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A DNA hybridization assay to identify toxic dinoflagellates in coastal waters: detection of *Karenia brevis* in the Rookery Bay National Estuarine Research Reserve

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Abstract

A DNA hybridization assay was developed in microtiter plate format to detect the presence of toxic dinoflagellates in coastal waters. Simultaneous detection of multiple species was demonstrated using *Karenia brevis*, *Karenia mikimotoi*, and *Amphidinium carterae*. Molecular probes were designed to detect both *K. brevis* and *K. mikimotoi* and to distinguish between these two closely related species. The assay was used to detect *K. brevis* in coastal waters collected from the Rookery Bay National Estuarine Research Reserve. Assay results were verified by species-specific PCR and sequence analysis. The presence/absence of *K. brevis* was consistent with microscopic observation. Assay sensitivity was sufficient to detect *K. brevis* in amounts defined by a regional monitoring program as ''present'' ($\leq$1000 cells/L). The assay yielded quick colorimetric results, used a single hybridization temperature, and conserved the amount of genomic DNA utilized by employing one set of PCR primers. The microplate assay provides a useful tool to quickly screen large sample sets for multiple target organisms.

Keywords: *Amphidinium carterae*; DNA hybridization; *Karenia brevis*; *Karenia mikimotoi*; Molecular probes; Rookery Bay NERR

1. Introduction

Coastal water quality is critical to human and ecosystem health, as well as to the fishing, aquaculture, and tourism industries. Toxic algae threaten many coastal states and can impact large geographic areas. The frequency, duration, and geographic extent of harmful algal blooms (HABs) appear to be increasing (Hallegraeff, 1993; Scholin et al., 1994; Magaña et al., 2003). Blooms of non-toxic algae also can cause harm by causing oxygen depletion, habitat alteration, and the displacement of indigenous species.

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Karenia species are unarmored, toxic dinoflagellates responsible for red tide outbreaks in various parts of the world (Horstman et al., 1991; Matsuyama et al., 1999; Chang et al., 2001; Godhe et al., 2001; Guillou et al., 2002). *Karenia brevis* (= *Gymnodinium breve* = *Ptychodiscus brevis*) is responsible for recurrent red tides in the Gulf of Mexico, including the coasts of Florida and Texas. Blooms can last more than 2 months and have occurred as far north as North Carolina (Summerson and Peterson, 1990; Tester et al., 1991). Blooms are responsible for fish, bird, and mammal mortality, including the endangered West Indian manatee (Forrester et al., 1977; Bossart et al., 1998; Landsberg, 2002). *K. brevis* releases brevetoxin, a lipid soluble molecule that activates sodium channels (Asai et al., 1984; Baden et al., 1995). Crashing waves aerosolize brevetoxin, which can induce respiratory distress (Hopkins et al., 1997; Kirkpatrick et al., 2004) and trigger asthma attacks (Watanabe et al., 1988). Cooking does not destroy the toxin, and ingestion of contaminated shellfish can cause neurotoxic shellfish poisoning (NSP) (Hopkins et al., 1997). Water column blooms can reach concentrations as high as $1.8x10^8$ cells/L producing massive fish kills (Steidinger, 1983). Possible respiratory irritation and closure of shellfish harvesting can occur at ''very low'' levels of 1000–5000 cells/L (http://floridamarine.org/). Closures of shellfish harvesting and loss of tourism cost local businesses millions of dollars annually (Anderson, 1994; Tester and Steidinger, 1997; Anderson et al., 2000).

Public health agencies, responsible for assuring that recreational water and seafood are safe, close fisheries and beaches impacted by HABs. However, it is often reports from citizens and physicians that alert public health agencies to a problem with toxic species (Hopkins et al., 1997); therefore, detection schemes that allow early warning are needed. Health agencies are responsible for large areas of coast with limited funds, and management decisions are based on time and labor intensive assays. Identification of HAB species requires considerable taxonomic expertise that is not broadly available, and many HAB species are delicate and difficult to identify when preserved (Faust et al., 1996). Detection methods that allow species identification without reliance on microscopic expertise also would benefit ecological research. Although, blooms are often thought of as a single species, this is an oversimplification of bloom dynamics (Smayda, 1995) as closely related, but hard to distinguish species are often present.

Molecular techniques are a promising means to improve HAB identification. In New Zealand, incorporation of rRNA-targeted probes into a marine biotoxin monitoring program led to a decrease in shellfish harvesting closures because the technique distinguished between toxic and non-toxic strains of *Pseudo-nitzschia* (Rhodes et al., 1998). Molecular phylogeny may indicate relationships better than morphology and may be more reliable than immunology because that can change with environmental conditions (Adachi et al., 1994).

Ribosomal RNA and DNA (rRNA, rDNA) sequences are widely used to distinguish organisms at the species level. In eukaryotes, rRNA genes are present as tandem repeats containing the 18S rDNA small subunit (SSU), an internal transcribed spacer (ITS1), the 5.8S rDNA, another internal transcribed spacer (ITS2), and the 28S rDNA large-subunit (LSU). This study targeted the D1/D2 region of the large subunit (LSU, 28S). This region contains conserved and hypervariable regions that are useful for distinguishing species and strains among dinoflagellates and for examining sequence heterogeneity among different strains (Scholin et al., 1994; Scholin and Anderson, 1996; Daugbjerg et al., 2000; Guillou et al., 2002).

The goal of this study was to develop a rapid and convenient molecular technique to identify multiple dinoflagellate species. PCR provides a sensitive and selective means of detection that has been used to identify dinoflagellate species from environmental samples (Guillou et al., 2002). In addition, real-time PCR has been successfully used to quantify toxic dinoflagellates (Gray et al., 2003). However, the equipment required is too expensive for many environmental laboratories or aquaculture companies. The procedure outlined here, which uses a standard thermocycler, can be utilized with a minimal amount of molecular biology experience.

The study site was the Rookery Bay National Estuarine Research Reserve (NERR). The Rookery Bay NERR is located five miles south of Naples, Florida at the northern end of the 10,000 Islands. The study site is a mangrove estuary containing both fresh and salt-water wetlands. The Reserve is connected to the Gulf of Mexico and is tidally influenced, with an

average tidal range of 0.6 m. The primary freshwater source is Henderson Creek, located at the northeastern corner of the Reserve. The water flow from Henderson Creek is managed to control drainage of the Belle Meade Water District. The creek experiences salinity fluctuations depending on tide, season, and water release (Shirley et al., submitted).

2. Materials and methods

2.1. Sequence of probes and primers

Probes were designed to hybridize within amplicons targeting the D1/D2 region of the rDNA large subunit (LSU). Probes and PCR primers were designed based on GenBank sequences for dinoflagellates, including those for *K. brevis* (accession no. U92248), *K. mikimotoi* (AF318223), and *A. carterae* (AF260380). Dinoflagellate sequences were aligned using Megalign (DNAstar) software. Probe and primer sequences were checked for hairpin, duplex, and dimer formation and compatible melting temperatures with MacVector and Oligo 4.0 software. Sequences were blasted against the GenBank database to test the specificity of primers and probes for their target. Probe and primer sequences are listed in Table 1. A probe termed "Amp" was designed to detect *A. carterae*, "Karenia" to detect *K. brevis*/*K. mikimotoi*, and "Brevis" to detect *K. brevis* but not *K. mikimotoi*. Species-specific PCR utilized the probe sequence as the forward primer and R-635 as the reverse, resulting in amplicons of size 338 bp, 206 bp and 320 bp, for Amp, Karenia and Brevis, respectively.

2.2. Production of microplates

Synthesis of the species-specific probes (Sigma Genosys) required an HPLC purification step to achieve the desired specificity. Species-specific probes were T-tailed in our laboratory prior to immobilization onto 96-well microtiter plates (polystyrene, amine surface; Costar 2388, Corning). The poly-T tails positioned the oligonucleotide probe above the plate surface, making the probe available for hybridization (Cinek et al., 2000; Kiesling et al., 2002a). Terminal transferase enzyme (4000 U; Roche Applied Science) was added to the following solution to make T-tailed probe in a final volume of 200 μl:100 μl autoclaved Milli-Q water, 1.2X terminal transferase buffer (Roche Applied Science), 4.8 mM dTTP (Roche Applied Science), 2.5 nmol oligonucleotide probe, and 0.25 mg/ml inorganic pyrophosphatase (Sigma #I-1643). The mixture was incubated in a 37 °C water bath for 4 h. Following incubation, 42.5 μl of autoclaved EDTA (0.5 M, pH 8.0) and 2.25 ml of filter-sterilized 1X TE (pH 7.4) were added, giving a final probe concentration of 1 pmol/μl. This volume was sufficient to covalently link T-tailed probe to 25 plates. T-tailed probe was stored at −20 °C.

To immobilize probes, 100 μl of T-tailed probe was combined with 10 ml filter-sterilized 1X TE (pH 7.4). The mixture (100 μl) was added to each well, yielding 1 pmol of T-tailed probe per well. Plates were dried in an oven (37–50 °C) overnight or until dry. Blocking buffer (5X Denhardt's solution, 1X phosphate buffered saline) was added to the plates (200 μl/well), and plates were incubated at room temperature for 1–4 h. Plates were turned over and shaken on absorbent towels to remove buffer and then dried upside down at room temperature until completely dry (∼1–2 h). Plates were sealed and stored in plastic or foil bags containing desiccant at 4 °C until use.

2.3. DNA amplification and sequencing

Genomic DNA in the D1/D2 region of the rDNA large subunit (LSU) was PCR amplified with the

Table 1
Probe and primer sequences

Name	Target	Sequence 5′ → 3′
D1R	LSU	ACC CGC TGA ATT TAA GCA TA
R-635	LSU	GGT CCG TGT TTC AAG ACG G
Amp	*Amphidinium carterae*	TTT GCC CAA GGA GGA TTA CG
Karenia	*Karenia brevis/mikimotoi*	CTA GGG ACA TGG TAA TTT GCT TC
Brevis	*Karenia brevis*	TGT TGT CTA AGG TGA TAG CTT GC

general eukaryotic primers DIR (Scholin et al., 1994) and R-635 (Fell et al., 2000; Kiesling et al., 2002b). Amplicons were approximately 700 bp in length and contained the species-specific probe sequences. DNA amplification was performed on a thermocycler (MJ Research PTC-100 Hotbonnet, Eppendorf Mastercycler, or ISC Bioexpress GenMate) using the following program: 94 °C hot start for 1 min, 29 cycles of anneal, denature, and extend (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min), a final 8 min extension step at 70 °C, and hold at 4 °C. PCR reactions consisted of 5 μl of 10X DyNAzyme II optimized buffer (1.5 mM $MgCl_2$, final), 0.2 mM dNTPs, 50 pmol of each primer (biotin-labeled), 1 μl genomic DNA, 1 U of DNA polymerase (DyNAzyme II; Finnzyme), and autoclaved Milli-Q water to bring the reaction volume to 50 μl. Amplicons used in the DNA hybridization assay were created with universal primers 5′-end labeled with biotin (Sigma-Genosys). Amplicons used to verify assay results by gel electrophoresis were created with a species-specific forward primer and no biotin label.

Cloning and sequencing of DNA extracted from seawater samples provided additional verification of results. DNA was amplified with D1R (forward) and R-635 (reverse) or D1R (forward) and Karenia (reverse) primer sets. Amplification used *Taq* polymerase to create T-A overhangs. Reactions consisted of 5 μl of 10X buffer, 2 mM $MgCl_2$, 0.1 mM dNTPs, 25 pmol of each primer, 2 μl DNA, 2.5 U of DNA polymerase (Promega), and autoclaved Milli-Q water to bring the reaction volume to 50 μl. Amplicons were cloned using the PCR2.1 vector (Invitrogen) according to manufacturer's instructions. At least twenty clones were selected from each sample. IR-labeled sequencing primers targeted the M13 region of the cloning vector. Sequencing was performed on an automated sequencer (Li-Cor Long Readir 4200) with the standard Li-Cor protocol with IRD800 and IRD700 conjugate primers and the Thermo Sequenase Primer Cycle Sequencing Kit with 7-deaza dGTP (Amersham). Sequences were read using e-Seq 1.2.1 and edited with AlignIRv1.2, and Editseq (DNAstar) software.

2.4. DNA hybridization

A DNA hybridization procedure (Cinek et al., 2000; Kiesling et al., 2002a) was modified to identify toxic phytoplankton. Hybridization Solution (0.43 M phosphate buffer, pH 6.8, 0.1% SDS, 5X Denhardt's), High Stringency Buffer (3 M tetramethylammonium chloride solution (TMA), 0.1% SDS), and Conjugate Dilutant (5X SSC, 0.1% SDS, 5X Denhardt's) were preheated to 37 °C. During method development, hybridization steps were carried out at either 55 of 60 °C. A hybridization temperature of 60 °C was needed for the Brevis probe to distinguish between *K. brevis* and *K. mikimotoi*, thus this is the temperature given in the protocol recommended below.

Aliquots of PCR reaction (10 μl) were placed in wells of a microtiter plate. Denaturation solution (5 μl; 0.25 N NaOH) was added to each well and the plate was incubated at room temperature for 2 min. Hybridization Solution (85 μl) was added, and the plate was sealed and incubated in a 60 °C water bath for 30 min. After incubation, the plate was washed 3 times at room temperature with 1X Wash Solution (0.5X SSC, 0.1% Tween 20, pH 7.0) using a microtiter plate strip washer (Bio-Teck ELP-40). High Stringency Buffer (200 μL) was added to each well, and the plate was sealed and incubated for 20 min in a 60 °C water bath. After incubation, the plate was washed 3 times at room temperature with the 1X Wash Solution. Streptavidin-POD conjugate (Roche Applied Science) was diluted 1:5000 in Conjugate Dilutant, yielding 0.5 U/μl. Diluted conjugate (100 μl) was added to each well. The plate was sealed and incubated in a 37 °C water bath for 10 min. After incubation, the plate was washed 3 times at room temperature with the 1X wash solution. Colorimetric HRP substrate (100 μL; TMB 1 Component HRP Microwell Substrate, BioFX Laboratories) was added to each well. Plates were sealed and incubated in a 37 °C water bath for 5–10 min. Stop Solution (100 μl; 0.5 M HCl) was added to each well. A positive reaction was indicated by development of yellow color. Color was detected visually and measured on a plate reader (absorbance at 450 nm) within 10 min after stopping the reaction.

2.5. Microplate assay quality control

Replicate wells were run for every reaction to verify consistent probe immobilization and to confirm results. Positive and negative controls were used to assess the success of PCR reactions and hybridization. DNA amplified from pure culture was utilized as the

positive control. Each microplate employed a dilution series of the positive control amplicon. Two negative controls were used – a blank (all chemistry except for PCR amplicon) and a PCR control (no DNA added to PCR reaction). As an additional control, DNA hybridization was periodically compared to gel electrophoresis of DNA amplified with a species-specific forward primer. The consistency of the DNA extraction procedure was evaluated by splitting a sample filter, performing separate DNA extractions, and comparing microplate results.

In this study, the desired sensitivity for the microplate assay was a positive result for *K. brevis* reported as "present" by a regional monitoring network and a negative result for "not present." A reaction was considered positive for sample:blank ratios $\geq$3. Using a ratio rather than raw data simplified interpretation of the results and normalized for variations between thermocyclers, individual PCR reactions, and microplate readers.

2.6. Culture and sample information

Cultures of *K. brevis* (Davis) Hansen et Moestrup CCMP 718 (Wilson clone) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). *Amphidinium carterae* culture was obtained courtesy of L. Brand (U. Miami/RSMAS). Cultures were maintained in the laboratory under the following conditions: L1 medium (Guillard and Hargraves, 1993) with no added silicate, pH 8.0, 19 °C, 14:10 h light:dark at 1220–2030 lux, and transferred to new medium between 22 and 30 days. Pellets of *K. brevis* strain NOAA1 and *K. mikimotoi* strain NOAA2 were obtained courtesy of S. Morton (NOAA, Charleston), and DNA from *K. brevis* strain Texas SP3 (Loret et al., 2002) was obtained courtesy of L. Campbell and T. Villareal (Texas A&M). DNA from cultures was extracted from 5 μm membrane filters (MEC, Millipore) using spin kits (Bio101 kits for soil, Qbiogene, Inc.) according to manufacturer's instructions. Cultures received as pellets were extracted using genomic DNA purification kits (Wizard, Promega) as per manufacturer instructions for gram-negative bacteria.

Environmental samples were obtained from the Rookery Bay NERR from February 2002 through October 2003. Samples were collected from the following sites as indicated in Fig. 1: Caxambas Pass, Fakahatchee Bay, Faka Union River, Goodland, Henderson Creek, Marco Pass, and the 951 Launch Ramp. Water was collected in sterile whirlpacks by Rookery Bay staff and volunteers and shipped overnight according to procedures outlined in Steidinger and Melton Penta (Steidinger and Melton Penta, 1999). Samples also were collected from New Pass and Siesta Beach in the Tampa Bay area during July 2002, the Florida Keys during a "black water" event in February 2002 (Hu et al., 2002), and a red tide event on the east coast of Florida from several sites between Melbourne and Cocoa Beach during November 2002.

In the laboratory, samples were vacuum filtered onto 5 μm, 47 mm mixed esters of cellulose membranes (Millipore). Samples from each site were processed with separate, autoclaved filter holders and ethanol-cleaned forceps. The 5 μm filters were stacked upon 0.2 μm filters so that bacterial DNA could be collected for other studies. Filters were stored at −20 °C until DNA extraction. The stacked filters were cut in two with scissors that were disinfected with bleach and ethanol. A kit (QBiogene, BIO 101) was used to extract each half separately, according to manufacturer's instructions.

3. Results

A DNA hybridization assay in microtiter plate format (Cinek et al., 2000; Kiesling et al., 2002a) was adapted to detect toxic dinoflagellates. The assay provided colorimetric identification of several toxic dinoflagellates, including *K. brevis*, the organism responsible for red tide in the Rookery Bay NERR. The hybridization technique was rapid, taking approximately 100 min to complete. DNA extraction was the rate-limiting step for the entire process (i.e., sample filtration to colorimetric result). Detection of multiple species was accomplished with one set of PCR primers and a single hybridization condition. This procedure conserved the amount of genomic DNA used and minimized steps in the process.

3.1. Cultures and enrichment samples

The unarmored, toxic dinoflagellates *K. brevis* and *A. carterae* (Steidinger, 1983; Jeong et al., 2001)

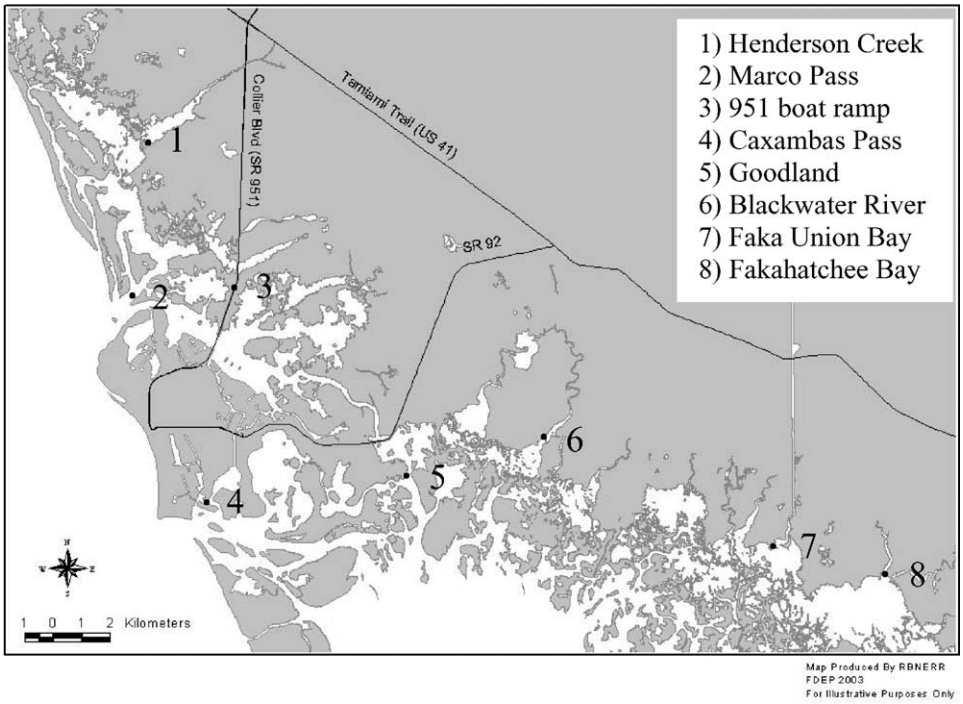

Fig. 1. Locations of sampling sites within the Rookery Bay National Estuarine Research Reserve.

were used as model organisms for this study. *K. brevis* was the causative agent of red tide in the study site, whereas *A. carterae* was easily grown in the laboratory and provided an ample supply of DNA. Simultaneous detection of *K. brevis* and *A. carterae* was achieved at 55 °C (Table 2) and 60 °C (not shown), and a colorimetric signal was achieved only when the DNA target matched the probe. The colorimetric signal correlated with the amount of amplicon added to a well, and replicate wells showed reproducible optical density (Fig. 2, Table 3).

Table 2
Detection of two dinoflagellate species using one DNA hybridization temperature (55 °C)

Immobilized probe: supplied target	Optical density, 450 nm avg ± S.D. (n=4)	
	Amp	Karenia
A. carterae	1.93 ± 0.10	0.09 ± 0.13
K. brevis	0.12 ± 0.013	3.75 ± 0.07

Table values are colorimetric signal strength quantified by a microplate reader.

Immobilized Karenia probe detected *K. brevis* DNA from cultures of *K. brevis* strain CCMP 718 and from enrichment cultures started from environmental samples (Table 3). *K. brevis* predominated in the original red tide samples but enrichment cultures quickly became overgrown with a variety of eukaryotic and prokaryotic organisms. Within days, *K. brevis* was no longer detected by microscopic examination, species-specific PCR, or the microplate assay. When amplicon from *K. brevis* was added, the colorimetric signal was equivalent to that of pure amplicon; therefore, the complex of amplicons did not inhibit hybridization. Optical density values for *A. carterae* were similar to those of the blank, showing that there was no significant hybridization to the Karenia probe. In addition to *K. brevis* strain CCMP 718, the assay was tested using *K. brevis* strains collected from Texas (*K. brevis* Texas SP3) and Florida (*K. brevis* NOAA-1). The assay successfully detected these strains, and replicate wells produced consistent optical density measurements (Table 4).

Immobilized Karenia probe also detected the toxic dinoflagellate *K. mikimotoi* (Table 4). *K. mikimotoi* is a

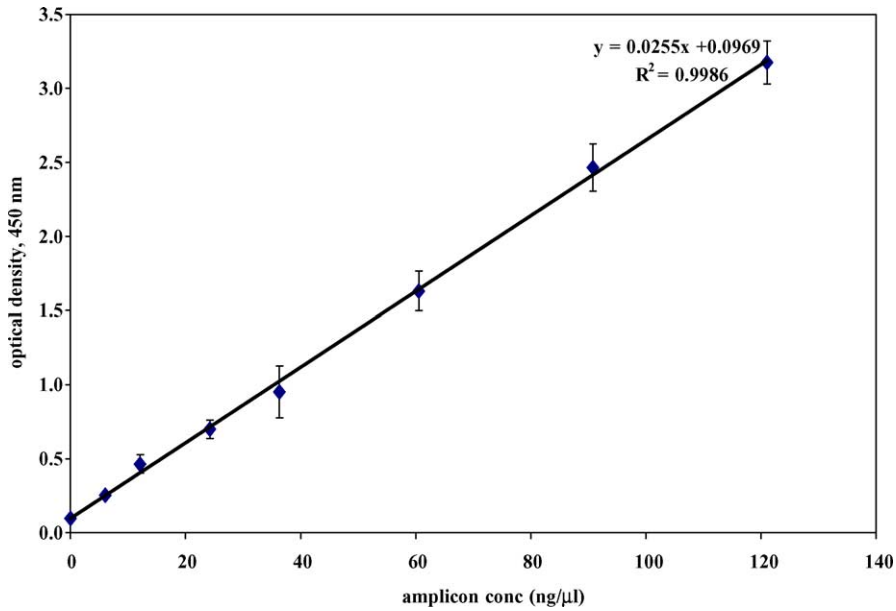

Fig. 2. Linear relationship between concentration of *K. brevis* amplicon and optical density measurements using immobilized ''Karenia'' probe.

close phylogenetic relative of *K. brevis*, differing by only 2 base pairs in the region of the probe. *K. mikimotoi* causes harmful algal blooms in various parts of the world and co-occurs in Florida red tide blooms (Steidinger et al., 1988). Simultaneous detection of *K. brevis* and *K. mikimotoi* can be achieved using immobilized Karenia probe. However, the ability to distinguish *K. brevis* from *K. mikimotoi* may be important for certain applications. The ''Brevis'' probe was designed for this purpose. When used as a PCR primer, Brevis amplified both species. When used in the microplate assay at a 60 °C hybridization temperature, Brevis detected various strains of *K. brevis* but not *K. mikimotoi*, thus providing the specificity needed to resolve the 2 bp difference between these species (Fig. 3; Table 5).

3.2. Analysis of environmental samples

Microplate analysis was performed on 110 separate samples collected during the course of the study. From these coastal samples, 60 were positive for *K. brevis* and 50 were negative. These determinations were based on the analysis of 196 PCR reactions, with each reaction evaluated using at least two replicate wells. A subset of Rookery Bay NERR samples was tested for

Table 3
Hybridization of *K. brevis* DNA in serial dilution and in a complex of amplicon

Amplicon	Optical density, 450 nm average ± S.D. (n = 2)	Value ratio sample/blank
222 ng *K. brevis* CCMP 718	3.67 ± 0.19	76
111 ng *K. brevis* CCMP 718	2.41 ± 0.04	50
22 ng *K. brevis* CCMP 718	0.76 ± 0.21	16
Enrichment culture, positive for *K. brevis*	0.45 ± 0.16	9
Enrichment culture, negative for *K. brevis*	0.11 ± 0.33	2
Negative culture + 111 ng *K. brevis* CCMP 718	2.67 ± 0.44	55
A. carterae	0.056 ± 0.001	1
PCR blank	0.048 ± 0.003	1

The assay utilized the Karenia probe at 60 °C.

Table 4
DNA hybridization utilizing the Karenia probe at 60 °C

Sample	Optical density, 450 nm average ± S.D.	Value ratio sample/blank
K. brevis *CCMP 718*	3.54 ± 0.06	17
K. brevis Texas SP3	3.71 ± 0.03	18
K. brevis NOAA-1	2.88 ± 0.41	14
K. mikimotoi NOAA-2	1.58 ± 0.32	8
PCR blank	0.20 ± 0.04	1
Water blank	0.16 ± 0.04	0.8

Table 5
DNA hybridization utilizing the Brevis probe at 60 °C to distinguish between *K. brevis* and *K. mikimotoi*

Sample	Optical density, 450 nm average ± S.D. (n = 4)	Value ratio sample/blank
K. brevis CCMP 718	2.88 ± 0.02	38
K. brevis Texas SP3	3.02 ± 0.15	26
K. mikimotoi NOAA-2	0.07 ± 0.01	0.9
PCR blank	0.08 ± 0.003	1

A. carterae. This organism was not identified via the microplate assay or by species-specific PCR, indicating that *A. carterae* was not present in those samples.

Significant effort was made to evaluate the reproducibility of the microplate assay using environmental samples. Fifty-two of the samples were analyzed repeatedly, yielding 138 PCR reactions. DNA extraction, PCR, and hybridization conditions were evaluated. The microplate assay produced consistent results. Only 4 samples had a discrepancy that needed further evaluation. Differences were attributed to increased stringency arising from using HPLC purified probe or a 60 °C hybridization temperature. Separate DNA extractions from the same sample produced matching microplate results.

3.3. Comparison to other molecular methods

Microplate assay identification was consistent with species-specific PCR as evaluated by gel electrophoresis. Microplate assay identification also was consistent with DNA cloning and sequencing (Table 6). In addition to confirming the presence of *K. brevis*, sequencing illustrated the complexity of the amplicons obtained from the universal primers. Amplification of environmental samples using D1R and R-635 as PCR primers yielded sequences from a large variety of organisms in addition to members of the Dinophyceae. Cloning of amplicons generated sequences from diatoms, aquatic fungi, and plants. Sequences from representatives of the genera *Carteria*, *Chrysolepidomonas*, *Guillardia*, *Hypochytrium*, *Paeonia*, *Rhodotorula*, *Skeletonema*, and *Uronema* were observed. These results indicate the complex milieu in which hybridization to the probe successfully occurred. Using Karenia and D1R as a PCR primer pair prior to cloning and sequencing allowed for more efficient screening of environmental samples for sequences related to *K. brevis*. From both sets of primers, readable sequences similar to *K. brevis* were obtained for 93 clones. An exact match to *K. brevis* GenBank accession no. U92248 was observed for 50 sequences, with 40 differing by 1–4 bp. The remaining 3 sequences had differences of 8, 69 and 97 bp. *K. mikimotoi* sequence was not observed. Sequences obtained from a culture of *K. brevis* CCMP 718 also showed variation. From 11 clones, 2 matched U92248 while the others differed by 1–5 bp. In contrast, direct sequencing (no cloning) of a *K. brevis* CCMP 718 culture yielded a sequence matching U92248.

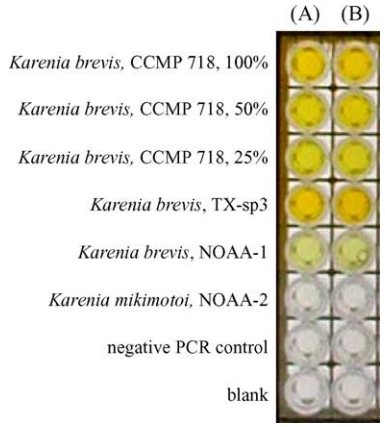

Fig. 3. Microplate assay using immobilized "Brevis" probe to distinguish between the closely related species *K. brevis* and *K. mikimotoi*. Two replicate strips of wells are shown.

3.4. Comparison to microscopic observation

Assay sensitivity allowed detection of *K. brevis* when it was "present" in the water (<1000 cells/L), as defined by the Florida Marine Research Institute (FMRI) (http://floridamarine.org). Microscopic enu-

Table 6
Detection of *K. brevis* in environmental samples by microplate assay and comparison to other molecular methods of detection and microscopic enumeration

Sample[a]	Microplate[b]	Species-specific PCR	Sequencing	Microscopic counts[c]
951 ramp	+	+	+	Low
Big Marco Pass	+	+	+	Low
Caxambas Pass	–	–	NA	Not present
Fakahatchee Bay	–	–	NA	Not present
Goodland	+	+[e]	+	Not present
Rookery Bay dock[d]	+	+	+	NA
K. brevis culture	+	+	+	
Negative controls	–	–	NA	

NA = data not available.
[a] Samples collected 2/23/02, 2/27/02 or 3/13/02.
[b] Probe was not HPLC purified.
[c] Data provided by FMRI. Definition at time of collection: low = >10,000 to <100,000 cells/L.
[d] Triplicate filters.
[e] Faint band.

meration was available from FMRI for 53 of the 110 environmental samples collected in this study. Microplate assay results were consistent with microscopic observation. Using probes that had been HPLC purified, the match to microscopic observation was 98%, with a complete match for reports of ''not present'' (19/19). Probes purified with standard desalting provided increased sensitivity over microscopic observation in some instances (Table 6; Goodland sample). The microplate assay returned a negative result for one sample in which *K. brevis* was identified via microscopy; however, the optical density measurement was borderline positive (2.9).

4. Discussion

The microplate assay allowed rapid identification of several species of toxic dinoflagellates without the microscopic expertise normally required. The technique did not require expensive instrumentation and had several advantages over species-specific PCR or cloning and sequencing of total extracted DNA. The assay gave immediate visual results, was more specific and convenient than a series of species-specific PCR reactions, and was faster, easier and less expensive than cloning. The technique conserved the amount of genomic DNA utilized, which can be critical to certain applications (Kiesling et al., 2002b).

The microplate assay was consistent with the sensitivity of microscopic observation. In this study, the objective was to calibrate the sensitivity of the assay so that a negative result was consistent with a report of ''not present'' provided by a regional monitoring program. When HPLC purified probes were used, this objective was met in every case tested. Use of HPLC purified probes is known to increase the stringency of the assay (Cinek et al., 2000) and is the protocol recommended here.

The microplate assay was tested with DNA probes that were synthesized with standard desalting. These probes appeared to provide greater sensitivity than microscopic observation for 3 of the 11 environmental samples tested. Interestingly, one of these samples was tested by species-specific PCR and sequencing, and the presence of *K. brevis* DNA was verified (Table 6; Goodland). Guillou et al. (2002) found PCR identification to be more sensitive than microscopy; however, a species was sometimes identified microscopically if a larger volume of water was examined. For this study, 1 ml or less of sample was routinely used for microscopic inspection (Steidinger and Melton Penta, 1999), whereas the microplate assay filtered 50–500 ml of water, depending on sample turbidity.

Sequences of rDNA from the D1/D2 region of the LSU amplified from environmental samples matched *K. brevis* sequences deposited in GenBank (accession nos. U92248, AY355455, AY355456, AY355457,

AY355458, AY355459, AF200677). In addition, many sequences (40/99 clones) differed by 1–4 bp from U92248, but did not match GenBank entries, including recently described species that may co-occur with *K. brevis* (Haywood et al., 2004). The frequency of this variation is higher than that expected from *Taq* polymerase errors (Cline et al., 1996). This variation could reflect the presence of different species or strains that have yet to be described. Variation may also reflect intraspecies variability. Direct sequencing of *K. brevis* CCMP 718 yielded a match to U92248. Cloning, in contrast, yielded 9 out of 11 clones with sequence variations of 1–5 bp; this frequency was again higher than that expected from PCR error. *In silico* analysis of the *K. brevis* probe against other described *Karenia* species (Haywood et al., 2004), indicates that it should be specific; it will be interesting to test actual cross-reactivity once these species become more widely available.

Studies have observed a high degree of conservation for *Karenia* rDNA isolated from various parts of the world (Hansen et al., 2000; Loret et al., 2002; Guillou et al., 2002; Mikulski et al., in press). In contrast, some *Alexandrium* species display large genetic heterogeneity (Scholin et al., 1994; Scholin and Anderson, 1996). The conservative nature of *Karenia* DNA suggests that the molecular probes used in this study should be applicable to *Karenia brevis/mikimotoi* strains from various geographic regions.

The microplate assay can be adapted to a variety of applications. The utility lies in the ability to simultaneously screen for a variety of targets and to distinguish between close molecular phylogenetic relatives. In addition to the dinoflagellate identification demonstrated here, the assay has been used to identify closely related copepods (Kiesling et al., 2002b) and marine yeasts (Kiesling et al., 2002a) and to detect prokaryotic organisms (K.D. Goodwin, unpublished data). Potential applications include monitoring of aquaculture or ballast water to quickly alert the presence of objectionable organisms or invasive species. The assay could be a tool to map the distribution of organisms, including cryptic species, and to utilize microbes to monitor aquatic ecosystems (Paerl et al., 2003).

If the U.S. follows the trend set by other countries, the burden of HAB monitoring will continue to grow and new methods will be needed to address the demand. Identification of species with molecular methods can make sample testing accessible to more laboratories, whereas microscopic expertise is not widely available and is seldom included in educational curricula, molecular techniques are now introduced in many high schools. The microplate assay can be used to process large sample sets containing complex assemblages of organisms. Sample throughput could be increased and the burden of microscopic examination reduced by using the assay to quickly identify the smaller subset of samples requiring enumeration. Increased throughput could allow more widespread screening of waters, helping protect health while avoiding blanket closures of coastal waters (Tyrrell et al., 1997).

5. Conclusions

A DNA hybridization assay in microplate format was used to detect *K. brevis* in waters collected from the Rookery Bay NERR. The assay can detect multiple dinoflagellate species simultaneously and distinguish between closely related species. It is easy to add and adapt probes to the assay as additional *Karenia* cultures and phylogenetic sequences become available. Overall, the microplate assay offers the sensitivity and specificity of molecular analysis in a convenient, adaptable, and relatively inexpensive format.

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References

Adachi, M., Sako, Y., Ishida, Y., 1994. Restriction fragment length polymorphism of ribosomal DNA internal transcribed spacer

and 5.8S regions in Japanese *Alexandrium* species (Dinophyceae). J. Phycol. 30, 857–863.

Anderson, D.M., 1994. Red Tides. Sci. Am. 271, 62–68.

Anderson, D.M., Hoagland, P., Kaoru, Y., White, A.W., 2000. Estimated annual economic impacts from harmful algal blooms (HABs) in the United States. Technical Report WHOI-2000-11, Woods Hole Oceanographic Institute, Woods Hole, MA.

Asai, S., Krzanowski, J.J., Lockey, R.F., Anderson, W.H., Martin, D.F., Polson, J.B., Bukantz, S.C., Szentivanyi, A., 1984. The site of action of *Ptychodiscus brevis* toxin within the parasympathetic axonal sodium channel h gate in airway smooth muscle. J. Allerg. Clin. Immunol. 73, 824–828.

Baden, D.G., Fleming, L.E., Bean, J.A., 1995. Marine toxins. In: de Wolff, F.A. (Ed.), Handbook of Clinical Neurology. Elsevier Science, Amsterdam, pp. 141–175.

Bossart, G.D., Baden, D.G., Ewing, R.Y., Roberts, B., Wright, S.D., 1998. Brevetoxicosis in manatees (*Trichechus manatus latirostris*) from the 1996 epizootic: gross, histologic, and immunohistochemical features. Tox. Path. 26, 276–282.

Chang, F.H., Chiswell, S.M., Uddstrom, M.J., 2001. Occurrence and distribution of *Karenia brevisulcata* (Dinophyceae) during the 1998 summer toxic outbreaks on the central east coast of New Zealand. Phycologia 40, 215–222.

Cinek, O., Wilkinson, E., Paltiel, L., Saugstad, O.D., Magnus, P., RØnningen, K.S., 2000. Screening for the IDDM high-risk genotype. A rapid microtitre plate method using serum as source of DNA. Tiss. Antigens 56, 344–349.

Cline, J., Braman, J.C., Hogrefe, H.H., 1996. PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases. Nucl. Acids Res. 24, 3546–3551.

Daugbjerg, N., Hansen, G., Larsen, J., Moestrup, O., 2000. Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. J. Phycol. 39, 302–317.

Faust, M.A., Morton, S.L., Quod, J.P., 1996. Further SEM study of marine dinoflagellates: the genus *Ostreopsis* (Dinophyceae). J. Phycol. 32, 1053–1065.

Fell, J.W., Boekhout, T., Fonseca, A., Scorzetti, G., Statzell-Tallman, A., 2000. Biodiversity and systematics of basidiomycetous yeasts as determined by large subunit rDNA D1/D2 domain sequence analysis. Int. J. Syst. Evol. Microbiol. 50, 1351–1371.

Forrester, D.J., Gaskin, J.M., White, F.H., Thompson, N.P., Quick Jr., J.A., Henderson, G.E., Woodard, J.C., Robertson, W.D., 1977. An epizootic of waterfowl associated with a red tide episode in Florida. J. Wildl. Dis. 13, 160–167.

Godhe, A., Otta, S.K., Rehnstam-Holm, A.-S., Karunasagar, I., Karunasagar, I., 2001. Polymerase chain reaction in detection of *Gymnodinium mikimotoi* and *Alexandrium minutum* in field samples from southwest India. Mar. Biotechnol. 3, 152–162.

Gray, M., Wawrik, B., Paul, J., Casper, E., 2003. Molecular detection and quantitation of the red tide dinoflagellate *Karenia brevis* in the marine environment. Appl. Environ. Microbiol. 69, 5726–5730.

Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32, 234–236.

Guillou, L., Nézan, E., Cueff, V., Erard-Le Denn, E., Cambon-Bonavita, M.-A., Gentien, P., Barbier, G., 2002. Genetic diversity and molecular detection of three toxic dinoflagellate genera (*Alexandrium*, *Dinophysis*, and *Karenia*) from French coasts. Protist 153, 223–238.

Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32, 79–99.

Hansen, G., Daugbjerg, N., Henriksen, P., 2000. Comparative study of *Gymnodinium mikimotoi* and *Gymnodinium aureolum*, Comb. Nov. (=*Gyrodinium aureolum*) based on morphology, pigment composition, and molecular data. J. Phycol. 36, 394–410.

Haywood, A.J., Steidinger, K.A., Truby, E.W., Bergquist, P.R., Bergquist, P.L., Adamson, J., Mackenzie, L., 2004. Comparative morphology and molecular phylogenetic analysis of three new species of the genus *Karenia* (Dinophyceae) from New Zealand. J. Phycol. 40, 165–179.

Hopkins, R.S., Herber, S., Hammond, R., 1997. Water-related disease in Florida: Continuing threats require vigilance. J. Florida M. A. 84, 441–445.

Horstman, D.A., McGibbon, S., Pitcher, G.C., Calder, D., Hutchings, L., Williams, P., 1991. Red tides in False Bay, 195901989, with particular reference to recent blooms of *Gymnodinium* sp.. Trans. Roy. Soc. S. Afr. 47, 611–628.

Hu, C., et al., 2002. Satellite images track "black water" event of Florida coast. Eos 83, 281, 285.

Jeong, H.J., Kang, H., Shim, J.H., Park, J.K., Kim, J.S., Song, J.Y., Choi, H.-J., 2001. Interactions among the toxic dinoflagellate *Amphidinium carterae*, the heterotrophic dinoflagellate *Oxyrrhis marina*, and the calanoid copepods *Acartia* spp.. Mar. Ecol. Prog. Ser. 218, 77–86.

Kiesling, T., Diaz, M., Statzell-Tallman, A., Fell, J.W., 2002a. Field identification of marine yeasts using DNA hybridization macroarrays. In: Hyde, K. (Ed.), Marine Mycology: The Organisms, Ecology and Applied Aspects. Fungal Diversity Press, Hong Kong, pp. 69–80.

Kiesling, T.L., Wilkinson, E., Rabalais, J., Ortner, P.B., McCabe, M.M., Fell, J.W., 2002b. Rapid identification of adult and macular stages of copepods using DNA hybridization methodology. Mar. Biotechnol. 4, 30–39.

Kirkpatrick, B., Fleming, L.E., Squicciarini, D., Backer, L.C., Clark, R., Abraham, W., Benson, J., Cheng, Y.S., Johnson, D., Pierce, R., 2004. Literature review of Florida red tide: implications for human health effects. Harmful Algae 3, 99–115.

Landsberg, J.H., 2002. The effects of harmful algal blooms on aquatic organisms. Rev. Fish. Sci. 10, 113–390.

Loret, P., Tengs, T., Villareal, T.A., Singler, H., Richardson, B., McGuire, P., Morton, S., Busman, M., Campbell, L., 2002. No difference found in ribosomal DNA sequences from physiologically diverse clones of *Karenia brevis* (Dinophyceae) from the Gulf of Mexico. J. Plank. Res. 24, 735–739.

Magaña, H., Contreras, C., Villareal, T.A., 2003. A historical assessment of *Karenia brevis* in the western Gulf of Mexico. Harmful Algae 2, 163–171.

Matsuyama, Y., Uchida, T., Honjo, T., 1999. Effects of harmful dinoflagellates, *Gymnodinium mikimotoi* and *Heterocapsa circularisquama*, red-tide on filtering rate of bivalve molluscs. Fish. Sci. 65, 248–253.

Mikulski, C.M., Morton, S.L., Doucette, G.J., in press. Development and application of LSU rRNA probes for *Karenia brevis* in the Gulf of Mexico, USA. Harmful Algae, in press.

Paerl, H.W., Dyble, J., Moisander, P.H., Noble, R.T., Piehler, M.F., Pinckney, J.L., Steppe, T.F., Twomey, L., Valdes, L.M., 2003. Microbial indicators of aquatic ecosystem change: current applications to eutrophication studies. FEMS Microbiol. Ecol. 46, 233–246.

Rhodes, L., Scholin, C., Garthwaite, I., 1998. *Pseudo-nitzschia* in New Zealand and the role of DNA probes and immunoassays in refining marine biotoxin monitoring programmes. Nat. Toxins 6, 105–111.

Scholin, C., Anderson, D.M., 1996. LSU rDNA-based RFLP assays for discriminating species and strains of *Alexandrium* (Dinophyceae). J. Phycol. 32, 1022–1035.

Scholin, C.A., Herzog, M., Sogin, M., Anderson, D.M., 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. J. Phycol. 30, 999–1011.

Shirley, M., McGee, V., Jones, T., Anderson, B., Schmid, J., submitted. The influence of altered freshwater inflow on the relative abundance of stenohaline and euryhaline oyster-reef crab populations, submitted.

Smayda, T.J., 1995. Environmental monitoring. In: Hallegraeff, G.M., Andersen, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO, Paris, pp. 283–293.

Steidinger, K.A., 1983. A re-evaluation of toxic dinoflagellate biology and ecology. Prog. Phycol. Res. 2, 147–278.

Steidinger, K.A., Melton Penta, H.L., 1999. Harmful microalgae and associated public health risks in the Gulf of Mexico. US EPA, Gulf of Mexico Program, EPA Grants MX004729-95-0.

Steidinger, K.A., Vargo, G.A., Tester, P.A., Tomas, C.R., 1988. Bloom dynamics and physiology of *Gymnodinium breve* with emphasis on the Gulf of Mexico. In: Andersen, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer-Verlag, Berlin, pp. 133–153.

Summerson, H.C., Peterson, C.H., 1990. Recruitment failure of the bay scallop, *Argopecten irradians concentricus*, during the first red tide, *Ptychodiscus brevis*, outbreak recorded in North Carolina. Estuaries 13, 322–331.

Tester, P.A., Steidinger, K.A., 1997. *Gymnodinium breve* red tide blooms: initiation, transport, and consequences of surface circulation. Limnol. Oceanogr. 42, 1039.

Tester, P.A., Stumpf, R.P., Vukovich, F.M., Fowler, P.K., Turner, J.T., 1991. An expatriate red tide bloom: transport, distribution, and persistence. Limnol. Oceanogr. 36, 1053–1061.

Tyrrell, J., Bergquist, P., Saul, D., MacKenzie, L., Bergquist, P., 1997. Oligonucleotide probe technology as applied to the study of harmful algal blooms. NZJ Mar. Fresh. Res. 31, 551–560.

Watanabe, T., Lockey, R.F., Krzanowski Jr., J.J., 1988. Airway smooth muscle contraction induced by *Ptychodiscus brevis* (red tide) toxin as related to a trigger mechanism of bronchial asthma. Immunol. Allerg. Pract. 10, 185–192.