Note

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Temporal changes in the abundance of the fish-killing dinoflagellate *Karlodinium veneficum* (Dinophyceae) in Tongyeong, Korea

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The toxic dinoflagellate *Karlodinium veneficum* has been implicated in numerous fish kill events around the world. Since this species commonly co-occurs with other morphologically similar dinoflagellates, field monitoring of this species in natural waters via light microscopy only has been problematic. In this study, we investigated temporal changes in *K. veneficum*'s abundance in the waters of Obido, Tongyeong, using a species-specific real-time polymerase chain reaction (PCR) assay. The field survey, from April to December 2010, revealed *K. veneficum* occurred at low densities (12 to 425 cells L^{-1}) during this time and that cell numbers peaked in June (early summer in Korea), indicating this species generally occurs in the warmer season (mostly at 16.9-22.3°C and 33.4-34.5‰) in the Obido area.

Key Words: dinoflagellate; *Karlodinium veneficum*; Obido; real-time PCR; red tide

INTRODUCTION

Karlodinium veneficum (Ballantine) J. Larsen, a mixotrophic dinoflagellate with a worldwide distribution, has been implicated in numerous fish kill events around the world (Place et al. 2008). *K. veneficum* produces water-soluble toxins that kill fish through gill disruption; however, researchers have successfully determined the structure of these karlotoxins (KmTxs), as they occur in *K. veneficum* (Kempton et al. 2002, Place et al. 2008). *K. veneficum* superficially resembles other small dinoflagellates < 15 µm in size, including *Pfiesteria piscicida* and *P. shumwayae*, which commonly co-occur with *K. veneficum* in the marine environment (Litaker et al. 2005, Garcés et al. 2006). These species are characterized by their distinct Kofoidian thecal plate formula. Definitive identification of these dinoflagellates relies on ultrastructural analyses via scanning electron microscopy (SEM), which are labor-intensive and unsuitable for rapid sample processing (Bergholtz et al. 2006, Garcés et al. 2006). The real-time polymerase chain reaction (PCR) technique very sensitively detects and quantifies DNA over a broad dynamic range (Walker 2002). Certain studies have used this method for detecting and quantifying a number of dinoflagellates (Bowers et al. 2000, Park et al. 2009). A previous study developed the TaqMan and SYTO9 format real-time PCR probes for *K. veneficum*, and these probes showed comparable results and high detection sensitivities (Park et al. 2009). In this study, we used a TaqMan-based real-time PCR probe that does not require a melting curve analysis to investigate temporal variations of *K. veneficum* abundances in Obido waters, Tongyeong, the location of many finfish farms.

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MATERIALS AND METHODS

Culture and analysis of environmental factors

We obtained *Karlodinium veneficum* (CCMP 415) from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). The culture was maintained in 30‰ f/2 medium (Guillard and Ryther 1962), without sodium silicate, at 20°C, under cool white fluorescent lamps (100-μmol photons m^{-2} s^{-1}) on a 12 : 12-h light : dark cycle. Surface water temperature and salinity were measured *in situ* with a water quality monitor (YSI 600 XL; YSI Nanotech, Yellow Spring, OH, USA).

Water sample collection and DNA extraction

From April through December 2010, we collected 250 mL surface water samples, at 1 month intervals, from each of 12 stations in Obido, Tongyeong, Korea (Fig. 1). We filtered the water samples onto a 1.2 μm pore, 25 mm diameter glass microfiber GF/C filter (Whatman Ltd., Maidstone, England), placed each filtered sample in a 2 mL microcentrifuge tube, and stored it at -70°C until DNA extraction. To prevent target DNA degradation, we processed these filtering samples upon a research vessel and extracted the samples genomic DNA from the samples within 2 months, using a phenol-chloroform extraction protocol (Hosoi-Tanabe and Sako 2005).

Real-time PCR condition

We used the following reagents to create the reaction mixture, to a final volume of 10 μL : 5 μL of platinum quantitative PCR supermix-UDG (Invitrogen, Eugene, OR, USA); forward and reverse primers, each at a final concentration of 0.3 μM; fluorogenic probe at a final concentration of 0.1 μM; 0.5 μL of template DNA (Park et al. 2009); and PCR grade water (Table 1). The thermal cycling condition comprised 2 min at 50°C and 2 min at 95°C, followed by 45 ten-s cycles at 95°C and 45 s at 60°C. All samples were analyzed in triplicate. We collected the fluorescence data at the end of each cycle, while a Rotor-Gene 6000 instrument (Corbett Research, Sydney, Australia) automatically determined the cycle threshold. To remove the surface waters' PCR inhibitors, we diluted the template DNA tenfold before use.

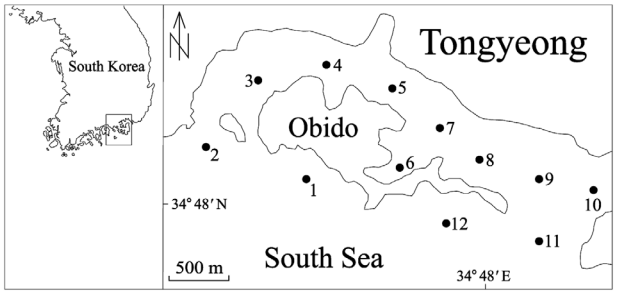

Fig. 1. Surface water sample collection locations for this study.

Construction of the standard curve for cell quantification

Before harvesting the cells (12,000 cells), we collected the laboratory-cultured *K. veneficum* and estimated its cell numbers via light microscopy (LM). After extracting the DNA, we used 10-fold serial dilutions of the DNA extracts to construct the standard curve (triplicate measurements by means of real-time PCR). To evaluate the success of the real-time PCR measurements, we calculated the correlation coefficient (r^2), reaction efficiency [$-1 + 10^{(-1/slope)}$], slope (M), and intercept (B), using the Rotor-Gene software version 1.7 (build 61; Corbett Research, Cambridge, UK). In the environmental samples, we calculated the target species' cell numbers as C_t values and measured them via comparison with the standard curve. In addition, we observed the surface water samples via LM to check the *K. veneficum* cell numbers.

Table 1. Primers and TaqMan probe for the *Karlodinium veneficum*-specific real-time polymerase chain reaction assay

Dinoflagellate	Forward / Reverse / Probe	Primer / Probe name	Sequence (5'→3')	Reference
Karlodinium veneficum	Forward	KVITSF3	CTGTGAACTTCTTTGTGAGCTCTT	Park et al. 2009
	Reverse	KVITSR3	TAGCGATAGCTTCGCAGACA	
	Probe	KVITSP3	FAM-AGGTGAATCCCAATGCTGCTCCACTA-TAMRA	

Primers / probes are labeled: F, forward; R, reverse; P, probe.

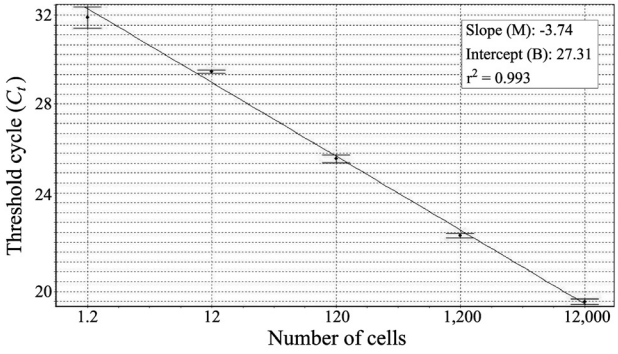

Fig. 2. A standard *Karlodinium veneficum* curve, showing the linear relationship between the C_t values and cell numbers from the *K. veneficum*-specific assay (r^2 = 0.993). The standard errors from each set of three measurements are shown as error bars.

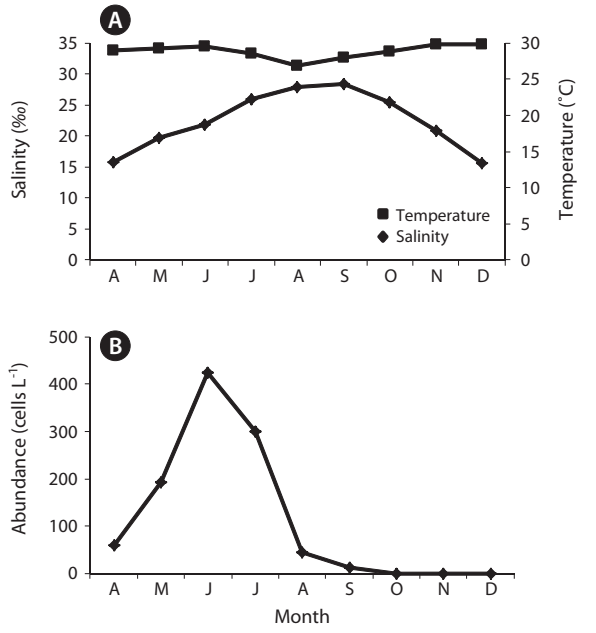

Fig. 3. (A) Temporal variations in temperature and salinity of surface waters in Obido. (B) Temporal variations in *Karlodinium veneficum* abundance in Obido, Tongyeong from April through December 2010, as quantified by real-time polymerase chain reaction. The values are the means of the triplicate wells.

RESULTS

We constructed a standard curve with known cell numbers and established a strong linear relationship between the C_t and the log of the starting cell number, with a correlation coefficient (r^2) ≥ 0.993 (Fig. 2). The assay could detect less than one *K. veneficum* cell in a reaction.

The field survey of the Obido waters showed that *K. veneficum* cell concentrations were generally low (Table 2, Fig. 3). The 12 sampling stations' average cell numbers ranged from 12 to 425 cells L^{-1}, and these cell numbers increased to 425 cells L^{-1} in June. *K. veneficum* was more abundant in early summer in the Obido area. The microscopic analysis revealed low *K. veneficum* and *Karlodinium* spp. cell abundances during the survey, and we observed these species only in samples that tested positive via the real-time PCR assay (Table 2). Surface water temperature and salinity ranged from 13.4-24.3°C and 31.3-34.8‰, respectively (Fig. 3).

DISCUSSION

Researchers have used a number of molecular methods, including real-time PCR, fluorescent *in situ* hybridization (FISH), sandwich hybridization, competitive PCR, a heteroduplex mobility assay, and fluorescent fragment PCR, to identify dinoflagellates rapidly (Oldach et al. 2000, Haywood et al. 2007, Park et al. 2007). TaqMan based real-time PCR probes have been previously designed to the internal transcribed spacer (ITS) rDNA and small subunit rDNA for the quantitative detection of *K. veneficum* (Handy et al. 2008, Park et al. 2009). Studies have extensively tested those species-specific assays against related organisms for their assay specificity and have successfully used these assays specifically for detecting *K. veneficum* in environmental samples (Handy et al. 2008, Park et al. 2009). The real-time PCR assay this study used specifically and sensitively detected *K. veneficum*, allowing us to identify low quantities of this dinoflagellate in our water samples (below one cell per reaction;

Table 2. *Karlodinium veneficum* abundance in Obido, Tongyeong, from April through December 2010, as measured by real-time polymerase chain reaction

Date	Cells L^{-1}	LM result
April	60	+
May	192	+
June	425	+
July	301	+
August	45	+
September	12	−
October	0	−
November	0	−
December	0	−

Values are the positive detection means: +, positive detection; −, negative detection by light microscopy (LM) analysis.

the real-time PCR could theoretically detect even one copy of the dinoflagellate's DNA). The rDNA-based real-time PCR probe possesses such a high sensitivity because the rDNA in most eukaryotes is repeated in tandem at a high copy number. For example, one study reports 100 to 200 copies in one *P. piscicida* cell (Saito et al. 2002).

K. veneficum commonly co-occurs with morphologically similar species, such as *Pfiesteria* species, and research has correlated *K. veneficum* with numerous fish kill events in many countries (Fensin 2006, Place et al. 2008). Small gymnodinioid dinoflagellates, including *K. veneficum*, have been associated with toxic activity since the 1950s (Ballantine 1956, Place et al. 2008). This species produces a suite of compounds with hemolytic, cytotoxic, and ichthyotoxic properties (Deeds et al. 2002) which are often known as KmTxs. In laboratory bioassays, the purified toxins cause the deaths of supportive cells in menhaden gills and eventually result in fish mortality (Deeds et al. 2006). In the present study, a temporal survey of *K. veneficum* cell densities in Obido waters shows *K. veneficum* (maximum 425 cells L^{-1}) mostly peaked in June, indicating potential, early-summer KmTx fish kills in the Obido area (16.9-22.3°C and 33.4-34.5‰) if blooms occur during favorable environmental conditions. In *K. veneficum* cells, the KmTxs' toxicities are KvTX1, 11.6 ± 5.4 ng mL^{-1} and KvTX2, 47.7 ± 4.2 ng mL^{-1} at 4.0×10^6 cells L^{-1} (Galimany et al. 2008). Researchers have generally reported that fish kill events in nature correlate with this species when it is at over 300 cells mL^{-1}. Although studies have also reported negative effects on marine animals, such as mussels, at pre-bloom concentrations (Galimany et al. 2008), the cell amounts that this study found might have negligible effects on aquaculture animals such as oysters, mussels, and finfishes in Obido waters.

In conclusion, the real-time PCR assay was highly sensitive and specific for detecting and quantifying *K. veneficum* in the environment, and we successfully implemented this real-time PCR assay in a distributional study of this species in the Obido area. The results of this field survey via real-time PCR suggests *K. veneficum* occurs commonly in the warmer season in the Obido area and that researchers need to make ongoing investigations of the relationship between environmental factors and cell occurrences to illuminate the bloom dynamics of *K. veneficum*.

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