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Temporal Changes in Abundances of the Toxic Dinoflagellate *Alexandrium minutum* (Dinophyceae) in Chinhae Bay, Korea

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Abstract

Marine dinoflagellate *Alexandrium minutum* producing paralytic shellfish toxins is responsible for paralytic shellfish poisoning (PSP). To investigate its temporal distributions in Chinhae Bay where PSP occurs annually, SYBR Green Ⅰ based *A. minutum*-specific real-time PCR probe was developed on the LSU rDNA region. Assay specificity and sensitivity were tested against related species, and its specificity was further confirmed by sequencing of field-derived samples. Ten months field survey in 2008 (a total 100 surface water samples) by using the real-time PCR probe showed that *A. minutum* was detected at very low densities of 1-4 cells L^{-1} in May and June being spring in Chinhae Bay, Korea.

Key Words : *Alexandrium minutum*, Dinoflagellate, PSP toxins, Real-time PCR

1. Introduction

The mixotrophic dinoflagellate *Alexandrium minutum* Halim produces the potent neurotoxin and is responsible for paralytic shellfish poisoning (PSP)[1,2]. About 30 species are included within the genus *Alexandrium* Halim and the majority of PSP events have been caused by *A. tamarense*-complex and the *A. minutum* species group[2,3]. The PSP outbreaks occur globally and the range and the frequency of the blooms seem to be increasing world-wide[2]. The first known bloom of *Alexandrium* species and PSP event in Korean waters occurred in Chinhae Bay in 1978[4], since then the PSP events have recurred along the southern coasts of Korea.

The classification of species within *Alexandrium* is primarily based on details of the thecal plate pattern. Most *Alexandrium* species have relatively thin and smooth thecal plates, and their generic characteristics include the thecal plate formula Po, 4, 6, 5, 2, 6c and 9-10s[5]. For morphological identification of *Alexandrium* species, light microscopy and/or scanning electron microscopy (SEM) analyses on laboratory-cultured or field-derived cells are required. These conventional identification methods are labor intensive and not suitable for rapid sample processing. To overcome these difficulties, molecular identification methods have been applied for quantitative detection of target organisms[6,7]. A number of approaches have been used for identification of dinoflagellates. Real-time quantitative PCR offers highly sensitive and rapid analysis as well as high-sample throughput analysis. This method has been used for detection and quantification of a number of dinoflagellates including *Cryptoperidiniopsis brodyi* Steidinger et Litaker, *Cochlodinium polykrikoides* Margalef, *Karlodinium veneficum* (Ballantine) J. Larsen, and *Pfiesteria piscicida* Steidinger et Burkholder[6～10].

For understanding the ecological roles of *A. minutum*, information on the abundances of this dinoflagellate in environments is needed. However its tem-

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poral and geographic distributions are poorly understood. In the present study, *A. minutum*-specific real-time PCR assay using a TaqMan probe was developed based on the large subunit (LSU) rDNA. Temporal and spatial distributions of *A. minutum* in surface waters of Chinhae Bay, Korea were investigated using the newly designed real-time PCR probe.

2. Materials and Methods

2.1. Cultures

Cultures were obtained from NFRDI's collection of microalgae, University of Tasmania, and CCMP (Provasoli-Guillard National Center for Culture of Marine Phytoplankton) (Table 1). Mixotrophic/phototrophic cultures were maintained in f/2 medium of 28 ‰ of salinity[11] without sodium silicate at 24°C, with cool white fluorescent lamps of 100 µmol photons $m^{-2}s^{-1}$ on a 12 : 12-h light : dark cycle. Heterotrophic dinoflagellates were grown in f/2 medium of 15‰ of salinity at 24°C in the dark and *Rhodomonas salina* (Wislouch) Hill et Wetherbee was supplied to them as food.

2.2. Collection of surface water samples from Chinhae Bay

Surface water samples of 250 mL were collected monthly at 10 stations from February to November 2008 in Chinhae Bay, Korea (Fig. 1). The water samples were filtered onto a 1.2 µm pore-size, 25 mm diameter glass microfibre GF/C filter (Whatman, Ltd. Maidstone, England). The filtered sample was placed in a 2 mL microcentrifuge tube, and stored at 70°C until DNA extraction. To prevent degradation of the target DNA, filtering samples were processed on a research vessel.

2.3. DNA extraction

A phenol-chloroform extraction protocol was used for extraction of genomic DNAs from the surface water samples and laboratory cultures[12]. Filter samples were suspended in 900 µL of TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0) and ground with wooden applicator sticks. The sample was boiled

Table 1. Cultures used in this study

Culture code	Date of isolation	Locality	Identification
CCMP113	September, 1987	Ria de Vigo, Spain	*Alexandrium minutum*
NFFACA1	April, 2000	Japan	*Alexandrium catenella*
CCMP1911	September, 1998	Sequim Bay, USA	*Alexandrium catenella*
CCMP116	June, 1984	Ria de Vigo, Spain	*Alexandrium tamarense*
CCMP112	November, 1985	Ria de Vigo, Spain	*Alexandrium affine*
CCMP1718	January, 1987	Massachusetts, USA	*Alexandrium andersoni*
CCMP1719	November, 1985	New Hampshire, USA	*Alexandrium fundyense*
CCMP1773	1986	Fjorden, Denmark	*Alexandrium ostenfeldii*
CCMP1888	1962	Laguna Obidos, Portugal	*Alexandrium lusitanicum*
CCMP2082	June, 1985	Uchiumi Bay, Japan	*Alexandrium insuetum*
CCMP2215	January, 1984	Kanagawa, Japan	*Alexandrium hiranoi*
NFFCPO1	September, 2003	Gijang, South Korea	*Cochlodinium polykrikoides*
NFFCPO2	August, 2005	Yeosu, South Korea	*Cochlodinium polykrikoides*
NFFGIMI	May, 1998	Narodo, South Korea	*Gymnodinium impudicum*
CCMP1678	1993	East Victoria, Australia	*Gymnodinium impudicum*
PPSB25	May, 2003	Surabaya, Indonesia, Ballast water	*Pfiesteria piscicida*
CCMP2807	December, 2002	North Carolina, USA	*Pfiesteria shumwayae*
CBWA12	February, 2003	Brunswick River, Western Australia	*Cryptoperidiniopsis brodyi*
CBHU1	February, 2003	Huon River, Tasmania, Australia	*Cryptoperidiniopsis brodyi*
CCMP2229	August, 2001	Florida, USA	*Karenia brevis*
CCMP1974	1995	Chesapeake Bay, USA	*Karlodinium veneficum*
CCMP415	1976	Norway	*Karlodinium veneficum*
CCMP1589	September, 1992	Rhode Island, USA	*Prorocentrum micans*
CCMP1170	February, 1982	Victoria, Australia	*Rhodomonas salina*

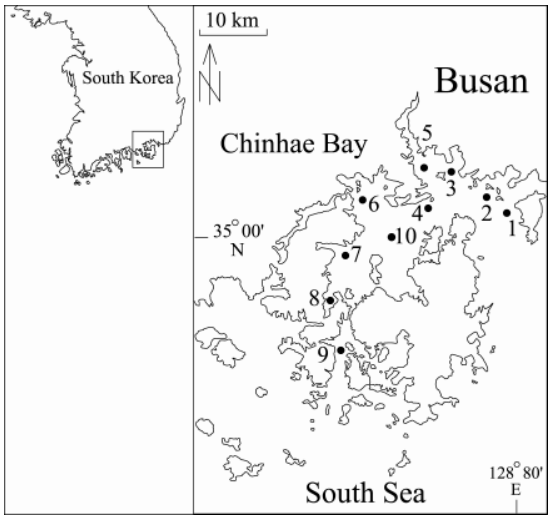

Fig. 1. Locations where surface water samples were collected for this study. Water samples were obtained from 10 stations in Chinhae Bay from February to November 2008.

at 100°C for 5 min, followed by adding 900 μL of phenol : chloroform : isoamyl alcohol (25 : 24 : 1). The sample was then mixed thoroughly and centrifuged at 14,000 rpm for 10 min. The supernatant transferred to a new tube, and 30 μL of 3 M sodium acetate (pH 5.2) and 700 μL of 99.5% of ethanol (20°C) were added, followed by incubation of the sample at 20°C for 30 min. The DNA samples were centrifuged at 14,000 rpm for 20 min at 4°C, and was rinsed twice with 70% of ethanol. They were then dried and dissolved in 100 μL of TE buffer.

2.4. Design of a SYBR Green Ⅰ-based real-time PCR probe specific for *A. minutum*

The LSU rDNA sequences of *A. minutum* available from GenBank were aligned with sequences of other organisms (about 100 species were compared) using the program ClustalX[13]. Unique sequences were manually searched from the alignments for designing a SYBR Green Ⅰ-based species-specific real-time PCR probe. The *A. minutum* specific probe forms 129-bp amplicon in size, and it was designed on the D2 of LSU rDNA region because the region was highly variable between species but conserved within the species (Table 2). The sequences of the primers had at least 20-bp mismatches with sequences of closely related organisms. The sequences of the primers were also checked against published sequences in GenBank by BLAST homology search. Optimal melting temperature and secondary structure of primer and probe sequences were predicted by Primer 3 (Whitehead Institute and Howard Hughes Medical Institute, Maryland) and Oligo analyzer 3 (Intergrated DNA Technologies, Inc., Iowa) software. The primer set was synthesized by Sigma-Proligo (Paris, France).

2.5. Assay specificity of the *A. minutum*-selective real-time PCR probe

A total of 24 species was used for testing assay specificity (Table 1). Specificity of the forward and reverse primers was tested using standard format PCR which does not require a fluorogenic probe. PCR amplicons of the target DNA (129-bp) in a 2% agarose gel stained with ethidium bromide was inspected to confirm the absence of nonspecific reactions. Subsequently, specificity of the SYBR Green Ⅰ-based real-time PCR probe was tested against other relatives. When applied to field samples, positive samples by real-time PCR were further analyzed by gel electrophoresis using 2% agarose gels for confirmation of assay specificity.

Table 2. Primers for species-specific real-time PCR

Dinoflagellate	Forward/ Reverse/Probe	Name	Sequence (5'→ 3')	Reference
Alexandrium minutum	Forward	AMLSUF	TTCTGCAAATCATTACCCTTGC	This study
	Reverse	AMLSUR	CCCTTCGACAAAAGAGCATATACA	
Cryptoperidiniopsis brodyi	Forward	CNITSF	TTGACACGTTGAAGTGAWGGA	8)
	Reverse	CBITSR	ACAGCCAATGAAAGAGTKATGACAA	
	Probe	CBITSP	FAM-CATCTCATCGCTCGCCGTCGAT-BHQ1	

2.6. Verification of assay specificity by sequencing of environmental samples

One of positives (Station 1, May 2008) was used for sequencing analysis. The rDNA (129-bp in size) of positive sample was amplified with primers AMLSUF-AMLSUR in a standard PCR platform using Takara *EX Taq* DNA polymerase with 39 cycles of 94°C for 1 min, 60°C for 1 min 30 s, 72°C for 2 min. The DNA band was visualized on 2% agarose gel stained with ethidium bromide, and the PCR product was cloned and insert-containing plasmid DNA was purified. Three clones of the purified product were selected for sequencing, and their analyses were conducted with pUC/M13 primers (Promega, Madison, WI, USA) by Macrogen (Seoul, Korea). Expectation values (E-values) and sequence similarity of the field sequences were estimated by a nucleotide BLAST program.

2.7. Real-time PCR conditions

Primers AMLSUF-AMLSUR had a similar melting temperature (Tm) of approximately 60°C when tested by Primer 3 computer program. Subsequently, 60°C was chosen for the annealing temperature. The following reagents were added for TaqMan based real-time PCR: 5 μL of platinum quantitative PCR supermix-UDG (Invitrogen, Eugene, Oregon, USA), primers at a final concentration of 0.2 μM, fluorogenic probe at a final concentration of 0.15 μM, 1 μL of template DNA, and PCR grade water to a final volume of 10 μL. For SYBR Green I based real-time PCR, the following components were added: 5 μL of platinum SYBR Green quantitative PCR supermix-UDG (Invitrogen, Eugene, Oregon, USA), primers at a final concentration of 3.0 μM, 1 μL of template DNA, and PCR grade water to a final volume of 10 μL. The thermal cycling conditions consisted of 2 min at 50°C (uracil DNA glycosylase incubation for prevention of the reamplification of carryover PCR products) and 2 min at 95°C following by 40 cycles of 15 s at 95°C and 60 s at 60°C. DNA melting curves were monitored from 70°C to 85°C in 1°C increments using a 5 s hold at each step. The assay was analyzed in the FAM channel (excitation/emission maxima of 470 nm-510 nm) of the Rotor Gene instrument. Fluorescence data were collected at the end of each cycle by the real-time PCR instrument (Rotor Gene 6000, Corbett Research, Sydney, Australia), and they were analyzed using the Rotor Gene software (v1.7 build 61).

2.8. Standard PCR conditions

Standard PCR was performed using Takara *EX Taq* DNA polymerase (Takara Mirus Bio, Madison, WI, USA) with 32 cycles of 94°C for 1 min, 60°C for 1 min 30 s, 72°C for 1 min. Positive DNA bands were visualized on 2% agarose gel stained with ethidium bromide. The following reagents were added for standard PCR: 0.1 μL of *EX Taq* DNA polymerase, primers at a final concentration of 300 μM, 2 μL of *EX Taq* buffer, 2.5 mM of dNTP mixture, 0.5 μL of template DNA, and PCR grade water to a final volume of 20 μL.

2.9. Standard curves for enumeration of cell numbers

Laboratory-cultured *A. minutum* was used for construction of a standard curve. Cell numbers were estimated by light microscopy using a Sedgwick-Rafter counting chamber before harvesting the cells (total 25,530 cells). Genomic DNA was extracted, and 10-fold serial dilutions of the DNA extracts were used to construct the standard curve. The curve was constructed by triplicate measurements using real-time PCR. The cell number of *A. minutum* in surface water samples was calculated as C_T values, and was measured by comparison with the standard curve. The accuracy of the standard curves was evaluated using known concentrations of *A. minutum* (18,300, 3,660 and 732 cells; n = 3) spiked into sterile-filtered field samples (0.2 μm membrane filter; MFS, California). After DNA extraction, cell numbers estimated by real-time PCR were compared to the cell numbers estimated by light microscopy.

2.10. PCR inhibitor removal/template DNA dilution from surface waters

A real-time PCR probe specific for *Cryptoperidiniopsis brodyi* Steidinger et Litaker was used for checking the inhibitors because *C. brodyi* has not been reported in Korean waters. Serial DNA dilutions of field sam-

ples(non-dilution, 10-fold, and 50-fold dilutions) spiked with *C. brodyi* DNA (CBWA12; 1.5 ng μL^{-1}) were amplified using real-time PCR, and their C_T values were compared. *C. brodyi* DNA without the addition of the field DNA was used for a positive control. Water samples collected in May from Chinhae Bay were used for template DNA. The absence of inhibitors was confirmed in 10-fold and 50-fold DNA dilutions. Ten other field samples were randomly chosen and were tested to confirm the absence of the inhibitors. Subsequently, 10-fold dilution of field-derived DNA was used for removal of PCR inhibitors.

3. Results

3.1. Specificity of *A. minutum*-selective real-time PCR assay

Specificity of forward and reverse primers was tested using SYBR Green I format real-time PCR. When the PCR product was visualized on agarose gel, only one amplicon of the expected size (129-bp) for the target species was produced and there was no non-specific reactions against related species (Fig. 2). Assay specificity was then tested in SYBR Green I format real-time PCR against related species. Since SYBR Green I format real-time PCR is amplicon sequence non-specific method, this dye detects any double-strand DNA molecules. The formation of non-specific amplicons was checked by melting curve analysis. *A. minutum* gave a strong positive fluorescent signal after 13 cycles, while related species produced faint signals only after 30 cycles (result not shown). The mean value of melting temperature (^oC) for positives was 79.7 and standard deviation was 0.05, while that of negative controls/related species was 75 to 77^oC (Fig. 3) indicating that the weak signals from the negative control observed after 30 cycles were likely to be primer dimers. Assay specificity was further confirmed by sequencing of a field-derived sample positive for *A. minutum*-specific real-time PCR assay. The partial LSU rDNA sequences (129-bp) obtained from Station 1, May, 2008 were identical to documented *A. minutum* sequences (GenBank accession number, AY831408) and low expectation values (E-value) of less than 1e-5 were estimated by a nucleotide BLAST program indicating that assay was specific for *A. minutum*.

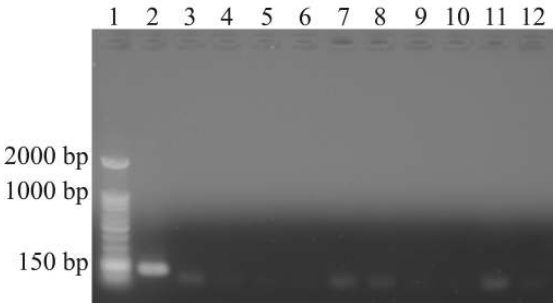

Fig. 2. Agarose gel analysis showing *A. minutum*-selective real-time PCR product (129-bp). Assay specificity was tested against related species (Table 1). Lanes: 1, 2 kbp-ladder molecular size marker; 2, *A. minutum* 3, *A. tamarense* 4, *A. affine* 5, *A. hiranoi* 6, *A. andersoni* 7, *A. fundyense* 8, *A. ostenfeldii* 9, *A. lusitanicum* 10, *A. insuetum* 11, *A. catenella* 12, no-template control.

3.2. Standard curve and detection limit

A standard curve was constructed using 10-fold serial dilutions of DNA extracts from *A. minutum*. A strong linear correlation between log (known cell number) and C_T value was yielded for the assay (correlation coefficient R^2 of 0.990). The values of slope (M) and intercept (B) were -3.161 and 24.409, respectively (Fig. 4). Detection limit of the assay was 2.5 cells per reaction within the dynamic range of cell numbers in the linear. The accuracy of standard curve was also tested. When DNA extracts from known cell numbers of *A. minutum* (18,300, 3,660 and 732 cells)

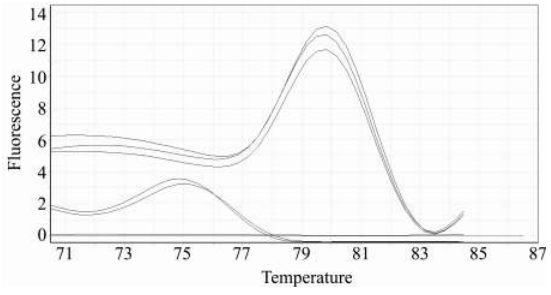

Fig. 3. Melting curves of SYBR Green I -based assay with *A. minutum* and *A. insuetum* DNA extracts and no-template. Melting temperature (^oC) of *A. minutum* was 79.7 ± 0.05 (n=3).

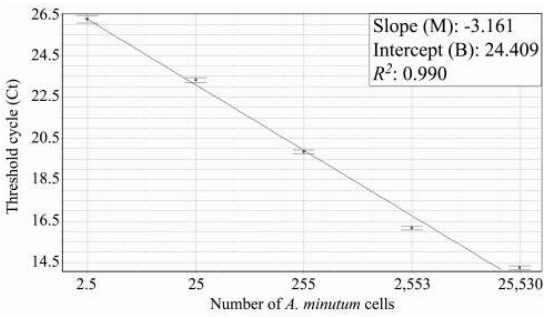

Fig. 4. Linear relationship between the C_T values and the cell numbers for *A. minutum* (R^2 = 0.990). The standard errors from three measurements are shown as error bars.

spiked with environmental samples were compared to the cell standard curve, cell numbers of the 10-fold diluted spiked cells (2,450 ± 620, 511 ± 141, and 58 ± 49 cells, respectively; n = 3; $P > 0.05$ by Student's *t* test) did not significantly differ from those of standard curve (1,830, 366 and 73 cells).

3.3. Temporal changes in *A. minutum* abundances in Chinhae Bay

Of 100 water samples analyzed, only 3 were positive for *A. minutum* (Table 3). The DNA of *A. minutum* was detected at 1 to 4 cells L^{-1} in May and June. The absence of *A. minutum* in the other samples was checked by melting curve analysis and gel electrophoresis using 2% agarose gels. Melting curve analysis was conducted for all samples, and gel electrophoresis was performed with 30 samples randomly chosen from negatives. All of the samples except for the 3 positives showed melting temperature of less than 77°C and no positive bands on gel indicating that there was no false negative reaction.

4. Discussion

4.1. Quantitative detection of *A. minutum* in natural environments

Information on geographic and temporal distributions of dinoflagellates is necessary to understand their bloom dynamics. Although *Alexandrium* species are responsible for paralytic shellfish poising for decades, their accurate detection has been problematic due to difficulty in rapid identification of these species. To address this issue, a number of molecular methods have been developed for quantitative detection of the cells. Real-time PCR is a validate method that offers highly sensitive and accurate detection of dinoflagellates. This method also reduces the analytical time to as little as 3 h from the time a sample arrived at the laboratory. The SYBR Green I format real-time PCR has been used for a number of dinoflagellates including the genus *Alexandrium*[14]. The *A. minutum* specific real-time PCR probe developed in this study provided a high sensitivity that allows the detection limit of less than 2.5 cells per reaction, which is similar to the results reported in other studies[6-8]. When applied to environmental samples, false-negative results caused by PCR inhibitors coextracted with target DNA may be problematic. Surface water contains PCR inhibitors such as phenolic compounds, heavy metals, and humic acids[15]. Dilution of template DNA obtained from 250 mL of surface water effectively removed the inhibitors indicating that there were no false negative reactions in the present study. For further confirmation of assay specificity, identification of field samples by culture dependent and independent methods may be desirable. Since a mono culture of *A. minutum* was not established during this survey, field-derived samples were

Table 3. Occurrences of *A. minutum* in Chinhae Bay, Korea during February and November 2008 measured by species specific real-time PCR assay. Surface water samples were collected from 10 stations at 1 month intervals (a total of 100 water samples). The primer set for real-time PCR is shown in Table 2. Only positive detection is shown. Values are the mean ± standard deviation of triplicate wells

Sampling site	Sampling date and cells liter^{-1}									
	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.
1	N/D	N/D	N/D	4±3	N/D	N/D	N/D	N/D	N/D	N/D
8	N/D	N/D	N/D	N/D	1±0.6	N/D	N/D	N/D	N/D	N/D
21	N/D	N/D	N/D	1±0.4	N/D	N/D	N/D	N/D	N/D	N/D

sequenced for further confirmation of assay specificity. The sequence result indicates that this assay provides *A. minutum* specific reaction.

4.2. Temporal distributions of *A. minutum*

For 10 months survey in Chinhae Bay, *A. minutum* was only found at very low densities (1-4 cells L^{-1}) in May and June. Since the difficulty in rapid identification of this species in natural samples, cell numbers estimated by real-time PCR did not compared to those counted by light microscopy. To verify the accuracy in the quantification of cells by the real-time PCR, known concentrations of *A. minutum* spiked into sterile-filtered field samples were compared to the standard curve, and the result indicated that *A. minutum* in field samples was accurately quantified in the present study. One possibility to explain the low cell density is the real-time PCR probe developed in this study may not detect all of the genotypes in the species. There has been known several genotypes in *A. minutum* worldwide, and there are high genetic variations within the LSU rDNA locus of this species[16]. Since the high genetic variation, the real-time PCR probe from this study showed high assay specificity and may reduce false-positive reactions when applied to natural samples. This assay can detect most of genotypes including strains from the U.S., Hong Kong, China, North Atlantic Ocean, Spain, Italy and France, which have identical sequences with those of *A. minutum*-specific primers. However, the primer set showed sequence differences with strains from Cape Town harbor in South Africa (GenBank accession numbers, DQ453522, DQ453524) and Fleet Lagoon in the U.K. (AY268598, AY705869) indicating that numbers of *A. minutum* may be underestimated by the real-time PCR probe if there exists various genotypes in Chinhae Bay. Other sequence regions such as mitochondrial cytochrome b successfully applied to detection of *Pfiesteria* species[17] may be an alternative choice for designing a species-specific probe. PSP events have caused the closure of shellfish harvesting grounds in Chinhae Bay when shellfish exceed the quarantine toxin level of 80 μg STXeq 100 fresh weight g^{-1} [18]. Since the lack of information on the correlation between *A. minutum* abundances and PSP events in Chinhae Bay, it is difficult to understand its association with PSP or its bloom dynamics. However, the present study showed the occurrences of *A. minutum* in spring when PSP occurs in Chinhae Bay.

5. Conclusion

In summary, the present study developed *A. minutum*-specific real-time PCR probe based on the LSU rDNA region. The assay was sensitive and specific to the target cells, and it was successfully applied to investigate temporal changes in abundances of this species. The occurrences of *A. minutum* at very low density indicate that this dinoflagellate is a minor component of the plankton community in Chinhae Bay.

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