

MOLECULAR DETECTION OF THE BREVETOXIN-PRODUCING DINOFLAGELLATE *KARENIA BREVIS* AND CLOSELY RELATED SPECIES USING rRNA-TARGETED PROBES AND A SEMIAUTOMATED SANDWICH HYBRIDIZATION ASSAY¹

Allison J. Haywood²

Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, 100 Eighth Ave. S.E., St. Petersburg, Florida 33701-5020, USA

Florida Institute of Oceanography, 830 1st St. South, St. Petersburg, Florida 33701, USA

Christopher A. Scholin, Roman Marin III

Monterey Bay Aquarium Research Institute, 7700 Sandholdt Rd., Moss Landing, California 95039-0628, USA

Karen A. Steidinger

Florida Institute of Oceanography, 830 1st St. South, St. Petersburg, Florida 33701, USA

Cynthia Heil

Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, 100 Eighth Ave. S.E., St. Petersburg, Florida 33701-5020, USA

and Jason Ray

Saigene Corporation, 3110 Judson St., Gig Harbor, Washington 98335, USA

Brevetoxins produced by the marine dinoflagellate *Karenia brevis* (C. C. Davis) G. Hansen et Moestrup cause neurotoxic shellfish poisoning (NSP) in human consumers and also endanger a variety of coastal wildlife. In the eastern Gulf of Mexico the presence and abundance of this species have traditionally been monitored using light microscopy (LM) observations of whole water samples. Various molecular probe methods now enable detection of multiple species from a single sample, allowing rapid sample analysis. We describe the development of sandwich hybridization assays (SHAs) for *Karenia brevis*, *K. selliformis* Haywood, Steid. et L. MacK., *K. mikimotoi* (Miyake et Kominami ex M. Oda) G. Hansen et Moestrup, *K. papilionacea* Haywood et Steid., the Karlotoxin-producer *Karlodinium veneficum* (D. Ballant.) J. Larsen (= *K. micrum*), and *Gymnodinium aureolum* (Hulburt) G. Hansen, comb. nov. The assays require no nucleic acid purification and use LSU rRNA-targeted probes and a semiautomated, 96-well plate format. Probes tested in matrix format were specific relative to rRNAs of all nontarget species used. The response of the SHA for a constant number of *K. brevis* cells per unit volume of homogenate depended on the growth status of a culture, decreasing for senescent cells relative to actively growing cells. The results of preliminary field tests of the *K. brevis* SHA indicated that cells collected from natural populations tended

to return a lower signal than those harvested from laboratory cultures, but these results are nonetheless very encouraging. These preliminary field studies show that robust standards are required for cell identification and enumeration, with which new methods can be compared.

Key index words: brevetoxin; *Karenia brevis*; red tide; rRNA probe; sandwich hybridization

Abbreviations: NSP, neurotoxic shellfish poisoning; PbTx, polyether brevetoxin; SHA, sandwich hybridization assay

Karenia brevis is a marine dinoflagellate that is well known to residents along the west coast of Florida, USA, for producing multiple, almost annual bloom events that are locally known as red tides (Steidinger et al. 1998). Blooms of *K. brevis* cause significant ecological and economic damage to coastal regions of Florida and other coastal states bordering the Gulf of Mexico (Anderson et al. 2000). Cyclic polyether compounds, called brevetoxins, produced by *K. brevis* can induce respiratory distress and eye and nasal membrane irritation in humans, principally via aerosols delivered by sea spray (Pierce 1986, Pierce et al. 1990). Brevetoxin accumulation in shellfish can also induce neurotoxic shellfish poisoning (NSP) in human consumers (after Baden and Mende 1982, Baden et al. 1982, Hallegraeff 2003). In addition to these human health impacts, polyether brevetoxins

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²Author for correspondence: e-mail ajhaywood@xnet.co.nz.

(PbTx) are associated with extensive fish, dolphin, manatee, and sea turtle mortalities (Landsberg and Steidinger 1998, Landsberg 2002, Flewelling et al. 2005). Mouse bioassays (Baden et al. 1982, Baden and Tomas 1989) and antibody-based techniques (Naar et al. 2002) are used to monitor PbTx concentrations in shellfish for public and environmental health purposes, respectively. In addition to the detection of PbTx, many monitoring and resource consent management activities require the rapid identification and enumeration of toxigenic *K. brevis* cells. Here we report on the development of new methods for detecting *K. brevis* and closely related species based on the use of species-specific oligonucleotide probes targeting LSU rRNA sequences. Historically, microscopic observations of live and preserved samples has been required to monitor species known to produce PbTx. The lower limit of detection (LLD) for light microscopy (LM) depends on the specific method used, but for routine phytoplankton monitoring in Florida, LLD is considered to be a background cell concentration of $\sim 10^3$ cells \cdot L $^{-1}$ (Geesey and Tester 1993). However, the recording frequency of low cell concentrations is probably underestimated by LM, and considerable taxonomic expertise is required to distinguish between closely related species (Steidinger et al. 1989, 1996, Haywood et al. 2004). The detection of *K. brevis* to such background cell concentrations allows an early warning of a possible toxic event, before the 5×10^3 *K. brevis* cells \cdot L $^{-1}$ trigger level is reached, at which commercial shellfish beds are closed. The reason this limit was chosen is that the current FDA-approved mouse bioassay used to detect PbTx takes 24 h, about the same amount of time required for shellfish to accumulate enough toxin to pose a risk of NSP when exposed to trigger-level cell densities (Naar et al. 2002). Large blooms of *K. brevis* are considered to initiate 18–74 km offshore (Steidinger and Haddad 1981, Tester and Steidinger 1997) and are often readily detected visually as rusty-colored water when cell concentrations exceed 5–10 million cells \cdot L $^{-1}$. Slow growth of these offshore populations followed by physical concentration at fronts, for example, can lead to large pulses of up to 80 million cells \cdot L $^{-1}$ moving onshore, providing a second means for PbTx accumulation above regulatory limits to occur within a time frame of 24 h or less. Unfortunately, on a spatial scale, small and even medium-scale blooms (of generally $\sim 1 \times 10^5$ – 1×10^6 cells \cdot L $^{-1}$) are less readily detectable visually and may not lead to any noticeable water discoloration. Blooms may also be patchy, leading to high and background cell concentrations being present in close proximity. Consequently, methods for detecting causative species must be able to cover this wide and dynamic range of cell concentrations.

A variety of cell-detection methods exist beyond those that employ traditional LM, varying in scale from optical methods and remote sensing (Millie

et al. 1997, Stumpf et al. 2003, Hu et al. 2005) to single-cell analyses (Sebastian and O’Ryan 2001). All have limitations; either they cannot provide species-level detection at the required 1×10^3 – 5×10^3 cells \cdot L $^{-1}$ LLD, the time and materials required for sample processing are an obstacle, or they may not be representative of the species, as in single-cell analyses. Satellite sensors, for example, are very useful for detecting large *K. brevis* blooms but currently have resolution levels (10^5 cells \cdot L $^{-1}$ from Tester et al. 1998, and 5×10^4 – 10^5 cells \cdot L $^{-1}$ and 0.5–1 mg \cdot m $^{-3}$ chl according to Hu et al. 2005) at least an order of magnitude above the trigger level for shellfish bed closures and at cell concentrations that could lead to fish kills (Steidinger et al. 1998). Cloud cover can also obscure satellite imagery, and the spectral resolution does not allow discrimination of dinoflagellates to the species level, leading to the use of chl concentration anomalies to identify possible HABs (Stumpf et al. 2003, Hu et al. 2005). At the other extreme, electron microscopy (EM) can be used to identify single cells and assist in the taxonomic identifications of even small gymnodinoids (Steidinger et al. 1996), but it is impractical for routine sample analyses. Similar problems are associated with monitoring many other harmful algae (e.g., Babin et al. 2005). In response to such challenges, many investigators have explored the use of molecular probes as tools to enhance our capability for rapidly detecting low concentrations of microalgae and to complement remote sensing and traditional microscopy cell-detection methods (e.g., Scholin et al. 2003). The application of rRNA-targeted probes in whole-cell and cell-free formats figures prominently in this regard (e.g., Bowers et al. 2000, Penna and Magnani 2000, Bolch 2001, Litaker and Tester 2002, Gray et al. 2003, Casper et al. 2004, Galluzzi et al. 2004, Goodwin et al. 2004, Groben et al. 2004, Ellison and Burton 2005, Lundholm et al. 2006, Metfies et al. 2006, Godhe et al. 2007, Scholin et al. in press).

In the Gulf of Mexico and internationally, the need for species-specific cell-detection methods has increased due to the cosmopolitan nature of some species of *Karenia* and other dinoflagellates known or considered to be toxic or harmful to humans and wildlife. *Karenia selliformis*, for example, produces gymnodimine (Seki et al. 1996) and was isolated (1994) and described from New Zealand waters (Haywood et al. 2004), but it is also found in Kuwait where it was associated with a large fish kill (Glibert et al. 2001, 2002, Heil et al. 2001). *Karenia selliformis* also appears to be in Chile and Tunisia, as isolates from these regions attributed to the genus *Gymnodinium* differ by only 2–4 bp in the D1–D2 region of the LSU rRNA gene (Guillou et al. 2002) relative to that of *K. selliformis* from New Zealand. Aside from *K. brevis*, at least three other species of *Karenia*—*K. mikimotoi*, *K. selliformis*, and *K. papilionacea*—were recorded in the Gulf of Mexico in 2002 using fluorescent in situ hybridization (FISH) assays

(A. J. Haywood, C. A. Scholin, and K. Petrik, unpublished data). These species may possibly have been misidentified as *K. brevis* in the past due to morphological similarities between the species (detailed in Haywood et al. 2004), although *K. mikimotoi* was previously recorded (K. Steidinger, unpublished data). Toxic and nontoxic species may be present concurrently yet look superficially very similar (particularly *K. brevis* and *K. papilionacea*, and *K. mikimotoi* and *K. selliformis*), leading to difficulties ascribing overall toxicity to known concentrations of individual species. *Karenia mikimotoi*, for example, is the only other species of the genus currently thought to contain appreciable concentrations of brevetoxin. *Karenia mikimotoi* is thought to produce ~60% of the PbTx of *K. brevis* based on receptor binding data and bronchoconstrictor activity (D. J. Baden, pers. comm.), although this figure is yet to be determined quantitatively by analytical methods. In New Zealand, *Gymnodinium aureolum* co-occurs with *K. mikimotoi* and is superficially similar in size and shape (A. J. Haywood, unpublished data). An isolate of *Gym. aureolum* is included in this study because any misidentifications could lead to errant warnings of potential NSP outbreaks.

Molecular methods developed for identifying *K. brevis* and related organisms ideally should allow for simultaneous detection of multiple species in a single sample and require minimal sample handling and no specialized laboratory equipment, apart from the semiautomated processor. In this regard, the sandwich hybridization assay (SHA) as described by Scholin et al. (1999) offers some advantages. In this method, the identification of target species is based on the detection of rRNA sequences in a

crude homogenate, and no purification of nucleic acids is required. The assay can be used in ship-board surveys to provide estimates of target cell concentrations in near real time (Anderson et al. 2005, C. A. Scholin, R. Marin III, and B. Keafer, unpublished data). SHAs for detecting species of *Alexandrium*, *Pseudo-nitzschia*, *Heterosigma*, *Fibrocapsa*, and *Chattonella* and a variety of invertebrates have all undergone both laboratory and field testing, so the benefits and limitations of the method are understood (Scholin et al. 1997, 1999, 2000, Tyrrell et al. 2001, Anderson et al. 2005, Goffredi et al. 2005, O'Halloran et al. 2006). Lastly, the SHA is highly amenable to automation and can be applied in a probe array format in both the laboratory and in situ, offering opportunities for detecting a wide variety of species simultaneously (Babin et al. 2005, Goffredi et al. 2005, Greenfield et al. 2006, Metfies et al. 2006). For these reasons, we chose to explore the development and testing of SHAs for the detection of a variety of species of *Karenia* as well as some closely related dinoflagellates. We describe development of SHAs for cultured representatives of *K. brevis*, *K. papilionacea*, *K. selliformis*, *K. mikimotoi*, *Gym. aureolum*, and one species of *Karlodinium*, *K. veneficum*. An example of using the SHA for *K. brevis* to analyze natural samples is also given. Concurrent work to develop FISH assays targeting whole cells will be reported separately.

MATERIALS AND METHODS

Cultures and cell enumeration. Cultures used in this study (Table 1) were maintained in modified 50% general-purpose (GP) medium as previously described (Haywood et al. 2004). Cell densities were determined using an S52 Sedgwick-Rafter

TABLE 1. Strains used for assay development and cross-reactivity testing.

| Species name, order (G, Gymnodiniales; P, Peridinales; PR, Prorocentrales) | Strain name | Isolated from | GenBank accession no. ^a |
|--|---------------------------|---|------------------------------------|
| <i>Karenia brevis</i> (G) | CCFWC 268 Wilson 1953 | John's Pass, Florida, USA | U92248 |
| <i>Karenia brevis</i> (G) | CCFWC 256 Charlotte A3 | Charlotte Harbor, Florida, USA | N/A |
| <i>Karenia mikimotoi</i> (G) | CAWD63 | Waimangu, Point/North Island, New Zealand | U92249 |
| <i>Karenia papilionacea</i> (G) | CAWD91 | Hawke's Bay, North Island, New Zealand | U92252 |
| <i>Karenia bidigitata</i> (G) | CAWD92 | Hawke's Bay, North Island, New Zealand | U92251 |
| <i>Karenia selliformis</i> (G) | CAWD79 | Foveaux Strait, South Island, New Zealand | U92250 |
| <i>Karenia brevisulcata</i> (G) | CAWD82 | Wellington Harbor, North Island, New Zealand | AY243032 |
| <i>Karlodinium veneficum</i> (G) | CAWD83 | Marlborough Sounds, South Island, New Zealand | U92257 |
| <i>Gymnodinium aureolum</i> (G) | CAWD87 | Coromandel, North Island, New Zealand | U92255 |
| <i>Gymnodinium simplex</i> (G) | CAWD86 | Tauranga, North Island, New Zealand | N/A |
| <i>Gyrodinium instriatum</i> (G) | CCMP431 | Lisbon, Portugal | N/A |
| <i>Amphidinium carterae</i> (G) | CCMP124 | Puerto Peñasco, Sonora, Mexico | AY460584 |
| <i>Coolia monotis</i> (P) | CCMP1345 | Knight Key, Florida, USA | AJ491339 ^b |
| <i>Heterocapsa pygmaea</i> (P) | CCMP1322 | Galveston Channel, Texas, USA | AB08409 ^b |
| <i>Prorocentrum micans</i> (PR) | CCMP1589 | Narragansett Bay, Rhode Island, USA | N/A |
| <i>Prorocentrum rhathymum</i> (=mexicanum; PR) | CCMP687 | Knight Key, Florida, USA | N/A |
| <i>Scrippsiella trochoidea</i> (P) | CCMP1599 | Perch Pond, Falmouth, Massachusetts, USA | N/A |
| <i>Takayama tasmanica</i> (G) | W040831-004C4 | Ft. Pierce, Florida, USA | N/A |

^aLSU rDNA except where indicated; ^bITS 1 & 2, 5.8S.

N/A: either not sequenced or not publicly available.

slide with 1 μL grids (Electron Microscopy Sciences, Washington, PA, USA). Cultures were swirled gently to ensure that cells were evenly distributed, and then a 10 mL subsample was withdrawn, fixed with unacidified Lugol's Iodine (50 g KI and 25 g I_2 in 500 mL ddH_2O used at a final concentration of 2%) in a 15 mL Falcon tube (BD Biosciences, San José, CA, USA), and gently inverted 10 times before a 1 mL sample was withdrawn and dispensed into the slide for counting. The slide was visually checked for any nonrandom distribution due to cell clumping. Cells were counted in the top, middle, and bottom scans of the slide, and the mean cell count was recorded, or sufficient fields were observed for a total of at least 250 cells for each position on the slide, and the mean count was corrected for 1 mL. Three separate slides of 1 mL were counted and averaged to estimate cell concentrations and to calculate culture volume to harvest to reach a desired number of cells on a filter for SHA analysis.

Capture and signal oligonucleotide probes. Partial LSU rDNA sequences (~700 bp encompassing D1–D2 domains) were obtained and compiled as previously described (Haywood et al. 2004, Table 1). The alignment was examined by eye for variable regions (avoiding insertions and deletions) from which target-specific probes could be designed. Beginning with *K. brevis*, probes were designed with a goal of maximizing base pair mismatches between target and nontarget species, with ideally no fewer than three mismatches. G (probe):U (rRNA) pairings can be stable in the hybridization solutions employed in the SHA as described by Scholin et al. (1996, see also Van Ness and Chen 1991) and so were not counted as a mismatch. Based on oligonucleotide design software termed "Oligotech" provided free by Oligos Etc. (Eugene, OR, USA), the theoretical melting temperature (T_m) of probe:target hybrids was required to be ~75°C (based on default settings of the Oligotech software), and generally probes could not have a propensity for intra- or intermolecular hybridization (e.g., the T_m for intramolecular stem-loop formation and/or homodimer formation was limited to ~20°C based on default Oligotech calculations). However, these general guidelines were not always followed. For example, due to the difficulty in designing a species-specific probe for *K. brevis*, we evaluated one probe with a theoretical stem-loop T_m of 60°C, which would provide at least 2 bp mismatches relative to other

Karenia species. Probe sequences were checked in silico for potential cross-reactivity using the BLAST algorithm (Altschul et al. 1990, v 2.2.10). Capture and signal probes (see Scholin et al. 1996) for *K. brevis* selected for testing (Table 2) were synthesized with a 5' biotin (capture) and either fluorescein (FITC) or dioxygenin (DIG) labels (5' or multiple label) for signals. Based on the initial work targeting *K. brevis*, probe development efforts were then expanded to include a variety of *Karenia* species as well as *K. veneficum* and *Gym. aureolum*, using the same general probe design guidelines as above, with stem-loop T_m tolerated up to ~60°C (Table 3). In all cases, capture probes were designed to be species-specific to the maximum extent possible, given available sequences and BLAST analyses, while signal probes were designed to target a wider taxonomic group of unarmored gymnodinioid dinoflagellates, including representative species of *Karenia*, *Karlodinium*, *Gymnodinium*, and *Gyrodinium* in particular. Probes were synthesized by Oligos Etc., and lyophilized stocks were resuspended in RNase-free ddH_2O , aliquoted into 0.5 mL tubes and vacuum desiccated (Thermo Savant DNA110 Speedvac; Thermo Electron Corp., Somerset, NJ, USA) for long-term storage, and then reconstituted into working stocks (typically 100 $\text{ng} \cdot \text{mL}^{-1}$ in 1 \times TE) as required (after Tyrrell et al. 2001). Probes were named using the nomenclature suggested by Wheeler Alm et al. (1996).

SHA. SHAs were carried out using a robotic processor (Saigene Corp., Gig Harbor, WA, USA) described previously (Scholin et al. 1999, Tyrrell et al. 2001) as per modifications given by Anderson et al. (2005) and Goffredi et al. (2005). The SHA yields a positive reaction when a sandwich of capture probe, target rRNA, and signal probe form during a series of reactions. A mechanical arm carries a plastic strip of 12 biotin-coated prongs (Saigene Corp. and Orca Research Inc., Bothwell, WA, USA) in a timed sequence through different rows of a 96-well plate (Evergreen, Los Angeles, CA, USA). Two plates can be loaded per processor. A heater maintains the temperature of the plates to a preset value that is held constant throughout sample processing. The sample and reagents are dispensed at 250 μL per well and are loaded in the following order: Row H = sample, Row G = capture probe [400 $\text{ng} \cdot \text{mL}^{-1}$ in a sodium phosphate buffer with recombinant streptavidin (RSA), a proprietary formulation provided by Saigene Corp.]. Row F contains the signal cocktail [one or

TABLE 2. Oligonucleotide probe combinations used in pair-wise combinations of capture and signal for *Karenia brevis* sandwich hybridization assay (SHA) development.

| Probe name ^a | Probe sequence (5'–3') | Target sequence aligned position (5'–3' rRNA) |
|--|-------------------------------------|---|
| <i>Signal (FITC-labeled)</i> | | |
| L*-Kbrv-265-(Gymno)-A-27 | ATA TTT AgC TTT AgA TgA AAT TTA CCA | 239–265 |
| L*-Kbrv-298-(Gymno)-A-25 | TCA Tgg TAC TTg TTT gCT ATC ggT C | 274–298 |
| L*-Kbrv-322-(Gymno)-A-24 | TTC AAA gTC CTT TTC ACC TTT CCC | 299–322 |
| L*-Kbrv-334-(Gymno)-A-25 | TTT AAC TCT CTT TTC AAA gTC CTT T | 310–334 |
| L*-Kbrv-359-(Gymno)-A-25 | TTC CCT TTC AgC AAT TTC Agg CAC T | 335–359 |
| L*-Kbrv-407-(Gymno)-A-23 | CAC YTT AgA CAA CAC CAA TAC TC | 385–407 |
| L*-Kbrv-448-(Gymno)-A-23 | TCA AAA CCT gCC ACC ACA CTT gC | 426–448 |
| L*-Kbrv-513-(Gymno)-A-27 | TAg AgT TTT CCT CAA TTA TgA ACT gAC | 490–513 |
| <i>Capture (biotin-labeled)</i> | | |
| L-S-Kbrv-594-(<i>K. brevis</i>)-A-18 | CgA CCA gAC ACA CAg TgA | 576–594 |
| L-S-Kbrv-539-(<i>K. brevis</i>)-A-21 | CgC CCg gAA gCA AAT TAC CAT | 519–539 |
| L-S-Kbrv-553-(<i>K. brevis</i>)-A-20 | Tgg ggA CAT TCA gTC gCC Cg | 534–553 |
| L-S-Kbrv-410-(<i>K. brevis</i>)-A-21 | TAT CAC CTT AgA CAA CAC CAA | 390–410 |

^a Probe nomenclature follows Tyrrell et al. (2001) first outlined in Wheeler Alm et al. (1996). L denotes LSU rDNA, where S indicates species-specific, and *indicates nonspecific or higher taxonomic group. The four-letter code represents the target dinoflagellate, and the three-digit code represents the 5' position of the probe relative to the aligned gymnodinioid rDNA sequences. Bracketed text is the full name of the target dinoflagellate, where "Gymno" represents a noncoherent taxonomic group of gymnodinioid dinoflagellates targeting at least the genera *Karenia*, *Karlodinium*, *Gymnodinium*, and *Gyrodinium*.

TABLE 3. The probe sequence and position relative to the respective target sequences for the combination of signal probes used with each of the species-specific capture probes in sandwich hybridization assay (SHA) analyses for five dinoflagellates.

| Probe name ^a | Probe sequence (5'–3') | Target sequence aligned position (5'–3' rRNA) |
|--|-------------------------------------|---|
| <i>Signal (FITC or DIG-labeled)</i> | | |
| L-*Kbrv-334-(Gymno)-A-25 | TTT AAC TCT CTT TTC AAA gTC CTT T | 310–334 |
| L-*Kbrv-407-(Gymno)-A-23 | CAC YTT AgA CAA CAC CAA TAC TC | 385–407 |
| L-*Kbrv-513-(Gymno)-A-27 | TAg AgT TTT CCT CAA TTA TgA ACT gAC | 490–513 |
| <i>Capture (biotin-labeled)</i> | | |
| L-S-Kbrv-553-(<i>K. brevis</i>)-A-20 | Tgg ggA CAT TCA gTC gCC Cg | 534–553 |
| L-S-Ksel-561-(<i>K. selliformis</i>)-A-25 | gTT CCA CTA gAg ACA TTC AgT CAC C | 537–561 |
| L-S-Kpap-209-(<i>K. papilionacea</i>)-A-21 | gTg ACT CTT AgA AAg CgC ACC gTg | 186–209 |
| L-S-Kmik-206-(<i>K. mikimotoi</i>)-A-21 | ACT CTT AgA ACA TgC ACC gTg CAC | 183–206 |
| L-S-Kave-559-(<i>Karlodinium veneficum</i>)-A-24 | TTC TAC CAg gCA CAT TCA ATC ACT CgC | 533–559 |
| L-S-Gaur-575-(<i>Gymnodinium aureolum</i>)-A-27 | gCA ATA TgA CAg TgA gTT CTg CAA ggC | 575–549 |

^aK. represents the genus *Karenia*. Probe nomenclature follows Tyrrell et al. (2001) first outlined in Wheeler Alm et al. (1996). L denotes LSU rDNA, and S, species-specific. An asterisk denotes that the probe is nonspecific or encompasses a higher taxonomic group. The four-letter code represents the target dinoflagellate, and the three-digit code represents the 5' position of the probe on the antisense strand relative to the aligned gymnodinioid sequences. Bracketed text is the full name of the target dinoflagellate, where "Gymno" represents a noncoherent taxonomic group of gymnodinioid dinoflagellates targeting at least the genera *Karenia*, *Karlodinium*, *Gymnodinium*, and *Gyrodinium*.

more probes at 150 ng · mL⁻¹ in 0.5 M guanidinium thiocyanate (GuSCN) buffer as described by Goffredi et al. 2005], Row E = wash buffer (50 mM Tris pH 7.5, 0.15 M NaCl, 0.05% Tween 20), and Row D = anti fluorescein or anti digoxigenin (DIG) antibody + HRP [ImmunoPure[®] Peroxidase Conjugated IgG antibodies diluted 1:1000 in Guardian[™] Peroxidase Conjugate Stabilizer/Diluent/Blocker (Pierce Biotechnology Inc., Rockford, IL, USA)]. Rows C and B contain wash buffer (same as Row E), and Row A, the HRP substrate (1-Step[™] Ultra TMB-ELISA; Pierce Biotechnology Inc.). The sandwich hybridization protocol proceeds with the biotin-coated prong moving through different rows of wells as follows: (1) Row G, 8 min application of capture probe/RSA complex to solid support, followed by (2) Row H, 10 or 20 min capture of target rRNA, and (3) Row F, 8 min exposure of capture probe:target hybrid to signal probe cocktail, and (4) Row E, 2 min wash, and (5) Row D, 5 min application of anti fluorescein or anti-DIG HRP antibody, and (6 and 7) Rows C and B, 2 min wash, and (8) Row A, 7 min with HRP substrate. The entire SHA protocol was carried out at 25°C–30°C, with a total processing time of ~1 h with a 20 min sample hybridization step. All reagents used in the SHA are available from the Saigene Corp. Upon completion of the SHA, the optical densities (OD) of reaction products in Row A were recorded immediately using an Emax plate reader (Molecular Devices, Sunnyvale, CA, USA) at 650 nm, and then again at 450 nm after acidification with 50 µL of 10% H₂SO₄ (v/v). The ODs of the blue (650 nm, low sensitivity) and yellow (450 nm, high sensitivity) reaction products are proportional to the amount of rRNA captured from a sample, which, in turn, can be related to an estimate of cell density based on standard curves derived from cultured and/or natural samples, the volume the sample was lysed in, and the volume of water filtered (Scholin et al. 1999, 2000).

To prepare samples for SHA, cells were collected onto 5 µm pore-size hydrophilic Durapore membranes (Millipore, Bedford, MA, USA) using gentle (<100 mm Hg) vacuum filtration. The filter was then transferred immediately to a 2 mL polypropylene cryovial (Nalgene Nunc International, Rochester, NY, USA) with the sample side of the filter facing inward, and either frozen in liquid N for storage or advanced to the lysis step. To process frozen samples, cryovials were removed from liquid N and allowed to thaw for several minutes to near room temperature before proceeding with lysis. To lyse

samples, typically 1–1.25 mL of GuSCN lysis buffer was added [3 M GuSCN, 50 mM Tris, 15 mM EDTA, 2% Sarkosyl and 0.2% SDS (v/v), at pH 8.5 and modified from Scholin et al. 1999]; the vial was capped tightly, gently vortexed, and then placed in an 85°C heating block for 5 min with brief, gentle vortexing midway through the heating step at ~2.5 min. The vials were removed from the heating block and allowed to cool for several minutes; then the lysate was poured into a 3 mL syringe (Becton Dickinson and Co., Franklin Lakes, NJ, USA) fitted with a 0.45 µm pore-size hydrophilic Durapore syringe filter (Millipore), and the filtered lysate was recovered in a fresh polypropylene tube. In cases where replicate samples were used to generate >1–1.25 mL of lysate, contents of individual cryovials were combined. The cooled, filtered lysate was added to sample wells (Row H) of the 96-well plate, and sample processing was completed as described above.

SHA standard curves and cross-reactivity testing. Capture-signal probe combinations were initially tested pair-wise in the absence of the target organism to ensure there was no reactivity between probes. These tests were repeated when signal probes were used in combination (i.e., three probes each at 150 ng · mL⁻¹), again ensuring no inherent cross-reactivity between a given capture probe and signal probe cocktail. For initial tests, *K. brevis* was added at a concentration of ~20,000 cells per 250 µL lysate (per well) from fresh cultures (~10,000 cells · mL⁻¹, both 1 week postsubculture and 2 weeks postsubculture), and results of the SHA assay were recorded for each single signal probe. Following these tests, the capture probes that performed the best were then tested with combinations of signal probes coupled with FITC at the either the 5' and 3' ends (bi-fluor) or at the positions of 5', mid-probe, and 3' (tri-fluor). As this work progressed, the label on the signal probes was changed to DIG for increased sensitivity and to improve lot-to-lot SHA consistency (see also Anderson et al. 2005). The same procedure developed for screening individual, candidate capture probes was then applied to other species with the same cocktail of signal probes used for the *K. brevis* SHA. Consequently, all SHA reagents for preparing samples and detecting species considered here are common, and only the choice of capture probe solution loaded into replicate wells of Row G dictated the specificity of the assay for a corresponding sample.

For cross-reactivity testing, cultured samples listed in Table 1 were harvested in logarithmic growth phase to achieve a

concentration of $\sim 20,000$ cells per well (i.e., per 250 μL of lysate). For standard curves, cells were also harvested to yield a maximum concentration of $\sim 20,000$ cells per well from samples taken between 1 and 2 weeks after subculturing, including a sufficient volume of lysate that was required for serial dilutions (stepwise 1:1 with lysis buffer) to generate a suite of samples with known concentrations of the targeted species. Standard curves for each species were generated, and each point on the curve was represented by six replicate wells per cell concentration. Three processors were run in parallel so that all samples for a given standard curve were processed at the same time using a single master lysate. Negative controls containing only the lysis buffer were included to establish the baseline or zero value. The Charlotte A3 strain of *K. brevis* was also used for standard curve generation to verify that the Wilson strain (1953, CCFWC268; Table 1) culture is representative of that species in general. The Charlotte A3 strain is both relatively recently isolated and from an area of the central West Florida coast that frequently experiences *K. brevis* blooms.

Growth-phase experiment. A batch culture was used to test the signal from the SHA for *K. brevis* (Wilson 1953 strain, using capture probe Kbrv 553 and signal probes listed in Table 3) over the different culture growth phases. Cells were harvested as above to give a standard concentration of 20,000 cells \cdot well $^{-1}$, and that lysate was then diluted serially as above to create a standard curve for intervals of 1, 3, and 4 weeks after inoculation. Cell counts were made using the Sedgwick Rafter method as previously explained.

Analysis of natural samples using the *K. brevis* SHA. Two 4 d cruises were run in the months of August and November 2004, with sample collection every 5 nm in a southwest direction from Sarasota, according to a preset station schedule in a zigzag pattern (for latitudes and longitudes of November 2004 MERHAB data, see the Data S1 in the supplementary material). Areas of high phytoplankton concentrations were identified using chl *a* readings from an underway Conductivity-Temperature-Depth (CTD; Sea-Bird SBE-25 from Sea-Bird Electronics Inc., Bellevue, WA, USA)/Cyclops-7 submersible fluorometer (Turner Designs Inc., Sunnyvale, CA, USA) system linked to the shipboard computer. The instruments were immersed in a drum fitted with incoming and outgoing hoses that allowed incoming seawater to be integrated over the distance traveled.

To achieve the LLD of ~ 1000 cells \cdot L $^{-1}$, the standard volume collected was 250 mL, reduced to 50 mL when high cell numbers ($>1 \times 10^6$ cells \cdot L $^{-1}$) were present as evident from a visual inspection of the sample. The integrated underway sample collected between stations was split into two lines, one comprising the inlet for the optical phytoplankton discriminator (OPD; see Robbins et al. 2006, based on the methods of Kirkpatrick et al. 2000), and the other clamped until the collection point of 5 nm between stations was reached. The boat was slowed, and a 10–15 L sample was collected into a container, with the collection start and stop times recorded by computer to match the data gathered by the OPD. The container was mixed thoroughly, and 3×500 and 1×250 mL subsamples were withdrawn, the former for SHA and the latter for addition to a 250 mL amber bottle (Nalgene Nunc International) containing 2% final volume unacidified Lugol's Iodine. Samples were also collected on station (every 10 min) with a carousel water sampler (Sea-Bird SBE-32; Sea-Bird Electronics Inc.) and 3×8 L Niskin bottles per depth. The three replicate bottles were poured into a 25 L bucket and mixed thoroughly by gentle stirring. From that stirred sample, a 1.5 L volume was collected as three replicate 500 mL samples per depth for SHA. In addition, a 250 mL sample was retained for traditional cell counts as stated above. All sample bottles were prerinsed with incoming sample seawater before use.

Shipboard cell counts were performed with a CK2 inverted binocular light microscope (Olympus America Inc., Melville,

NY, USA) using a 3 mL fixed subsample withdrawn by a 5 mL disposable pipette (Corning Incorporated Life Sciences, Acton, MA, USA) and settled in a marked chamber of a 24-well polystyrene plate (Nalgene Nunc International). All cells present in the chamber thought to be *K. brevis* were counted, with sample dilution (0.22 μm filtered GoMx seawater) as necessary. Six replicate filters for SHA were routinely collected for each depth. Three of those were homogenized immediately in 1.25 mL lysis buffer in separate cryovials, heated, 0.45 μm syringe filtered and combined, and then dispensed into the sample row of the 96-well plate as previously described. The remaining three replicate filters were frozen in liquid nitrogen, resulting in a total of six archived filters per station per single cryocane representing both surface (~ 1 m depth) and bottom (~ 1 m from the seafloor) replicates, or for underway samples, two different midstation, integrated surface samples per cane.

For near real time, shipboard analysis of natural samples, SHA plates were configured with four species-specific probes in triplicate wells per plate, comprising *K. brevis* (wells G1–3), *K. veneficum* (wells G4–6), *K. papilionacea* (wells G7–9), and *K. selliformis* (wells G10–12).

RESULTS

Probe evaluation, cross-reactivity tests, and standard curves. The aim of this research was to develop a set of SHAs for detecting naked, harmful dinoflagellates from the genus *Karenia* and the related species *K. veneficum* and *Gym. aureolum*. All of these dinoflagellates are relatively fragile and visually difficult to distinguish from each other. Our long-term goal is to achieve an LLD of $\sim 10^3$ cells \cdot L $^{-1}$ or less for all species in natural samples, since this is already achievable with cultured cells. Initial work centered on *K. brevis*, with the pair-wise evaluation of signal and capture probes listed in Table 1. Four signal probes and a single capture probe emerged from this testing as being reactive toward homogenates of *K. brevis* (Table 2). Three of the four signal probes offered roughly equivalent reactivity and were used in combination to improve sensitivity of the assay for subsequent testing (Fig. 1a). Negative controls (lysis buffer only, no cell homogenate) proved that there was no reaction between the capture and individual signal probes or a cocktail of multiple signal probes. The sole reactive capture probe for *K. brevis* is predicted to have a stem-loop structure with a theoretical T_m of $\sim 56^\circ\text{C}$ based on the Oligotech software, but nonetheless showed good reactivity toward *K. brevis* and no cross-reactivity with the other species examined in this study, most notably *K. mikimotoi* (the most closely related species, which differs from the *K. brevis* probe sequence by 3 bp). This initial result led us to reassess our criteria for probe design for the other species, in particular allowing intramolecular stem-loop T_m to 60°C , the predicted temperature indicated for the *K. mikimotoi* capture probe.

While there was only one reactive capture probe for *K. brevis* of several synthesized (Table 2), more than one of the capture probes designed for

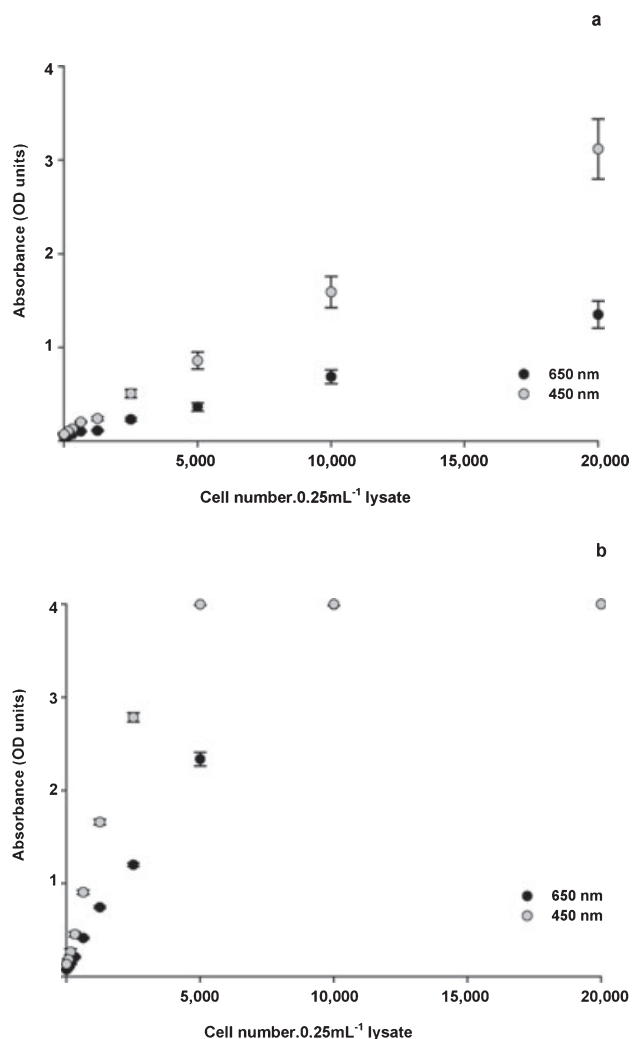


FIG. 1. Sandwich hybridization assay (SHA) standard curves using the Wilson 1953 strain of *Karenia brevis*: (a) from 2002 for combination of three tri-FITC-labeled signal probes, and (b) from 2004 for a combination of three tri-DIG-labeled signal probes, both with the same *K. brevis*-specific capture probe. Symbols represent absorbance mean values in optical density (OD) units per 0.25 mL lysate; error bars are standard deviations ($n = 6$).

K. selliformis, *K. mikimotoi*, *K. papilionacea*, *Ka. veneficum*, and *Gym. aureolum* were reactive with their intended target. Probes for *K. selliformis* and *Ka. veneficum* targeted the same site as that used for *K. brevis* but did not show a propensity for stem-loop formation. For all of the probes tested, homodimers were predicted either to be unstable or to have theoretical T_m values below 10°C. The most reactive probes for each species were selected for further evaluation, and those that performed best and were chosen for further study are shown in Table 3. Tests of those probes gave results that paralleled what was obtained for *K. brevis*. All reacted vigorously with their intended target, showed no evidence of capture:signal probe hybridization in lysis buffer negative controls, and did not cross-react with lysates of

nontarget species from several genera (Table 4). Representative species were chosen for cross-reactivity testing on the basis of both their similarity in rRNA sequences relative to the target species as identified in BLAST searches, the number of bp mismatches indicated from these analyses, and their availability for testing.

Initial standard curves relating SHA reactivity (OD) toward variable numbers of *K. brevis* per unit volume showed a relatively linear response when using the FITC-labeled signal probes (Fig. 1a). Subsequent work with the tri-DIG labeled signal probe cocktail led to a much more sensitive assay, with saturation at $\sim 5 \times 10^3$ cells \cdot 250 μ L lysate⁻¹ (well⁻¹) at 450 nm, and $\sim 1 \times 10^4$ cells \cdot well⁻¹ at 650 nm (Fig. 1b). The extended capture hybridization time from 10 to 20 min contributed to the elevated response, as did changes to the underlying chemistry of the assay and prong shape (see also Anderson et al. 2005). Probes listed in Table 3 were used with the 20 min sample hybridization time and tri-DIG signal probe cocktail for cross-reactivity testing (Table 4) and to generate standard curves. Probes for all species except *K. papilionacea* yielded curves of a similar shape and dynamic range (Fig. 2), although the standard curves generated from the 2-week-old subcultures were slightly less reactive than 1-week-old samples.

Comparisons of different strains of K. brevis and growth-phase experiment. Overall, the two strains of *K. brevis* available returned similar standard curves, with no significant difference in reactivity as a function of the number of cells per volume homogenate (not shown). In the growth-phase experiment, there was a clear decline in reactivity over time for a constant number of cells (Fig. 3a). After 2 weeks from inoculation, the signal from the SHA was 60%–100% of the starting values (1 week after inoculation). After 4 weeks from inoculation, there was a dramatic decline to between 7% and 50% of the initial values, corresponding with a parallel drop in cell concentration that indicated decline of the culture and onset of senescence (Fig. 3b).

Shipboard whole-water sampling and analysis. *Karenia* species were not abundant during the August 2004 cruise, and, correspondingly, only very low SHA positives (of $< 2 \times 10^4$ cells \cdot L⁻¹) were recorded (data not shown). In contrast, higher densities of *K. brevis* (=very low on the Fish and Wildlife Research Institute [FWRI] scale; see Discussion) were found during the November 2005 cruise off Cape Romano, equating to the southernmost sampling stations in our cruise plan, with very low densities of *K. papilionacea*, *K. selliformis*, and *Ka. veneficum*. A comparison of cell-density estimates based on LM observations versus estimates based on the SHA for *K. brevis* for that time is shown in Figure 4, with the *K. brevis* standard curve (450 nm = high sensitivity data, and 650 nm = low sensitivity data) included for reference.

TABLE 4. Absorbance data at 450 nm expressed categorically from optical density (OD) units (–, ≤ 0.2 ; +, 0.3–1.0; ++, 1.1–2; +++, 2.1–3; +++, 3.1–4). All comparisons were performed at 2×10^4 cells $250 \mu\text{L}^{-1}$ of the relevant culture and with tri-DIG-labeled probes.

| Dinoflagellate | L-S-Kbrv-556- (K. brevis) -A-20 | L-S-Kpap-209- (K. papilionacea) -A-21 | L-S-Kmik-206- (K. mikimotoi) -A-21 | L-S-Ksel-560- (K. selliformis) -A-25 | L-S-Kven-559- (Karlodinium veneficum) -A-24 | L-S-Gaur-575- (Gymnodinium aureolum) -A-27 |
|---|---------------------------------|---------------------------------------|------------------------------------|--------------------------------------|---|--|
| <i>Karenia brevis</i> (Wilson 1953) | ++++ | – | – | – | – | – |
| <i>Karenia papilionacea</i> | – | +++ | – | – | – | – |
| <i>Karenia mikimotoi</i> | – | – | ++++ | – | – | – |
| <i>Karenia selliformis</i> | – | – | – | ++++ | – | – |
| <i>Karlodinium veneficum</i> | – | – | – | – | ++++ | – |
| <i>Gymnodinium aureolum</i> | – | – | – | – | – | ++++ |
| <i>Amphidinium carterae</i> | – | – | – | – | – | – |
| <i>Coolia monotis</i> | – | – | – | – | – | – |
| <i>Gymnodinium simplex</i> | – | – | – | – | – | – |
| <i>Gyrodinium instriatum</i> | – | – | – | – | – | – |
| <i>Heterocapsa pygmaea</i> | – | – | – | – | – | – |
| <i>Karenia bidigitata</i> | – | – | – | – | – | – |
| <i>Prorocentrum micans</i> | – | – | – | – | – | – |
| <i>Prorocentrum rathymum</i> (=mexicanum) | – | – | – | – | – | – |
| <i>Scrippsiella trochoidea</i> | – | – | – | – | – | – |
| <i>Takayama tasmanica</i> | – | – | – | – | – | – |

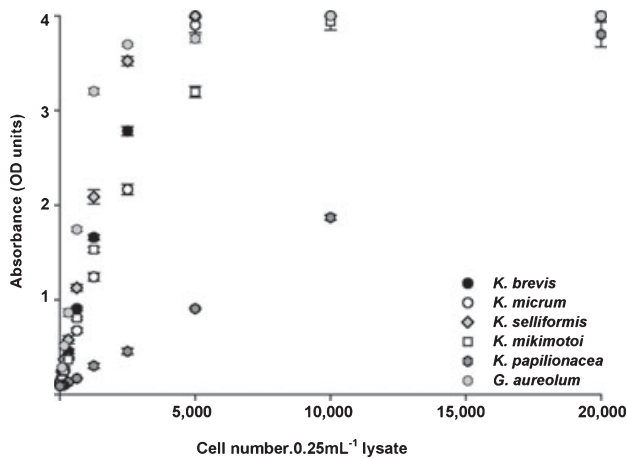


FIG. 2. Sandwich hybridization assay (SHA) standard curves for optimal 5' biotin-labeled capture probes for *Karenia brevis*, *Karenia selliformis*, *Karlodinium veneficum*, *Karenia mikimotoi*, *Karenia papilionacea*, and *Gymnodinium aureolum* each paired with three tri-DIG-labeled signal probes against their target species. Symbols represent absorbance mean values in optical density (OD) units per 0.25 mL lysate (per species as indicated); error bars are standard deviations ($n = 6$).

Overall, estimates of *K. brevis* density based on the 450 nm SHA standard curve relative to microscopy-based counts showed the laboratory cultures to return a greater signal than cells collected from natural populations, with assay saturation being $\sim 5 \times 10^3$ cells per well for cultures and $\sim 1 \times 10^4$ cells per well for field samples. Given the sample and lysis solution volumes employed, this equates to $\sim 1 \times 10^5$ and $\sim 2 \times 10^5$ cells $\cdot \text{L}^{-1}$, respec-

tively. As would be expected, the 650 nm (low sensitivity for low cell numbers, and high sensitivity for high cell numbers) values show that there is a continuing upward trend in assay reactivity beyond those densities.

DISCUSSION

Microscopic counts have traditionally provided important information about the spatial scale and intensity of *K. brevis* blooms, and more recently, satellite imagery (Stumpf et al. 2003, Tomlinson et al. 2004, Hu et al. 2005) and in-water optical sensors (using methods reported in Kirkpatrick et al. 2000) are complementing those measurements. In this work, we strove to provide additional tools for identifying *K. brevis* and related species that can be difficult to discriminate using LM and that have high LSU rDNA sequence similarities. The SHA method uses rRNA-targeted probes in a format that can be applied in a laboratory, aboard ships, and within autonomous sensor systems (Greenfield et al. 2006). Here we report on the details of developing a suite of SHAs for cultured species and provide an example of applying the *K. brevis* probes to natural samples. A more thorough consideration of the utility of these assays for analysis of field samples will be presented in a separate communication.

Development of the SHAs. The SHA chemistry as applied here was initially devised for detecting human pathogens in a clinical setting (Briselden and Hillier 1994). The constraints imposed for that application parallel those associated with analysis of environmental samples, especially the need for rapid detection of a variety of specific organisms and minimal requirements for sample handling

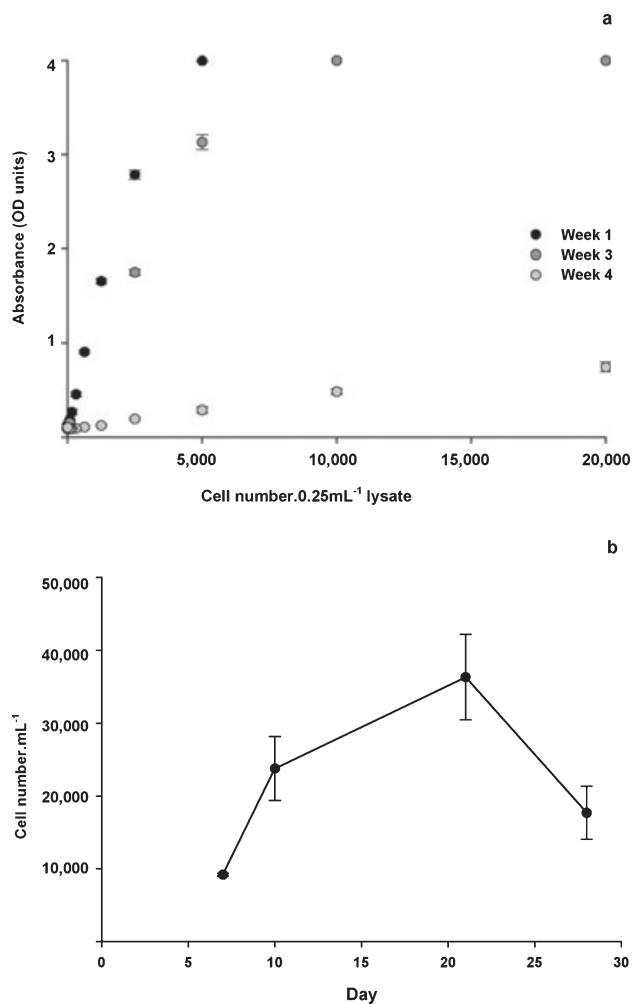


FIG. 3. Sandwich hybridization assay (SHA) batch culture experiment for *Karenia brevis* (Wilson 1953). Standard curves and counts for weeks 1, 3, and 4 after inoculation. Error bars are standard deviations ($n = 3$). (a) Reactivity decreases over time as shown by lower absorbance readings (OD units at 450 nm) from the SHA. (b) Cell number per week of the batch culture experiment. The days on which SHAs were run are indicated.

(e.g., direct detection of target molecules in a crude cell homogenate and no requirement for PCR). Evaluation and modification of this technology for environmental research and monitoring purposes has taken place over the past decade and has showed promise as a means for detecting HAB species representing three different classes of algae—Dinophyceae, Bacillariophyceae, and Raphidophyceae (Scholin et al. 1996, 1999, Tyrrell et al. 2001, Anderson et al. 2005, O'Halloran et al. 2006). Many issues regarding the details of how this and other molecular-probe-based assays can be integrated into routine monitoring programs remain for consideration (Anderson et al. 2005, Scholin et al. in press), although it is noteworthy that in New Zealand, the SHA has gained international accreditation and can be used to monitor certain

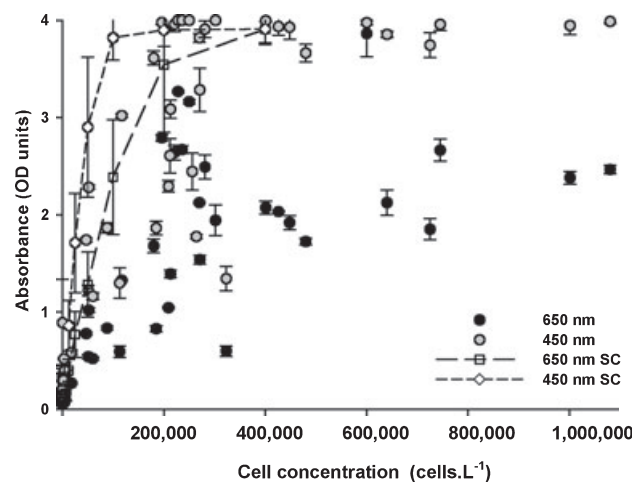


FIG. 4. Shipboard results (MERHAB Eastern Gulf of Mexico Sentinel Program) for the *Karenia brevis*-targeted sandwich hybridization assay (SHA) from November 2004 (450 nm = high sensitivity, and 650 nm = low sensitivity) relative to the mean of three standard curves for *K. brevis* (SC = standard curve) expressed in cells per L. All errors are standard deviations ($n = 3$). The top end of the standard curve error bar represents culture samples taken 1 week, and bottom end 2 weeks, after sub-culturing (every 3 weeks), both with a starting cell concentration of $\sim 1 \times 10^4$ cells \cdot L⁻¹.

species of *Pseudo-nitzschia* to regulate commercial shellfish harvests (Ayers et al. 2005). The chemistry underlying the SHA is amenable to application in a variety of formats beyond the 96-well-plate version considered here. For example, the "front end" sample collection and homogenization methodology is compatible with a variety of "downstream" nucleic acid purification methods (C. A. Scholin, personal observation), and the SHA approach can be used in a variety of array formats (Ahn et al. 2006, Greenfield et al. 2006). Consequently, the core methodology offers a rich landscape for future research and development activities. Here, our primary goal was to first develop SHAs for a variety of unarmored dinoflagellates and then establish a protocol biased toward detecting cells at low concentrations in field samples. We then plan to translate these assays into an array format consistent with in-water autonomous operation in support of the regional monitoring and event response program outlined below.

Probe characteristics and specificity. Using our stated design criteria, probes required a theoretical T_m of no less than 75°C to give the best reactivity and minimize cross-reactivity with closely related nontarget species. Probes successfully discriminated between the target and nontarget species, including those representative of three separate orders of dinoflagellates (Gymnodiniales, Peridinales, and Prorocentrales), with hybridization temperatures of $\sim 25^\circ\text{C}$ – 30°C . Software used to aid probe design indicated that the *K. brevis* capture probe contained

significant secondary structure (a stem-loop structure stable to 56°C), that if generally avoided would prematurely exclude the probe from further evaluation. Indeed, we suspect that stem-loop structures may actually confer some advantages, since under appropriate conditions the probe may be more prone to undergo an intramolecular interaction rather than to hybridize to sequences in solution, unless the match is exact, similar to molecular beacons (Schofield et al. 1997). Regardless, past experience has shown that the most reactive and specific probes in the SHA format as used in this study are not always obvious based on predictions generated by computer algorithms, and use of strict design criteria tuned primarily for NaCl-based buffers can potentially exclude very useful probes from consideration (see also Tyrrell et al. 2001).

For those reasons, it is advisable to test a variety of probes for a given target when developing SHAs, even though they may not appear to be ideal, especially where there is limited intraspecific variation in sequences, as is evident between the species of *Karenia*. Probes should be designed to bracket potentially good sites since it is known that moving probes a few to 10 bp “left” or “right” of a given position can have a profound effect on their performance (Tyrrell et al. 2001 and references therein). This coupled theoretical and empirical approach was applied here. For example, predicted stem-loop formation temperature for both the *K. brevis*-targeted and *K. mikimotoi*-targeted capture probes exceeded our initial limit of 20°C by ~3-fold, yet these probes performed very well. Additionally, all of the closely related species considered here share a high degree of similarity at the rRNA sequence level, so one might expect that the most reactive probes would tend to target the same region of the molecule. However, this was not always the case. The best capture probes designed for *K. selliformis* and *Ka. veneficum* do target the same region as that for *K. brevis*, while that for *Gym. aureolum* overlaps this region by 10 bp relative to the probe sequence for *K. brevis*, and those for *K. mikimotoi* and *K. papilionacea* target a completely different region (Table 3).

Although in our design criteria we aimed for no fewer than 3 bp mismatches between closely related species, in practice this was not possible for all species considered in the alignment. In some cases, there are only single base pair mismatches between the probe sequence and nontarget rRNA sequence [for example, the *K. mikimotoi* probe relative to *K. bidigitata* Haywood et Steid. and *K. brevisulcata* (F. H. Chang) G. Hansen et Moestrup, and *K. selliformis* relative to *K. brevisulcata*]. These near matches were not necessarily of the most closely related species as would be determined phylogenetically. For example, while the probe for *K. brevis* was at least 2 bp different to closely related *Karenia* rRNA sequences, it was 3 bp different from

K. mikimotoi, the nearest relative according to a comparison of the LSU sequences. Despite those complications, we were able to achieve species-specific capture probes for all five species targeted relative to the closely and distantly related cultures tested (Tables 1, 4). Cultures used for testing were chosen not only to represent different numbers of rRNA sequence mismatches and different families, but also to represent species found in the Gulf of Mexico region, where possible and as publicly available.

While the choice of capture probe for *K. brevis* was self-evident since only one of the candidate probes tested proved reactive, the choice of probes selected for the other species (Table 3) rested on their reactivity toward their target and lack of cross-reactivity with closely related nontarget species thought to be present in Florida and New Zealand. Probes being used in this study may not prove to be specific globally, particularly as sequences from different geographic locations were only available for *K. mikimotoi* at the time of probe design. The potential need to “tune” probes for use in specific geographic areas is well known (e.g., Scholin et al. 2003, Lundholm et al. 2006). To address any such intraspecific sequence variability and also any possible cross-reactivity issues in the future, we will continue to test the assays with lysates from cultured target and nontarget isolates of different strains and species as these become publicly available. At the same time, we will continue to make field testing a priority, since intra- and interspecific genetic diversity of rRNAs present in natural samples will always exceed what can be presented to the SHAs via cultures. The probes must also be tested with lysates from natural seawater samples, in which they are to be routinely used to identify as early as possible any cross-reactivity with species that cannot be cultured. Parallel field testing of these SHAs is also underway in New Zealand.

The dynamic range of the SHA can be adjusted by altering sample volume, lysate volume, hybridization time, and the number of signal probes and signal moieties attached to each signal probe (Fig. 1, a and b; Scholin et al. 2003). The assays developed for four of the five species (Table 3) show similar reactivity and dynamic range (Fig. 2), which is advantageous since a single adjustment used to alter the dynamic range of the assay will result in similar detection levels of *K. brevis*, *K. selliformis*, *K. mikimotoi*, *Gym. aureolum*, and *Ka. veneficum*. The SHA for *K. papilionacea* is similarly specific but not as reactive toward the target species as the other assays. The reasons for this are not yet apparent and are under further study. Apart from the SHA for *K. papilionacea*, however, our results to date suggest that we could theoretically meet the required LLD of $\sim 10^3$ (1250) cells \cdot L⁻¹ if 250 mL of natural seawater is filtered and then homogenized in 1 mL of lysis buffer for the other five SHAs, with a couple of provisos. To achieve this LLD, we assumed both that

cells collected from natural samples will have a reactivity similar to that in pure culture as recorded here for *K. brevis*, and that *K. brevis* is always accurately identified in shipboard cell counts. However, early results suggest that either some cells from whole coastal seawater samples were less reactive than cultured cells, the shipboard cell count was not always accurate, or the presence of other contributing factors (see "Preliminary analysis of coastal whole-water samples using SHA" below).

Intraspecific variation and effect of growth phase on SHA reactivity for K. brevis. No intraspecific variation was seen for the Wilson 1953 and Charlotte A3 strains of *K. brevis*. However, we suspected that minor variations between the two seen on occasion might be related to the physiological status and/or growth state of the cultures at the time cells were harvested. For example, Miller et al. (2004) observed that the reactivity of an SHA for a culture of *Pseudo-nitzschia multiseries* (Hasle) Hasle grown in continuous culture varied depending on the limiting nutrient. For this reason, we sought to explore the potential impact that culture status might have on apparent reactivity of the SHA. Indeed, growth phase of the culture clearly impacted the slope of the standard curve as shown in Figure 3, with reactivity for a constant number of cells decreasing over time, especially as the culture began to decline. Associated with that sharp decrease in reactivity, cells appeared unhealthy and had lost discriminative morphological features, consistent with senescence. Similar decreases in SHA reactivity have been observed for both *Heterosigma akashiwo* (Hada) Hada ex Y. Hara et Chihara and *Fibrocapsa japonica* Toriumi et Takano with the onset of stationary phase (Tyrrell et al. 2001). Although these observations were probably due to lowered amounts of rRNA per cell, we cannot rule out the possibility that some of that decline is related to increased cell lysis of unhealthy cells during filtration, fragmentation of the rRNA such that capture and signal probes can no longer hybridize to a contiguous molecule to give rise to a signal, or some type of substance produced by senescent cells that inhibits capture of the rRNA from the outset. In any case, growth status of *K. brevis* will impact estimates of cell abundance using the SHA and a standard curve derived from rapidly growing cells. Whether this presents serious problems for routine applications in monitoring programs remains to be determined. For example, this could be a useful attribute of the assay, as some studies have indicated that the production of the most potent toxins by *K. brevis* (PbTx-A and PbTx-B) is associated with rapid cell growth, while in the stationary phase there is a shift to the less potent derivatives (Roszell et al. 1990, Wright and Cembella 1998).

There is also variability associated with standard curves constructed using cultures harvested at similar densities. A culture approaching stationary

phase (beyond 3 weeks) does not recover reactivity immediately when subcultured, even when left for a week in fresh medium (not shown). Unfettered and rapid growth decreases such fluctuations, and frequent culture transfers can avoid changes in the reactivity of the SHA that result from culture conditions. Neither extreme may be a good representation of natural populations, and the standard curve used should probably reflect some average taken over the growth phases, ideally between 2 and 3 weeks after subculturing based on the information we currently have.

Preliminary analysis of coastal whole-water samples using SHA. The analysis of field samples for *K. brevis* indicated that cells collected from natural populations are not generally as reactive as those held in pure culture (Fig. 4), but these preliminary results are nonetheless very encouraging. The cell count is more difficult to do accurately aboard ship than in the laboratory because of the difficult working conditions. Cells on their side cannot be flipped for examination, for example, and there are also issues with engine-induced vibrations on station and the identification of small cells as previously discussed. These factors could have contributed to a lower (or higher) perception of SHA reactivity relative to a given cell concentration than actually exists. However, the fixed replicate samples can be analyzed later if cell counts and SHA results differ substantially. Other contributing factors included equipment issues as outlined below. Regardless, given the batch culture experiment discussed above, variation in the reactivity of cells collected from natural samples over wide spatial and temporal scales was actually expected. The patch of *K. brevis* occurring off Cape Romano was considerably further south of patches found in previous years, and this southern location may have ensued from warmer waters and multiple, late-season hurricanes. The full extent to which the range of reactivity observed in culture will manifest itself in natural populations remains to be determined and, we believe, can only be assessed empirically by repeated analysis of the kind presented here. Indeed, the range of reactivity achieved in culture may exceed what could be measured in natural populations. For example, cells that are senescent or under severe nutrient limitation may not persist in nature but could survive in unialgal culture(s). There is also the likelihood that different life-cycle stages may be encountered in natural populations that react differently from, and are not typical of, asexual vegetative cells maintained in culture. Beyond those SHA-centric considerations, errors associated with the microscopy counts can also confound the apparent relationship between cell-density estimates (see below). For all these reasons, repeated analysis of natural samples as outlined here is required to develop an empirical basis for interpreting the SHA data and casting reasonable estimates of species presence and abundance.

The results shown in Figure 4 were also impacted by problems with equipment that were ongoing at the time of the cruise. The processors used on the cruise employed an older style of prong arm where two separate anodized aluminum arms are used for the two plates run per processor (see Scholin et al. 1999). The detailed machining and marginally off-set alignment of the arms relative to the plates caused a variety of problems, such as well-to-well signal variability and depressed signal intensities. Since that time, a new mono arm was made that supports processing of the two plates. The new arm is made of stainless steel and facilitates the fit of the prong to the plate, consistently aligning the prong properly with the wells. Some of the archived samples used to construct Figure 4 during the cruise were reanalyzed in the laboratory with the new arm. The SHA values obtained were closer to the predictions based on the standard curve, and well-to-well variations were lower. The relevant standard curves were less affected.

The advantages of early detection and unequivocal identification. There are several reasons that the unequivocal detection of *K. brevis* and related species at low, even background concentrations is desirable. The first motivation relates to PbTx risk assessment and the co-occurrence of multiple species of *Karenia*. *Karenia* spp. described from New Zealand waters—*K. papilionacea*, *K. selliformis* (Haywood et al. 2004), and *K. mikimotoi* (cosmopolitan)—have also been found in the eastern Gulf of Mexico based on LM with confirmatory FISH assays (A. J. Haywood, C. A. Scholin, and K. Petrik, pers. obs.). The latter species was known to co-occur with *K. brevis* in Gulf waters on occasion according to records of microscopic observations (Fish and Wildlife Research Institute in St. Petersburg, Florida) and as documented by Heil et al. (2004). The presence of *K. papilionacea* was only suspected prior to confirmatory molecular studies in the region, as possibly the species reported in Steidinger et al. (1966). Cells resembling *K. papilionacea* and *K. brevis* have led to yellow-colored blooms with no associated toxicity in the Florida Keys (K. Steidinger, unpublished data). Microscopic observations and pigment profiles were used to identify a different species of *Karenia* in at least one offshore cruise in 2001 (Pederson et al. 2004). Only *K. selliformis* is known to produce gymnodimine (Seki et al. 1996), although previous reports cite *K. mikimotoi* as the causative species (Wright and Cembella 1998). While the toxicity of these organisms in Florida remains unknown, synergistic toxic effects could result from the co-occurrence of multiple species, highlighting the importance of unequivocal species identification and the capacity to detect multiple species in addition to *K. brevis*. *Gymnodinium aureolum* was included in our testing both because it co-occurs with *K. mikimotoi* in New Zealand (A. J. Haywood, unpublished data), and because in

earlier, predominantly European literature, *K. mikimotoi* was often referred to as *Gyrodinium aureolum* (see Hansen et al. 2000).

Techniques for detecting low cell concentrations could help to identify bloom initiation sites. Although microscopy can be used for this purpose, there is likely to be considerable error associated with those estimates. Currently, three 1 mL aliquots of a sample are examined, and cells enumerated in the laboratory (which gives a theoretical detection of $333 \text{ cells} \cdot \text{L}^{-1}$, or one cell in one of the 1 mL aliquots). Those counts are then used to generate red-tide reports. Experienced workers can find identifying specific *Karenia* species problematic even in the laboratory when large numbers of samples must be examined over a short period of time. The difficulties of handling large sample numbers are compounded aboard ship by the inherent vibrations and motion encountered. In addition, the widely varying cell numbers that can occur over small spatial scales may require sample dilution(s) followed by back-calculation(s) to achieve the final estimates. Although preserved samples are always taken for later confirmation of the dominant species if required, the SHA could provide a means for screening many samples for multiple species far more rapidly than what might be reasonably achieved using microscopy alone.

How precise do cell-density estimates need to be, and what role can the SHA play to meet that requirement? The relative risk associated with PbTx outbreaks scales with order-of-magnitude (not incremental) changes in *K. brevis* cell densities. Red-tide reports from the Florida Fish and Wildlife Research Institute available online (<http://www.floridamarine.org>) cite $10^3 \text{ cells} \cdot \text{L}^{-1}$ as “present,” $<10^4 \text{ cells} \cdot \text{L}^{-1}$ as “very low,” $10^5 \text{ cells} \cdot \text{L}^{-1}$ as “low,” $>10^5$ but $<10^6 \text{ cells} \cdot \text{L}^{-1}$ as “medium,” and $>10^6 \text{ cells} \cdot \text{L}^{-1}$ as “high” (although cell numbers can easily reach $80 \times 10^6 \text{ cells} \cdot \text{L}^{-1}$ or more in a bloom event). Cellular PbTx content is thought to vary from 16.6 to 6.6 $\text{pg} \cdot \text{cell}^{-1}$ (Baden and Tomas 1989), consequently requiring $\sim 5 \times 10^3$ – $12 \times 10^3 \text{ cells} \cdot \text{L}^{-1}$ to reach the PbTx regulatory limit in shellfish (Baden and Mende 1982) in the day it takes to run the mouse bioassay, based on a mean filtration rate for oysters. Based on those values, closure of a shellfish-harvesting area is set at $5 \times 10^3 \text{ cells} \cdot \text{L}^{-1}$ and is thus conservative for this species as it assumes the highest, not mean, toxin content recorded per cell and the presence of the most potent toxins rather than toxin derivatives. Although the operation of the SHA may be adjusted to accommodate either lower or higher levels of detection, we aimed for the $\sim 10^3 \text{ cells} \cdot \text{L}^{-1}$ level to be consistent with monitoring practices in place today. This level provides for some advance warning of a potential NSP outbreak but does not push the sensitivity to a level that does not offer information applicable for routine analyses.

The SHA assay could also be used to complement traditional counts or other methods where rapid assessment of species' presence in complex natural samples is desired. For example, as discussed above, *Karenia* seen in natural samples are often of multiple shapes and sizes, potentially representing diel cell-cycle variations, different life-cycle stages, or pleomorphisms. Such cells are not necessarily the same shape as the vegetative cells used to describe the species and that are commonly observed in cultures. These problems combined with the presence of other microorganisms and detrital material that may obscure cells made detection of some species at low densities difficult. In such cases, the SHA may offer some advantages since the standard sample volume is increased (e.g., ~250:1 or 83:1 relative to microscopy if 250 mL for SHA and either 1 mL or 3 mL for microscopy are used), visualization of the target species is not required, and relatively large numbers of samples can be screened rapidly aboard ship if desired.

Monitoring and event response for harmful algal blooms (MERHAB) program. The Eastern Gulf of Mexico Sentinel Program was established as a multi-institute, multi-investigator initiative, under the auspices of the MERHAB program (NOAA) in late 2002. One of the main aims of the Sentinel Program was to detect *K. brevis* using both the SHA and optical detection methods. In conjunction with these biosensors, bottom-stationed ocean profilers (BSOPs, Langebrake et al. 2002) would collect physical parameters, with the ultimate aim of deployment of both the biosensors and BSOPs to allow concurrent collection of biological and physical data. Biophysical models have already been developed to predict the conditions under which coastal upwelling occurs in the hot spot for *K. brevis* blooms. Near real-time detection of *Karenia* spp. would then be used to provide initiation data for the models, and over time, a predictive system could be established to forecast potential *K. brevis* outbreaks.

Implementation of this vision is still in its early stages. Tests of the kind undertaken during the November 2004 cruise illustrate the potential utility of the *K. brevis* SHA and some of the challenges that lie ahead. Overall, the *K. brevis* concentration estimates based on microscopy agreed well with those obtained using the SHA, with some exceptions as seen in Figure 4. Clearly, additional field studies are needed to assess the utility of the SHA. Independently, our results highlight the critical need to establish criteria for ascribing species designations and reference cell counts that are considered the "gold standards" against which all new methods are compared. In our case the gold standard for cell enumeration is a microscopy-based cell count from unacidified Lugol's Iodine-fixed samples, and the species are identified using conservative morphological characters that in the majority of cells are visible upon fixation. The statistical problems with

accuracy and precision relating to cell counts are known and have been described, and procedures for mitigating these factors have been suggested (Sournia 1978). In some circumstances and with certain microalgae, these problems may be difficult if not impossible to overcome, leading to outliers with respect to both whole-cell-based and cell-homogenate-based assays. Similarly, physiological effects could lead to some variability in reactivity of the SHA relative to cell counts. For all of these reasons, we foresee an urgent need to advance an understanding of what information molecular methods actually provide relative to the current standards. Given that understanding, the research and resource management communities must arrive at a consensus as to how new methods are best integrated into routine monitoring programs.

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Supplementary Material

The following supplementary material is available for this article:

Appendix S1. Latitudes and longitudes for November 2004 MERHAB data.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1529-8817.2007.00407.x>.

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