutrient loading from anthropogenic influences (e.g. Burkholder et al., 1997). In addition, the general expansion of HAB research over the past two decades has increased our awareness of algal blooms (Halle-graeff, 1995).

Blooms of HAB species can have varying impacts on fish and human health activities, including closure of coastal recreational areas and shellfish beds, with detrimental effects on local economies. In Maryland fish kills and lesion events associated with Pfiesteria spp. blooms in 1997 resulted in economic losses estimated at $ 43 million in lost seafood sales and another $ 2.2 million in recreational fishing on charter boats, all due to the public's perception of seafood safety and water quality during these events (Lipton, 1998).

In response to the growing concern of impacts from HAB species, monitoring programs have been implemented in several states, including Maryland (Maryland Department of Natural Resources; MDNR), and employ both morphological and genetic methods for HAB species identification in the water column (Magnien, 2001). Until

recently, monitoring efforts in Maryland concentrated on targeting distribution of motile forms of these species (i.e. zoospores). However, some HAB species, including Pfiesteria spp., have life cycles that include benthic cysts. Thus a better understanding of the distribution of these life forms requires assessment of sediment samples as part of monitoring programs.

Resident cyst populations and seed beds have been identified for several species, including Alexandrium tamarense and A. fundyense in the Gulf of Maine (Anderson, 1997, 1998) and A. fundyense (Lebour) Balech (Protogonyaulax tamarensis [Lebour] Taylor) in the St. Lawrence estuary (Cembella et al., 1988), both associated with paralytic shellfish poisoning. It has been suggested that bloom development of these red tide species is dependent on the presence of seed beds containing a benthic resting cyst stage (e.g. Anderson and Wall, 1978). Seed beds are commonly studied in relation to ocean currents which can transport cysts to other areas that favor bloom initiation. Other research has suggested the use of algal cyst assemblage data as an indication of environmental changes including factors such as water temperature and salinity, climate (Dale, 1996; Smol and Cumming, 2000) and eutrophication (i.e. Dale, 1996).

In order to better understand the bloom dynamics and distribution of Pfiesteria spp. in the Chesapeake Bay and Coastal Bays (Maryland), a comprehensive collection of sediment samples was incorporated into MDNR's monitoring efforts from 2000 to 2003. Samples were tested with real-time PCR (polymerase chain reaction) assays (Taqman technology; Holland et al., 1991; Wittwer et al., 1997) specific for the 18S rRNA gene of Pfiesteria piscicida Steidinger et Burkholder and Pfiesteria shumwayae Glasgow et Burkholder (Oldach et al., 2000; Bowers et al., 2000; see erratum 2002 for corrected primer sequences). Recently, these assays were also used to confirm the presence of both species in sediment and culture material derived from sediment samples collected from the Oslofjord region in Norway (Jakobsen et al., 2002).

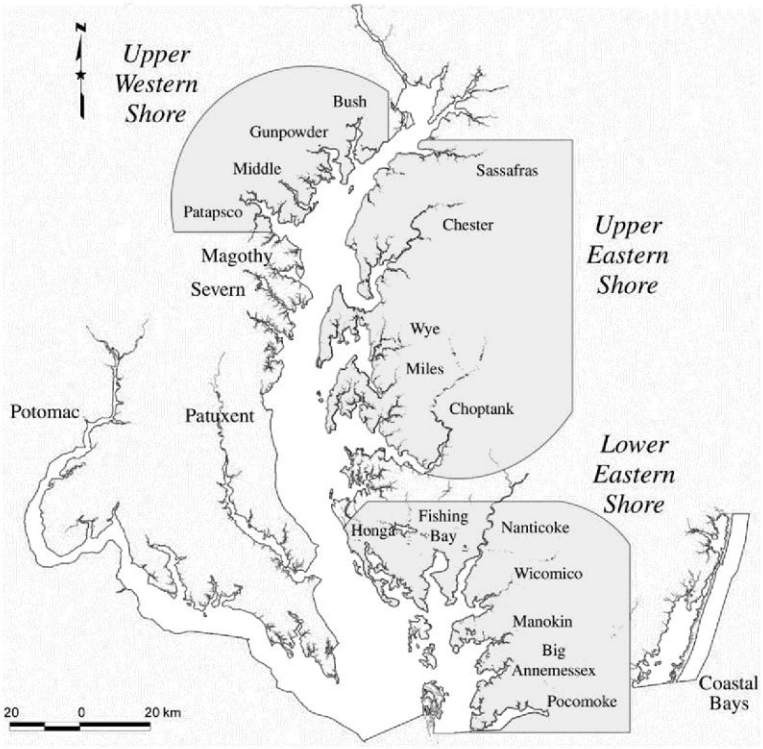

Fig. 1. Location of tributaries in the Chesapeake Bay where surface sediment samples were collected from 2000 to 2003.

PCR has been shown to be a sensitive method for detection of various microorganisms in sediment, although there are difficulties with inhibiting substances in sediment material (e.g. Schaefer, 1997; Stults et al., 2001; Saito et al., 2002). Several strategies have been used to overcome the problem of PCR inhibition by contaminating substrates present in the sediment. Stults et al. (2001) and Saito et al. (2002) diluted template DNA prior to PCR assays. Additionally, commercial purification kits are available that remove inhibitors and are easier to use than traditional DNA extraction methods (e.g. Smalla et al., 1993). Effects of inhibitors on PCR can be further reduced by adding bovine serum albumin (BSA) to PCR (Kreader, 1996; Schaefer, 1997).

In this study, we utilized all three methods: DNA extraction and purification with a commercial kit, dilution of DNA (1:10 and 1:100 dilutions), and the addition of BSA to PCR.

2. Materials and methods

2.1. Collection of surface sediment samples

Surface sediment samples were collected from representative tributaries in Chesapeake Bay, MD (Fig. 1; Table 1). Tributaries chosen for sampling were categorized according to the following criteria based on the MDNR's four levels of monitoring (Table 1): Level I sites — previously sampled (water column) in response to an adverse fish and/or human health event (n = 8); Level II — previously closed due to fish/human health events associated with Pfiesteria spp. (n = 6); Level III — determined by risk assessment to be conducive for blooms of Pfiesteria spp. (n = 8); and Level IV — not in Levels I–III and currently monitored as part of the MDNR's long-term water quality program (n = 9). Some tributaries were sampled in multiple years to investigate the persistence of Pfiesteria spp. and to determine if annual distribution coincided with results from prior years' water sample data. Coordinates for each station may be found at http://mddnr.chesapeakebay.net/hab/bowers.html.

Table 1
Distribution of tributaries sampled for the sediment study

	Total sediment samples		
	Level	Collected	Year sampled[a]
Western Shore Tributary			
Magothy River	IV	24	2, 3
Patuxent River	IV	24	1, 2
Potomac River	IV	12	1
Severn River	IV	24	2, 3
Upper Western Shore			
Bush River	IV	12	3
Colgate Creek (trib. of Patapsco River)	I	24	2
Gunpowder River	IV	18	3
Middle River	III	48	1, 2, 3
St. Mary's River (trib. of Potomac River)	I	12	1
Stoney Creek (trib. of Patapsco River)	I	12	2
Upper Eastern Shore Tributary			
Chester River	IV	12	3
Choptank River	IV	24	1, 3
Miles River	I	6	3
Sassafras River	IV	12	2
Wye River	I	6	3
Lower Eastern Shore Tributary			
Back Creek (trib. of Manokin River)	II	12	2
Big Annemessex River	III	12	2
Chicamacomico River (trib. of Fishing Bay)	II	12	2
Fishing Bay	II	12	1
Honga River	I	12	1
Manokin River	II	12	2
Nanticoke River	III	12	1
Pocomoke River	II	24	1, 3
Pokata Creek (trib. of Fishing Bay)	I	12	2, 3
Transquaking River (trib. of Fishing Bay)	II	12	2
Wicomico River	III	24	2, 3
Coastal Bays Tributary			
Herring/Turville Creek	III	12	2
Marshall Creek	III	24	1, 2
Scarboro Creek	I	5	3
St. Martin River	III	12	1
Trappe Creek	III	12	1

Level I: previously sampled (water column) in response to an adverse fish and/or human health event; level II: previously closed due to fish/human health events presumptively associated with *Pfiesteria* spp.; level III: determined by risk assessment (MDNR) to be conducive for blooms of *Pfiesteria* spp.; level IV: currently monitored as part of MDNR's long-term water quality program.

[a] Year 1: November 2000–May 2001; year 2: November 2001–April 2002; year 3: March 2003–May 2003.

Within selected tributaries, sampling zones of 3– 12 ppt surface salinity from July through October were delineated using the most recent 3–5 years of available salinity data. Salinity data sources included records from long-term, continuous, water quality mapping, routine fishery, and HAB rapid response monitoring conducted by the MDNR. These spatial and temporal zones represent the general distribution of Pfiesteria spp. found during summertime water column sampling in Chesapeake Bay. In some instances, such as in the Coastal Bays,

areas of higher salinity were sampled since the target salinity zones were much more spatially restricted and temporally variable than in the Chesapeake Bay. In other instances where there were limited salinity data, boundaries of sampling zones were located on best judgment or salinity data drawn from a longer period of record. The sampling zones were further restricted to the 2 m or less bathymetric contour since shallow waters were believed to be the primary habitat of this dinoflagellate. This sampling strategy also provided greater comparability in sample depths across the spectrum of large and small tributaries that were included in this study.

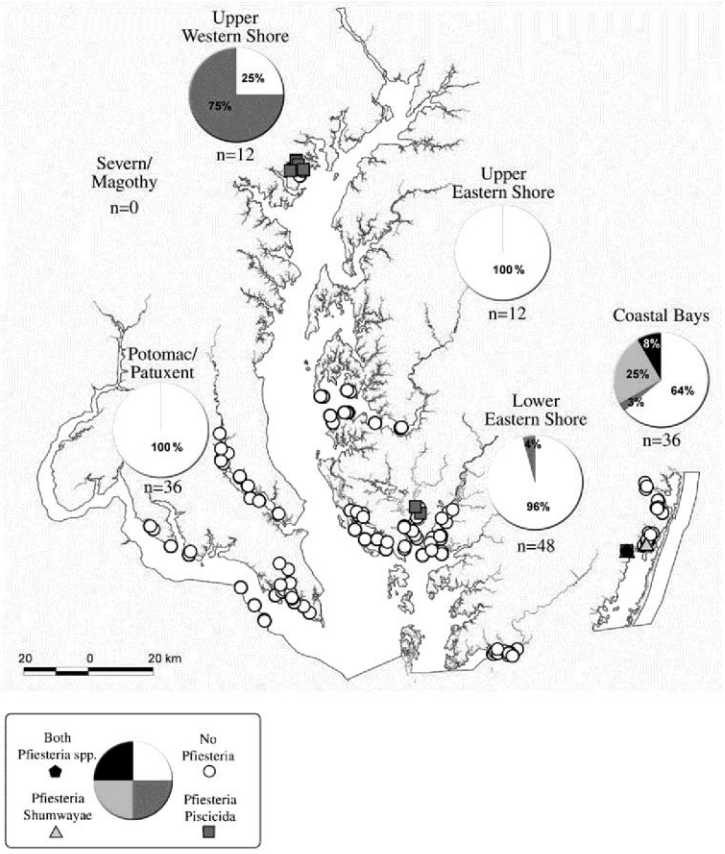

Fig. 2. Location of surface sediment samples collected during the first sampling year (November 2000–May 2001). The pie graphs are shaded corresponding to PCR results: both *Pfiesteria* spp. present, no *Pfiesteria* spp. present, only *P. piscicida* present, or only *P. shumwayae* present.

Sets of 50 sequentially numbered random sample sites were selected apriori within each tributary's salinity and bathymetric zones using GIS software. Depending on the size of the tributary, the first 6 or 12 accessible, sequential random sites were sampled. Samples were collected by MDNR personnel, during late fall through early spring, using a cellulose acetate butyrate-lined corer with a 6.7 cm internal diameter. A syringe was used to collect sediment from the undisturbed 2 cm surface layer in the sample core container for transfer into a 50 ml conical tube. Samples were stored at 4 °C until analyzed (generally within 4 weeks).

2.2. DNA extraction

Approximately 5 ml of 0.3 M NaCl were added to each 50 ml conical tube containing sediment. Tubes were mixed vigorously to re-suspend the sediment and then centrifuged for 10 min at approximately 1000 × g. Supernatant was decanted and a sterile spatula was used to transfer approximately 0.25 g of the top layer of sediment to a tube supplied with the UltraClean Soil DNA Kit (MoBio Laboratories Inc.; Solana Beach, California) containing beads and lysis buffer. Manufacturer's instructions were followed to remove PCR inhibitors, and to extract, precipitate, wash and elute total DNA. 1: 10 and 1: 100 dilutions of stock DNA were prepared in sterile deionized water for each sample.

2.3. Polymerase chain reaction

Real-time PCR assays with probes based on Taqman technology (Holland et al., 1991; Wittwer et al., 1997) were used to screen total DNA derived from sediment samples for P. piscicida and P. shumwayae (Bowers et

al., 2000; erratum, 2002) using either the Light-cycler™ (Idaho Technology; Idaho Falls, Idaho). Each reaction contained the following: forward primer ('107' for P. piscicida [50-CAGTTAGATTGTCTTTGGTGG-TCAA-30] or 'Pshumfor' for P. shumwayae [50- TGCATGTCTCAGTTTAAGTCCCA-30]) and reverse primer ('320' for P. piscicida [50-AGCTGATAGGT-CAGAAAGTGATATGGTA-30]or 'Pshumrev' for P. shumwayae [50-TCGATCATCAAATACACTAAAA-CTGTTTT-30]) at a final concentration of 0.2 μM each (Operon, Alameda, California); 'P. piscicida' [50- CATGCACCAAAGCCCGACTTCTCG-30] or 'P. shumwayae' [50-TACGGCGAAACTGCGAATGGCT-CAT-30] probe labeled 50FAM (carboxyfluorescein) and 30 TAMRA (carboxytetramethylrhodamine) at a final concentration of 0.3 μM (Operon, Alameda, California); Taq polymerase at a final concentration of 0. 1 U/μl (Life Technologies, Rockville, Maryland); $MgCl_2$ at a final concentration of 4 mM (Life Technologies, Rockville, Maryland); dNTP mixture at a final concentration of 0.2 mM each (Bioline, Reno, Nevada); BSA at a final concentration of 0.25 mg/ml (Idaho Technologies, Idaho Falls, Idaho), PCR buffer at a final concentration of 1 × (Life Technologies, Rockville, Maryland); and 1–3 μl template. The following quantification cycling protocol was used on the Lightcycler™: 50 cycles at 94 °C for 0 s; 60 °C for 20 s, with a temperature transition time of 20 °C s^{-1}. Fluorescence acquisition was 100 ms after each 60 °C incubation and display mode was CH1 e^{-1} with gain set at 1.

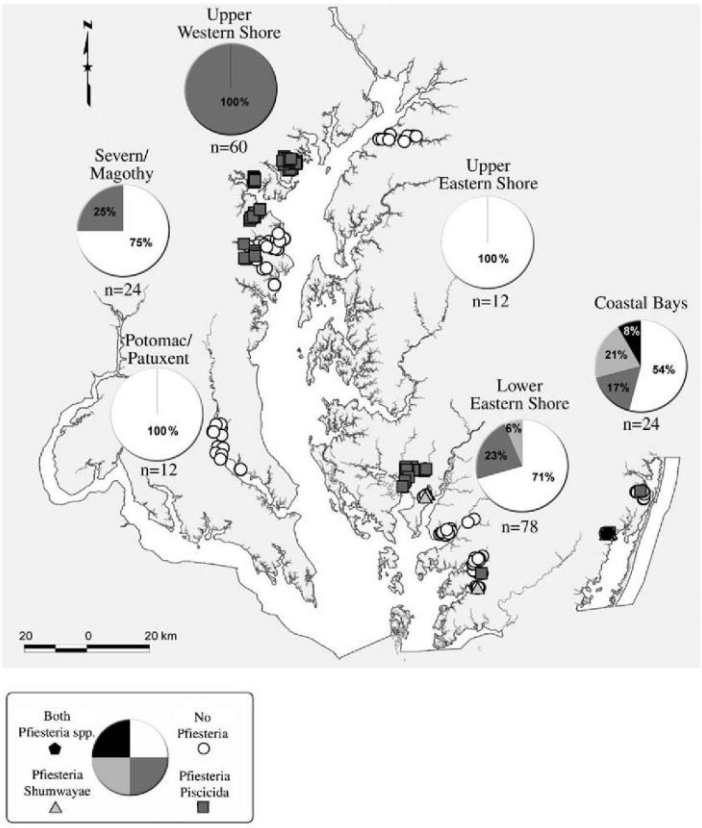

Fig. 3. Location of surface sediment samples collected during the second sampling year (November 2001–April 2002). The pie graphs are shaded corresponding to PCR results: both *Pfiesteria* spp. present, no *Pfiesteria* spp. present, only *P. piscicida* present, or only *P. shumwayae* present.

3. Results

3.1. Sample collection and distribution

Sediment samples were intentionally collected during winter months at a time when Pfiesteria spp. in the water column were thought to be less likely to be detected. Based on PCR results for P. piscicida and P. shumwayae derived from >8000 water column samples collected by MDNR as part of their routine monitoring program, (monthly to bi-monthly collections from various tributaries throughout the Bay from 1999 to 2003), the temporal distribution for water column detection of these organisms is late summer to early fall.

A total of 491 sediment samples were collected during this 3-year study (Table 1): 144 samples during the first year (November 2000 to May 2001; Fig. 2), 210 samples during the second year (November 2001 and April 2002; Fig. 3), and 137 samples during the third year (March 2003 to May 2003; Fig. 4). Sampling months during the third year were shifted due to weather constraints. The 491 samples were divided between the

various tributary levels as follows (Table 1): 89 samples were collected from tributaries in the level I group; 72 samples were collected from tributaries in the level II group; 156 samples were collected from tributaries in the level III group; and 162 samples were collected from tributaries in the level IV group.

3.2. Detection of Pfiesteria spp. by PCR

Positive PCR results for surface sediment samples were found in each year of sampling (Figs. 2–4). PCR was initially performed on undiluted DNA template derived from the sediment samples. If a sample set from a tributary contained both positive and negative samples, all negative samples from that set were re-run with 1: 10 and 1: 100 dilutions to ensure that there were no false negative samples (undiluted DNA noted to occasionally suppress PCR whether through presence of inhibitors or excess DNA, data not shown). If all samples in a given set were negative, PCR was performed on 1:10 and 1:100 dilutions from a representative one-third of the samples in that set to evaluate if there were false negatives in the original PCR.

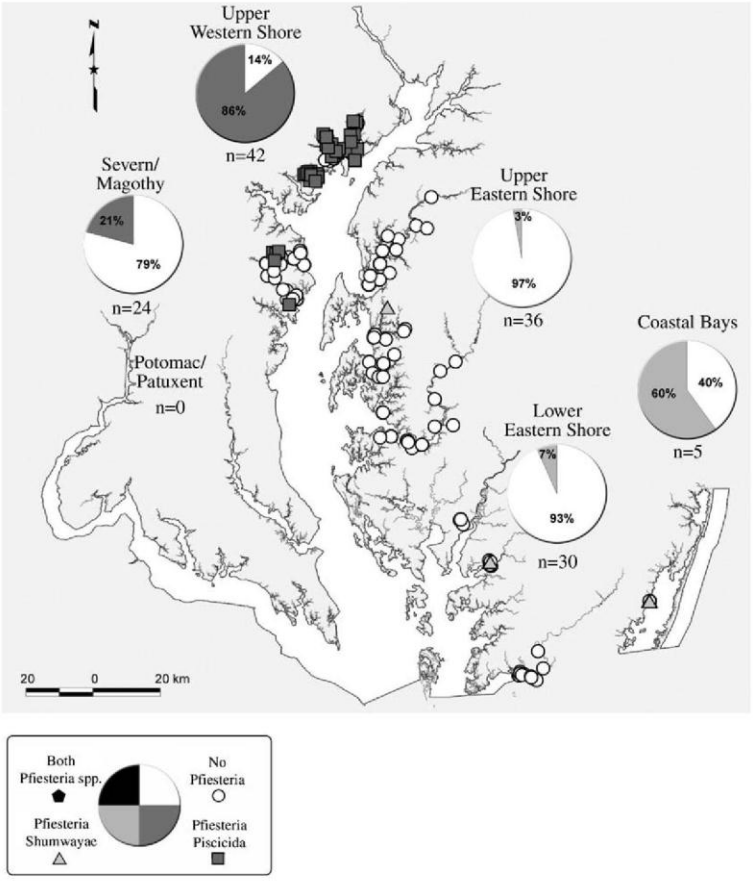

Fig. 4. Location of surface sediment samples collected during the third sampling year (March 2003–May 2003). The pie graphs are shaded corresponding to PCR results: both *Pfiesteria* spp. present, no *Pfiesteria* spp. present, only *P. piscicida* present, or only *P. shumwayae* present.

In year 1, all samples positive for P. piscicida (n = 15) were obtained from undiluted material. In year 2, 90 samples were positive for P. piscicida, with 77 from undiluted DNA and 13 from DNA diluted 1: 10. In the third sampling year, 41 samples were positive for P. piscicida; 38 from undiluted DNA, two from DNA diluted 1:10 and one from DNA diluted 1:100. For P. shumwayae, in years 1 and 3 all positive samples (n = 12 and n = 6, respectively) were from undiluted material. In year 2, 12 samples were positive for P. shumwayae; 10 from undiluted DNA, one from DNA diluted 1: 10 and one from DNA diluted 1: 100.

The tributary with the highest percentage of positive sediment samples (94%) was Middle River (located on the upper western shore). Fig. 5 represents seasonal distribution of this organism in the water column based on samples collected between 1999 and 2003. The dates for sediment collection are indicated.

4. Discussion

Until recently monitoring for Pfiesteria spp. in the Chesapeake Bay (Maryland) has primarily involved sampling of the water column, which captures the motile stages in the dinoflagellates' life cycle. This study assessed

sediment samples in order to better understand the distribution of resting stages of these species (Burkholder and Glasgow, 1997), and to provide assessment of the utility of the molecular approach used, since application of PCR to sediment can be problematic due to inhibitory contaminants. We found that the UltraClean Soil DNA Kit (MoBio Laboratories Inc.) was generally successful in removing such inhibitors, as evidenced by the low percentage of samples that needed to be diluted before detection was possible. Dilution of DNA template for PCR is an accepted form of overcoming the issue of inhibition (e.g. Stults et al., 2001; Saito et al., 2002), however, it does not entirely rule out the possibility of a major contaminating factor that may have affected all samples (even diluted DNA) from a given region. Another method for assessing inhibition involves analysis of a subsample that has been spiked with a known amount of Pfiesteria DNA (Zhang and Lin, 2002).

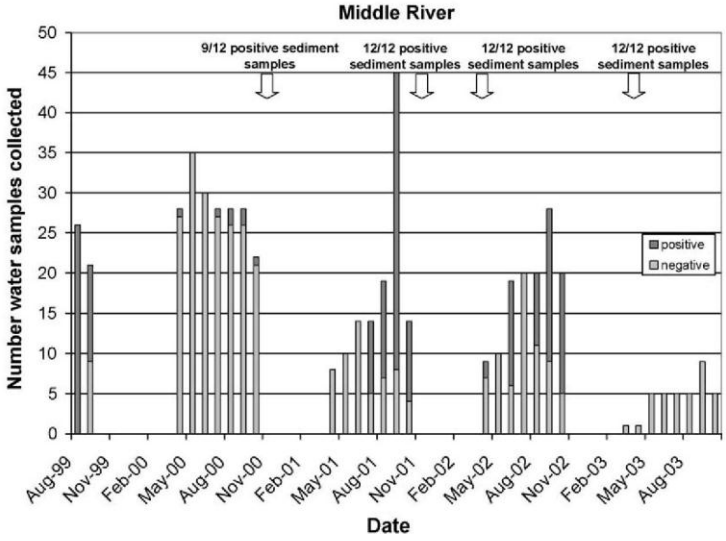

Fig. 5. Graph indicating number of positive water column samples from Middle River (upper western shore) between 1999 and 2003 (samples were collected as part of MDNR's routine monitoring program). Dates for collection of surface sediment samples are indicated.

The study design provided sufficient spatial coverage and yearly replications to observe significant regional trends in the data. Occurrence of P. piscicida was most prevalent in upper Chesapeake Bay western shore tributaries, with other 'hot spots' occurring in select lower Eastern Shore tributaries and the Coastal Bays. Major tributaries such as the Potomac, Patuxent and Choptank Rivers were negative for Pfiesteria spp. Positive results for P. shumwayae were limited mainly to the lower Eastern Shore and Coastal Bays. Yearly replicates in select tributaries showed similar positive and/or negative results, indicating that this molecular approach allows for reproducible results and that the heterogeneous Pfiesteria spp. distributions that we found may persist in the sediments from year-to-year despite short-term fluctuations in environmental variables and zoospore population dynamics. Larger tributaries may harbor localized 'seed beds' of Pfiesteria spp. that were missed by this survey, but in general the sample design represents a broad survey of Pfiesteria spp. distribution in comparable sediment habitats (<2 m depth) throughout a wide range of tributary sizes and locations.

PCR results from this study do reveal key points about the presence of Pfiesteria spp. throughout the Chesapeake Bay. First, the data show that P. piscicida is detected more often in environmental samples than P. shumwayae. Similar results have been found in water column PCR data generated since 1999 as part of MDNR's comprehensive monitoring program (>8000 samples analyzed; unpublished results Oldach and Rublee laboratories). Second, although no water column samples were collected and analyzed simultaneously with the sediment samples in this study, inferences can be drawn from PCR data generated from water column samples collected throughout the Bay as part of Maryland DNR's comprehensive monitoring program (1999-present; results posted at http:// www.dnr.state.md.us). During this long-term monitoring program, all of the tributaries included in the current sediment study were monitored at some level, from one-time sampling events to routine monthly monitoring.

As expected, there was a strong positive association between distribution of Pfiesteria species in estuarine sediments and their presence in past water column samples. For example, sediment samples from Middle River (all 3 years, from 2000 to 2003) revealed a high prevalence of P. piscicida and P. shumwayae. Conversely,

sediment samples negative for Pfiesteria spp. were from the same tributaries where these species have not been routinely detected in the water column in the past. For example, water column samples were analyzed routinely from 2000 to 2003 (at least once monthly from April through October) from tributaries such as the Patuxent, Choptank and Nanticoke Rivers with zero samples positive from the Patuxent and Choptank River's (n = 102 and 77, respectively) and only one positive sample from the Nanticoke River (n = 120). Also, no positive results from sediment samples were observed in the Pocomoke River, the location of a Pfiesteria outbreak in 1997, but this agrees with a paucity of positive water column samples observed in this system since the initial outbreak.

Overall, the PCR data from this study show that DNA derived from Pfiesteria spp. is present in sediments at times when environmental conditions are not considered favorable (based on past water column data) to sustain zoospore populations in the water column (late fall to early spring). Unfortunately, funding limitations did not allow for cyst identification from these sediment samples, however, the PCR results do identify areas for future collection of samples for cyst analyses and determination of associated environ-mental factors. Water quality parameters (temperature, dissolved oxygen, salinity) and sediment parameters (grain size analyses and measurement of nitrogen, carbon and phosphorus content) collected during this study are currently being analyzed with PCR results. Recently, analysis by Glibert et al. (2004) revealed high concentrations of urea in PCR-positive sediment samples from this study. The PCR data acquired from this 3-year study can be combined with other areas of Pfiesteria spp. and HAB research to better understand the ecology of these organisms.

5. Conclusions

Current real-time PCR methodology allows for rapid and specific detection of DNA derived from Pfiesteria spp. in sediment. Commercially available kits are sufficient for producing clean templates for use in real-time PCR. Although it is widely recognized that contaminants in sediment can inhibit PCR, we found that only a small number of samples needed to be diluted to eliminate reporting of false negative results.

This study demonstrated the need to include sediment sampling in monitoring efforts of HAB species that include a benthic and/or cyst stage in their life cycle. Monitoring of the water column is useful in determining presence or absence of the motile forms of the species, particularly in association with an adverse fish or human health event, however, it does not take into account the fate of the organism when environ-mental conditions are not favorable. Results from this study indicate Pfiesteria spp. persist in surface sediment likely in the form of cysts. Ongoing analyses relating water quality and sediment parameters will enhance our understanding of its life cycle and persistence in the Chesapeake Bay, and future studies should explore cyst identification and dynamics.

References

Anderson, D.M., Wall, D., 1978. The potential importance of benthic cysts of Gonyaulax tamarensis and G. excavata in initiating toxic dinoflagellate blooms. J. Phycol. 14, 224–234.
Anderson, D.M., 1997. Bloom dynamics of toxic Alexandrium species in the northeastern U.S. Limnol. Oceanogr. 42, 1009–1022.
Anderson, D.M., 1998. Physiology and bloom dynamics of toxic Alexandrium species, with emphasis on life cycle transitions. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms, NATO ASI Series, vol. G41. Springer, Berlin, pp. 29–48.
Bowers, H.A., Tengs, T., Glasgow Jr., H.B., Burkholder, J.M., Rublee, P.A., Oldach, D.W., 2000. Development of real-time PCR assays for rapid detection of Pfiesteria piscicida and related dinoflagellates. Appl. Environ. Microbiol 66, 4641–4648 (Erratum: Appl. Environ. Microbiol. 68, 3180).
Burkholder, J.M., Mallin, M.A., Glasgow Jr., H.B., Larsen, L.M., McIver, M.R., Shank, G.C., Deamer-Melia, N., Briley, D.S., Springer, J., Touchette, B.W., Hannon, E.K., 1997. Impacts to a coastal river and estuary from rupture of a large swine waste holding lagoon. J. Environ. Qual. 26, 1451–1466.
Burkholder, J.M., Glasgow Jr., H.B., 1997. Pfiesteria piscicida and other Pfiesteria-like dinoflagellates: behavior, impacts, and environmental controls. Limnol. Oceanogr. 42, 1052–1075.
Cembella, A.D., Turgeon, J., Therriault, J.C., Beland, P., 1988. Spatial distribution of Protogonyaulax tamarensis resting cysts in near-shore sediments along the north coast of the lower St. Lawrence estuary. J.

Shellfish Res. 7, 597–609.
Dale, B., 1996. Dinoflagellate cyst ecology: modeling and geological applications. In: Jansonius, J., McGregor, J. (Eds.), Palynology: Principles and Applications, vol. 3. American Association of Stratigraphic Palynologists Foundation, pp. 1249– 1275.
Glibert, P.M., Alexander, J., Trice, T.M., Michael, B., Magnien, R.E., Lane, L., Oldach, D., Bowers, H., 2004. Chronic urea nitrogen
loading: a correlate of Pfiesteria spp. in the Chesapeake and Coastal Bays of Maryland, USA. In: Steidinger, K.A., Lands-berg, J.H., Tomas, C.R., Vargo, G.A. (Eds.), Proceedings of the Xth International Conference on Harmful Algae. Florida Fish and Wildlife Conservation Commission and Intergovernmental Oceanographic Commission of UNESCO, pp. 74–76.
Hallegraeff, G.M., 1995. Harmful algal blooms: a global overview. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. IOC Manuals and Guides No. 33. UNESCO, pp. 1–22.
Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 50–30 exonuclease activity of Thermus aquaticus DNA polymerase. Proc. Natl. Acad. Sci. 88, 7276–7280.
Jakobsen, K.S., Tengs, T., Vatne, A., Bowers, H.A., Oldach, D.W., Burkholder, J.M., Glasgow Jr., H.B., Rublee, P.A., Klaveness, D., 2002. Discovery of the toxic dinoflagellate Pfiesteria in northern European waters. Proc. R. Soc. Lond. 269, 211–214.
Joachimsthal, E.L., Ivanov, V., Tay, J.H., Tay, S.T., 2003. Flow cytometry and conventional enumeration of microorganisms in ships' ballast water and marine samples. Mar. Pollut. Bull. 46, 308–313.
Kreader, C.A., 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl. Environ. Microbiol. 62, 1102–1106.
Lewis, P.N., Hewitt, C.L., Riddle, M., McMinn, A., 2003. Marine introductions in the Southern Ocean; an unrecognised hazard to biodiversity. Mar. Pollut. Bull 46, 213–223.
Lipton, D.W., 1998. Pfiesteria's economic impact on seafood industry sales and recreational fishing. In: Gardner, B.L., Koch, L. (Eds.), Proceedings of the Conference, Economics of Policy Options for Nutrient Management and Pfiesteria, Center for Agricultural and Natural Resource Policy, University of Maryland, College Park, pp. 35–38.
Magnien, R.E., 2001. State monitoring activities related to Pfiesteria-like organisms. Environ. Health Persp. 109, 711–714.
Oldach, D.W., Delwiche, C.F., Jakobsen, K.S., Tengs, T., Brown, E.G., Kempton, J.W., Schaefer, E.F., Bowers, H.A., Glasgow Jr., H.B., Burkholder, J.M., Steidinger, K.A., Rublee, P.A., 2000. Heteroduplex mobility assay-guided sequence discovery: elucidation of the small subunit (18S) rDNA sequences of Pfiesteria piscicida and related dinoflagellates from complex algal culture and environmental sample DNA pools. Proc. Natl. Acad. Sci. 97, 4303–4308.
Saito, K., Drgon, T., Robledo, J.A.F., Krupatkina, D.N., Vasta, G.R., 2002. Characterization of the rRNA locus of Pfiesteria piscicida and development of standard and quantitative PCR-based detection assays targeted to the nontranscribed spacer. Appl. Environ. Microbiol. 68, 5394–5407.
Schaefer, E. F., 1997. A DNA assay to detect the toxic dinoflagellate Pfiesteria piscicida, and the application of a PCR-based probe. Master's Thesis, University of North Carolina at Greensboro.
Smalla, K., Cresswell, N., Mendonca-Hagler, L.C., Wolters, A., van Elsas, J.D., 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. J. Appl. Bacteriol. 74, 78–85.
Smol, J.P., Cumming, B.F., 2000. Tracking long-term changes in climate using algal indicators in lake sediments. J. Phycol. 36, 986–1011.
Stults, J.R., Snoeyenbos-West, O., Methe, B., Lovley, D.R., Chandler, D.P., 2001. Application of the 5' fluorogenic exonuclease assay (Taqman) for quantitative ribosomal DNA and rRNA analysis in sediments. Appl. Environ. Microbiol. 67, 2781–2789.
Wittwer, C.T., Herrmann, M.G., Moss, A.A., Rasmussen, R.P., 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 22, 130–138.
Zhang, H., Lin, S., 2002. Detection and quantification of Pfiesteria piscicida by using the mitochondrial cytochrome b gene. Appl. Environ. Microbiol. 68, 989–994.