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Integration of the nuclease protection assay with sandwich hybridization (NPA-SH) for sensitive detection of *Heterocapsa triquetra*

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Abstract

Microalgae are photosynthetic microorganisms that function as primary producers in aquatic ecosystems. Some species of microalgae undergo rapid growth and cause harmful blooms in marine ecosystems. *Heterocapsa triquetra* is one of the most common bloom-forming species in estuarine and coastal waters worldwide. Although this species does not produce toxins, unlike some other *Heterocapsa* species, the high density of its blooms can cause significant ecological damage. We developed a *H. triquetra* species-specific nuclease protection assay sandwich hybridization (NPA-SH) probe that targets the large subunit of ribosomal RNA (LSU rRNA). We tested probe specificity and sensitivity with five other dinoflagellates that also cause red tides. Our assay detected *H. triquetra* at a concentration of 1.5×10^4 cells/mL, more sensitive than required for a red-tide guidance warning by the Korea Ministry of Oceans and Fisheries in 2015 (3.0×10^4 cells/mL). We also used the NPA-SH assay to monitor *H. triquetra* in the Tongyeong region of the southern sea area of Korea during 2014. This method could detect *H. triquetra* cells within 3 h. Our assay is useful for monitoring *H. triquetra* under field conditions.

Key words: nuclease protection assay sandwich hybridization, *Heterocapsa triquetra*, red tide, monitoring

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1 Introduction

Microalgae, which occur as single cells or chains, are photosynthetic microorganisms and primary producers in aquatic ecosystems (Debelius et al., 2009; Ebenezer et al., 2012; Morel and Price, 2003). Microalgae can be utilized in producing commercial products, such as cosmetics, food, biofuels, and health-promoting medications (Priyadarshani and Rath, 2012; Spolaore et al., 2006). Some microalgae can grow quickly under favorable environmental conditions (Naito et al., 2005). The rapid growth of microalgae makes it possible to mass-produce commercial products, but also cause harmful algal blooms in marine ecosystems.

Heterocapsa triquetra is a common bloom-forming dinoflagellate species present in estuarine coastal waters and brackish waters worldwide (Tas, 2015; Baek et al., 2011). Although it does not release toxins, high-density blooms can lead to ecological damage (Litaker et al., 2002a, b). *Heterocapsa triquetra* blooms occur regularly in the North Sea, Atlantic Ocean, the Mediterranean Sea, and the eastern Pacific Ocean (Litaker et al., 2002a). In Korea, *H. triquetra* is a dominant species in the Masan Bay and the Jinhae Bay from autumn to winter (Baek et al., 2011; Lee and Han, 2007). *Heterocapsa triquetra* blooms can harm the commercial shellfish and fish industries (Archambault et al., 2004; Lu and Hodgkiss, 2004). Therefore the identification and monitoring of *H. triquetra* are needed for characterization of their distribution and favorite blooming conditions.

The traditional method for identification and quantitation of microalgal species is observation of morphology by microscopy, but this requires an experienced biologist and is time-consuming. Moreover, different biologists may report different results when microalgae are identified by this traditional method (Suh et al., 2016), and changes in the appearance and size of microalgae under different environmental conditions or different growth stages can make identification difficult (Xin et al., 2005). Therefore, there is increasing interest in several molecular-based detection methods, such as the polymerase chain reaction (PCR), fluorescence *in situ* hybridization (FISH), real-time PCR, restriction fragment length polymorphism (RFLP), flow cytometry and microscopy (FlowCAM), and the sandwich hybridization assay (SHA) (Godhe et al., 2001; Chen et al., 2013; Antonella and Luca, 2013; Jedlicki et al., 2012; Hyka et al., 2013; Diercks et al., 2008b).

The rRNA-targeted sandwich hybridization assay (SHA) uses

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two probes, a capture probe and signal probe. The capture probe (rRNA) and signal probe are combined like a sandwich (Tyrrell et al., 2002). Previous researchers developed SHA-detection methods for several microalgae: *Heterosigma akashiwo*, *Fibrocapsa japonica*, *Alexandrium minutum*, *Gymnodinium catenatum*, *Lingulodinium polyedrum*, and *Protoceratium reticulatum* (Tyrrell et al., 2002; Ayers et al., 2005; Diercks et al., 2008a, b). Subsequently, problems with this method appeared when targeting unstable RNA with limited specificity and reproducibility. This led to development of a nuclease protection assay that is based on sandwich hybridization (NPA-SH) (Cai et al., 2006; Zhen et al., 2007). This newer method uses the same two probes as the SHA method, as well as a third NPA probe. More specifically, the NPA-SH method uses S1 nuclease after hybridization of the target RNA and NPA probe. The S1 nuclease degrades the sample to single stranded oligonucleotides, resulting in perfectly matched NPA probes. This method makes many copies of the target DNA, and the NPA probe is more stable and specific than the rRNA used for the SHA probe (Suh et al., 2016). Previous researchers have already developed NPA-SH detection methods for *Cochlodinium polykrikoides*, *Prorocentrum minimum*, *Prorocentrum micans*, *Prorocentrum donghaiense*, *Skeletonema costatum*, and *Phaeocystis globosa* (Suh et al., 2016; Cai et al., 2006; Zhen et al., 2008, 2009). However an NPA-SH method for detection of *H. triquetra* has not yet been developed. This species is currently detected by a beta-methylamino-L-alanine (BMAA) method that employs ultra-high pressure liquid chromatography coupled with mass spectroscopy (UHPLC-MS/MS).

In this study, we developed a *H. triquetra* NPA-SH species-specific probe in an effort to easily and rapidly detect this species, comparable to other NPA-SH assays for microalgae. Then we used the method for field monitoring near Tongyeong, in the southern sea area of Korea. Tongyeong is located near the Masan Bay and the Jinhae Bay, where blooms of *H. triquetra* are common (Park et al., 2013). However compared with other places near Tongyeong, such as the Masan Bay and the Jinhae Bay, data on *H. triquetra* is poor near Tongyeong. Thus, we monitored *H. triquetra* near Tongyeong using species specific probes and the NPA-SH method from January to December of 2014.

2 Materials and methods

2.1 *Microalgae cultures*

Ten species of microalgae (*Asterionellopsis glacialis*, *Chattonella marina*, *Chaetoceros curvisetus*, *Eucampia zodiacus*, *Heterosigma akashiwo*, *Leptocylindrus danicus*, *Prorocentrum minimum*, *Scrippsiella trochoidea*, *Skeletonema marinoi*, and *Thalassiosira nordenskioeldii*) were collected in Tongyeong, and cultivated at the Korea Institute of Ocean and Science Technology (KIOST) in Geoje, Korea. *Cochlodinium polykrikoides* was obtained from the Library of Marine Samples in KIOST and *H. triquetra* was from a laboratory at KIOST (Baek et al., 2011). All 12 species were cultured in f/2 medium with salinity 30 at 20°C under 12 h light-dark cycle.

2.2 *Microalgae RNA prep and sequencing*

RNA was extracted according to a modification of the method described by Venugopalan and Kapoor (Venugopalan and Kapoor, 1997), and was used to produce cDNA using the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA). The LSU rDNA genes were subjected to PCR with a pair of primers (forward 5′-CGGAGGAAAAGAAACTAAC, reverse 5′-AGCTACTAGATGGTTCGAT) (Zhen et al., 2007). PCR amplification was conducted using a 20 μL reaction mixture that contained 2 μL of 10× reaction buffer, 2 μL of 2.5 mmol/L dNTPs, 2.5 units of Taq DNA polymerase (TaKaRa, Japan), 1 μL of 30 ng/μL total DNA, and 1 μL each of 10 μmol/L forward primer and reverse primer. The PCR amplification protocol was 10 min at 94°C, 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and then a 5 min extension at 72°C. The PCR products were separated by 1% agarose gel electrophoresis, and then purified using the MEGA-spin™ Agarose Gel DNA Extraction Kit (Intron, Korea). Products were then cloned into the pGEM-T-Easy Vector (Promega, Madison, WI, USA) and used for transformation of *E. coli* DH5α cells. LSU rRNA genes were sequenced by Bioneer Corporation (Daejeon, South Korea), and sequences were analyzed using DNAstar and MEGA6 software.

2.3 *NPA-SH specific probes and detection*

The sequenced LSU rDNA of *H. triquetra* was confirmed by search of BLASTn (http://www.ncbi.nlm.nih.gov/Blast.cgi). Then, the sequences of all 12 species were aligned, using Clustal W in MEGA6, and the most variable regions were identified for use in making an NPA probe for *H. triquetra*. Three probes were designed: a ~60-mer NPA probe targeting LSU rRNA; a 25-mer capture probe that was labeled with biotin at the 5′ end and had a 3′ terminal region that was complementary to the NPA probe; and a 25-mer signal probe that was labeled with fluorescein at the 3′ end and was complementary to the 5′ terminal region of the NPA probe. The NPA probe for *H. triquetra* was 5′-CCACGCTTGCGCTGAAGCAGCAGGCAATCACATTAGCACGCACCAATCTTGCCAAGAAGC; the capture probe was 5′-biotin-GCTTCTTGGCAAGATTGGTGCGTGC; and the signal probe was 5′-GCCTGCTGCTTCAGCGCAAGCGTGG-fluorescein (Table 1). All probes were chemically synthesized by Bioneer Corporation (Daejeon, Korea). The NPA-SH analysis was modified from Cai et al. (2006) (Zhen et al., 2007). Cultured *H. triquetra* were collected in a 1.5 mL Eppendorf tube after centrifugation to remove growth medium. Then, 950 μL of lysis buffer (80% formamide, 450 mmol/L NaCl, 5 mmol/L Na_2EDTA, 1 mg/mL yeast tRNA, 1% SDS, pH 6.4) and 50 μL of 10 mg/mL yeast t-RNA was added, and the sample was sonicated for 10 s with 50% duty cycle and 450 W output sets. The sample was centrifuged again to precipitate cell debris. Then, 30 μL of lysate, 3 μL of NPA probe solution (500 nmol/L NPA probes in lysis buffer), and 50 μL of mineral oil were mixed in a 1.5 mL Eppendorf tube. The sample was denatured at 98°C for 5 min and then cooled to room temperature to allow hybridization of the NPA probe with the 28S rRNA. Then, 30 μL of S1 nuclease mix was added (60 units S1 nuclease in 1.4 mol/L sodium chloride, 22.5 mmol/L zinc sulfate, 250 mmol/L sodium acetate, pH 4.5) (Promega, USA), and the sample was incubated for 1 h at 42°C to restrict non-hybridized regions. The reaction was stopped by adding 150 μL of a nuclease stop solution (62.5 mmol/L sodium hydroxide, 30 mmol/L EDTA, and 1× phosphate-buffered saline (PBS), pH 7.2). The mixture was then denatured at 98°C for 5 min, and used before it had cooled. The sample was coated with biotin-labeled capture probes in a 96-well streptavidin-coated microplate (Pierce Biotechnology, Inc. Rockford, IL.), with each well containing 100 μL of S1 nuclease-treated sample. The plate was cooled to room temperature for 5 min, washed three times with PBS and 0.5% Tween-20. Each well was filled with 100 μL of 5 nmol/L signal probes in a hybridization buffer (4× SSC, 10% formamide, 0.02% SDS pH 7.2), and the plate was incubated at 50°C for 20 min with shaking (130 r/min). After washing three times with PBST (3.2 mmol/L Na_2HPO_4, 0.5

Table 1. NPA sandwich hybridization specific probes

Detection method	Probe	Sequence	Length/bp
NPA-SH	Capture probe	Biotin-GCTTCTTGGCAAGATTGGTGCGTGC	25
	NPA probe	CCACGCTTGCGCTGAAGCAGCAGGCAATCACATTAGCACGCACCAATCTTGCCAAGAAGC	60
	Signal probe	GCCTGCTGCTTCAGCGCAAGCGTGG-FAM	25

mmol/L KH_2PO_4, 1.3 mmol/L KCl, 135 mmol/L NaCl, 0.5% Tween20, pH 7.4), 100 μL of an anti-fluorescein-POD (Roche, USA, 1:6 000 dilution in PBS, 2% goat serum) was added to each well. Then, the plate was incubated at 37°C for 10 min, and washed three times with PBS. Finally, 100 μL/well of 3, 3′, 5, 5′-tetramethylbenzidine (TMB, Sigma, USA) substrate was added, and the sample was incubated at 37°C for 10 min to allow blue color development. The reaction was stopped by adding 50 μL of 2 mol/L H_2SO_4 per well, causing the color to change to yellow. Absorbance was measured at 450 nm and 620 nm using a plate reader (FLUOstar, BMG Thermo Fisher Scientific Inc, USA) and the $A_{450\,nm}/A_{620\,nm}$ ratio was calculated.

The specificity was checked with six cultured microalgae: *C. marina*, *C. polykrikoides*, *H. triquetra*, *H. akashiwo*, *P. minimum*, and *S. trochoidea*. The sensitivity was tested by counting *H. triquetra* under light microscopy using serial dilutions. Three replicates of each dilution were analyzed. The absorbance of NPA-SH and microscopic data were compared.

2.4 *Field sample collection and test*

Natural seawater was collected monthly near Tongyeong, Korea (34°45′97.58″N, 128°22′54.62″E) from January to December 2014 (Jan. 24, Feb. 14, Mar. 10, Apr. 9, May 15, Jun. 19, Jul. 22, Aug. 27, Sep. 19, Oct. 16, Nov. 18, and Dec. 11). Samples were collected in surface seawater (1 L) using a net with a pore size of 0.2 μm. At the same time, water temperature, salinity, pH, and dissolved oxygen (DO) were measured by YSI instrument. The sample was immediately placed on ice, and then carried to the laboratory. Then, the sample was centrifuged at 3 000 r/min for 10 min, the supernatant was removed, and the pellet was stored at –70°C until use. Stored samples were used for the *H. triquetra* NPA-SH assay. Only lysis buffer and yeast t-RNA were added to the frozen samples, and the samples were sonicated for lysis of microalgae, using the same parameters as for NPA-SH lysis.

2.5 *Statistical analysis*

NPA-SH assay was performed at least three times in quadruplicate for each experiment. All data were presented as means±SE. A student's *t*-test was performed to test differences between controls and each experimental group.

3 Results

3.1 *Probe specificity*

We isolated total RNA of microalgae that are responsible for red tides (*C. marina*, *C. polykrikoides*, *H. triquetra*, *H. akashiwo*, *P. minimum*, and *S. trochoidea*), and of which the most abundant species were near Tongyeong (*A. glacialis*, *C. curvisetus*, *E. zodiacus*, *L. danicus*, *S. marinoi*, and *T. nordenskioeldii*). Then, we analyzed the LSU sequences of all species to design *H. triquetra* species-specific probes for NPA-SH. The results show the signals of sixmicroalgae samples, a negative control, and a mixed sample of *C. marina*, *C. polykrikoides*, *H. triquetra*, *H. akashiwo*, *P. minimum*, and *S. trochoidea* (Fig. 1). The signal of each sample ($A_{450\,nm}/A_{620\,nm}$) expressed relatively to the negative control. *Heterocapsa triquetra* had the strongest signal (1.59), consistent with its yellow color, and all the other samples had signals less than 1.06. This result means that the *H. triquetra* NPA-SH oligonucleotide probe can distinguish *H. triquetra* from five other microalgae that are also responsible for red tides.

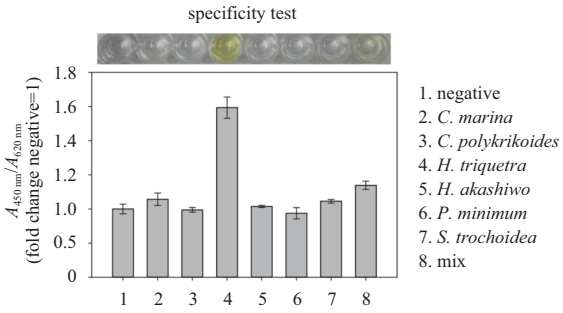

Fig. 1. Specificity of the *H. triquetra* sandwich hybridization probe. The *H. triquetra* sample and the mixed sample (which included *H. triquetra*) appeared yellow (above) and had $A_{450\,nm}/A_{620\,nm}$ values greater than all other samples.

3.2 *Probe sensitivity*

We tested probe sensitivity by measuring the signal with different numbers of cells (Fig. 2). The results show that the signal was above baseline when the cell concentration was 1.5×10^4 cells/mL (Fig. 2). This is lower than 3.0×10^4 cells/mL standard threshold for a red-tide warning issued by the Korea Ministry of Oceans and Fisheries in 2015. We also established a standard curve for the range of 3.0×10^3 to 1.5×10^5 cells/mL. The least-squares linear regression equation was $y=0.526\,4x+0.353\,5$, $r^2=0.893\,2$ (Fig. 2).

3.3 *Probes test at field sample*

Finally, we tested the NAP-SH method using field samples collected from natural seawater near Tongyeong, in the southern sea area of Korea (Fig. 3). We analyzed three samples per month from January to December of 2014. Based on the standard regression equation above, the $A_{450\,nm}/A_{620\,nm}$ value of 1.11 in September corresponds to 2 874 cells/mL, and the $A_{450\,nm}/A_{620\,nm}$ value of 1.23 in December corresponds to 3 330 cells/mL. All other months had no detectable *H. triquetra* (Fig. 4).

4 Discussion

Detection of red tide species in seawater samples is important for monitoring the occurrence of red tides. For example, identification of the onset of a harmful algal bloom may allow implementation of measures that can reduce economic and/or environmental damage. Previously, identification and detection of microalgae depended on viewing the morphological characteristics by microscopy (Suh et al., 2016; Ki and Han, 2006). Though many molecular detection methods are now available for detecting red tide species, only one chemical method is currently available for detection of *H. triquetra*. This motivated us to develop NPA-SH probes to detect *H. triquetra* in natural field samples (Jiang and Ilag, 2014).

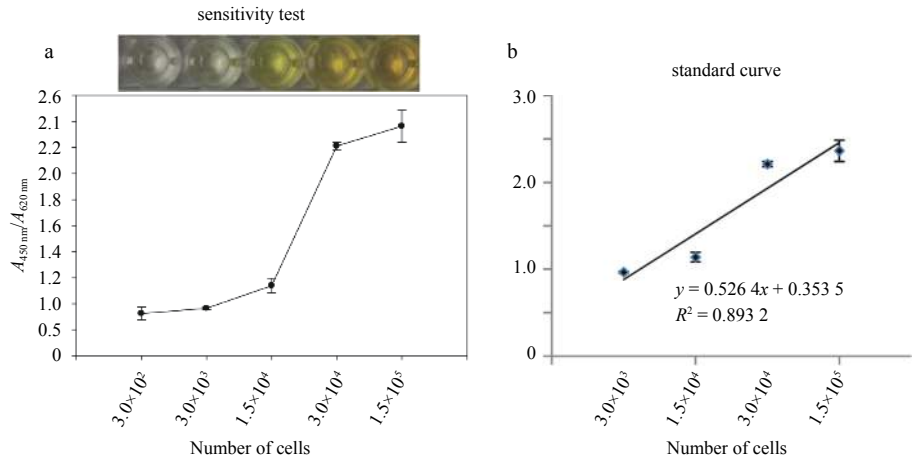

Fig. 2. Sensitivity of *H. triquetra* sandwich hybridization probe. a. Samples with fewer than 3.0×10^2 cells/mL had $A_{450\,nm}/A_{620\,nm}$ values of about 1.0, and were colorless. As cell numbers increased, they became yellow and the $A_{450\,nm}/A_{620\,nm}$ values increased. b. A standard curve for cells in the concentration range of 3.0×10^3 to 1.5×10^5 cells/mL.

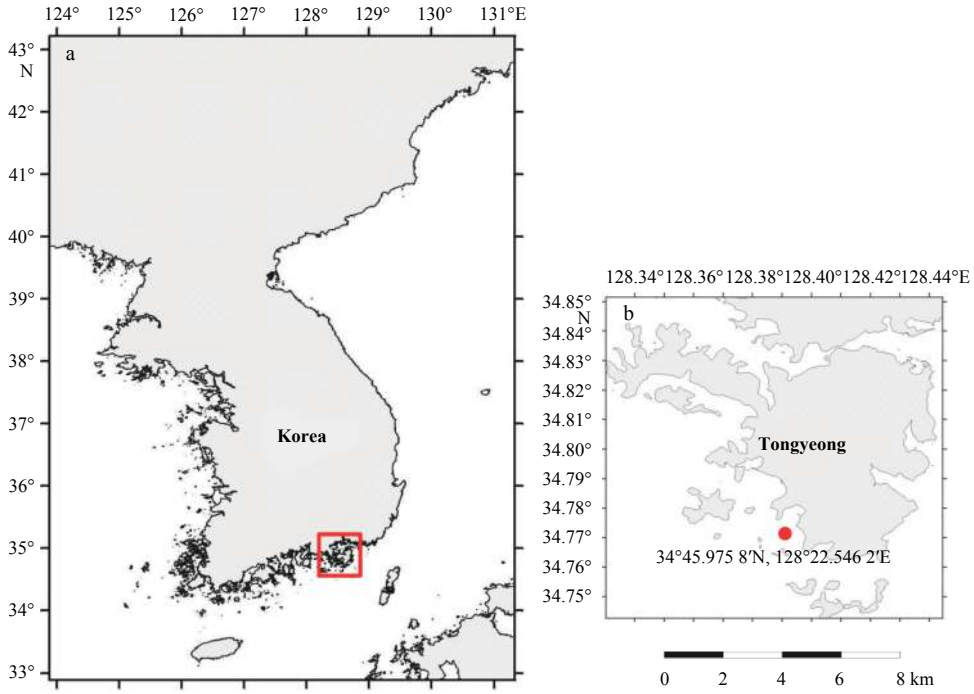

Fig. 3. Sampling locations near Tongyeong, in the southern sea area of Korea. a. The red square shows the location of Tongyeong. b. Expansion of Tongyeong region. The red circle shows the sampling location.

The NPA-SH method is an elaboration of the more simple SHA method. Researchers have used the rRNA-targeted SHA method to detect phytoplankton since 1996. In particular, researchers who used this method in New Zealand to monitor microalgae received international accreditation in 2004 (Ayers et al., 2005). However, the limitations of this method, such as use of degradative RNA, low specificity and low reproducibility, led to the development of the newer NPA-SH method (Zhen et al., 2007). Skipping the RNA extraction step also reduces the time needed for the NPA-SH method. Additionally, we also reduced hybridization times for the capture probe and NPA probe, and the NPA probe and signal probe. This reduced the total time by 4 h (Cai et al., 2006). The principle of probe hybridization is the same as the PCR annealing step. Thus, probe hybridization requires just a few seconds to a few minutes after denaturation. By reducing the hybridization time to about 5 min, we were able to detect *H. triquetra* within 3 h of sample collection. Another benefit of the NPA-SH method is that the results can be checked visually, in that the yellow color is evident on streptavidin-coated plates (Figs 1, 2 and 4). This is a major advantage of the NPS-SH method when it is in need of detecting *H. triquetra* in the field.

We developed *H. triquetra* specific-probes for NPA-SH, and monitored it over 2014 near Tongyeong. *Heterocapsa triquetra* were detected in September and December of 2014 near Tongyeong (Figs 3 and 4). Normally, *H. triquetra* is a major species from autumn to winter in the Masan Bay and the Jinhae Bay, which are located near Tongyeong (Lee and Han, 2007; Lee et al., 2005). In the Masan Bay and the Jinhae Bay, *H. triquetra*

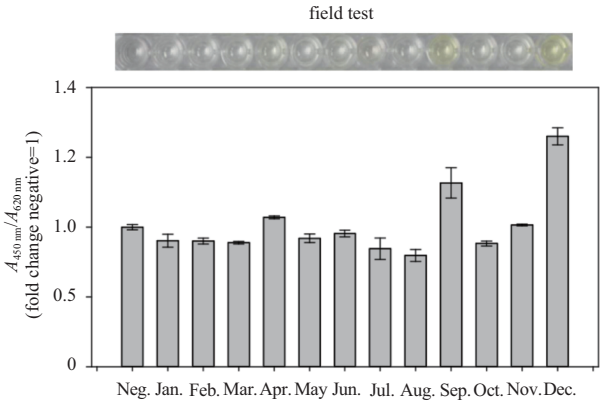

Fig. 4. *Heterocapsa triquetra* field sampling by NPA-SH during 2014. *Heterocapsa triquetra* appeared during September and December.

bloomed at extremely low salinities (below 25) after heavy rainfall (Lee and Limand, 2006). However, in the Mediterranean Sea near Turkey, this species appeared in January to April, and was undetectable in May (Tas, 2015). Normally, *H. triquetra* grows between 10°C and 25°C at salinity of 15 to 40, although it can adapt to a broad range of temperatures and salinities (Baek et al., 2011). Thus, *H. triquetra* blooms may depend on environmental conditions, such as nitrogen influx from heavy rain and the influx of freshwater.

The seawater temperature and salinity near Tongyeong were 22.5°C and 33.61 in September 2014, 13.9°C and 33.93 in December 2014. The temperatures were favorable for *H. triquetra*, but the salinity levels were a bit higher than those at the Masan Bay and the Jinhae Bay during the bloom season (Lee and Han, 2007; Lee and Limand, 2006). *Heterocapsa triquetra* is a mixotrophic dinoflagellate that does not produce toxins (Battocchi et al., 2010), but blooms can increase the pH to above 9.0, thereby disrupting ecosystem function. At the sampling times, we found no evidence of blooms based on surface observations, the seawater pH was always below 9.0, and there were no red-tide warnings. Although *H. triquetra* blooms did not appear in 2014, continuous monitoring is needed to better understand the environmental factors that influence *H. triquetra* blooming. Also, regular monitoring makes it possible to provide early warnings before the occurrence of serious red tides.

5 Conclusions

We developed species-specific probes using the NAP-SH method to detect and identify *H. triquetra* from environmental samples. This technique was effective in identification of *H. triquetra* in cultured microalgae samples and in natural seawater samples. We can detect cells at a concentration of 1.5×10^4 cells/mL, a greater sensitivity that required by the Korea Ministry of Oceans and Fisheries guidelines. We successfully used this method to monitor environmental samples for *H. triquetra* and found it could detect *H. triquetra* in the field without the use of laboratory equipment. The NPA-SH method described here can detect *H. triquetra* from field samples within 2 h without requiring expert knowledge in microalgal morphology. It is promising for the development of an easy-to-use *H. triquetra* detection kit.

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