# Development of Real-Time PCR Assays for Rapid Detection of *Pfiesteria piscicida* and Related <u>Dinoflagellates</u>

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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. shumwavae* (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).

Opposition or collection site       Source: strain(s)       P. picotidad PCR       P. shummapper sp. nov. PCR         Procentum minimum       CCMP: 699       Negl       Negl       Negl         Amphidulum caterate       CCMP: 1314       Neg       Negl       Negl         Amphidulum caterate       CCMP: 1314       Neg       Negl       Negl         Amphidulum caterate       CCMP: 1314       Neg       Negl       Negl         Amphidulum caterate       CCMP: 711       Negl       Negl       Negl         Amphidulum caterate       CCMP: 713       Negl       Negl       Negl         Gymodatin ghydram       CCMP: 415       166       Negl       Negl         Gymodatin ghydram       CCMP: 415       116       Negl       Negl         Gymodatin ghydram       CCMP: 717       Negl       Negl       Negl         Porocertum freinfum       CCMP: 710       Negl       Negl       Negl         Colaid month       CCMP: 711       Negl       Negl       Negl         Amoutodic cluders       CCMP: 724       Negl       Negl       Negl         Colaid monthi       CCMP: 7160       Negl		TABLE 1. Specificity of species-selective primers			
Processing injurant       CCMP; 699       Neg*       Neg         Iderocapa injurant       CCMP; 449       Neg       Neg         Amphidulum carene       CCMP; 1314       Neg       Neg         Amphidulum carene       CCMP; 1314       Neg       Neg         Amphidulum carene       CCMP; 713       Neg       Neg         Amphidulum carene       CCMP; 713       Neg       Neg         Genomiants       CCMP; 713       Neg       Neg         Genomiants       CCMP; 713       Neg       Neg         Genomiants       CCMP; 717       Neg       Neg         Genomiants       CCMP; 717       Neg       Neg         Processitis       Neg       Neg       Neg         Arcostis       CCMP; 717       Neg       Neg         Processitis       Neg       Neg       Neg         Arcostis       CCMP; 717       Neg       Neg         Arcostis       CCMP; 713       Neg       Neg         Arcostis       CCMP; 713       Neg       Neg         Arcostis       CCMP; 713       Neg       Neg         Arcostis       <	Organism or collection site and date (mo/yr)	Source; strain(s)	P. piscicida PCR	P. shumwayae sp. nov. PCR	
Idencepts triguetra       CCMP, 449       Neg       Neg       Neg         Katodhimum rotundatum       CCMP, 1314       Neg       Neg       Neg         Katodhimum rotundatum       CCMP, 1321       Neg       Neg       Neg         Combum tongipes       CCMP, 1371       Neg       Neg       Neg         Langohatiniam polyedram       CCMP, 407       Neg       Neg       Neg         Chattonella subsala       CCMP, 718       Neg       Neg       Neg         Chattonella subsala       CCMP, 415, 416       Neg       Neg       Neg         Granodnium gatabeurum       CCMP, 717, 72, 735       Neg       Neg       Neg         Scrippsiella groomsas       CCMP, 718       Neg       Neg       Neg         Scrippsiella groomsas       CCMP, 717, 735       Neg       Neg       Neg         Adenoidscus toxicus       CCMP, 718       Neg       Neg       Neg         Coldia monsitus       CCMP, 718       Neg       Neg       Neg         Adenoidscus toxicus       CCMP, 718       Neg       Neg       Neg         Coldia monsitus       CCMP, 1391       Neg       Neg	Prorocentrum minimum	ССМР; 699	Neg <sup>b</sup>	Neg	
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Advantant mutuatum       CLM: 154-2       Neg       Neg         Communit mutuatum       CLM: 154-2       Neg       Neg         Langulatinam polyadrum       CCMF: 1770       Neg       Neg         Cambani Insights       CCMF: 407       Neg       Neg         Cambani Insights       CCMF: 1710       Neg       Neg         Chattonells subsits       CCMF: 171       Neg       Neg         Chattonells subsits       CCMF: 171       Neg       Neg         Chattonells subsits       CCMF: 711       Neg       Neg         Fornocensum folgenmanum       CCMF: 772, 735       Neg       Neg         Scrippstella forensus       CCMF: 711       Neg       Neg         Scrippstella forensus       CCMF: 713       Neg       Neg         Colain anotati       CCMF: 713       Neg       Neg         Adenotation writers       CCMF: 1301       Neg       Neg         Adenotation writers       CCMF: 142       Neg       Neg         Orain anotation       CCMF: 142       Neg       Neg         Adenotation writers       CCMF: 142       Neg       Neg         Adenotation writers<	Amphidinium carterae	CCMP; 1314	Neg	Neg	
Contain       CCMP; 1770       Neg       Neg         Control       CCMP; 4071       Neg       Neg         Gymandhium breve       CCMP; 718       Neg       Neg         Catanolla subslas       CCMP; 717       Neg       Neg         Gymandhium gathbenum       CCMP; 171       Neg       Neg         Froccentum folfmannaum       CCMP; 415, 416       Neg       Neg         Froncentum instathenum       CCMP; 717, 715       Neg       Neg         Gombardicus traista       CCMP; 717, 715       Neg       Neg         Forocentum instathenum       CCMP; 717, 715       Neg       Neg         Forocentum instathenum       CCMP; 717, 715       Neg       Neg         Forocentum instathenum       CCMP; 1891       Neg       Neg         Forocentum instathenum       CCMP; 1891       Neg       Neg         Gomodhium mikinnoti       CCMP; 421       Neg       Neg         Gymandhium mikinnoti       CCMP; 1361       Neg       Neg         Gymandhium mikinnoti       CCMP; 1373       Neg       Neg         Gymandhium mikinnoti       CCMP; 1360       Neg       Neg <t< td=""><td>Katoainium rotunaatum</td><td>CCMP; 1542 CCMP: 731</td><td>Neg</td><td>Neg</td></t<>	Katoainium rotunaatum	CCMP; 1542 CCMP: 731	Neg	Neg	
Linguistation projection       CCMP, 417       Neg       Neg         Constraintina polation       CCMP, 718       Neg       Neg         Grantonium berwe       CCMP, 217       Neg       Neg         Constraintian galathearum       CCMP, 215, 416       Neg       Neg         Grantonium infestimum       CCMP, 633       Neg       Neg         Procentum freistimum       CCMP, 700       Neg       Neg         Scrippsielia fromensae       CCMP, 771       Neg       Neg         Procentum freistimum       CCMP, 783       Neg       Neg         Scrippsielia resta       CCMP, 780       Neg       Neg         Colar montois       CCMP, 830       Neg       Neg         Adenoidas cludens       CCMP, 801       Neg       Neg         Grantonium miktonoi       CCMP, 421, 400       Neg       Neg         Procentum operulatum       CCMP, 1342       Neg       Neg	Caratium longings	CCMP; 751 CCMP: 1770	Neg	Neg	
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Chattonilia subalia       CCMP; 217       Neg       Neg         Gymnodhium salatheanum       CCMP; 415, 416       Neg       Neg         Procentum forfinamianum       CCMP; 633       Neg       Neg         Procentum forfinamianum       CCMP; 700       Neg       Neg         Strippidel forfinamianum       CCMP; 771       Neg       Neg         Strippidel forfinamianum       CCMP; 771       Neg       Neg         Strippidel forfinamianum       CCMP; 771       Neg       Neg         Strippidel forfinamianum       CCMP; 717       Neg       Neg         Strippidel forfinamianum       CCMP; 717       Neg       Neg         Strippidel forfinamianum       CCMP; 711       Neg       Neg         Colar anontal       CCMP; 803       Neg       Neg         Gymnodhium miknotoi       CCMP; 421, 400       Neg       Neg         Procentum operculatum       CCMP; 1342       Neg       Neg         Procentum operculatum       CCMP; 1342       Neg       Neg         Procentum operculatum       CCMP; 1342       Neg       Neg         Oprocentum operculatum       CCMP; 1342       Neg       Neg </td <td>Gymnodinium breve</td> <td>CCMP: 718</td> <td>Neg</td> <td>Neg</td>	Gymnodinium breve	CCMP: 718	Neg	Neg	
(Heterokontophysa)       CCMP; 415, 416       Neg       Neg         Provcentrum hoffmannisuum       CCMP; 633       Neg       Neg         Provcentrum tristitum       CCMP; 700       Neg       Neg         Scripsiella faroensae       CCMP; 772, 735       Neg       Neg         Scripsiella faroensae       CCMP; 772, 735       Neg       Neg         Gambienducas tasicus       CCMP; 1600       Neg       Neg         Froitecentian relicutum       CCMP; 1601       Neg       Neg         Gambienducas tasicus       CCMP; 1601       Neg       Neg         Gomadnium varians       CCMP; 1421       Neg       Neg         Gomadnium varians       CCMP; 421       Neg       Neg         Gomadnium nukimotoi       CCMP; 1421       Neg       Neg         Prozocentrum trait       CCMP; 1421       Neg       Neg         Prozocentrum nukimotoi       CCMP; 1412       Neg       Neg         Prozocentrum sp.       CCMP; 1421       Neg       Neg         Prozocentrum nukimotoi       CCMP; 1430       Neg       Neg         Prozocentrum sp.       CCMP; 1646, 605, 1739       Neg       Neg </td <td>Chattonella subsalsa</td> <td>CCMP; 217</td> <td>Neg</td> <td>Neg</td>	Chattonella subsalsa	CCMP; 217	Neg	Neg	
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Constant       CCMP: 202       Ng       Ng         Granodinium nikimotoi       CCMP: 211       Ng       Ng         Granodinium nikimotoi       CCMP: 421       Ng       Ng         Amphidnium operculatur       CCMP: 421       Ng       Ng         Amphidnium operculatur       CCMP: 1342       Ng       Ng         Amphidnium operculatur       CCMP: 1342       Ng       Ng         Parosonium sp.       CCMP: 1341       Ng       Ng         Parosonium operculatur       CCMP: 1341       Ng       Ng         Parosonium sp.       CCMP: 1300       Ng       Ng         Alexandrium tonnerws       CCMP: 1300       Ng       Ng         Orphin marine (Alexalar)       CCMP: 1306       Ng       Ng         Organius collectur       CCMP: 1306       Ng       Ng         Granodinium finitur       CCMP: 1306       Ng       Ng         Granodinium singurun       CCMP: 1300       Ng       Ng         Granodinium singurun       CCMP: 1310       Ng       Ng         Granodinium instriatur       CCMP: 1310       Ng       Ng         Granodinium instriatur <t< td=""><td>Adancidas aludans</td><td>CCMP: 1801</td><td>Neg</td><td>Neg</td></t<>	Adancidas aludans	CCMP: 1801	Neg	Neg	
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Gramoditium mikimotoi       CCMP: 429, 430       Neg       Neg         Amphidinium opercultum       CCMP: 447       Neg       Neg         Amphidinium opercultum       CCMP: 1342       Neg       Neg         Parocentrum sp.       CCMP: 1541       Neg       Neg         Parocentrum compressum       CCMP: 1641       Neg       Neg         Proceditium inclinatum       CCMP: 1786       Neg       Neg         Atexandrium tamarense       CCMP: 1900       Neg       Neg         Opritiu marine (Alecolata)       CCMP: 1920       Neg       Neg         Orginatium foliaceum       CCMP: 1920       Neg       Neg         Orginatium ingliacum       CCMP: 1200       Neg       Neg         Orgonatus cochila       CCMP: 1200       Neg       Neg         Orgonatus cochila       CCMP: 1310       Neg       Neg         Gronodinium instriatum       CCMP: 1311       Neg       Neg         Gronodinium instriatum       CCMP: 1310       Neg       Neg         Gronodinium instriatum       CCMP: 767       Neg       Neg         Proocentrum brainflow       CCMP: 767       Neg       Neg	Gymnodinium varians	CCMP: 421	Neg	Neg	
<i>ifetocopsa niei</i> CCMP; 447NegNeg <i>Amphilatium operculatum</i> CCMP; 1342NegNeg <i>Procontrum</i> sp.CCMP; 1342NegNeg <i>Procontrum</i> sp.CCMP; 1786NegNeg <i>Procontrum</i> numarrseCCMP; 1786NegNeg <i>Recardium inclinatum</i> CCMP; 1786NegNeg <i>Recardium inclinatum</i> CCMP; 160, 605, 1739NegNeg <i>Oprinis maring</i> (Abcolata)CCMP; 1522NegNeg <i>Gomadular cochlaa</i> CCMP; 1522NegNeg <i>Gomadular cochlaa</i> CCMP; 1522NegNeg <i>Gomadular cochlaa</i> CCMP; 1520NegNeg <i>Gronodular cochlaa</i> CCMP; 1678NegNeg <i>Gronodular cochlaa</i> CCMP; 1678NegNeg <i>Grodular inpulcium</i> CCMP; 1678NegNeg <i>Grodular inpulcium</i> CCMP; 1678NegNeg <i>Grodular inpulcium</i> CCMP; 1310NegNeg <i>Grodular inpulcium</i> CCMP; 1310NegNeg <i>Grodular inpulcium</i> CCMP; 1370NegNeg <i>Grodular inpulcium</i> CCMP; 1370NegNeg <i>Froocentrum mesicanan</i> CCMP; 1370NegNeg <i>Rodonnonas</i> sp.CCMP; 1371NegNeg <i>Procentrum mesicanan</i> CCMP; 1371NegNeg <i>Procentrum mesicanan</i> CCMP; 1371NegNeg <i>Procentrum mesicanan</i> CCMP; 1372NegNeg <i>Procentrum mesicanan</i> CCMP; 1373NegNeg <td>Gymnodinium mikimotoi</td> <td>CCMP; 429, 430</td> <td>Neg</td> <td>Neg</td>	Gymnodinium mikimotoi	CCMP; 429, 430	Neg	Neg	
Amphidizium operculatum       CCMP; 1342       Neg       Neg         Perocentrum compressun       CCMP; 1786       Neg       Neg         Precadinium inclinatum       CCMP; 1786       Neg       Neg         Precadinium inclinatum       CCMP; 1786       Neg       Neg         Alexandrium tanarense       CCMP; 160       Neg       Neg         Opritis marine (Albeolata)       CCMP; 1617       Neg       Neg         Gonyaldax cochlea       CCMP; 1326       Neg       Neg         Gonyaldax cochlea       CCMP; 1326       Neg       Neg         Gonyaldax cochlea       CCMP; 1417       Neg       Neg         Forocentrum sp.       CCMP; 1678       Neg       Neg         Grodinium inpudicum       CCMP; 1310       Neg       Neg         Grodinium inpudicum       CCMP; 141       Neg       Neg         Grodinium incenteum       CCMP; 1310       Neg       Neg         Grodinium incenteum       CCMP; 768       Neg       Neg         Floatering piscicida       NCSU; 97-1       Pos       Neg         Presertar piscicida       NCSU; 97-1       Pos       Neg         <	Heterocapsa niei	CCMP; 447	Neg	Neg	
Procentrum $p_{corr}$ CCMP; 1541NegNegProconctrumcompressumCCMP; 1890NegNegRecandrinu inclinatumCCMP; 1800NegNegAlexandrinu inclinatumCCMP; 1800NegNegCaynthis marina (Alveolata)CCMP; 106NegNegCorrelationCCMP; 1326NegNegCorrelationCCMP; 1326NegNegCorrelationCCMP; 1326NegNegCorrelationCCMP; 1326NegNegCorrelationCCMP; 1320NegNegCorrelationCCMP; 1320NegNegCorrelationCCMP; 1320NegNegCorrelationCCMP; 1320NegNegCorodinium instriatumCCMP; 1678NegNegGrodinium instriatumCCMP; 1310NegNegGronodinium actentumCCMP; 1310NegNegGronodinium actentumCCMP; 767NegNegRhodononas sp.CCMP; 767NegNegProsciediaNCSU; 102-1Pos'NegPisetra piscicidaFL DEP; MDFDEPMR23PosNegPisetra piscicidaNCSU; 97-1PosNegPisetra piscicidaFL DEP; MMRCO981020BR01C5PosNegPisetra shumwayae sp. nov.NCSU; 172-1PosNegPisetra shumwayae sp. nov.NCSU; 187-2NegNegPisetra shumwayae sp. nov.NCSU; 187-3NegNegPisetra shumwayae sp. nov. </td <td>Amphidinium operculatum</td> <td>CCMP; 1342</td> <td>Neg</td> <td>Neg</td>	Amphidinium operculatum	CCMP; 1342	Neg	Neg	
Procentrum compressumCCMP; 1786NegNegThecadnium inclinatumCCMP; 1890NegNegAlexandrium tamarenseCCMP; 106NegNegOprihis narina (Alveolata)CCMP; 1326NegNegPerilatium foliaceumCCMP; 1326NegNegGonyaluk zcochleaCCMP; 1326NegNegGonyaluk zcochleaCCMP; 1326NegNegFroncentum balticumCCMP; 1326NegNegFroncentum balticumCCMP; 1370NegNegGrodinium inputicumCCMP; 1371NegNegGrodinium inputicumCCMP; 1431NegNegGrodinium inputicumCCMP; 1310NegNegGrodinium inputicumCCMP; 141NegNegGrodinium incatenumCCMP; 767NegNegGrodinium actenumCCMP; 767NegNegRhodonnas sp.CCMP; 768NegNegPrescrita piscicidaNCSU; 102-1Pos'NegPisetra piscicidaNCSU; 97-11PosNegPisetra piscicidaNCSU; 97-11PosNegPisetra piscicidaNCSU; 77-88NegPosPisetra piscicidaNCSU; 728-7NegNegPisetra piscicidaNCSU; 728-7NegPosPisetra piscicidaNCSU; 728-7NegNegPisetra piscicidaNCSU; 728-7NegNegPisetra piscicidaNCSU; 728-7NegNegPisetra piscicidaNCSU; 728-7<	Prorocentrum sp.	CCMP; 1541	Neg	Neg	
Thecadinum inclinatumCCMP; 1890NegNegAlexandrium tranarenseCCMP; 164, 605, 1739NegNegOpyrhis marina (Aleolata)CCMP; 1326NegNegOpyrhis marina (Aleolata)CCMP; 1326NegNegGonyaltax cochleaCCMP; 1326NegNegGonyaltax cochleaCCMP; 1326NegNegGronodniums anguineumCCMP; 1471NegNegProncentrum sp.CCMP; 1768NegNegGrondnium inguidicumCCMP; 1411NegNegGrondnium inguidicumCCMP; 1411NegNegGynodnium instriatumCCMP; 141NegNegGynodnium instriatumCCMP; 1310NegNegGynodnium instriatumCCMP; 1370NegNegFroocentrum mexicanumCCMP; 1370NegNegProocentrum mexicanumCCMP; 767NegNegProocentrum mexicanumCCMP; 768NegNegPiesteria piscicidaPLDP; MDFDEPMR23PosNegPiesteria piscicidaCCMP; 768NegNegPiesteria piscicidaCCMP; 1831PosNegPiesteria piscicidaCCMP; 1827NegNegPiesteria piscicidaCCMP; 1827NegNegPiesteria piscicidaCCMP; 1827NegNegPiesteria piscicidaCCMP; 1827NegNegPiesteria piscicidaCCMP; 1827NegNegPiesteria piscicidaCCMP; 1827NegNeg <tr< td=""><td>Prorocentrum compressum</td><td>CCMP; 1786</td><td>Neg</td><td>Neg</td></tr<>	Prorocentrum compressum	CCMP; 1786	Neg	Neg	
Alexandrium tamarenseCCMP; 116NegNegOrynris marina (Alveolata)CCMP; 1326NegNegPeridinium foliaceumCCMP; 1326NegNegGonyaulax cochleaCCMP; 1592NegNegGronzolarium sanguineumCCMP; 1592NegNegProrocentrum sp.CCMP; 703NegNegGrondinium inpudicumCCMP; 1678NegNegGrondinium inpudicumCCMP; 1678NegNegGrondinium instraitumCCMP; 1310NegNegGrondinium instraitumCCMP; 1310NegNegGrondinium instraitumCCMP; 1370NegNegGrondinium catenatumCCMP; 1678NegNegFroocentrum mexicanumCCMP; 1707NegNegFroocentrum mexicanumCCMP; 1768NegNegFleateria piscicidaFL DEP; MDFDEPMR23Pos'NegFleateria piscicidaCCMP; 1831Pos'NegFleateria piscicidaCCMP; 1831NegPos'Fleateria piscicidaCCMP; 1827NegPosFleateria shumwayae sp. nov.NCSU; J-28-TNegNeg<	Thecadinium inclinatum	CCMP; 1890	Neg	Neg	
Oxyrhis marina (Alveolata)CCMP; 604, 605, 1739NegNegPerdinium (Diaceum)CCMP; 1326NegNegGonyaulax cochleaCCMP; 1326NegNegGonyaulax cochleaCCMP; 1326NegNegProzoentrum balicumCCMP; 1260NegNegProzoentrum sp.CCMP; 1078NegNegGyrodinium ingrudicumCCMP; 1078NegNegGyrodinium ingrudicumCCMP; 119NegNegGyrodinium instriatumCCMP; 1310NegNegGyrodinium uncatenumCCMP; 1310NegNegGyrodinium uncatenumCCMP; 1370NegNegGyrodinium atenatumCCMP; 767NegNegFrozoentrum mexicanumCCMP; 768NegNegFisteria pisciciaP. CCMP; 768NegNegProsticiaP. CCMP; 768NegNegPisteria pisciciaP. CCMP; 768NegNegPisteria pisciciaP. DEP; MDFDEPMR23PosNegPisteria pisciciaCCMP; 1831PosNegPisteria pisciciaCCMP; 1831PosNegPisteria pisciciaNCSU; 97-11PosNegPisteria pisciciaCCMP; 1871NegPosPisteria pisciciaNCSU; 97-11PosNegPisteria pisciciaPosNegPosPisteria pisciciaCCMP; 1872NegPosPisteria pisciciaPosNegPosPisteria pisciciaPosNeg <td>Alexandrium tamarense</td> <td>CCMP; 116</td> <td>Neg</td> <td>Neg</td>	Alexandrium tamarense	CCMP; 116	Neg	Neg	
Pertainum jolaceumCCMP; 1320NegNegGonyauka CochkaCCMP; 1592NegNegGymodinium sanguheumCCMP; 1592NegNegProzentrum sp.CCMP; 1260NegNegGymodinium instriatumCCMP; 130NegNegGyrodinium instriatumCCMP; 1310NegNegGyrodinium instriatumCCMP; 1310NegNegGymodinium uncatenumCCMP; 1310NegNegGymodinium autoatenumCCMP; 1310NegNegGymodinium actenutumCCMP; 767NegNegFroocentrum mexicanumCCMP; 768NegNegRhodomonas sp.CCMP; 768NegNegPiesteria piscicidaNCSU; 102-1PosNegPiesteria piscicidaNCSU; 97-1PosNegPiesteria piscicidaNCSU; 97-1PosNegPiesteria piscicidaNCSU; 97-1PosNegPiesteria piscicidaNCSU; 97-1PosNegPiesteria piscicidaNCSU; 97-1PosNegPiesteria piscicidaNCSU; 97-1PosNegPiesteria piscicidaNCSU; 102-1PosNegPiesteria piscicidaNCSU; 199-1PosNegPiesteria piscicidaNCSU; 97-28-1PosNegPiesteria piscicidaNCSU; 199-1PosNegPiesteria shumwayae sp. nov.NCSU; 12-28-1NegPosPiesteria shumwayae sp. nov.NCSU; 12-28-1NegNegOrgo	Oxyrrhis marina (Alveolata)	CCMP; 604, 605, 1739	Neg	Neg	
Onlyada: UchiedCCMP; 132NegNegProcentrum sanguineumCCMP; 1260NegNegProcentrum sp.CCMP; 1260NegNegGyrodinium instriatumCCMP; 1678NegNegGyrodinium instriatumCCMP; 1678NegNegGyrodinium instriatumCCMP; 1310NegNegGyrodinium instriatumCCMP; 1310NegNegGyrodinium incatenumCCMP; 1310NegNegGyrodinium incatenumCCMP; 1370NegNegFroocentrum meticanumCCMP; 767NegNegRhodomonas sp.CCMP; 768NegNegPfesteria piscicidaFL DEP; MDFDEPMR23PosNegPfesteria piscicidaNCSU; 97-1PosNegPfesteria piscicidaNCSU; 97-1PosNegPfesteria piscicidaCCMP; 1831PosNegPfesteria piscicidaNCSU; 97-1PosNegPfesteria piscicidaNCSU; 12-28-TNegPosPfesteria shumwayae sp. nov.NCSU; 12-28-TNegPosPfesteria shumwayae sp. nov.NCSU; 12-28-TNegNegPfesteria shumwayae sp. nov.NCSU; 12-28-TNegNegPfesteria shumwayae sp. nov.NCSU; 12-8-TNegNegPfesteria shumwayae sp. nov.NCSU; 12-8-TNegNegPfesteria shumwayae sp. nov.NCSU; 12-8-TNegNegPfesteria shumwayae sp. nov.NCSU; 12-8-TNegNegPfesteria shumwayae sp	Periainium jouaceum	CCMP; 1326 CCMP: 1502	Neg	Neg	
Opmodunium ballicumCCMP; 117FregFregProrocentrum ballicumCCMP; 1260NegNegProrocentrum sp.CCMP; 1260NegNegGyrodnium inpulicumCCMP; 419NegNegGyrodnium instriatumCCMP; 431NegNegGyrodnium instriatumCCMP; 1310NegNegGyrodnium uncatenumCCMP; 1310NegNegGyrodnium catenumCCMP; 1310NegNegGyrodnium catenumCCMP; 1370NegNegFroocentrum mexicanumCCMP; 767NegNegRhodomonas sp.CCMP; 768NegNegPflesteria piscicidaPLDEP; MDFDEPMR23Pos'NegPflesteria piscicidaNCSU; 102-1Pos'NegPflesteria piscicidaNCSU; 97-1PosNegPflesteria piscicidaCCMP; 1831PosNegPflesteria piscicidaCCMP; 1831PosNegPflesteria siscicidaCCMP; 1821PosNegPflesteria siscicidaCCMP; 1821NegPosPflesteria siscicidaCCMP; 1821NegPosPflesteria siscicidaCCMP; 1827NegPosPflesteria siscicidaCCMP; 1827NegPosPflesteria siscicidaCCMP; 1872NegNegPflesteria siumwayae sp. nov.NCSU; BPNegNegPflesteria siumwayae sp. nov.NCSU; BPNegNegPflesteria siumwayae sp. nov.NCSU; BPNegNeg <t< td=""><td>Gonyaulax cocniea</td><td>CCMP; 1592 CCMP: 417</td><td>Neg</td><td>Neg</td></t<>	Gonyaulax cocniea	CCMP; 1592 CCMP: 417	Neg	Neg	
Notice in the instructionCCMP: 1000NegNegGyrodinium impulicumCCMP: 1678NegNegGyrodinium instriatumCCMP: 431NegNegGyrodinium instriatumCCMP: 431NegNegGyrodinium instriatumCCMP: 419NegNegGyrodinium incatenumCCMP: 1310NegNegGyrodinium neatenatumCCMP: 1370NegNegRhodomonas sp.CCMP: 767NegNegProtocentrum mexicanumCCMP: 767NegNegRhodomonas sp.CCMP: 7668NegNegPfesteria piscicidaFL DEP: MDFDEPMR23PosNegPfesteria piscicidaCCMP: 1831PosNegPfesteria piscicidaCCMP: 1831PosNegPfesteria piscicidaFL DEP: MMRCO981020BR01C5PosNegPfesteria piscicidaFL DEP: MMRCO981020BR01C5PosNegPfesteria shurmwayae sp. nov.NCSU: B-VandemereNegPosPfesteria shurmwayae sp. nov.NCSU: B-VandemereNegNegPfesteria sh	Prorocentrum balticum	CCMP: 1260	Neg	Neg	
Gyrodinium inpudicumCCMP; 1678NegNegGyrodinium instriatumCCMP; 431NegNegGyrodinium instriatumCCMP; 431NegNegGyrodinium instriatumCCMP; 431NegNegGyrodinium catenatumCCMP; 1310NegNegGyrodinium catenatumCCMP; 1310NegNegForocentrum mexicanumCCMP; 1370NegNegRhodomonas sp.CCMP; 767NegNegPlesteria piscicidaNCSU; 102-1Pos'NegPlesteria piscicidaNCSU; 97-1Pos'NegPlesteria piscicidaCCMP; 1831PosNegPlesteria piscicidaCCMP; 1831PosNegPlesteria piscicidaCCMP; 1831PosNegPlesteria siscicidaCCMP; 1827aNegPosPlesteria siscicidaPosNegPosPlesteria siscicidaCCMP; 1827aNegPosPlesteria siumwayae sp. nov.NCSU; P-2a-TNegPosPlesteria siumwayae sp. nov.NCSU; BPNegNegPlesteria siumwayae sp. nov.NCSU; BPNegNegPlesteria siumwayae sp. nov.CCMP; 1827aNegNegPlesteria siumwayae sp. nov.CCMP; 1827bNegNegPlesteria siumwayae sp. nov.CCMP; 1873NegNegPlesteria siumwayae sp. nov.CCMP; 1877NegNegVilmington River (Ga.) 11/98CCMP; 1877NegNegVilmington River (Ga.) 11/98	Prorocentrum sp.	CCMP: 703	Neg	Neg	
Gyrodinium instriatumCCMP; 431NegNegGyrodinium simplexCCMP; 419NegNegGyrodinium uncatenuumCCMP; 1100NegNegGyrodinium catenatumCCMP; 1110NegNegGyronodinium catenatumCCMP; 1370NegNegProrocentrum mexicanumCCMP; 767NegNegRhodomonas sp.CCMP; 767NegNegPiesteria piscicidaNCSU; 102-1Pos'NegPiesteria piscicidaFL DEP; MDFDEPMR23PosNegPiesteria piscicidaCCMP; 1831PosNegPiesteria piscicidaCCMP; 1831PosNegPiesteria piscicidaCCMP; 1831PosNegPiesteria piscicidaCCMP; 1831PosNegPiesteria piscicidaCCMP; 1821NegPosPiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPiesteria shumwayae sp. nov.NCSU; BPNegPosPiesteria shumwayae sp. nov.NCSU; BPNegNegPiesteria shumwayae sp. nov.NCSU; BPNegNegPiesteria shumwayae sp. nov.NCSU; BPNegNegPiesteria shumwayae sp. nov.NCSU; BPNegNegPiesteria shumwayae sp. nov.CCMP; 1827aNegNegPiesteria shumwayae sp. nov.CCMP; 1873NegNegWilmington River (Ga.) 11/98CCMP; 1873NegNegWilmington River (Ga.) 11/98CCMP; 1874NegNegWilmington	Gyrodinium impudicum	CCMP: 1678	Neg	Neg	
Gymondinium simplexCCMP; 419NegNegGynodinium uncatenuumCCMP; 1310NegNegGymodinium catenatumCCMP; 1310NegNegProrocentrum mexicanumCCMP; 1370NegNegRhodomonas sp.CCMP; 767NegNegRhodomonas sp.CCMP; 767NegNegPfesteria piscicidaFL DEP; MDFDEPMR23PosNegPfesteria piscicidaRCSU; 97-1PosNegPfesteria piscicidaCCMP; 1831PosNegPfesteria piscicidaCCMP; 1831PosNegPfesteria piscicidaCCMP; 1831PosNegPfesteria piscicidaCCMP; 1831PosNegPfesteria piscicidaCCMP; 1831PosNegPfesteria piscicidaCCMP; 1827aNegPosPfesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfesteria shumwayae sp. nov.NCSU; B-VandemereNegNegPfesteria shumwayae sp. nov.NCSU; B-VandemereNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827aNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1872NegNegPfesteria shumwayae sp. nov.CCMP; 1873NegNegVilimington River (Ga.) 11/98CCMP; 1874NegNegWilimington River (Ga.) 11/98CCMP; 1875NegNegWilimington River (Ga.) 11/98CCMP; 1877NegNegWilimington River (Ga.) 11/98CCMP; 1877Neg <td< td=""><td>Gyrodinium instriatum</td><td>CCMP; 431</td><td>Neg</td><td>Neg</td></td<>	Gyrodinium instriatum	CCMP; 431	Neg	Neg	
Gyrodinium uncatenumCCMP; 1310NegNegGymnodinium catenatumCCMP; 1370NegNegProocentrum mexicanumCCMP; 767NegNegRhodomonas sp.CCMP; 768NegNegPhodomonas sp.CCMP; 768NegNegPflesteria piscicidaNCSU; 102-1Pos'NegPflesteria piscicidaNCSU; 97-1PosNegPflesteria piscicidaCCMP; 768NegNegPflesteria piscicidaNCSU; 97-1PosNegPflesteria piscicidaCCMP; 1831PosNegPflesteria piscicidaCCMP; 1831PosNegPflesteria piscicidaCCMP; 1831PosNegPflesteria piscicidaCCMP; 1827NegPosPflesteria shumwayae sp. nov.NCSU; 7-28-TNegPosPflesteria shumwayae sp. nov.NCSU; BPNegNegPflesteria shumwayae sp. nov.NCSU; BPNegNegPflesteria shumwayae sp. nov.NCSU; 1827aNegNegPflesteria shumwayae sp. nov.NCSU; BPNegNegPflesteria shu	Gymnodinium simplex	CCMP; 419	Neg	Neg	
Gymnodinium catenatumCCMP; 141NegNegProrocentrum mexicanumCCMP; 1370NegNegRhodomonas sp.CCMP; 767NegNegRhodomonas sp.CCMP; 767NegNegPfesteria piscicidaNCSU; 102-1Pos'NegPfesteria piscicidaFL DEP; MDFDEPMR23PosNegPfesteria piscicidaCCMP; 1831PosNegPfesteria piscicidaCCMP; 1831PosNegPfesteria piscicidaCCMP; 1831PosNegPfesteria piscicidaCCMP; 1831PosNegPfesteria piscicidaCCMP; 1831PosNegPfesteria shumwayae sp. nov.Species B' (GenBank AF218805)NegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; B-ZaNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827aNegNegGymnodinium galatheanumHPEL*; GENegNegPfiesteria-like isolate sitesNegNegVilinington River (Ga.) 11/98CCMP; 1872NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1878 <td>Gyrodinium uncatenum</td> <td>CCMP; 1310</td> <td>Neg</td> <td>Neg</td>	Gyrodinium uncatenum	CCMP; 1310	Neg	Neg	
Protocentrum mexicanumCCMP; 1370NegNegRhodomonas sp.CCMP; 767NegNegRhodomonas sp.CCMP; 767NegNegPfesteria piscicidaNCSU; 102-1Pos*NegPfesteria piscicidaFL DEP; MDFDEPMR23PosNegPfiesteria piscicidaCCMP; 1831PosNegPfiesteria piscicidaCCMP; 1831PosNegPfiesteria piscicidaCCMP; 1831PosNegPfiesteria piscicidaCCMP; 1831PosNegPfiesteria piscicidaCCMP; 1831PosNegPfiesteria shumwayae sp. nov.Species B' (GenBank AF218805)NegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; BTNegNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827aNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1873NegNegPfiesteria-like isolate sitesNegNegPfiesteria-like isolate sitesNegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.)	Gymnodinium catenatum	CCMP; 414	Neg	Neg	
Rhodomonas sp.CCMP; 767NegNegPhodomonas sp.CCMP; 768NegNegPfiesteria piscicidaFL DEP; MDFDEPMR23PosNegPfiesteria piscicidaRCSU; 97-1PosNegPfiesteria piscicidaCCMP; 1831PosNegPfiesteria piscicidaFL DEP; MMRCC981020BR01C5PosNegPfiesteria piscicidaFL DEP; MMRCC981020BR01C5PosNegPfiesteria shumwayae sp. nov.Species 78' (GenBank KF218805)NegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; 7-28-TNegPosPfiesteria shumwayae sp. nov.NCSU; 728-TNegNegPfiesteria shumwayae sp. nov.NCSU; 728-TNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827aNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827aNegNegPfiesteria-like isolate sitesNegNegNegPfiesteria-like isolate sitesNegNegNegWilmington River (Ga.) 11/98CCMP; 1873NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1879Neg	Prorocentrum mexicanum	CCMP; 1370	Neg	Neg	
Rhodomonas sp.CCMP; 768NegNegPfiesteria piscicidaNCSU; 102-1Pos <sup>6</sup> NegPfiesteria piscicidaFL DEP; MDFDEPMR23PosNegPfiesteria piscicidaNCSU; 97-1PosNegPfiesteria piscicidaCCMP; 1831PosNegPfiesteria piscicidaFL DEP; MMRCC981020BR01C5PosNegPfiesteria shumwayae sp. nov.Species 'B' (GenBank AF218805)NegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; BPNegNegPosPfiesteria shumwayae sp. nov.NCSU; BPNegNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827bNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1872NegNegPfiesteria-like isolate sitesNeuse River (N.C.) 10/98CCMP; 1872NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1877Neg	Rhodomonas sp.	CCMP; 767	Neg	Neg	
Tjesteria piscicidaNCSU; 102-1PosNegPfiesteria piscicidaFL DEP, MDFDEPMR23PosNegPfiesteria piscicidaCCMP; 1831PosNegPfiesteria piscicidaCCMP; 1831PosNegPfiesteria piscicidaFL DEP, MMRCC981020BR01C5PosNegPfiesteria piscicidaFL DEP, MMRCC981020BR01C5PosNegPfiesteria shurnwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shurnwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shurnwayae sp. nov.NCSU; BPNegPosPfiesteria shurnwayae sp. nov.NCSU; BPNegNegPoperidiniopsis sp. (gen. nov.)CCMP; 1827aNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827bNegNegPfiesteria-like isolate sitesNegNegNegWilmington River (Ga.) 11/98CCMP; 1872NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1878NegNegWilmington River (Ga.) 11/98CCMP; 1870NegNegWilmington River (Ga.) 11/98CCMP; 1880NegNegWilmington River (Ga.) 11/98CCMP; 1880Ne	Rhodomonas sp.	CCMP; 768	Neg De -f	Neg	
Pjesteria piscicidaPL DEP, MDPDEPMRESPosNegPjesteria piscicidaNCSU; 97-1PosNegPjesteria piscicidaCCMP; 1831PosNegPjesteria piscicidaFL DEP; MMRCC981020BR01C5PosNegPjesteria shumwayae sp. nov.Species 'B' (GenBank AF218805)NegPosPjesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPjesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPjesteria shumwayae sp. nov.NCSU; 7-28-TNegPosPjesteria shumwayae sp. nov.NCSU; BPNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827aNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827bNegNegPjesteria-like isolate sitesNegNegNegWilmington River (Ga.) 11/98CCMP; 1873NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1879NegNegNeuse River Isolate (N.C.) 12/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1870NegNegWilmington River (Ga.) 11/98CCMP; 1880NegNegWilmington River (Ga.) 11/98C	Pflesteria pisciciaa Biastaria pisciciaa	NCSU; 102-1 EL DED: MDEDEDMD22	Pos	Neg	
Tjesteria piscicidaNCSU; 971TOSNegPfiesteria piscicidaCCMP; 1831PosNegPfiesteria piscicidaFL DEP; MMRCC981020BR01C5PosNegPfiesteria shumwayae sp. nov.Species 'B' (GenBank AF218805)NegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827aNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827bNegNegGymnodinium galatheanumHPEL*; GENegNegPfiesteria-like isolate sitesNegNegNegWilmington River (Ga.) 11/98CCMP; 1872NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1871NegNegWilmington River (Ga.) 11/98CCMP; 1879NegNegWilmington River (Ga.) 11/	Pfiesteria piscicida	NCSU: 07.1	POS	Neg	
Preserva pisocicidaCCMP; 101103NegPfiesteria pisocicidaFL DEP; MMRCC981020BR01C5PosPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; BPNegPosPfiesteria shumwayae sp. nov.NCSU; BPNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827aNegNegGymnodinium galatheanumHPEL*; GENegNegPfiesteria-like isolate sitesNegNegWilmington River (Ga.) 11/98CCMP; 1872NegNegWilmington River (Ga.) 11/98CCMP; 1873NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegNeuse River Isolate (N.C.) 12/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1880NegNegWilmington River (Ga.) 11/98CCMP; 1880NegNegWilmington River (Ga.) 11/98CCMP; 1881NegNegWilmington River (Ga.) 11/98CCMP; 1882	Pfiastaria piscicida	CCMP: 1831	Pos	Neg	
PristeriaPrice B* (GenBank AF218805)NegPosPfiesteriashumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteriashumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteriashumwayae sp. nov.NCSU; 7-28-TNegPosPfiesteriashumwayae sp. nov.NCSU; BPNegPosPfiesteriashumwayae sp. nov.NCSU; BPNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827aNegNegGymnodinium galatheanumHPEL*; GENegNegPfiesteria-like isolate sitesNegNegWilmington River (Ga.)11/98CCMP; 1872NegNegWilmington River (Ga.)11/98CCMP; 1875NegNegWilmington River (Ga.)11/98CCMP; 1875NegNegWilmington River (Ga.)11/98CCMP; 1877NegNegWilmington River (Ga.)11/98CCMP; 1877NegNegWilmington River (Ga.)11/98CCMP; 1877NegNegNeuse River Isolate (N.C.)12/98CCMP; 1879NegNegNeuse River Isolate (N.C.)11/98CCMP; 1879NegNegWilmington River (Ga.)11/98CCMP; 1880NegNegWilmington River (Ga.)11/98CCMP; 1880NegNegWilmington River (Ga.)11/98CCMP; 1880NegNegWilmington River (Ga.)11/98CCMP; 1880NegNegWilmingto	Pfiesteria piscicida	FL DEP: MMRCC981020BR01C5	Pos	Neg	
Pfiesteria shumwayae sp. nov.NCSU; B-VandemereNegPosPfiesteria shumwayae sp. nov.NCSU; 7-28-TNegPosPfiesteria shumwayae sp. nov.NCSU; 7-28-TNegPosPfiesteria shumwayae sp. nov.NCSU; 3PNegNegPosPfiesteria shumwayae sp. nov.NCSU; 3PNegNegNegPfiesteria shumwayae sp. nov.NCSU; BPNegNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827bNegNegGymnodinium galatheanumHPEL*; GENegNegPfiesteria-like isolate sitesNegNegNegWilmington River (Ga.) 11/98CCMP; 1872NegNegWilmington River (Ga.) 11/98CCMP; 1873NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegNeuse River Isolate (N.C.) 12/98CCMP; 1877NegNegNeuse River Isolate (N.C.) 12/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1880NegNegWilmington River (Ga.) 11/98CCMP; 1881NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.	Pfiesteria shumwavae sp. nov.	Species 'B' (GenBank AF218805)	Neg	Pos	
Pfiesteria shumwayae sp. nov.NCSU; 7-28-TNegPosPfiesteria shumwayae sp. nov.NCSU; BPNegPosCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827aNegNegCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827bNegNegGymnodinium galatheanumHPEL*; GENegNegPfiesteria-like isolate sitesNegNegWilmington River (Ga.) 11/98CCMP; 1872NegNegWilmington River (Ga.) 11/98CCMP; 1874NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1878NegNegWilmington River (Ga.) 11/98CCMP; 1878NegNegWilmington River (Ga.) 11/98CCMP; 1878NegNegWilmington River (Ga.) 11/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1870NegNegWilmington River (Ga.) 11/98CCMP; 1881NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegPocomoke River (Md.) 1/98CCMP; 1882NegNegPocomoke River (Md.) 1/98CCMP; A8925PosNeg <td>Pfiesteria shumwayae sp. nov.</td> <td>NCSU; B-Vandemere</td> <td>Neg</td> <td>Pos</td>	Pfiesteria shumwayae sp. nov.	NCSU; B-Vandemere	Neg	Pos	
Pfiesteria shumwayae sp. nov.NCSU; BPNegPosCryptoperidiniopsis sp. (gen. nov.)CCMP; 1827aNegNegGymnodinium galatheanumCCMP; 1827bNegNegPfiesteria-like isolate sitesNegNegPfiesteria-like isolate sitesNegNegWilmington River (Ga.) 11/98CCMP; 1872NegNegWilmington River (Ga.) 11/98CCMP; 1873NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegNeuse River Isolate (N.C.) 12/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1880NegNegWilmington River (Ga.) 11/98CCMP; 1881NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Md.) 1/98CCMP; A8925PosNegChicamacomico River (Md.) 1/98CCMP; A8925PosNeg <td>Pfiesteria shumwayae sp. nov.</td> <td>NCSU; 7-28-T</td> <td>Neg</td> <td>Pos</td>	Pfiesteria shumwayae sp. nov.	NCSU; 7-28-T	Neg	Pos	
$\begin{array}{cccc} \dot{Cryptoperidiniopsis} \ {\rm sp.} \ ({\rm gen. nov.}) & {\rm CCMP}; 1827a & {\rm Neg} & {\rm Neg} \\ Cryptoperidiniopsis \ {\rm sp.} \ ({\rm gen. nov.}) & {\rm CCMP}; 1827b & {\rm Neg} & {\rm Neg} \\ \hline {\rm Gymnodinium galatheanum} & {\rm HPEL}^*; \ {\rm GE} & {\rm Neg} & {\rm Neg} \\ \hline {\rm Pfiesteria-like isolate sites} & & & & & & & & & & & & & & & & & & &$	Pfiesteria shumwayae sp. nov.	NCSU; BP	Neg	Pos	
Cryptoperidiniopsis sp. (gen. nov.)CCMP; 1827bNegNegGymnodinium galatheanumHPEL <sup>4</sup> ; GENegNegPfiesteria-like isolate sitesNegNegWilmington River (Ga.) 11/98CCMP; 1872NegNegWilmington River (Ga.) 11/98CCMP; 1873NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1880NegNegWilmington River (Ga.) 11/98CCMP; 1881NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegChicamacomico River (Md.) 1/98CCMP; A8925PosNegChicamacomico River (Md.) 1/98CCMP; A8922PosNeg	Cryptoperidiniopsis sp. (gen. nov.)	CCMP; 1827a	Neg	Neg	
Gymnodinium galatheanumHPEL*; GENegNegPfiesteria-like isolate sitesNeuse River (N.C.) 10/98CCMP; 1872NegNegWilmington River (Ga.) 11/98CCMP; 1873NegNegWilmington River (Ga.) 11/98CCMP; 1874NegNegWilmington River (Ga.) 11/98CCMP; 1875NegNegWilmington River (Ga.) 11/98CCMP; 1876NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1877NegNegWilmington River (Ga.) 11/98CCMP; 1878NegNegWilmington River (Ga.) 11/98CCMP; 1878NegNegWilmington River (Ga.) 11/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1880NegNegWilmington River (Ga.) 11/98CCMP; 1881NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegPocomoke River (Md.) 1/98CCMP; 1882PosNegChicamacomico River (Md.) 1/98CCMP; A8925PosNeg	Cryptoperidiniopsis sp. (gen. nov.)	ССМР; 1827b	Neg	Neg	
Pfiesteria-like isolate sites       Neg       Neg         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; 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; 1879       Neg       Neg         Wilmington River (Ga.) 11/98       CCMP; 1879       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         Wilmington River (Ga.) 11/98       CCMP; 1882       Neg       Neg         Pocomoke River (Md.) 1/98	Gymnodinium galatheanum	HPEL <sup>a</sup> ; GE	Neg	Neg	
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; 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; 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         Wilmington River (Ga.) 11/98       CCMP; 1882       Neg       Neg         Pocomoke River (Md.) 1/98       CCMP; A8925       Pos       Neg	Pfiesteria-like isolate sites				
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; 1875       Neg       Neg         Wilmington River (Ga.) 11/98       CCMP; 1876       Neg       Neg         Wilmington River (Ga.) 11/98       CCMP; 1877       Neg       Neg         Wilmington River (Ga.) 11/98       CCMP; 1878       Neg       Neg         Wilmington River (Ga.) 11/98       CCMP; 1879       Neg       Neg         Wilmington River (Ga.) 11/98       CCMP; 1870       Neg       Neg         Wilmington River (Ga.) 11/98       CCMP; 1870       Neg       Neg         Wilmington River (Ga.) 11/98       CCMP; 1880       Neg       Neg         Wilmington River (Ga.) 11/98       CCMP; 1882       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	Neuse River (N.C.) 10/98	CCMP; 1872	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         Wilmington River (Ga.) 11/98       CCMP; 1877       Neg       Neg         Wilmington River (Ga.) 11/98       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         Wilmington River (Ga.) 11/98       CCMP; 1882       Neg       Neg         Pocomoke River (Md.) 1/98       CCMP; A8925       Pos       Neg         Chicamacomico River (Md.) 1/98       CCMP; A8925       Pos       Neg	Wilmington River (Ga.) 11/98	CCMP; 1873	Neg	Neg	
Wilmington Kiver (Ga.) 11/98       CCMP; 18/5       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; 1877       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         Wilmington River (Ga.) 11/98       CCMP; 1882       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	Wilmington River (Ga.) 11/98	CCMP; 1874	Neg	Neg	
Wilmington River (Ga.) 11/98       CCMP; 18/6       Neg       Neg         Wilmington River (Ga.) 11/98       CCMP; 1877       Neg       Neg         Neuse River Isolate (N.C.) 12/98       CCMP; 1877       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         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	Wilmington River (Ga.) 11/98	CCMP; 1875	Neg	Neg	
wilmington Kiver (va.) 11/95       CCMP; 18/7       Nég       Nég         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         Wilmington River (Ga.) 11/98       CCMP; 1882       Neg       Neg         Pocomoke River (Md.) 1/98       CCMP; A8925       Pos       Neg         Pos       Neg       Neg       Neg       Neg	Wilmington River (Ga.) 11/98	CCMP; 18/0	Neg	Neg	
Netse Kiver (Sa.) 12/96CCMP; 16/6NegNegWilmington River (Ga.) 11/98CCMP; 1879NegNegWilmington River (Ga.) 11/98CCMP; 1880NegNegWilmington River (Ga.) 11/98CCMP; 1881NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegPocomoke River (Md.) 1/98CCMP; A8925PosNegChicamacomico River (Md.) 1/98CCMP; A8932PosNeg	Winnington Kiver (Ga.) 11/98 Neuse Biver Isolate (N.C.) 12/09	CCMP: 1879	neg Nca	Neg	
Wilmington River (Ga.) 11/26       CCMP; 1877       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	Wilmington River (Gn ) 11/08	CCMP: 1870	Neg	Ineg	
Wilmington River (Ga.) 11/98CCMP; 1881NegNegWilmington River (Ga.) 11/98CCMP; 1882NegNegPocomoke River (Md.) 1/98CCMP; A8925PosNegChicamacomico River (Md.) 1/98CCMP; A8932PosNeg	Wilmington River (Ga) 11/90	CCMP: 1880	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	Wilmington River (Ga.) 11/90	CCMP: 1881	Neg	Neg	
Pocomoke River (Md.) 1/98       CCMP; A8925       Pos       Neg         Chicamacomico River (Md.) 1/98       CCMP; A8932       Pos       Neg	Wilmington River (Ga) 11/98	CCMP: 1882	Neg	Neg	
Chicamacomico River (Md.) 1/98 CCMP: A8932 Pos Neo	Pocomoke River (Md.) 1/98	CCMP: A8925	Pos	Neg	
- · · · · · · · · · · · · · · · · · · ·	Chicamacomico River (Md.) 1/98	CCMP; A8932	Pos	Neg	

Continued on following page

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	

<sup>a</sup> HPEL, Horn Point Environmental Laboratories (University of Maryland Center for Environmental Studies).

<sup>b</sup> Neg, negative. <sup>c</sup> 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  $\mu$ m; 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 <sup>a</sup>	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	+	<u></u>
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	—	-

<sup>a</sup> 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- $\mu$ m-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  $\mu$ l of elution buffer and stored at  $-20^{\circ}$ 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  $5-\mu$ m-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  $\mu$ l, and 1  $\mu$  1/10 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<sup>-1</sup> (Life Technologies, Rockville, Md.); MgCl<sub>2</sub> 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<sup>-1</sup> (Idaho Technologies); PCR buffer at a final concentration of  $1 \times$  (Life Technologies); approximately 10 ng of

template DNA; and PCR grade water to a final volume of 10  $\mu$ l (Sigma). For a 10- $\mu$ l *P. shumwayae*-specific reaction, primers Pshumfor (5'-TGCATGTCTCAGTTTAAGTC A-3') and Pshumrev (5'-TCGATCATCAAATACACTAAAACTGTTTT-3') each at a final concentration of 0.2  $\mu$ M (Operon), were used. The probe used in this assay, at a final concentration of 0.30  $\mu$ M, was *P. shum* (5'-FAM-TACGG CGAAACTGCGAATGGCTCAT-TAMRA-3'). The same reagents and concentrations were used as described above to obtain a 10- $\mu$ l 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<sup>-1</sup>. Fluorescence acquisition was 100 ms after each incubation at 60°C, and the display mode was CH1 1<sup>-1</sup> with the gain set at 1.



FIG. 3. Single-cell specificity and sensitivity of *P. piscicida* real-time PCRbased 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  $\mu$ l of extracted DNA from 100  $\mu$ l 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<sup>-1</sup> (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<sup>-1</sup>). 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  $(10^1 \text{ to } 10^3 \text{ cells ml}^{-1} \text{ versus } 10^5 \text{ or more total phytoplankton cells ml}^{-1}$ ; 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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