

# Development and field application of rRNA-targeted probes for the detection of *Cochlodinium polykrikoides* Margalef in Korean coastal waters using whole cell and sandwich hybridization formats<sup>☆</sup>

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## Abstract

The dinoflagellate, *Cochlodinium polykrikoides* Margalef, has been responsible for mass mortalities of both wild and farmed fish along the Korean coast on virtually an annual basis since 1982. Economic impacts to the fishing and aquaculture industries are extensive, with a loss of USD \$95 million reported in 1995 alone. The use of taxon-specific molecular probes for harmful algal species is recognized as a promising approach for the early detection of bloom formation and as part of an effective mitigation strategy. We have developed and successfully applied large subunit ribosomal RNA (LSU rRNA)-targeted probes in both whole cell and sandwich hybridization assay (SHA) formats for the species-specific detection of *C. polykrikoides* in Korean coastal waters. Sequences of the D1–D3 variable regions used to design probes were identical between five Korean and one Hong Kong *C. polykrikoides* isolates, while sequences for several N. American *Cochlodinium* isolates differed to varying degrees from the former. The automated SHA detected *C. polykrikoides* at levels as low as ~1–3 cells/L in the field, demonstrating its suitability for detecting the target species at pre-bloom concentrations. This method should thus prove valuable to existing monitoring programs aimed at providing aquaculture interests with an early warning of frequently devastating bloom events.

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**Keywords:** *Cochlodinium polykrikoides*; Harmful algal blooms; Korea; LSU rRNA; Molecular probes; Sandwich hybridization

## 1. Introduction

The sale of fishery products on both domestic and foreign markets represents an important source of income to the Korean economy. However, recurring blooms of the dinoflagellate, *Cochlodinium polykrikoides* Margalef, first recorded off of the Korean coast in 1982, have resulted in widespread mass

mortalities of wild and farmed fish, and were designated by the government as natural disasters in 1990 (Kim, 1998). Losses attributed to these now virtually annual events reached a peak in 1995 of an estimated USD \$95 million (Kim, 1998). Blooms of *Cochlodinium* species and their associated impacts to finfish aquaculture have since been reported from a number of other countries (see Kudela et al., 2008). For example, in 1999, a bloom of *Cochlodinium* sp. along the west coast of N. America in British Columbia, Canada, caused losses to the farmed salmon industry of approximately CDN \$2 million (Whyte et al., 2001).

Due to the economic impacts of *C. polykrikoides* blooms, the Korean government currently supports a vigorous effort to control HABs and mitigate their devastating effects. In addition to direct control measures involving the application of clays to remove cells from the water column (Kim, 2006), a promising approach to mitigation has been the monitoring of phytoplankton assemblages for the presence and increasing

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abundance of harmful species. Early detection of bloom formation along with attempts to circumvent losses by moving net pens or early harvesting are critical aims of the aquaculture industry in Korea and throughout the world's coastal waters (Kim, 1998).

Detection of harmful algal species has traditionally been performed by light microscopic observation. More recently, the application of molecular probe-based approaches to organism detection has resulted in improved speed and efficiency of monitoring various HAB taxa (Tyrrell et al., 2001; Anderson et al., 2005). The use of LSU rRNA-targeted molecular probes in both a whole cell (WC) format using fluorescence in-situ hybridization (FISH) and a sandwich hybridization assay (SHA) using a semi-automated format have been demonstrated as effective means to detect the presence of harmful algae (Scholin et al., 1996, 1999; Tyrrell et al., 2002; John et al., 2005; Kim et al., 2005; Mikulski et al., 2005). The WC method involves fluorescently labeling intact cells with species-specific oligonucleotide probes and visualizing target cells using epifluorescence microscopy. This method increases the speed of cell enumeration and permits visual discrimination between morphologically similar species (Rhodes et al., 2004; Mikulski et al., 2005; John et al., 2005). The SHA method detects the presence of target rRNA in a sample lysate by employing a “sandwich” of probes, one that is species-specific and captures the target molecules on a solid support and a second (often less specific; e.g., genus level) ‘signal’ probe that binds to another site on the same rRNA molecule and facilitates quantification based on colorimetric detection (Scholin et al., 1996; Anderson et al., 2005). This latter method circumvents the need for time-consuming microscopic evaluation of material and enables detection of target species at very low ‘pre-bloom’ cell densities by concentrating large volumes of water prior to preparing the sample lysate. Oligonucleotide probes applied using WC and/or SHA have been employed successfully for HAB monitoring and extensive field data sets are available (Rhodes et al., 1998; Scholin et al., 1999; Matweyou et al., 2004; Anderson et al., 2005; Ayers et al., 2005; O’Halloran et al., 2006). The SHA has also been modified by inclusion of a nuclease protection assay for detecting species of the toxic dinoflagellate genus *Prorocentrum*, when the traditional SHA approach was unsuccessful (Cai et al., 2006).

The primary aim of the current study was to develop species-specific, LSU rRNA-targeted molecular probes for the detection of *C. polykrikoides* in Korean coastal waters. Our immediate objectives were to incorporate these probes into a rapid, reliable SHA assay capable of identifying this species while still a minor component of the phytoplankton assemblage and to develop a corresponding WC method for visual confirmation. Ultimately, these assays will be implemented by both surveillance and research programs to provide valuable near-real time data on the abundance and distribution of *C. polykrikoides*. This information will support efforts to monitor and model bloom populations, thereby providing the advanced warning needed for an effective management/mitigation strategy.

## 2. Materials and methods

### 2.1. Culture growth and maintenance

Isolates of *C. polykrikoides* originating from different locations along the Korean coast and obtained in different years were provided by the Korean National Fisheries Research and Development Institute (Busan, Republic of Korea) (Table 1). Three *C. polykrikoides* isolates from North America and one from Hong Kong were obtained from the Colección de Dinoflagelados Marinos (CODIMAR, La Paz, BCS, Mexico; isolate CPPV-1), the Woods Hole Oceanographic Institution (Woods Hole, MA, USA; isolates CPCB10 and HK), and the Dept. of Fisheries and Oceans Canada (Naniamo, BC, Canada; isolate CS01) for phylogenetic comparisons, probe design, and cross-reactivity testing. All cultures were maintained in 25 mL batch cultures in 50 mL borosilicate glass tubes on a 16 h:8 h light:dark cycle with a photon flux density of  $\sim 75 \mu\text{mol m}^{-2} \text{s}^{-1}$  (model QSL; Biospherical Instruments, San Diego, CA, USA). The five Korean isolates were maintained at 20 °C in seawater (30 psu) amended with *f/2* nutrients (–Si) and trace metals with 0.01  $\mu\text{M}$  selenium added (Guillard, 1975). Growth medium for the remaining isolates was amended with L1 nutrients (Guillard and Hargraves, 1993) and these cultures were maintained at appropriate temperatures – CS01/15 °C, CPPV-1/25 °C, HK and CPCB10/20 °C. Cultures of additional taxa used for cross-reactivity testing were maintained as described for the Korean *C. polykrikoides* isolates; silicate (104  $\mu\text{M}$ ) was added to diatom cultures.

### 2.2. DNA extraction, PCR amplification, and sequencing

Algal DNA was extracted according to a modified CTAB/PCI/CI protocol (Scholin et al., 1994b) described previously by Mikulski et al. (2005). Hypervariable regions D1–D3 of the LSU rDNA were amplified by polymerase chain reaction (PCR) using primers D1R and D3Ca (Lenaers et al., 1989). Details of the PCR protocol are given in Mikulski et al. (2005). Amplification products were purified (Wizard<sup>®</sup> PCR prep kit, Promega Corp., Madison, WI, USA) and sequenced in both directions using four internal primers (D2Ra, D2C, D1C, Scholin et al., 1994b; D3Rf2, Mikulski et al., 2005), in addition to the above PCR primers. Sequencing was performed on an ABI Prism 3730xl sequencer (Applied Biosystems, Foster City,

Table 1  
Details of *Cochlodinium* isolates employed and sequenced in this study

Strain	Date isolated	Location
<i>C. poly</i>	Sept. 2000	Namhae, Republic of Korea
PP-3	Sept. 2001	Tongyeong, Republic of Korea
PP-6	Sept. 2001	Busan, Republic of Korea
CP 2002	Aug. 2002	Busan, Republic of Korea
CP 2002-1	Aug. 2002	Namhae, Republic of Korea
CS01	Sept. 1999	Coal Harbour, BC, Canada
HK	Unknown	Hong Kong
CPCB10	Sept. 2001	Cotuit Bay, MA, USA
CPPV-1	Unknown	Bahía de La Paz, B.C.S., México

CA, USA) by SeqWright DNA Technology Services (Houston, TX, USA). Consensus sequences were generated and checked using Chromas (v.1.45; <http://www.technelysium.com.au/chromas.html>), and then aligned in BioEdit (v.5.0.9; Hall, 1999). GenBank accession numbers for all isolates used herein are given in Iwataki et al. (2008).

### 2.3. Whole cell (WC) hybridization probe design

Consensus sequences for all Korean *C. polykrikoides* isolates were aligned against each other, an additional *C. polykrikoides* isolate from GenBank (accession no. [AF067861](http://www.ncbi.nlm.nih.gov/GenBank/AF067861)), and other representatives of the Gymnodiniaceae to identify potential probe target sites unique to *C. polykrikoides*. Five oligonucleotide probes (18–22 bases) were designed and compared against the GenBank database using a nucleotide BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>). Probes were synthesized by Oligos Etc. (Wilsonville, OR, USA) with a 5' 6-carboxyfluorescein label and evaluated in a WC hybridization assay. Probes designated as Cp-C1, Cp-C2, and Cp-C4 targeted the D3 hypervariable region, while probes Cp-C3 and Cp-C5 targeted the D1 hypervariable region (Table 2).

#### 2.3.1. WC hybridization optimization and cross-reactivity testing

Fluorescently-labeled, *C. polykrikoides*-specific probes were tested in the WC format as modified from Miller and Scholin (2000) by Mikulski et al. (2005). Briefly, culture or field material was fixed for 1 h with an equal volume of saline ethanol solution [22 mL 95% ethanol, 5 mL deionized water, 3 mL 25× SET buffer (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris, pH 7.8)], followed by exposure to formaldehyde (3.7% final concentration) for at least 20 min prior to filtration (13 mm diameter, 2 µm black polycarbonate membrane; Osmonics/MSI Poretics™; Osmonics, Minnetonka, MN, USA) using a custom-made manifold (see Miller and Scholin, 1998). Cells were rinsed with 1 mL of hybridization buffer (5× SET, 0.1% IGEPAL-CA630, 25 µg/mL poly A) and 500 µL of probe (5 ng/µL) dissolved in hybridization buffer were added. Filters were hybridized for 1.5 h at 45 °C in a hybridization oven (Hybridiser HB-1D; Techne, Inc., Princeton, NJ, USA), washed

for 10 min at 47 °C with 1 mL of pre-warmed 5× SET to remove excess probe, and mounted on glass microscope slides with SlowFade Light antifade solution (Molecular Probes Inc., Eugene, OR, USA). Samples were observed on a Zeiss microscope (Axiovert S100; Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) fitted with a FITC filter set (excitation 465–495 nm, emission 515–555 nm, dichroic 505 nm; Chroma Technology Corp., Brattleboro, VT, USA) and digital images were obtained using a cooled CCD camera (Spot model 1.4.0; software version 2.1.2; Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

The labeling and signal strength of *C. polykrikoides*-specific probes applied in a WC format were evaluated initially using isolate PP-3, followed by all other available *C. polykrikoides* isolates (Table 1). Positive and negative control probes were applied concurrently with each novel probe tested (Miller and Scholin, 2000). Species-specificity was assessed using several members of the Gymnodiniaceae as well as other dinoflagellate, raphidophyte, and diatom taxa (Table 3), which included Korean isolates of species frequently present during *C. polykrikoides* blooms.

### 2.4. Sandwich hybridization assay (SHA) probe design

Those probes identified as optimal for the WC format were modified for use in the SHA by lengthening to obtain an estimated  $T_m$  (melting temperature) of 70–75 °C (OligoTech software). Four species-specific probes were synthesized with a 5' biotin label linked to a C9 spacer by Oligos Etc. for use as capture probes (Table 4). These same sequences were modified to produce signal probes by labeling the 5' end with digoxigenin (DIG) linked to a C9 spacer. Additional *Cochlodinium*-specific signal probes targeting the D1 and D3 regions were designed as described above. Signal probe target sequences were located approximately 100 bases (upstream or downstream) from corresponding capture probes. Two oligonucleotides of 62 and 77 bp and complementary to capture–signal probe combinations served as positive controls.

#### 2.4.1. SHA optimization

Capture–signal probe combinations were evaluated in the SHA as described previously in Scholin et al. (1999) and Tyrrell

Table 2  
Summary of oligonucleotide probes designed against *Cochlodinium polykrikoides*

Probe	Probe sequence 5'–3'	Aligned position	Variable region	Probe reactivity against <i>C. polykrikoides</i> PP-3
Cp-C1	<b>G</b> CCC <b>AAG</b> C <b>A</b> CT <b>C</b> GCACAT	803–820	D3	+
Cp-C2	GCGCATGG <b>G</b> TTT <b>G</b> C <b>A</b> GCCC	817–838	D3	++
Cp-C3	TGTAC <b>G</b> GAG <b>G</b> GCTGCAGA	210–227	D1	–
Cp-C4	CTC <b>G</b> CAATTGAT <b>C</b> A <b>G</b> TCCGGT	865–884	D3	+++
Cp-C5	GGTCTC <b>A</b> AAC <b>A</b> CGTATTTA	289–307	D1	+
Positive	GWATTACCGCGGCKGCTG	–	N/A	++
Negative	CAGCMGCCGCGUAAUWC	–	N/A	–

Probe sequences are the reverse complement of the aligned sequence. The aligned position refers to an alignment of *C. polykrikoides* isolate PP-3 against *Prorocentrum micans* (GenBank accession no. [AF260377](http://www.ncbi.nlm.nih.gov/GenBank/AF260377)) using BioEdit. Aligned positions may not correlate with the length of the probe due to inserted alignment gaps in either sequence. Bases in bold and underlined correspond to sequence differences between *C. polykrikoides* and the closely-related species, *Karenia brevis*. The positive control probe targets a SSU-targeted universal sequence and the negative control is the reverse complement of the positive (Miller and Scholin, 2000). N/A, not applicable.

Table 3  
Species and isolates used to test *Cochlodinium polykrikoides*-targeted probes for cross-reactivity and specificity

Species	Isolate	Isolation locale	Cp-C1	Cp-C2	Cp-C3	Cp-C4	Cp-C5	SHA
<i>Cochlodinium polykrikoides</i>	C. poly, PP-3 PP-6, CP 2002, CP 2002-1,HK	See Table 1	+	+++	–	+++	+	++
<i>Cochlodinium polykrikoides</i>	CPCB10, CPPV-1	See Table 1	++	++	–	+++	–	++
<i>Cochlodinium</i> sp.	CS01	See Table 1	–	–	–	–	–	–
<i>Alexandrium tamarense</i>	NF-F-ATA-3	Southern Coastal Sea, Korea	–	–	nt	–	–	–
<i>Gyrodinium impudicum</i> *	NF-F-GIM-1	Narodo, Korea	nt	nt	nt	–	nt	–
<i>Heterocapsa triquetra</i>	D-009	Yosu, Korea	nt	nt	nt	–	nt	–
<i>Prorocentrum micans</i> **	NF-F-PMC-1	Tongyeong, Korea	nt	nt	nt	–	nt	–
<i>Prorocentrum minimum</i> **	NF-F-PMI-1	Tongyeong, Korea	nt	nt	nt	–	nt	–
<i>Scrippsiella trochoidea</i>	D-083	Yosu, Korea	nt	nt	nt	–	nt	–
<i>Pyramimonas</i> sp.	NF-F-PYR-1	Busan, Korea	nt	nt	nt	–	nt	–
<i>Chattonella antiqua</i>	NF-F-CAN-1	Jangheung, Korea	–	–	nt	–	–	–
<i>Prymnesium parvum</i>	H-20	JeJudo, Korea	nt	nt	nt	nt	nt	–
<i>Chaetoceros didymus</i>	B-217	Namhae, Korea	nt	nt	nt	nt	nt	–
<i>Gymnodinium</i> sp.	NF-F-Gym-sp-2	Tongyeong, Korea	–	–	nt	–	–	nt
<i>Heterosigma akashiwo</i> **	NF-F-HAK-1	Masan, Korea	–	–	nt	–	–	–
<i>Heterosigma akashiwo</i>	CCMP 1870	Long Beach, CA, USA	nt	nt	nt	nt	nt	–
<i>Gonyaulax polygramma</i> **	NF-F-GPO-0	Busan, Korea	–	–	nt	–	–	nt
<i>Karenia brevis</i>	NOAA-1	Charlotte Harbor, FL, USA	–	–	–	–	–	–
<i>Karenia brevis</i>	C2	Charlotte Harbor, FL, USA	nt	nt	nt	nt	nt	–
<i>Karenia mikimotoi</i>	G303 ax-2	Suo Nada, Japan	nt	–	nt	–	nt	–
<i>Gymnodinium simplex</i>	CCMP420	Gulf of Tehuantepec, Mexico	nt	–	nt	–	nt	–
<i>Gymnodinium catenatum</i> *	SE-GC	Senzaki Bay, Japan	nt	–	nt	–	nt	–
<i>Alexandrium fundyense</i>	GTCA-29	Gulf of Maine, NH, USA	nt	–	nt	–	nt	–
<i>Akashiwo sanguinea</i>	95003	Florida Bay, FL, USA	nt	–	nt	–	nt	–
<i>Amphidinium operculatum</i>	CCMP119	Knight Key, FL, USA	nt	–	nt	–	nt	nt
<i>Pseudo-nitzschia</i> sp.	NF-F-PSE-1	Incheon, Korea	nt	nt	nt	nt	nt	–
<i>Alexandrium monilatum</i>	AM02	Gulf Port, MS, USA	nt	nt	nt	nt	nt	–
<i>Alexandrium tamarense</i>	OK905-5	Okkirai Bay, Japan	nt	nt	nt	nt	nt	–

Cp-C# refers to whole cell hybridization results. SHA refers to response in the sandwich hybridization assay. ‘\*’, morphologically similar species; ‘\*\*’, species that commonly co-occur during *Cochlodinium* blooms in Korean coastal waters; ‘–’, negative result (no binding for WC or no signal for SHA); ‘+’, positive result, number of ‘+’ symbols relative denotes strength of signal; ‘nt’, not tested.

Table 4  
Capture and signal probes for use in the sandwich hybridization assay

Probe	Sequence	LSU rDNA Variable region
5' Biotin-labeled capture probes		
Cp-C1	5' Biotin-ON – C9 – GCCCAAGCACTCGCACATATG -3'	D3
Cp-C2	5' Biotin-ON – C9 – GCGCATGGGTTTGAGCCC -3'	D3
Cp-C4	5' Biotin-ON – C9 – GTCTCGCAATTGATCAGTCGGT -3'	D3
Cp-C5	5' Biotin-ON – C9 – ATCGGTCTCAAACACGTATTTAGCTT -3'	D1
5' Digoxigenin-labeled signal probes		
Cp-S1	5' DIG – C9 – GCCCAAGCACTCGCACATATG -3'	D3
Cp-S2	5' DIG – C9 – GCGCATGGGTTTGAGCCC -3'	D3
Cp-S4	5' DIG – C9 – GTCTCGCAATTGATCAGTCGGT -3'	D3
Cp-S5	5' DIG – C9 – ATCGGTCTCAAACACGTATTTAGCTT -3'	D1
Cp-S6	5' DIG – C9 – GTCCTTTTCATCTTTCCCTCATGG -3'	D1
Cp-S7	5' DIG – C9 – ACTTTTAACTCTCTTTTCAAAGTCCTTTTCATC -3'	D1
Cp-S8	5' DIG – C9 – GACGGGTGGGAAAGAACATTTG -3'	D3
Cp-S9	5' DIG – C9 – TAGTTCACCATCTGGGTCCTA -3'	D3
Complementary control oligonucleotides		
CpCOMP-1	5' – AAGCTAAATACGTGTTTGAGACCGATAGCACACA AGTACCATGAGGGAAAGATGAAAAGGAC -3'	D1
CpCOMP-2	5' – GGGCTGCAAACCCATGCGCGCAACGAAAGTGACTGC TGAGATCTTTGACACGAAACCGACTGATCAATTGCGAGAC -3'	D3

Probe sequences are the reverse complement of the target sequence. The complementary control oligonucleotides are identical to the target sequences.

et al. (2001), with modifications as given by Anderson et al. (2005). Briefly, *C. polykrikoides* cells were filtered gently onto a 25 mm diameter, 0.45  $\mu\text{m}$  Durapore membrane (Millipore Corp., MA, USA) and either placed in a 2 mL microcentrifuge tube and processed immediately or transferred to a cryovial and stored over  $\text{LN}_2$  (vapor phase). Fresh samples or frozen filters warmed to room temperature were combined with 1.5 mL of lysis buffer (SSP; Saigene Corp., Seattle, WA, USA), incubated for 5 min at 85 °C (vortexing after 2.5 min), passed through a 0.45  $\mu\text{m}$  Durapore syringe filter, and cooled to room temperature. Three 250  $\mu\text{L}$  aliquots of this lysate diluted appropriately with lysis buffer were placed in adjacent wells of a 96-well plate (row H), which was then mounted on an automated sample processor (Saigene Corp.) to conduct the pre-programmed assay (total time 1 h). Rows of the 96-well plate (250  $\mu\text{L}$  per well) were filled as follows: Row A – horseradish peroxidase (HRP) substrate; Rows B and C – wash buffer E; Row D – anti-DIG antibody–HRP conjugate; Row E – wash buffer E; Row F – DIG-labeled signal probe; Row G – biotin-labeled capture probe; Row H – sample lysate (Anderson et al., 2005). All SHA reagents are available through Saigene Corp. Upon completion of the assay, signal strength was measured as optical density (OD) on a microplate reader (FLUOstar; BMG Labtechnologies, Durham, NC, USA) at 650 nm, followed by addition of 50  $\mu\text{L}$  of 10%  $\text{H}_2\text{SO}_4$  and measured again at 450 nm.

Four capture and eight signal probes were tested in 14 combinations (one capture/one signal) as constrained by location on the LSU rRNA molecule (e.g., pairing of D1 region capture and signal probes). Multiple signal probe combinations were also assessed for their ability to enhance signal strength. Complementary oligonucleotides (described above) were used to monitor performance of the assay and sandwich hybridization processor. Cell number (*C. polykrikoides*) in the lysate was held constant when comparing OD values among capture–signal probe combinations.

Sandwich hybridization assays performed at NFRDI, Busan, Korea, utilized sealed, pre-filled 96-well plates custom manufactured by Saigene Corp. containing *C. polykrikoides*-specific capture and signal probes described herein.

#### 2.4.2. Cross-reactivity and background testing for SHA

Cross-reactivity tests were performed utilizing the optimal combination of capture–signal probes (Section 2.4.1) and included all *Cochlodinium* cultures (Table 1), as well as representatives from several additional algal classes (Table 3). At least 5000 cells of each taxon contained in 250  $\mu\text{L}$  of lysate were placed in triplicate wells. Average OD readings (450 nm)  $\leq 0.01$  units above an SSP control (buffer only, no cells) were considered to be negative.

The potential background interference of natural seawater was evaluated using whole water samples collected near the NFRDI laboratory (Busan, Korea). Combinations of two volumes (5 L and 1 L) of this seawater and two quantities (15,000 cells and 1200 cells) of *C. polykrikoides* cells (i.e., 5 L/15,000 cells, 5 L/1200 cells, 1 L/15,000 cells, and 1 L/1200 cells) were filtered in triplicate and placed over  $\text{LN}_2$  for later

analysis. Triplicate samples consisting only of natural seawater or *C. polykrikoides* cells were stored as controls.

Signal interference from known amounts of a non-target organism was also assessed. The dinoflagellate, *Akashiwo sanguinea* (isolate 95003), was added at a range of levels (12,000, 1200, 120, and 12 cells) to a known quantity of *C. polykrikoides* cells (1200 cells), filtered onto triplicate 25 mm diameter, 0.45  $\mu\text{m}$  Durapore membrane filters, and stored over  $\text{LN}_2$ . Triplicate filters containing only *A. sanguinea* (24,000 cells per filter) or *C. polykrikoides* (1200 and 2400 cells per filter) were archived as controls.

#### 2.4.3. Standard curves and limit of detection for SHA

Standard curves were generated using laboratory cultures of *C. polykrikoides* (PP-3 isolate) by placing a known quantity of cells on a filter and preparing a lysate (see Section 2.4.1), serially diluting with SSP buffer to desired concentrations, and running in triplicate on the assay. Replicate cell counts ( $n = 3\text{--}5$ ) were performed on Lugol's fixed samples by two different individuals for greater accuracy. Cell concentrations varied from 78 to 5000 cells/mL and were within the empirically determined linear range of the assay, thus maximizing assay detection capability. Curves using a range of lower cell concentrations with finer scale resolution (16–2500 cells/mL) were produced in order to determine the assay limit of detection (i.e., LOD; OD indistinguishable from SSP control).

#### 2.5. Sandwich hybridization vs. whole cell hybridization

A comparison between the sandwich hybridization assay and whole cell assay was performed using laboratory cultures of *C. polykrikoides*. One aliquot was removed from the culture for microscope counts (see Section 2.4.3) and the remainder divided between sandwich hybridization and whole cell assays. Multiple independent experiments were conducted to compare the accuracy of quantification by the two methods ( $n = 3$  for SHA,  $n = 2$  for WC).

#### 2.6. SHA and WC testing on field samples

Field samples of known volume (80–100 L) were collected during two surveys off the southern Korean coast (19–23 June, 2006 and 12–16 July, 2006) (Fig. 1) aboard the R/V Tamgu 17 using a portable submarine pump fitted with a flow meter. Whole water was pre-screened through a 330  $\mu\text{m}$  mesh sieve, concentrated gently to about 300 mL on a 10  $\mu\text{m}$  sieve, and then reduced in volume to 10–20 mL over a smaller diameter 10  $\mu\text{m}$  sieve before filtering onto a 25 mm diameter, 0.45  $\mu\text{m}$  Durapore membrane filter and freezing in  $\text{LN}_2$  vapor onboard. Samples were transported to NFRDI and stored at  $-70$  °C prior to analysis by SHA. Duplicate samples were taken when weather permitted, archived at NFRDI, and sent on dry ice to NOAA (Charleston, SC, USA) for inter-laboratory comparison of SHA results.

Field material was also collected during the above surveys for direct light microscope counts and for WC hybridizations.

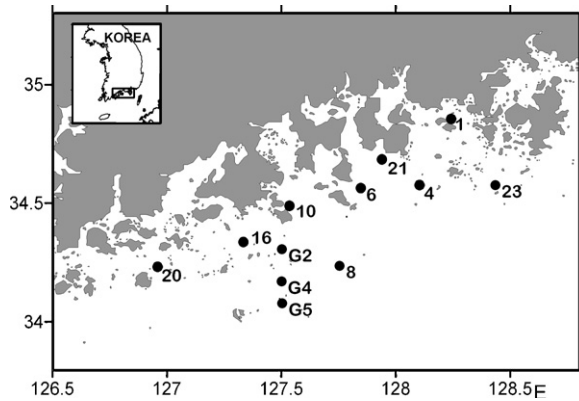


Fig. 1. Locations of field sampling stations along the south coast of Korea.

For direct counts, 1 L of seawater was concentrated to 1 mL and *C. polykrikoides* cells were enumerated in 100–200  $\mu\text{L}$  onboard. For WC hybridizations, 2 L of seawater were concentrated to 3 mL and combined with 3 mL of saline ethanol fixative and 600  $\mu\text{L}$  of 35% formalin. These samples were stored at 4 °C until analyzed at NFRDI according to the method described above (Section 2.3.1).

### 3. Results

#### 3.1. Sequence comparisons

The LSU rDNA sequences spanning the D1–D3 hyper-variable regions (955–980 bp) were identical for all six *C. polykrikoides* isolates originating from Korea and Hong

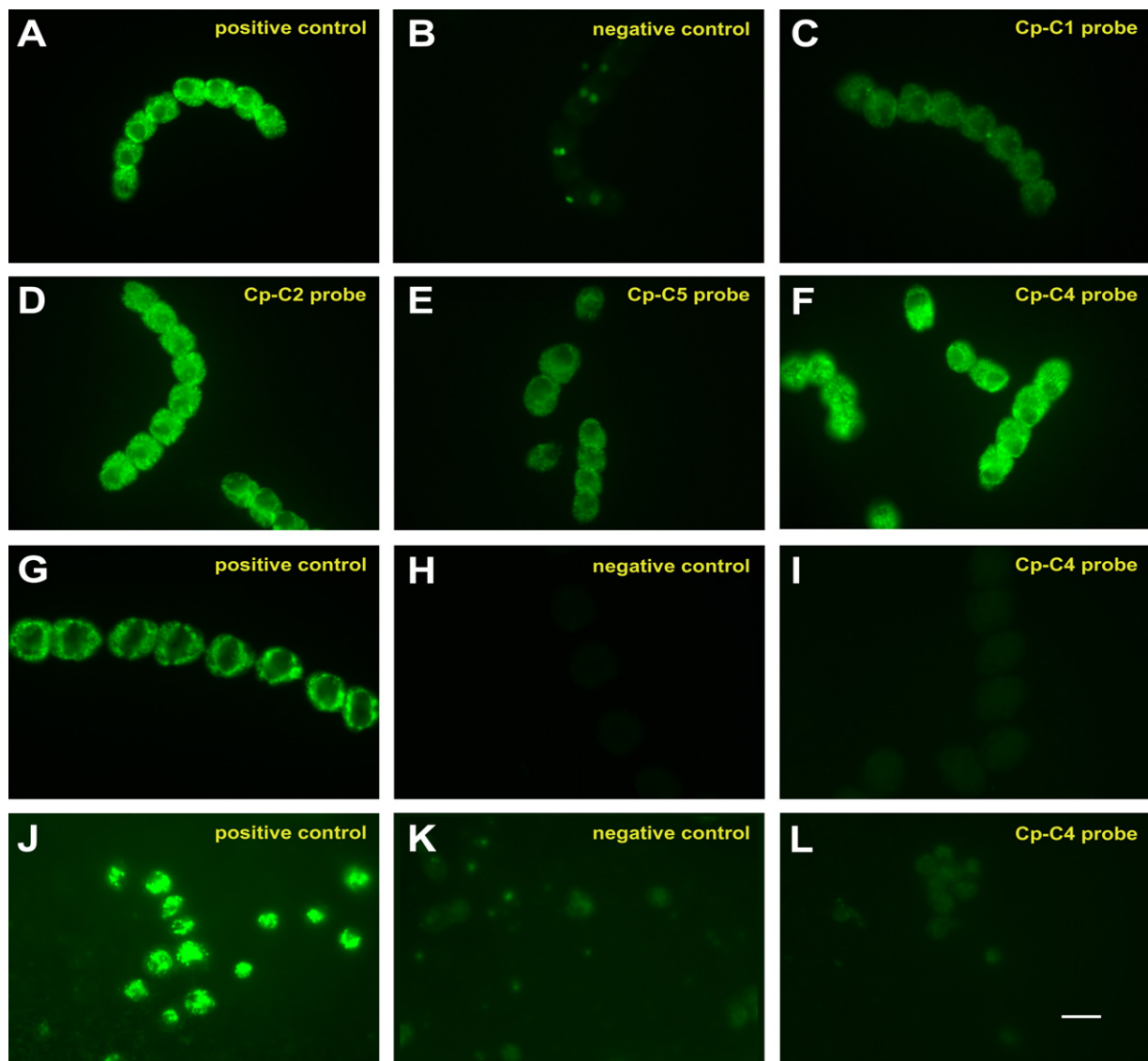


Fig. 2. Epifluorescence images showing signal from FITC-labeled probes applied in a whole cell hybridization format. (A–F) *Cochlodinium polykrikoides* (isolate PP-3). (A) Positive control probe targeting universally conserved SSU rRNA sequence. (B) Negative control probe (reverse complement of positive control; Miller and Scholin, 2000). (C–F) Four of five species-specific probes tested; result for probe Cp-C3 is not shown (equivalent to negative control (B)). (G–I) *Gymnodinium catenatum* and (J–L) *Heterosigma akashiwo* labeled with positive, negative, and Cp-C4 probes to demonstrate negative cross-reactivity test results for representative species. Scale bar located in Fig. 2L = 40  $\mu\text{m}$  and applies to all micrographs.

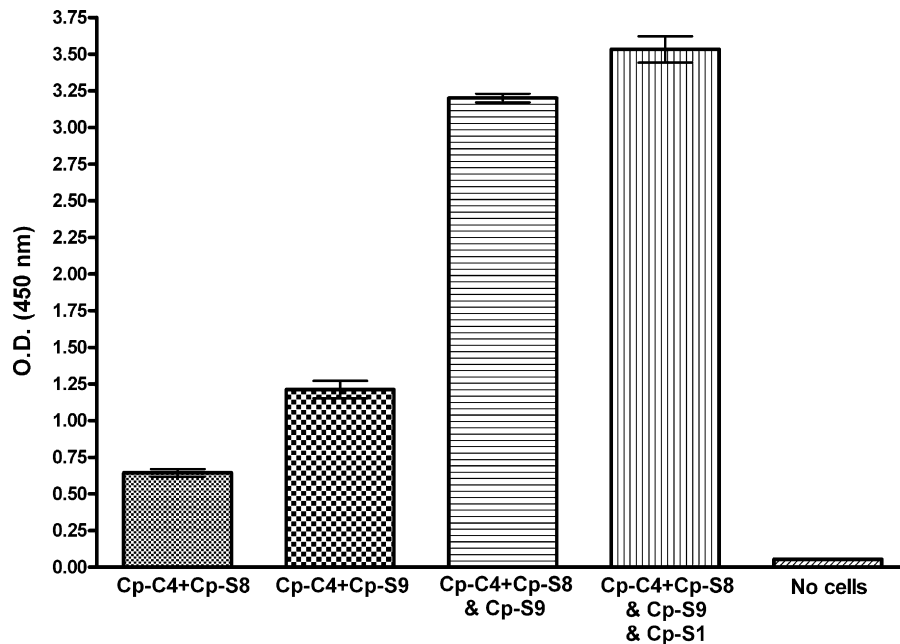


Fig. 3. Results of signal amplification experiments testing an increasing number of signal probes combined with the Cp-C4 capture probe. The same sample lysate was used to evaluate all capture–signal probe combinations. Values are mean ( $n = 3$ )  $\pm$  S.E.

Kong, as well as a partial *C. polykrikoides* sequence (D1, D2; 734 bp) deposited in Genbank (accession no. [AF067861](#)). Sequences for two of the N. American *Cochlodinium* isolates, CPPV-1 and CPCB10, were identical to each other, but showed only 91.3% similarity to the Korean isolates. The British Columbia isolate (CS01) was the most disparate with 76.4% similarity to the Korean isolates and 75.0% similarity to the other N. American isolates. Detailed phylogenetic analyses of these and other *Cochlodinium* strains are provided by Iwataki et al. (2008).

### 3.2. Whole cell (WC) hybridization probe design and testing

Each *C. polykrikoides*-specific probe sequence had at least three base pair differences from *Karenia brevis*, the taxon most similar phylogenetically for which sequence data were available (Table 2). Of the five LSU rRNA-targeted probes tested in the WC format, four successfully labeled *C. polykrikoides* isolate PP-3. The fluorescence signal strengths were as follows: Cp-C4 > Cp-C2 > Cp-C1 = Cp-C5, with the brightest labeling somewhat greater than the positive control (Fig. 2; Tables 2 and 3). Probe Cp-C5 did not label the N. American isolates CPCB10 and CPPV-1, yet the Cp-C4 signal was again brighter than the positive control (based on visual comparison). None of the probes showed binding to the British Columbia isolate (CS01), while Cp-C3 did not label any *Cochlodinium* isolate and was not evaluated further (Table 3). Among the other 13 genera and 19 species tested, representing dinoflagellates, diatoms, and raphidophytes, and including both morphologically similar (e.g., Fig. 2G–I) and frequently co-occurring (e.g., Fig. 2J–L) species, none showed cross-reaction with the probes tested (Table 3).

### 3.3. Sandwich hybridization assay (SHA) probe design and testing

Fourteen pairs of capture and signal probes (Table 4) were tested using the SHA, with the capture probe Cp-C4 and signal probe Cp-S9 pair yielding the strongest response. Multiple signal probes were then tested in eight permutations. A combination of three signal probes (Cp-S8, Cp-S9, and Cp-S1) paired with capture probe Cp-C4 produced the greatest signal strength (Fig. 3). This probe combination was employed in all subsequent SHAs and was included in the custom, pre-filled SHA plates produced by Saigene Corp. for use at NFRDI.

No cross-reactivity was observed among the 15 genera and 21 species of algae tested on the *C. polykrikoides*-specific SHA, including the *Cochlodinium* isolate from British Columbia, Canada (Table 3). Moreover, neither natural seawater (1 L and 5 L equivalents) nor varying amounts of *Akashiwo sanguinea* had any effect on quantification of *C. polykrikoides* by SHA (data not shown). Negative controls comprising 5 L and 1 L of Korean coastal water, or 24,000 *A. sanguinea* cells, filtered onto Durapore membranes did not generate an OD signal above the SSP control (i.e., no *C. polykrikoides* cells).

### 3.4. Standard curves and limit of detection for SHA

Two standard curves were generated using laboratory cultures of *C. polykrikoides*. The first was used as a quantification curve across a previously determined linear response range (78–5000 cells/mL, Fig. 4) and employed for estimating cell concentrations in samples from the field as well as laboratory experiments. The second curve was used to determine the SHA limit of detection (LOD) and covered a narrower range of 16–2500 cells/mL with greater resolution.

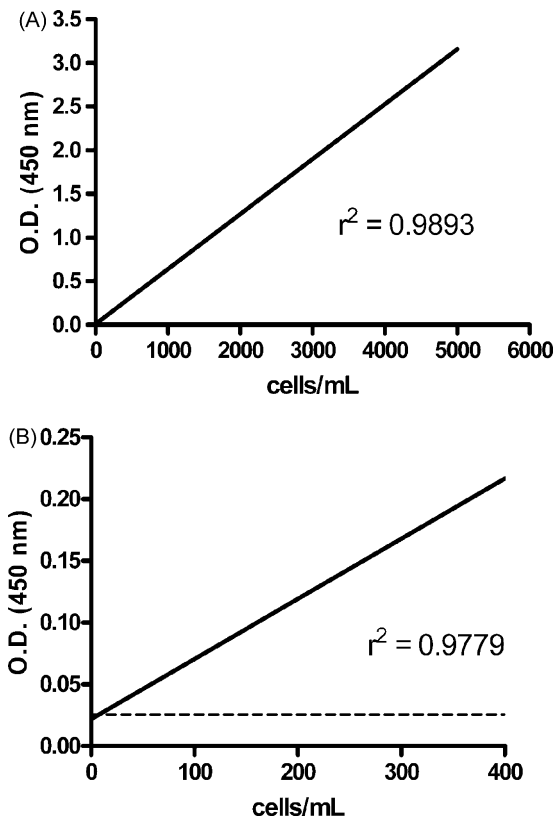


Fig. 4. (A) Sandwich hybridization assay calibration curve for *C. polykrikoides* generated using a laboratory culture (isolate PP-3,  $n = 3$ ). (B) Lower end of the limit of detection (LOD) curve generated using *C. polykrikoides* isolate PP-3 ( $n = 3$ ). Lower cell numbers and finer scale resolution were used for the LOD curve, whereas the calibration curve was used to maximize the upper limit of cell detection. A total of 250  $\mu$ L was placed in each well. (—) Lysis buffer only value.

The LOD, calculated based on a 3:1 signal:background ratio against the SSP control, was  $115 \pm 53$  cells/mL of extract (mean  $\pm$  S.D.,  $n = 3$ ), which equates to  $\sim 29$  cells/well (range 16–42 cells/well).

Table 5  
Results (cells/L) from two-field surveys conducted during June and July 2006

Sample type	Station designation											
	1	4	6	8	10	16	20	21	23	G2	G5/G4*	
Survey 1: June 19–23, 2006												
SHA	ND	2.4	ND	ND	2.9	ND	ND	ND	ND	1.4	ND	
WC	n/a	2	n/a	n/a	ND	n/a	n/a	n/a	n/a	ND	n/a	
LM	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
SHA NOAA	n/a	n/a	ND	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	
Survey 2: July 12–16, 2006												
SHA	ND	ND	ND	ND	200	ND	ND	ND	ND	400	180	
WC	n/a	n/a	n/a	n/a	23	n/a	n/a	n/a	n/a	525	416	
LM	ND	ND	ND	ND	ND	ND	ND	ND	ND	1100	230	
SHA NOAA	n/a	n/a	n/a	n/a	+	22.1	0.8	n/a	n/a	n/a	215	

SHA refers to 100 L water samples filtered onto 0.45  $\mu$ m Durapore membranes, frozen over LN<sub>2</sub>, and tested by SHA at NFRDI, Busan, Korea. WC counts refer to 2 L water samples concentrated to 3 mL and combined with 3 mL of saline ethanol fixative and 600  $\mu$ L of 35% formalin, with 1.65 mL samples filtered onto Durapore filters to generate lysate for hybridization (equivalent to 500 mL sample hybridized). LM counts refer to 1 L samples concentrated to 1 mL and enumerated onboard ship using light microscopy. SHA NOAA denotes duplicate samples taken during the survey, frozen over LN<sub>2</sub>, shipped on dry ice to NOAA, Charleston, SC, USA, and analyzed by SHA. 'ND', sample below limit of detection; 'n/a', duplicate samples not available; and, '+', positive sample, but above the linear range of the SHA calibration curve. \*, due to weather conditions, station G4 was substituted for G5 during the second survey.

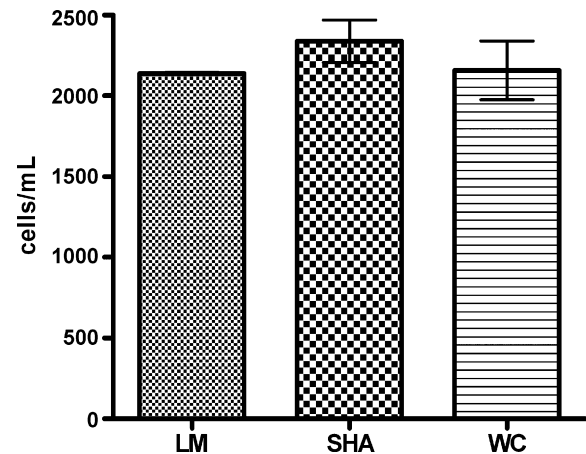


Fig. 5. Comparison of traditional direct light microscope counts (LM), sandwich hybridization assay (SHA), and whole cell (WC) hybridization results for quantification of *C. polykrikoides* isolate PP-3 (LM counts on five independent samples,  $n = 3$  for SHA,  $n = 2$  for WC). Values are mean  $\pm$  S.D.

### 3.5. Sandwich hybridization vs. whole cell hybridization

Quantification was consistent among the three methods evaluated (SHA, WC hybridization, and direct light microscope counts) using cultured *C. polykrikoides* (PP-3 isolate). The average value determined by direct counts was 2138 cells/mL ( $n = 5$ , S.D. =  $\pm 7.071$ ), whereas the means generated by SHA and WC hybridization were 2292 cells/mL ( $n = 2$ , S.D. =  $\pm 181.7$ ) and 2158 cells/mL ( $n = 3$ , S.D. =  $\pm 131.9$ ), respectively (Fig. 5).

### 3.6. Field Studies

Field samples were collected from 11 sites along the southern coast of Korea (Fig. 1). During the first survey (June 2006), *C. polykrikoides* cells were detected at three stations using the SHA at the following estimated concentrations: Stn. 4, 2.4 cells/L; Stn. 10, 2.9 cells/L; Stn. G2, 1.4 cells/L

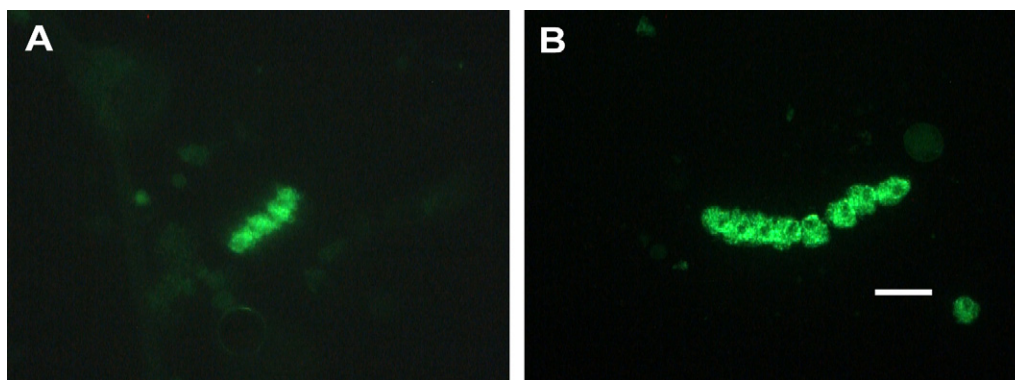


Fig. 6. Chains of *C. polykrikoides* were detected by whole cell hybridization (Cp-C4) in seawater samples collected at (A) Namhae, Stn. 4, June 20, 2006 and (B) Naro, Stn. G2, July 13, 2006. Scale bar located in Fig. 6B = 100  $\mu\text{m}$  and applies to both micrographs.

(Table 5). Direct light microscope counts of Lugol's preserved samples at these three stations did not detect any cells. A duplicate sample taken at Stn. 6 and analyzed at NOAA by SHA tested negative for *C. polykrikoides*, confirming the negative value obtained by NFRDI. WC hybridization showed 2 cells/L at Stn. 4, with no cells detected at Stns. 10 or G2. Over the second survey (July 2006), *C. polykrikoides* was also detected by SHA at three stations but at higher levels than in June: Stn. 10, 200 cells/L; Stn. G2, 400 cells/L; Stn. G4, 180 cells/L (Table 5). A duplicate Stn. 10 sample tested positive for *C. polykrikoides* at NOAA, but could not be quantified as the OD reading fell outside the linear range of the calibration curve and additional lysate was unavailable for further analysis. A duplicate sample from Stn. G4 contained 215 cells/L. Positive results were also obtained at NOAA for duplicate samples from Stn. 16 and Stn. 20 (22 and 0.8 cells/L, respectively), but tested negative at NFRDI. WC hybridization revealed 23 cells/L at Stn. 10, 525 cells/L at Stn. G2, and 416 cells/L at Stn. G4. While quantitative agreement with the SHA was variable, the presence of *C. polykrikoides* cells was confirmed by WC hybridization during both June and July surveys (Fig. 6). Light microscope counts yielded 1100 cells/L at Stn. G2 and 230 cells/L at Stn. G4, but did not detect cells at Stn. 10.

#### 4. Discussion

We have successfully developed species-specific LSU rRNA-targeted oligonucleotide probes for detection of *C. polykrikoides* from Korean waters using both WC and SHA formats. The WC method has the advantages of visualizing labeled, intact cells to verify species' morphology, excluding signal due to binding of free rRNA and/or non-target material, and quantifying cell number. Samples can also be prepared in the field and stored for later analysis. Nonetheless, this technique is not presently amenable to automation and, depending on biomass levels, can be limited in terms of sample volume filtered and analyzed. As an alternative complementary approach, *C. polykrikoides*-specific probes were integrated into an automated SHA, which was capable of detecting as few as  $\sim 30$  cells per well in a 96-well plate. The method, following sample preparation, is fully automated, requires little technical expertise, and allows analysis of

samples onboard ship in near-real time (1 h to complete) or archival at  $-70^\circ\text{C}$ . Furthermore, since material collected on a filter is taken up in a lysate, particles contained in large volumes of whole water can be tested in one sample. Herein, we have detected *C. polykrikoides* in the field at concentrations of 1–3 cells/L, given our ability to concentrate 100 L of seawater onto a single filter, or the equivalent of 100–300 cells/L had only 1 L been filtered. The SHA lower detection limit of several cells per liter is critical for identifying areas of *Cochlodinium* bloom initiation and tracking early bloom development, which will aid in formulating predictive models for this species in Korean coastal waters. At higher cell concentrations and correspondingly lower sample volumes, routine-monitoring efforts will be facilitated by the near-real time data provided by this assay. Limitations of the SHA include the inability to identify target organisms visually and the generation of signal from extracellular rRNA in the absence of intact cells. Overall, the species-specific probes and assays developed during this study represent powerful tools for the reliable and rapid detection of the fish-killing dinoflagellate, *C. polykrikoides*, over a range of concentrations relevant to both coastal managers and the research community.

##### 4.1. Sequence comparisons

The LSU rDNA regions targeted for probe development (D1–D3) were selected based on their ability to resolve species-level differences within microalgal groups (e.g., Scholin et al., 1994b; Lenaers et al., 1989). Nevertheless, Scholin et al. (1994a) reported that sequences of *Alexandrium* spp. isolated from the same geographic region were likely to be more similar than if compared with those from other areas, regardless of morphologically-based species designation. Indeed, a similar trend in LSU rDNA sequence divergence was apparent when comparing *Cochlodinium* isolates originating from Korea and Hong Kong relative to those from N. America (see Results Section 3.1), and this finding has been corroborated with additional LSU rDNA sequence data from these and other N. American *Cochlodinium* strains (Iwataki et al., 2008). Kim et al. (2004) also described such a pattern based on SSU rDNA sequences, whereby four Korean *C. polykrikoides* isolates obtained during three different years and locations were

identical over nearly 1800 bp. These authors postulated that highly variable gene loci, such as microsatellites, would be necessary to distinguish genetically among Korean isolates. The same conclusion would be adopted based on identical LSU rDNA sequences for the Korean and Hong Kong *C. polykrikoides* isolates examined during the present study.

The current work was focused on detection of *C. polykrikoides* in Korean waters, yet our sequence data and probe cross-reactivity test results yielded some useful insights on N. American strains of this organism. Sequences for two of the N. American *Cochlodinium* isolates (CPPV-1, B.C.S., Mexico; CPCB10, MA, USA) were identical, but both differed markedly from the third (CS01, BC, Canada) (see also Iwataki et al., 2008), indicating the potential to distinguish between these two groups using LSU rRNA-targeted probes. Future development of detection methods based on such probes will be important, since *Cochlodinium* red tides and associated farmed fish mortalities have been reported from locations on the west coast of N. America (Whyte et al., 2001; Gárate-Lizárraga et al., 2004). While similarly intense blooms have occurred along the U.S. east coast (Zubkoff et al., 1979; Gobler et al., 2008), there is no record of fish kills, although experiments using field material have demonstrated the potential for adverse effects on finfish as well as shellfish (Ho and Zubkoff, 1979; Gobler et al., 2008). This would suggest that apparent differences in ichthyotoxicity likely reflect the increased susceptibility of farmed fish unable to escape a bloom event, rather than reduced toxic activity among N. American *Cochlodinium* populations; however, further characterization of *Cochlodinium* toxicity is needed.

#### 4.2. Whole cell (WC) hybridization

Among the five probes designed for *C. polykrikoides*, only one (Cp-C3) did not bind to Korean isolate PP-3 in a whole cell format (Fig. 2), likely reflecting an inaccessibility of the binding site due to secondary ribosomal structure (see Miller and Scholin, 2000). This result is consistent with those for reported for other harmful species (e.g., Miller and Scholin, 1996; Tyrrell et al., 2001; Mikulski et al., 2005) and further emphasizes the need to empirically test whole cell probes. In addition, the labeling of two N. American isolates (CPCB10 and CPPV-1) by probe Cp-C2 despite a one base pair mismatch within the probe sequence, albeit yielding a weak signal (Table 3), confirmed the potential for target recognition regardless of a mismatch also as documented by others (e.g., Tyrrell et al., 2001; Mikulski et al., 2005). Cross-reactivity testing is thus imperative and evaluation of *C. polykrikoides*-specific probes herein against 14 algal genera revealed no non-specific labeling. Further, the best-performing Cp-C4 probe did not cross-react with either *Gyrodinium impudicum* or *Gymnodinium catenatum*, both morphologically similar to and potentially co-occurring with *C. polykrikoides* (Cho et al., 2001; Cho and Costas, 2004). The specificity data generated during this study for Cp-C4 in a WC format thus support its utility for the specific detection of *C. polykrikoides* in Korean coastal waters containing mixed phytoplankton assemblages.

#### 4.3. Sandwich hybridization assay (SHA)

Evaluation of 22 different capture–signal probe combinations demonstrated that three signal probes (Cp-S8, Cp-S9, Cp-S1) coupled with capture probe Cp-C4 yielded the maximum signal strength in the SHA (Fig. 3). Specificity tests of the SHA against 23 culture isolates representing 15 algal taxa, including another species of *Cochlodinium*, were all negative (Table 3, final column) and assays performed on a range of non-target cell concentrations (*A. sanguinea*, data not shown) produced no signal. Moreover, the material contained in samples of Korean coastal water obtained near the NFRDI laboratory (5 L and 1 L equivalents) did not affect the assay background level nor the quantification of *C. polykrikoides* cells added to a sample. Together these results indicate that the SHA for *C. polykrikoides* is a sufficiently specific test that shows minimal susceptibility to the matrix effects of natural water samples and non-target organisms.

The SHA limit of detection was determined to be about 29 cells per well or  $115 \pm 53$  (mean  $\pm$  S.D.) cells per mL of lysate (Fig. 4), which is very similar to that reported by Tyrrell et al. (2002) for a *Heterosigma akashiwo* SHA ( $\sim 30$  cells/well; based on 400 mL of ambient cell concentrations  $\geq 300$  cells/L). The relatively large standard deviation resulted from variation in the control (i.e., background) value between plates, coupled with calculation of the LOD based on a 3:1 signal:background ratio. In practice, cell abundances below this level can be detected, but not reliably quantified. Kim (1998) reported that Korea (i.e., NFRDI) uses a warning system when monitoring *C. polykrikoides*, which includes a “red tide attention” (50 cells/mL) and a “red tide alert” (1000 cells/mL) and allows sufficient response time for precautionary actions. Detection of *C. polykrikoides* cells at and below 50 cells/L is easily possible using the SHA developed herein, depending on the volume filtered (e.g., 10 L of 50 cells/L provides  $\sim 330$  cells per mL of lysate). Our LOD is also well below that reported by Cho et al. (2004) for detection of *C. polykrikoides* using a fluorescently-labeled antibody (i.e., 5000 cells/L), which is limited by the volume of water that can be filtered and visualized microscopically. Future efforts will be aimed at further decreasing the SHA detection limit by linking multiple DIG molecules to the signal probes as reported by other investigators (Anderson et al., 2005; Goffredi et al., 2006).

#### 4.4. Sandwich hybridization vs. whole cell hybridization

Very close agreement was demonstrated between the SHA, WC format, and direct light microscope counts for quantification of *C. polykrikoides* (Fig. 5), yet this comparison is based on a limited data set employing laboratory cultures and must be expanded. For example, Anderson et al. (2005) reported poor agreement between SHA and a WC assay below 25 cells/L (based on 8 L samples). These same methods developed for *C. polykrikoides* have not been evaluated at low cell concentrations. Moreover, previous studies have shown that growth phase and nutritional status can affect signal strength for both SHA and WC probes (Anderson et al., 1999; Tyrrell et al., 2001;

Miller et al., 2004). Similar experiments must be conducted using the *C. polykrikoides*-specific SHA and WC assay.

#### 4.5. Field studies

*C. polykrikoides* was detected off the southern coast of Korea by both SHA and WC hybridization (Table 5, Fig. 1). The former assay reported concentrations of 1–3 cells/L, demonstrating its potential application when *C. polykrikoides* cells are still a minor component of the phytoplankton assemblage. This degree of sensitivity is made possible by the ability to concentrate and process large sample volumes (100 L herein). Lysis of athecate *Cochlodinium* cells during concentration did not appear to be a problem with gentle filtration, but this possibility does warrant a more systematic evaluation. In high biomass areas, filter clogging may necessitate reducing the sample size, thereby increasing the SHA detection limit. This is certainly the case for WC assays and direct microscope counts. Excess non-target material can mask fluorescently-labeled cells in the former method and will be even more problematic for viewing unlabeled cells by light microscopy. Indeed, negative results by these two methods for field samples (corresponding to positive SHA tests) were due likely to limitations imposed by small sample volumes. From a practical standpoint, routine monitoring during typical bloom months will involve filtration of considerably lower volumes (~1–10 L), given the higher biomass levels as well as the need to minimize sampling time for optimizing spatial coverage. Once cell numbers increase above background levels and begin to approach the “red tide attention” levels noted above (i.e., 50 cells/mL; Kim, 1998), reductions in sample volume to less than 1 L should continue to yield a strong SHA signal.

Duplicate samples from selected stations sent to NOAA yielded a limited, inter-laboratory comparison of the SHA. While SHA values for Stn. G4 (July 2006) agreed very closely between laboratories (NFRDI, 180 cells/L; NOAA, 215 cells/L) and with direct microscope counts (230 cells/L), data for other stations were less consistent. In the case of Stn. G2 (July 2006), insufficient sample dilution resulted in an OD above the linear range of the calibration curve that translated into a ~3-fold lower SHA value than for the corresponding direct count. For Stn. 10 (July 2006), the difference between the SHA (200 cells/L), the WC (23 cells/L), and direct count (no cells detected) conducted by NFRDI may reflect a non-homogeneous cell distribution among the 100 L, 2 L, and 1 L samples, respectively, prior to concentration and sub-sampling for the latter two methods. Unfortunately, the duplicate SHA sample tested by NOAA was too concentrated and the additional sample needed to produce a more dilute lysate was not available. Additional factors leading to these and other discrepancies between NFRDI and NOAA SHA values include actual differences in cell number, variation in calibration curves, conduct of the assay, as well as archival of field samples over LN<sub>2</sub> and shipment on dry ice from NFRDI to NOAA. Archived samples were analyzed at NOAA within 3 months of the original sampling date. Anderson et al. (2005) reported that SHA samples are stable in LN<sub>2</sub> vapor for several months, but

further testing of long-term stability for *C. polykrikoides* samples stored over LN<sub>2</sub> or at –70 °C and shipped on dry ice is required. Interestingly, Tyrrell et al. (2002) showed that *H. akashiwo* samples can be fixed with acidic Lugol's for SHA and are stable for 3 months, which should also be tested for *C. polykrikoides* in the event that LN<sub>2</sub> is not available. For WC hybridization, *C. polykrikoides* samples were preserved using a protocol developed for another athecate dinoflagellate, *Karenia brevis* (Mikulski et al., 2005), which produced excellent results after 1.5 months storage at 4 °C (see their Fig. 6). Mikulski et al. (2005) reported strong labeling of *K. brevis* cells up to 7 months post-collection when stored under these conditions, but further testing is required to confirm a similar outcome for *C. polykrikoides*.

Comparisons of the *C. polykrikoides*-specific WC hybridization method and the SHA were limited to a total of six samples among both June and July 2006 surveys, with variable agreement between values. In addition to the issues outlined above that may have affected the SHA results, the potential effect of different sample volumes represented by the two methods (i.e., SHA – 100 L; WC – 0.5 L) must also be considered. Clearly, the WC assay will be important for verifying the presence of the target species when sufficient cell concentrations are present, especially with the co-occurrence of morphologically similar taxa; however, a much larger sample set is needed to evaluate the influence of potential error sources on both this method and the SHA. For example, an extensive, 4-year field validation and comparison of the SHA and WC formats for detection of *Alexandrium fundyense* was reported recently by Anderson et al. (2005) in the Gulf of Maine, USA, which ultimately showed the benefit of both approaches depending on the objective. More comprehensive field trials of both *C. polykrikoides* probe-based detection methods are currently underway.

To conclude, the *C. polykrikoides*-specific sandwich hybridization assay and whole cell hybridization method developed during this study were applied successfully against both laboratory cultures and field samples originating from Korean coastal waters. The SHA is capable of detecting target cells at levels far below bloom concentrations, is relatively inexpensive to perform (estimate of analytical costs approx. USD \$5/sample in triplicate; Tyrrell et al., 2002), has a potential throughput of 36–48 samples per 8 h day (Anderson et al., 2005), and is not technically demanding compared to methods such as quantitative real-time PCR (Popels et al., 2003; Coyne et al., 2005). This method is ideal for monitoring and other field applications, and the SHA technique has been accredited for the national monitoring program in New Zealand (Ayers et al., 2005). Application of species-specific, fluorescently-labeled probes in a WC format allows processing of up to three times the number of samples compared with direct light microscope counts (Anderson et al., 2005) and is preferred when both enumeration and morphological confirmation of an organism are required. Another benefit of the WC assay is its ability to differentiate morphologically similar organisms, such as *G. impudicum* and *G. catenatum*, that can occur within a *Cochlodinium* bloom. In addition, the *C. polykrikoides*-specific

oligonucleotide probes designed for the SHA will be amenable for re-formatting onto fully automated, in-situ instruments (e.g., the Environmental Sample Processor, Scholin et al., 1998; Greenfield et al., 2006), demonstrated recently to be capable of autonomous, sub-surface detection of harmful algal species in near-real time. The methods developed herein will enhance the monitoring capabilities for *C. polykrikoides* and mitigation of its potentially devastating impacts not only in Korean waters, but also in other regions where this and other *Cochlodinium* species (after probe re-design) are now established members of the coastal phytoplankton community (e.g., Hallegraef, 1992; Viquez and Hargraves, 1995; Whyte et al., 2001; Kudela et al., 2008 and references therein).

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