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QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION FOR *COCHLODINIUM FULVESCENS* (DINOPHYCEAE), A HARMFUL DINOFLAGELLATE FROM CALIFORNIA COASTAL WATERS[1]

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Harmful blooms formed by species of the dinoflagellate *Cochlodinium* have caused massive fish kills and substantial economic losses in the Pacific Ocean. Recently, prominent blooms of *Cochlodinium* have occurred in central and southern California (2004–2008), and *Cochlodinium* cells are now routinely observed in microscopical analysis of algal assemblages from Californian coastal waters. The first documented economic loss due to a *Cochlodinium* bloom in California occurred in Monterey Bay and resulted in the mortality of commercially farmed abalone. Increasing occurrences of *Cochlodinium* blooms, the fact that these cells preserve poorly using standard techniques, and the difficulty of identifying preserved specimens using morphological criteria make *Cochlodinium* species prime candidates for the development of a quantitative real-time polymerase chain reaction (qPCR) approach. The 18S rDNA gene sequenced from *Cochlodinium* cells obtained from California coastal waters, as well as GenBank sequences of *Cochlodinium*, were used to design and test a Molecular Beacon® approach. The qPCR method developed in this study is species specific, sensitive for the detection of *C. fulvescens* that has given rise to the recent blooms in the eastern Pacific Ocean, and spans a dynamic abundance range of seven orders of magnitude. Initial application of the method to archived field samples collected during blooms in Monterey Bay revealed no statistically significant correlations between gene copy number and environmental parameters. However, the onset of *Cochlodinium* blooms in central California was consistent with previously reported findings of correlations to decreased surface temperature and increased inputs of nitrogenous nutrients.

Key index words: **18S rDNA; *Cochlodinium*; harmful algal blooms; protist; qPCR**

Abbreviation: **BLAST, basic local alignment search tool**

Harmful blooms of dinoflagellates within the genus *Cochlodinium* have been increasing in frequency worldwide and have caused substantial economic losses and fish kills in the Pacific Ocean off the coasts of Japan (Onoue and Nozawa 1989, Yuki and Yoshimatsu 1989), China (Onoue and Nozawa 1989, Yuki and Yoshimatsu 1989), Korea (Kim 1998), Malaysia (Anton et al. 2008), the Philippines (Azanza et al. 2008), and Canada (Whyte et al. 2001). Blooms of this genus have also been reported in the eastern Pacific including Costa Rica (Vargas-Montero et al. 2006), Mexico (Morales-Blake et al. 2001, Gárate-Lizárraga et al. 2004), and

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the Caribbean Sea (Margalef 1961). A persistent bloom in 2008–2009 in the Arabian Gulf and Gulf of Oman had severe consequences including fish mortality, damage to coral reefs, impacts to coastal tourism, and most significantly, the forced closure of desalination plants in the region (Richlen et al. 2010).

The presence of *Cochlodinium* off the California coast has been unremarkable until recently. However, prominent blooms of *Cochlodinium* have been documented in the coastal waters of central and southern California in 2004 and from 2006 to 2008 (Curtiss et al. 2008, Kudela et al. 2008). Blooms in Monterey Bay during 2004 and 2006 and off San Diego during 2004 attained cell abundances of 10^4 cells·L^{-1} (Curtiss et al. 2008, Kudela et al. 2008). Abundances in Santa Monica Bay during 2006 reached an even greater cell abundance of 10^6 cells·L^{-1} and a chl *a* concentration of 67 mg·m^{-3} (Reifel 2009). Cells belonging to the genus *Cochlodinium* are now routinely identified as members of algal assemblages at many locations along the central and southern California coastline.

Concurrent with these recent outbreaks of *Cochlodinium*, harmful events presumably linked to these dinoflagellates have been documented. The first reported ecological damage and economic loss due to a *Cochlodinium* bloom in Californian coastal waters occurred in Monterey Bay (2007) and resulted in a loss of commercially farmed abalone (http://www.cencoos.org/documents/about/Abalone_success.pdf). Additional reports of mussel mortalities were observed in Monterey Bay during blooms (Curtiss et al. 2008). These increases in cell abundances of *Cochlodinium*, and their resulting harmful effects, raise concern for more effective monitoring of these species and the need for tools to study the ecology of this harmful organism.

Species of *Cochlodinium* are morphologically similar and difficult to differentiate by light microscopy except by an expert taxonomist. Recently, the morphologies of *C. polykrikoides* Margalef, *C. catenatum* Okamura, *C. convolutum* Kof. et Swezy, and *C. fulvescens* Iwataki, Kawami et Matsuoka were compared (Matsuoka et al. 2008), and phylogenetic relationships were also proposed for some *Cochlodinium* species based on DNA sequence information (Iwataki et al. 2007, 2008). Results of these studies confirmed that differences in morphological characteristics among *C. polykrikoides*, *C. convolutum*, and *C. fulvescens* species were subtle, whereas *C. catenatum* Okamura (reported from La Jolla CA by Kofoid and Swezy 1921) is a different species than *C. catenatum* sensu Okamura (Matsuoka et al. 2008). Phylogenetic results based on LSU rDNA gene sequences revealed that *C. polykrikoides* can be distinguished from *C. fulvescens*, but that they are closely related sister species. Samples from a 2006 bloom of *Cochlodinium* in Monterey Bay as well as samples from British Columbia, Canada were included in those studies and indicated that *C. fulvescens* was the species present in those waters (Iwataki et al. 2008). However, a robust culture of *C. fulvescens* isolated from North America has not yet been established despite numerous attempts by several groups (R. Kudela, personal observation and C. Tomas, personal observation) limiting material for comparative studies of morphology.

In addition to difficulties associated with morphological identification of live or preserved *Cochlodinium* species, cells of this genus often burst or become deformed during preservation (Curtiss et al. 2008). Therefore, determinations of cell abundances based on microscopy of preserved samples would be expected to underestimate actual cell abundances.

LM is currently the most commonly employed method for identifying and enumerating coastal phytoplankton including HAB-forming species in monitoring and research programs, but molecular techniques are being adopted rapidly as alternative or complementary approaches for determining the abundances of important protistan species including many HAB taxa. The latter methods require less taxonomic expertise for identification than microscopical analyses and are less time consuming for large numbers of samples, although they require considerable front-end work to design and verify approaches (Miller and Scholin 1996, Countway and Caron 2006). Quantitative real-time polymerase chain reaction (qPCR) based on rDNA can provide species-specific identification and has now been successfully developed and employed for a number of HAB species (Coyne et al. 2001, Moorthi et al. 2006).

The increasing frequency of *C. fulvescens* blooms on the United States, West Coast, the lack of an established culture of this species, and the difficulty of identifying *Cochlodinium* species based on morphological criteria made this species a prime candidate for the development of a qPCR approach. We exploited the unique sequence signature of the 18S rDNA gene from local *Cochlodinium* sp. as well as two GenBank sequences (AB288381 and AB288380, identified as *C. fulvescens* in GenBank) to design and test a Molecular Beacon® approach to identify and quantify abundance of *C. fulvescens* in natural water samples. The approach was then applied to samples from local coastal waters to begin to elucidate the environmental conditions conducive to algal blooms, such as temperature, nutrient concentrations, rainfall, and river runoff (Hallegraeff et al. 1995, Thompson et al. 2008), and to determine if any of these conditions correlate to blooms of *C. fulvescens* in California.

MATERIALS AND METHODS

Collection and processing of environmental samples. *Cochlodinium* cells were obtained from a natural seawater sample collected during a *Cochlodinium* bloom on 4 December 2006, in Santa Monica Bay in southern California (33°54.2′ N, 118°28.22′ W). Live single cells isolated from the bloom were identified as

Cochlodinium based on morphological features (C. Tomas, unpublished data). Samples for genetic analysis were collected at the same time by gently (<10 mmHg) filtering seawater onto GF/F filters (Whatman Inc., Florham Park, NJ, USA) and placing filters in 2 mL of 2x Lysis Buffer (100 mM Tris [pH 8], 40 mM EDTA [pH8], 100 Mm NaCl, 1% sodium dodecyl sulfate) and storing at −80ºC for subsequent molecular analysis (Countway and Caron 2006).

qPCR samples were collected as part of a time series monitoring project as described below and analyzed using the *C. fulvescens* qPCR assay developed in this study.

Cloning and sequencing. Bulk DNA was extracted from the filter collected on 4 December 2006, in Santa Monica as described in Countway and Caron (2006). As noted above, visual examination indicated that the sample was strongly dominated by *Cochlodinium* cells. Amplification of the full length 18S rDNA gene from the above isolated environmental DNA was achieved using the following eukaryotic specific primers, Euk A (5′-AACCTGGTTGATCCTGCCAGT-3′), Euk B (5′-GATCCTTC TGCAGGTTCACCTAC-3′) (Medlin et al. 1988, Countway and Caron 2006). PCR products were visualized on an agarose gel, checked for appropriate size, band isolated, recovered, purified, and cloned all as described in Countway and Caron (2006). Plasmids were then isolated and sequenced from eight individual clones, again as described in Countway and Caron (2006). Sequencing was conducted using a Beckman-Coulter CEQ8000 automated DNA sequencer using the internal plasmid primers M13F and M13R, 570F, (5′-GTAATTCCAGCTCCAATAGC-3′), 570R (5′-GCTATTGGAGCTGGAATTAC-3′), 1055F, (5′-GGTG GTGCATGGCCG-3′), 1055R (5′-CGGCCATGCACCACC-3′) (Weekers et al. 1994). Sequence fragments were assembled and aligned using Chromas Pro (2003–2009 Technelysium Pty. Ltd., Brisbane, Australia). The identity of the cloned and sequenced gene was confirmed as a *C. fulvescens* sequence by comparison to *Cochlodinium* sequences available from GenBank. The full length sequence was deposited into GenBank (accession number HQ896315). This 18S rDNA sequence and associated plasmid DNA was then used in further development of the qPCR assay.

Probe and primer design. A species-specific Molecular Beacon® probe and primer set (Eurofins, mwg/operon, Huntsville, AL, USA) was designed to target the California sequence generated in this study (HQ896315) and two *C. fulvescens* sequences from GenBank (AB288381, isolated from Japan in October 2004, and AB288380, isolated from Indonesia in December 2003) with which our sequence shared high similarity (see ''Results''). The three sequences were aligned using BioEdit Sequence Alignment Editor, version 7 (Hall 1999). The Molecular Beacon® probe and flanking primers were designed in Allele ID 6.01 (Premier Biosoft International, Palo Alto, CA, USA) and were then manually adjusted to increase specificity. The specificities of the probe and primers were evaluated using the basic local alignment search tool (BLAST, Altschul et al. 1997), probeCheck (http://131.130.66.200/cgi-bin/probe check/content.pl?id=citation; Loy et al. 2008), and via the manual alignment of 93 closely related dinoflagellates and common co-occurring algae and protozoa using BioEdit Sequence Alignment Editor, version 7. In addition, all other 18S sequences of other *Cochlodinium* species (mainly *C. polykrikoides*) were downloaded from GenBank and manually aligned (sequences included AY347309, DQ779984, DQ779985, DQ779986). A pair-wise alignment of the California sequence of *C. fulvescens* (HQ896315) with one of the 18S *C. polykrikoides* sequences (DQ779984) was conducted to ensure species-specificity of the primers and probe. A comparison with other *Cochlodinium* qPCR primers was not possible since no other primers exist for the 18S region of *Cochlodinium.*

The species-specific forward primer (CochloFw, 5′-CG AGTTCGTACCTCCCCTTGG-3′) targeted nucleotide positions 615–635 and the reverse primer (CochloRv, 5′-GCGTTTGCCT GCCTGAAACAC-3′) matched positions 738–758, resulting in a 143-bp amplification product. A Molecular Beacon® probe (specific sequences set apart by brackets) with seven complementary ''stem-forming'' nucleotides at the 5′ and 3′ ends was designed to anneal at base 687 (5′-CGCGATC[TGTATGACGT ATTCAACCCGGACTTTT]GATCGCG-3′).

Probe and primer optimization. qPCR reaction conditions were optimized using plasmid DNA containing the California strain 18S rDNA to test different Mg^{2+} concentrations (4–5.5 mM) and annealing temperatures (52ºC–62ºC). Total reaction volumes of 50 μL for each sample were composed of 5 μL of plasmid DNA diluted 1:100 (concentration 4.5 ng·μL^{-1}) and 45 μL of master mix consisting of 0.5 μM of each primer, 0.25 μM Molecular Beacon®, 1X Promega buffer B, 5 mM Promega $MgCl_2$, 250 μM Promega of dNTPs, 300 ng·μL^{-1} BSA (Sigma A-7030, St Louis, MO, USA), and 2.5 units of GoTaq polymerase in colorless buffer. Samples were processed on a Bio-Rad iCycler iQ (Hercules, CA, USA) using the following optimal thermal protocol: 1 cycle at 95°C for 3 min, 35 cycles at 95ºC for 30 s, 60ºC for 1 min, 72ºC for 30 s, and a hold at 4ºC.

A melt curve analysis was conducted of the plasmid DNA fragment generated by PCR using SYBR® Green dye (Molecular Probes, Eugene, OR, USA).

Cross-reactivity testing. The *C. fulvescens* probe and primers were also tested in real-time PCR reaction using culture lysates of 31 nontarget organisms from a variety of taxonomic groups to determine species specificity of the assay (see Table 1). The nontarget protistan cultures were grown in 250 mL sterile culture flasks in K medium (made from sterile seawater) modified with 36 μM PO_4^{2-}. Cell cultures were harvested after

TABLE 1. Culture lysates of 31 nontarget protists from a variety of taxonomic groups were tested to determine specificity of the assay within 35 cycles.

Taxonomy		Amplification
Alveolata;	*Akashiwo sanguinea*	−
Dinophyceae	*Alexandrium catenella*	−
	Cochlodinium fulvescens	+
	Gymnodinium beii	−
	Lingulodinium polyedrum	−
	Prorocentrum gracile	−
	Prorocentrum micans	−
	Scrippsiella nutricula	−
	Symbiodinium pulchrorum	−
Haptophyta;	*Isochrysis galbana*	−
Prymnesiophyceae	*Phaeocytis globosa*	−
Stramenopiles;	*Chaetoceros* sp.	−
Bacillariophyceae	*Coscinodiscus* sp.	−
	Ditylum brightwellii	−
	Minutocellus polymorphus	−
	Odontella sp.	−
	Pseudo-nitzschia australis	−
	Pseudo-nitzschia delicatissima	−
	Pseudo-nitzschia pseudo-delicatissima	−
	Thalassiosira rotula	−
	Thalassiosira weissflogii	−
Stramenopiles; Dictyochophyceae	*Pteridomonas* sp.	−
Stramenopiles; Pelagophyceae	*Aureococcus anophagefferens*	−
Stramenopiles;	*Chattonella marina*	−
Raphidophyceae	*Fibrocapsa japonica*	−
	Heterosigma akashiwo	−
Viridiplantae; Chlorophyceae	*Pyramimonas* cf. *tychotreta*	−

reaching a minimum abundance of $\sim 1.0 \times 10^3$ cells·mL^{-1} and filtered onto Whatman GF/F filters, lysed, and stored at −20°C. Prior to cross-reactivity testing, all culture lysates were amplified with universal eukaryote-specific PCR primers (Euk A, 5′-AAC CTGGTTGATCCTGCCAGT-3′ and Euk B, 5′-GATCCTTC TGCA GGTTCACCTAC-3′; Medlin et al. 1988) to ensure viable amplification. The culture lysates were then diluted 1:100 with sterile water, and 5μL of each lysate was used in triplicate reactions of the qPCR assay. Target plasmid containing the *C. fulvescens* gene was used as a positive control.

Standard curves. Sensitivity of the qPCR assay was determined using standard curves generated from serial dilutions of *C. fulvescens* plasmid DNA that spanned seven orders of magnitude (77–7.7×10^8 copies). The standard curves generated were comprised of triplicate PCR reactions for each concentration on the curve. A new standard curve was performed with each batch of field samples analyzed by the qPCR assay, and used to calculate the gene copy number in those field samples. Therefore, multiple standard curves were generated in this study. The amplification efficiency was calculated (using equation $E = 10^{[(-1/\text{slope}) - 1]} \times 100\%$) for each standard curve used to calculate gene copy numbers from the field samples.

Application of qPCR to environmental samples. As part of the California Program for Regional Enhanced Monitoring of PhycoToxins (Cal-PReEMPT), whole water samples were collected weekly from integrated surface water (0–2 m) throughout the year at the Santa Cruz Wharf (36°57.48′ N, 122°1.02′ W), in Monterey Bay, California. Chl *a* samples were collected on Whatman GF/F filters, extracted for 24 h in 7 mL of 90% acetone at −20°C, and analyzed using a Turner Designs 10-AU fluorometer (Sunnyvale, CA, USA) using the nonacidification method (Welschmeyer 1994). Nutrient samples (nitrate, ammonium urea, and phosphate) were filtered through Whatman GF/F filters and stored frozen in polyethylene bottles until analyzed. The nitrate plus nitrite (hereafter referred to as nitrate) and phosphate were analyzed using standard methods on a LaChat Instruments automated ion analyzer (8000 series) (Parsons et al. 1984). The ammonium and urea samples were stored frozen in 60 mL low-density polyethylene tubes and analyzed using the fluorometric ammonium (Holmes et al. 1999) and the diacetyl monoxime thiosemicarbizide technique (Goeyens et al. 1998), respectively.

Net tow samples were also collected weekly as part of the monitoring program, using a 20 μm mesh plankton net that was hauled five times between 3.3 m and the surface (total tow effort was 16.5 m). These samples were examined live in the laboratory using an Olympus SZH Steriozoom dissecting microscope (Olympus, Tokyo, Japan) and a record was kept of all species and genera observed based on the relative abundance (present = 1%–9% of the sample, common = 10%–49% of the sample, abundant = > 50% of the sample). When *Cochlodinium* was observed in these net tow samples, qPCR samples were collected on Whatman GF/F filters and frozen (−20°C). All environmental data during observed *Cochlodinium* presence at the Santa Cruz Wharf are presented in this study.

Stream flow data were downloaded from the USGS National Water Information System: Web Interface (http://waterdata.usgs.gov/nwis) for the San Lorenzo River in Santa Cruz, California (station identification is USGS 11161000, 36°59.27′ N, 122°01.51′ W). Precipitation data were downloaded from the National Atmospheric Deposition Program Web site (http://nadp.sws.uiuc.edu/sites/siteinfo.asp?net=NTN&id=CA66) for San Benito County, California (Station identification is CA66, Pinnacles National Monument, 36°29′ N, 121°09.25′ W).

Simple pair-wise correlation analyses were performed for the Monterey Bay 2007 data using the software package STATISTICA (StatSoft Inc. 2002), and relations between gene copy number and temperature, chl *a*, urea, nitrate, ammonium, phosphate, and streamflow were examined.

Also, as part of the Cal-PReEMPT program, weekly samples were collected at the Cal Poly Marine Science Research and Education Pier (35°10.20′ N, 120°44.46′ W) in San Luis Obispo Bay in 2007 and 2008. When *Cochlodinium* was present, additional samples for qPCR analysis were collected. All samples were collected and stored as described above for the Santa Cruz Wharf. Weekly temperature and nutrient data were available through the Southern California Coastal Ocean Observing System Harmful Algae and Red Tide Regional Monitoring Program (http://www.sccoos.org).

A field sample was also collected in Santa Monica Bay in Los Angeles (33°54.2′ N, 118°28.2′ W) on 4 December 2006, during a *Cochlodinium* bloom (all study locations shown in Fig. 1).

Several negative control samples were collected from each of the field sites. These negative controls were samples collected when *Cochlodinium* cells were not observed by microscopical examination.

RESULTS

Specificity and sensitivity of the real-time qPCR assay. Pair-wise sequence comparisons of the 18S rDNA gene sequence obtained from *Cochlodinium* cells collected from Californian coastal waters and two GenBank strains of *C. fulvescens* (AB288381 and AB288380) revealed 99% similarity across 1,747 nucleotide positions. All three strains matched the two primers and probe designed for this method with 100% similarity.

The BLAST results for the forward primer included the *C. fulvescens* strains from GenBank and the reverse primer BLAST results included both *C. fulvescens* sequences from GenBank as well as two uncultured eukaryote clones (EU500115 and AY665024). The Molecular Beacon® probe BLAST results included both *C. fulvescens* sequences from GenBank as well as one uncultured eukaryote clone (EU500115). A manual alignment with the GenBank sequence EU500115 revealed the Molecular Beacon® sequence was different at a single nucleotide position. No other *Cochlodinium* species were present in the BLAST and ProbeCheck results. In addition to BLAST comparisons, probe and primer sequences were also compared to closely related dinoflagellates by manual alignment of sequences and no nontarget sequences were revealed. The *C. polykrikoides* sequences that were manually aligned were 100% similar. The pair-wise alignment between the *C. fulvescens* sequence (HQ896315) and one of the *C. polykrikoides* sequences (DQ779984) revealed a 24% similarity.

The primer/probe design was tested against target (*C. fulvescens* plasmid) and nontarget DNA in triplicate qPCR amplifications. All the nontarget protistan culture lysates returned negative (below threshold) results for cross-reactivity tests for 35 cycles of amplification (Table 1). Serial dilutions of *C. fulvescens* plasmid DNA (4.5 ng·μL^{-1}, equal to $\sim 7.8 \times 10^6$ copies) were generated spanning seven orders of magnitude (77–7.7×10^8 copies; $r^2 = 0.99$;

FIG. 1. Map of California showing the three field sites: Santa Cruz Wharf in Monterey Bay, Cal Poly Pier in San Luis Obispo, and Santa Monica Bay in the Los Angeles region.

Fig. 2). One of the plasmid standard curves generated with the field samples and used to calculate 18S gene copy numbers present in field samples is shown in Figure 2. There were triplicate PCR reactions analyzed for each concentration and standard deviations (represented in Fig. 2 as the error bars) were calculated and ranged from 0 to 0.6. The amplification efficiencies calculated for each standard curve used to calculate gene copy numbers for the field samples ranged from 94% to 99%.

Field results. Santa Monica Bay: Microscopical analysis of water collected during a noticeable discoloration of coastal waters in December 2006 indicated that 98% of the microplankton was comprised of *Cochlodinium* cells. Cell abundance based on microscopic cell counts of a preserved sample was 2.2×10^6 cells·L^{-1} (Reifel 2009). For reasons noted above, this may represent an underestimation due to poor preservation of *Cochlodinium* cells. Application of the qPCR approach to this sample confirmed the presence of high numbers of rDNA genes from *C. fulvescens*; 35,600 copies·μL^{-1}.

Two negative control samples (samples in which *Cochlodinium* was not observed in live microscopical observations of whole water samples) were collected in February 2009 from the Los Angeles Harbor (33°43.4′ N, 118°7.13′ W) and the San Pedro Shelf (33°38.29′ N, 118°8.34′ W). *C. fulvescens* was not detected by the qPCR assay in either sample.

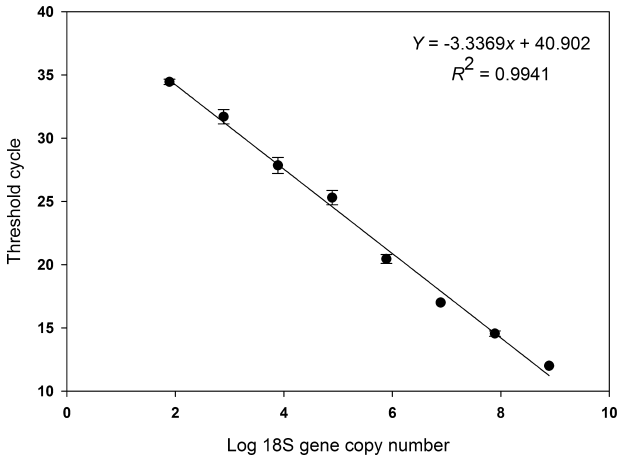

FIG. 2. Calibration of *C. fulvescens* qPCR method with a dilution series of plasmid DNA containing the 18S gene of the species. The range spans seven orders of magnitude, from 77 to 7.7×10^8 copies. The solid dots represent mean results of triplicate PCR reactions, and the error bars represent standard deviation of triplicate PCR reactions.

Monterey Bay: A *Cochlodinium* bloom in Monterey Bay began in late September and continued through mid-November 2007. The qPCR approach was applied to samples collected during this period and confirmed the presence of high copy numbers of rDNA genes for *C. fulvescens* in these samples. Gene copy numbers ranged over two orders of magnitude during this period from 647 to 44,900 copies·μL^{-1} (\Fig. 3a). *Cochlodinium* cells first appeared in high relative abundance (>50% of the microscopical observations of a net tow sample) in the weekly samples from the Santa Cruz Wharf on 26 September 2007, which had a corresponding gene copy number of 11,500 copies·μL^{-1}. *Cochlodinium* decreased in relative abundance and was characterized as common (10%–50% of the net tow sample) by 10 October 2007, with corresponding gene copy number decreasing to 647 copies·μL^{-1}. Gene copy numbers were highest at 44,900 copies·μL^{-1} during the following week and decreased in the weeks thereafter, but continued to be characterized as common (Fig. 3a).

Temperature during this time period decreased from 16°C to 10.9°C with 13.5°C corresponding to the period of maximum gene copy number (44,900 copies·μL^{-1}; Fig. 3a). Chl *a* ranged from 6.8 to 289 mg·m^{-3} (Fig. 3a). The maximum chl *a* concentration of 289 mg·m^{-3} occurred during mid-November when *Akashiwo sanguinea* was the dominant phytoplankton taxon. The highest gene copy number for *C. fulvescens* (44,879 copies·μL^{-1}) corresponded to chl *a* concentration of 70 mg·m^{-3}.

Concentrations of ammonium, nitrate and urea ranged from 0.02 to 2.6 μM, below detection to 5.7 μM, and 0.1 to 2.2 μM, respectively (Fig. 3c). Ammonium and nitrate concentrations were maximal 1 week prior to maximum gene copy number for *C. fulvescens*. Urea increased to high concentration (2.14 μM) 1 week prior to maximum detected gene copy number, and the highest urea concentration coincided with highest value for *C. fulvescens* gene copy number.

The measured daily stream flow from the San Lorenzo River was high prior to the onset of the *Cochlodinium* bloom (data not shown). The highest stream flow rate occurred 3 weeks prior to the highest gene copy concentrations and remained high for several weeks. The highest daily precipitation occurred 1 week prior to the onset of the bloom and increased again 1 week prior to the highest value of gene copy number.

There were no significant correlations between gene copy number and any of the environmental parameters measured as part of the Monterey Bay field study (Spearman correlation coefficients; $P > 0.05$).

A negative control sample was collected on 8 July 2008, from the Santa Cruz Wharf when *Cochlodinium* was not observed in live observations of plankton net tows. *C. fulvescens* was not detected by the qPCR assay for that sample.

San Luis Obispo Bay: Blooms of *Cochlodinium* occurred in San Luis Obispo Bay from September through mid-November during 2007 and 2008. Gene copy numbers during 2007 ranged from 79 to 42,728 copies·μL^{-1} (data not shown) and 796 to 294,490 copies·μL^{-1} during 2008 with the pronounced maximum during early October (Fig. 3b).

There were no simultaneous observations of environmental factors collected during the 2007 bloom; however, the daily temperature reported from the Diablo Canyon buoy during this period decreased from 16.2°C to 13.7°C (data not shown).

Temperature during the 2008 bloom decreased from 16°C to 12.5°C (Fig. 3b). Chl *a* ranged from 1.4 to 67 mg·m^{-3}. The maximum chl *a* concentration of 67 mg·m^{-3} occurred in mid-October when *Cochlodinium*, *Prorocentrum*, and *Ceratium* were the most abundant taxa observed (10%–49% of net tow samples). The highest gene copy number (294,490 copies·μL^{-1}) corresponded to a chlorophyll *a* concentration of 28 mg·m^{-3} during mid-September when *Prorocentrum* was also abundant (≥50% of net tow sample) and *Cochlodinium* was common in the plankton (10%–49% of net tow sample).

Concentrations of ammonium, nitrate, and phosphate ranged from 0.2 to 6.8 μM, 0.1 to 21.9 μM, and 0.2 to 2.9 μM, respectively, in 2008 (Fig. 3d). Peaks in the concentration of ammonium and nitrate preceded increases in chl *a* concentration, and minor peak in gene copy number. Phosphate concentration was highest (2.9 μM) 1 week prior to the maximum observed gene copy number of 294,490 copies·μL^{-1}).

A negative control sample was collected (when *Cochlodinium* was not observed in live observations of plankton tow material) from the Cal Poly Pier on

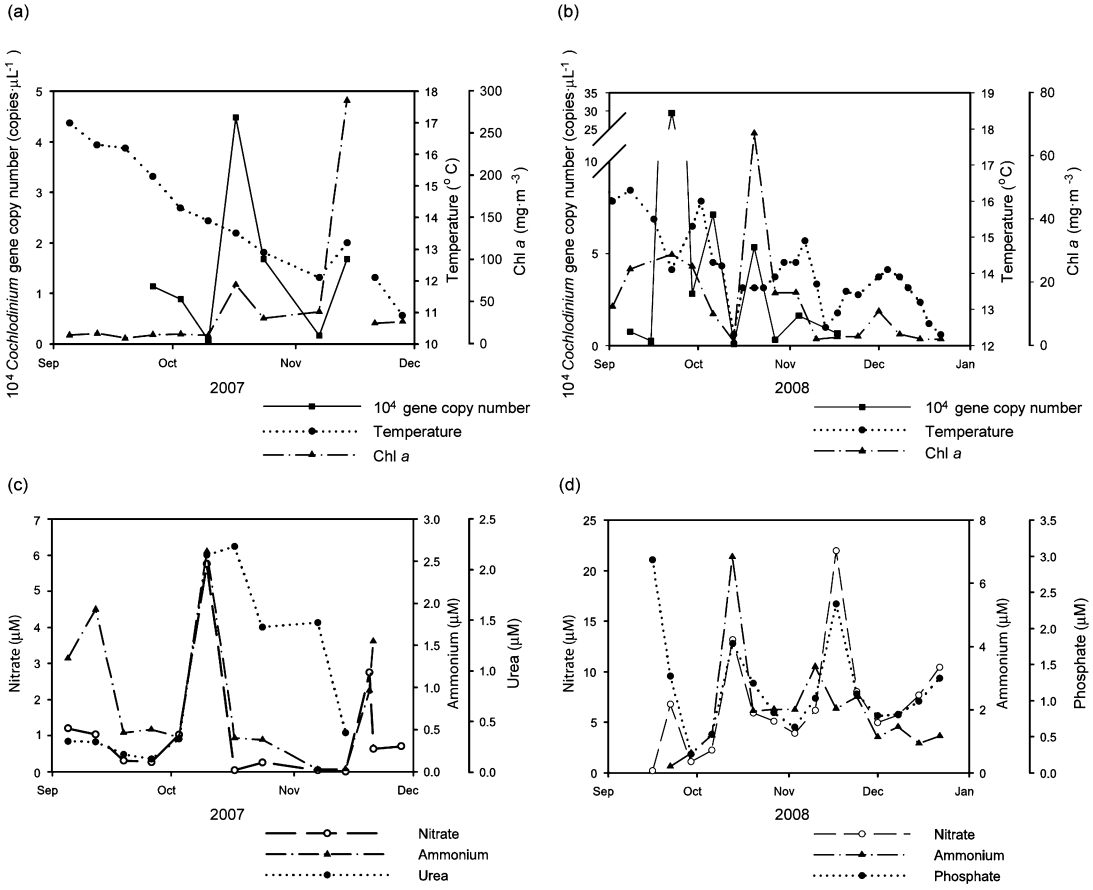

FIG. 3. Weekly field samples collected during a *Cochlodinium* bloom at the Santa Cruz Wharf, Monterey Bay, California, in 2007 (a) and during a *Cochlodinium* bloom at the Cal Poly Pier, San Luis Obispo, California, in 2008 (b). Gene copy numbers are indicated by a solid black line (■), temperature is indicated by a dotted line (•), and chl *a* is indicated by a dashed and dot line (▲). Weekly field samples collected during a *Cochlodinium* bloom at the Santa Cruz Wharf, Monterey Bay, California, in 2007 (c) and during a *Cochlodinium* bloom at the Cal Poly Pier, San Luis Obispo, California, in 2008 (d). Nitrate (μM) is indicated by a dashed line (○), ammonium (μM) is indicated by a dashed and dot line (▲), urea (μM) is indicated by a dotted line (•) (c only), and phosphate (μM) is indicated by a dotted line (•) (d only).

8 January 2008. No *Cochlodinium* cells were detected by the qPCR assay in this sample.

DISCUSSION

The use of real-time qPCR for identifying and quantifying HAB species (and other protistan taxa) is rapidly becoming a sensitive and accurate approach for augmenting traditional microscopical techniques for monitoring and studying population ecology and dynamics. The method developed and briefly applied here employed sequences of plasmid DNA obtained from a presently uncultured species of dinoflagellate, *C. fulvescens*, to improve identification and quantification of this species in natural samples.

The development of a real-time qPCR method for *C. fulvescens* is timely and desirable for several reasons. First, species of *Cochlodinium* are difficult to distinguish by morphological features except by a trained taxonomist. Second, preservation of *Cochlodinium* species from field samples is problematic and can lead to underestimation of cell abundances when using microscopical methods. Third, a healthy long-term laboratory culture for morphological comparisons with natural assemblages of *Cochlodinium* has not been established for *C. fulvescens* from California, despite repeated attempts from persistent blooms that have occurred in recent years. The qPCR method developed in this study avoids these hurdles in studying the ecology of this emerging HAB concern on the United States, West Coast by providing an approach that is species-specific and highly sensitive for the detection and identification of *C. fulvescens*. The method can be used to monitor and detect *C. fulvescens* over a range spanning seven orders of magnitude, ranging from 77 to 7.7×10^8 copies.

We report qPCR results in the environmental samples of this study in units of gene copy number, calibrated against a plasmid containing the *C. fulvescens* 18S gene, although cell abundances of the dinoflagellate are the preferred form of data for the study of phytoplankton ecology and other cell iden-

tification methodologies report results in this unit. Conversion of gene copy number directly to cell abundances has been accomplished for other protistan taxa using cultures, but cultures of the California strain of *C. fulvescens* have not yet been established. Until, cultures are available to directly correlate gene copy number with cell abundance, approximate cell abundances can be calculated from copy number in our study using information contained in the literature.

The range of 18S rDNA copy number reported for eukaryotes varies widely and correlates roughly with genome size (Prokopowich et al. 2003), cell length (Zhu et al. 2005, Godhe et al. 2008) and biovolume (Godhe et al. 2008). Reported copy numbers range from approximately 1 copy·cell^{-1} for picoplanktonic protists (*Nannochloropsis salina*) to >12,000 copies·cell^{-1} for large dinoflagellates (*Akashiwo sanguinea*). Other studies have determined rDNA gene copy numbers of 1,000 copies·cell^{-1} in *Alexandrium minutum* (Galluzzi et al. 2004), 100–200 copies·cell^{-1} in *Pfiesteria piscicida* (Saito et al. 2002), 61 (*Skeletonema marinoi*) to 36,896 (*Ditylum brightwelli*) copies·cell^{-1} for diatoms, and 1,057 (*Pentapharsodinium faeroense*) to 12,812 (*Lingulodinium polyedrum*) copies·cell^{-1} for dinoflagellates (Godhe et al. 2008).

The cell length of *C. fulvescens* is 37.5–57.5 μm, with a mean of 45.8 μm (Iwataki et al. 2007). Using the correlation between cell length and 18S rDNA copy number established by Godhe et al. (2008), an estimate of the number of copies per cell for *C. fulvescens* was made resulting in an estimation of 18S rDNA copy number of 5,620 copies·cell^{-1} for *C. fulvescens*. The cell abundances for the environmental samples examined in this study were subsequently estimated by dividing the copies·μL^{-1} in each sample (determined by the qPCR method) by the estimated rDNA copies per cell for *C. fulvescens* (converted to units of cells·L^{-1}). These rDNA copy numbers only provide a rough correlation to cell abundance and do not take into account other factors that have been shown to increase variability in gene copy number, such as intraspecies variability and growth phase (Galluzzi et al. 2010).

Ranges in cell abundances estimated in this manner for the three study sites were 6×10^6 cells·L^{-1} (Santa Monica Bay), 1×10^5 to 8×10^6 cells·L^{-1} (Monterey Bay 2007), 1×10^4 to 8×10^6 cells·L^{-1} (San Luis Obispo Bay 2007), and 1×10^5 to 5×10^7 cells·L^{-1} (San Luis Obispo Bay 2008). Estimation of copy number in *C. fulvescens* will improve when direct measurements of copy number in this species can be established from cultured cells or cells isolated from natural samples.

Cell abundance for the single Santa Monica Bay bloom sample was determined from microscopic counts based on a preserved sample (Reifel 2009). That value (2×10^6 cells·L^{-1}) agreed reasonably well with cell abundances estimated for the same sample based on gene copy number (from qPCR results) converted to cell abundance using the copy number per cell for *C. fulvescens* derived from the literature (6×10^6 cells·L^{-1}). The microscopical count reported for this sample likely underestimated the actual cell abundance due to the aforementioned preservation problems associated with *Cochlodinium* species (Curtiss et al. 2008) and presumably explains the 3-fold difference in cell abundance estimates between the two methods. No microscopical counts from preserved samples were performed for the samples from Monterey Bay or San Luis Obispo Bay, but the cell abundances based on our conversion of copy number to cell abundance for the 2007 Monterey Bay bloom reported in this study (10^5 to 10^6 cells·L^{-1}) were one to two orders of magnitude higher than previously reported bloom abundances for blooms in 2004 and 2006 based on microscopic counts of preserved samples (Curtiss et al. 2008, Kudela et al. 2008). The cell abundances estimated during the bloom in San Luis Obispo Bay (up to 10^7 cells·L^{-1}) are the highest reported in California to date. We speculate that the microscopical counts of *Cochlodinium* blooms have significantly underestimated cell abundances during these blooms due to losses of identifiable cells during preservation. The establishment of cultures of *C. fulvescens* will allow for a comparison between gene copy numbers and cell abundance which is a natural next step for this study.

Numerous studies have examined the ecology of *Cochlodinium* species, yet little is known regarding the ecophysiology of *C. fulvescens* and the specific environmental factors leading to bloom formation in California. In general, *Cochlodinium* species have been documented in both cool and warm waters, have been shown to be capable of utilizing both organic and inorganic nitrogen sources, and have been observed in association with moderate nutrient loading (Kudela et al. 2008). The primary goal of this study was the development and demonstration of a tool that could facilitate the collection of abundance data for *C. fulvescens* from environmental samples. Therefore, conclusive determination of the environmental factors promoting the development and maintenance of *C. fulvescens* blooms in California cannot be made from the modest amount of information provided here. However, the field results from Monterey Bay and San Luis Obispo Bay revealed that cooling of surface water temperatures corresponded to the onset and persistence of *C. fulvescens* blooms in these ecosystems. These results are in agreement with previously reported temperature conditions during blooms of *Cochlodinium* in Monterey Bay in 2004 and 2006 (Kudela et al. 2008). The observed increases in nitrogenous nutrients (including nitrate, ammonium and urea), presumably due to river runoff from the San Lorenzo River, preceded the peak copy number concentrations of *C. fulvescens*. These results from Monterey Bay provide anecdotal evidence that decreased surface

temperatures and the injection of nitrogenous nutrients that often accompany upwelling events and/or river runoff provide favorable growing conditions for *C. fulvescens* in central California.

The qPCR method developed in this study provides a new tool that can be used to identify and quantify *C. fulvescens* in natural populations. The use of this method in future field studies will be valuable for providing new insights regarding the ecophysiology of *C. fulvescens* and specific environmental conditions that lead to the development and maintenance of blooms in California. The factors that lead to bloom formation are critical to HAB management as well as to developing predictive models for HAB events.

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