Research Article

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An advanced tool, droplet digital PCR (ddPCR), for absolute quantification of the red-tide dinoflagellate, *Cochlodinium polykrikoides* Margalef (Dinophyceae)

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To quantify the abundance of the harmful dinoflagellate *Cochlodinium polykrikoides* in natural seawaters, we developed the innovative procedure using a droplet digital PCR (ddPCR) with *C. polykrikoides*-specific primers targeting the internal transcription sequence (ITS). The abundance of *C. polykrikoides* was estimated by the specific copy number of target ITS DNA segments per cell in cultures and natural water samples. The copy number per *C. polykrikoides* cell as acquired by ddPCR was 157 ± 16, which was evaluated against known cell numbers through a simplified protocol preparing DNAs. The abundances of *C. polykrikoides* in the waters of different locations estimated by ddPCR agreed with the number of cells visually counted under a microscope. This protocol was used to measure the abundance of *C. polykrikoides* close to and further off the southern coast of Korea in August of 2016 and 2017. The practical application showed that this method can reduce time for analysis and increase accuracy.

Key Words: abundance; *Cochlodinium polykrikoides*; copy number; ddPCR; dinoflagellate; quantification

Abbreviations: CA, cellulose acetate; ddPCR, droplet digital polymerase chain reaction; EPS, exopolysaccharide; gDNA, genomic DNA; GFF, glass-fiber filter; HAB, harmful algal bloom; ITS, internal transcribed spacer; PCR, polymerase chain reaction; qPCR, quantitative real-time polymerase chain reaction

INTRODUCTION

Red tides or harmful algal blooms (HABs) have caused large scaled mortality of fish and great loss in aquaculture industry (Park et al. 2013). Thus, governments of many countries have spent a large budget in reducing the loss. Detecting and monitoring the species causing HABs are critical steps in managing and minimizing losses in aquaculture industry (Sellner et al. 2003, Park et al. 2013, McKibben et al. 2015). A variety of dinoflagellates are known to be causative species of many HABs in the world ocean have been caused by (Richlen et al. 2010, Kudela and Gobler 2012, Lee et al. 2013, Jeong et al. 2016). Of these, the dinoflagellate *Cochlodinium polykrikoides* has often caused mass mortality of farmed fish and shellfish in many countries (Shahraki et al. 2013, 2014, Rountos et al. 2014). To reduce the economic and environmental impacts caused by *C. polykrikoides*, it is necessary to quickly

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identify it and accurately measure its cell abundance before its HAB occur. However, it is generally difficult to identify *C. polykrikoides* based only on its morphological features because it is similar to other *Cochlodinium* species that are found in Korean waters (Cho et al. 2001, Matsuoka et al. 2008, Iwataki et al. 2010, Marcoval et al. 2013). Furthermore, identifying HAB species under a microscope may require a long time for training beginners, and is a time-consuming, labor-intensive, and error-prone process even for experts. Thus, new methods of identifying and quantifying the abundance of a HAB have been developed.

Pigments have been suggested to be useful biomarkers for detecting particular phytoplankton groups, however, they alone are inadequate for identify a species among related species (Jeffrey and Vesk 1997). Metabolites, such as toxins, are also used as biomarkers in environmental monitoring programs, along with plastid or mitochondrial DNA, *rbcL*, *tufA*, and *COI*, but their quantities can be influenced by the physiological status of the species (Bereiter-Hahn 1990, Sato et al. 2003, Park et al. 2016). Genomic DNA (gDNA) found in the nucleus of eukaryotic organisms represents the complete set of genetic information of an organism, and exhibits features that differ in size and complexity among species (Coleman and Mai 1997, Buchheim et al. 2011, Hong et al. 2016). Although gDNA is not accessible for all marine species, the sequences of several rDNA regions of many marine species have been obtained for phylogenetic analyses. In addition, these regions are used for designing species-specific primers to identify and enumerate species via polymerase chain reaction (PCR)-based tools. Two conditions must be met to ensure accurate quantification of HABs using PCR with species-specific primers in the environmental samples: neither gDNA extraction nor the PCR tool used for quantifying the amount of target gDNA are affected by any possible contaminants in the sample, such as dirt or salts.

The quantitative real-time PCR (qPCR) method is most commonly used for identifying and monitoring HAB species. However, it can also be used as a tool for measuring the relative amount of species with fold changes in the sample based on the internal and external controls, or by simply comparing threshold cycles (*Ct*) to predefined standard *Ct* values that are generated using a known number of cells (Coyne et al. 2005, Park et al. 2014, Lee et al. 2017). The quantity based on a standard curve offers reliable values for interpreting the abundance of particular species in complex marine communities. However, the quantity measured using qPCR may not be an accurate representation of cell abundances, because the *Ct* values in qPCR are highly susceptible to PCR inhibitors in seawater samples, which may contain salts, pigments, exopolysaccharides (EPSs), humic acids, and other substances (Flekna et al. 2007, Ellison et al. 2011). Thus, new molecular methods may be needed.

In contrast to qPCR, a major advantage of this droplet digital polymerase chain reaction (ddPCR) is that it is relatively insensitive to the existence of PCR inhibitors in environmental samples, and thus optimizes reproducibility and consistency when attempting to obtain the necessary amount of target DNA fragments. Moreover, standard curves or references used to estimate the number of target DNAs or for interpreting the abundance of specific species are not required in a ddPCR assay (Sanders et al. 2011, Scollo et al. 2016). For accurate estimation using these, the target DNA fragments must be randomly distributed across a large number of partitions, and 20,000 nanoliter droplets in each ddPCR reaction and the absolute copy number of target DNA should be calculated statically based on the ratio of droplets carrying target DNA fragments. The process should provide precise estimates of the abundance of a species with higher speed, accuracy, and sensitivity than microscopic observation or qPCR assays.

In this study, we constructed a *C. polykrikoides*-specific primer by using the sequences of the internal transcribed spacer (ITS) region between 18S and 28S ribosomal DNA. Using this primer and the ddPCR method, we quantified the abundances of *C. polykrikoides* in coastal waters of the southern Korea in 2016 and 2017. The results of this study provide a basis on using the ddPCR method for early detection and monitoring of *C. polykrikoides* blooms.

MATERIALS AND METHODS

Cochlodinium polykrikoides isolate, culture, and sampling

Cultures of *C. polykrikoides* isolated from Yeosu Bay, southern Korea, were grown at 23°C in enriched f/2 seawater media under the illumination of ~300 μmol photons m^{-2} s^{-1} and a 12 : 12 h light : dark cycle provided by four 36 W daylight fluorescent lamps (Dulux L 36W/865; Osram, Münich, Germany). The cultures were transferred to new media every week.

To validate inter-sample variability and the consistency of the ddPCR assay, we collected various seawater samples from different locations along the southern

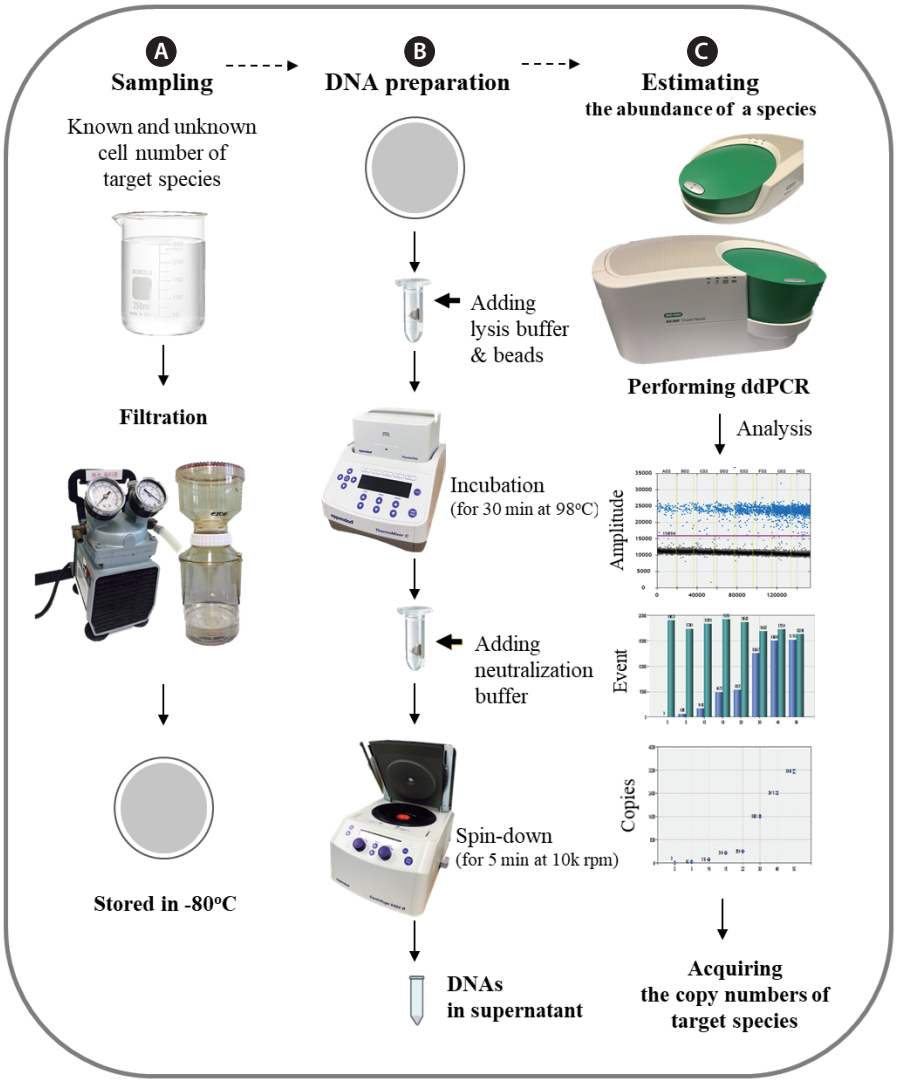


Fig. 1. Schematic diagrams of genomic DNA (gDNA) preparation and droplet digital polymerase chain reaction (ddPCR) analysis for estimating the abundance of target species. (A) Filtering seawater samples in which *Cochlodinium polykrikoides* abundances are known and unknown through a cellulose acetate filter at low vacuum. (B) gDNA (as a PCR template) preparation by lysis with beading and boiling. (C) Performing ddPCR with 5 μL of the gDNA prepared in (B) and determination of the specific copy number of target DNA based on the *C. polykrikoides* ITS-specific primer set.

coast of Korea (Muan, 35.02° N, 126.37° E; Wando, 34.31° N, 126.76° E; Taean, 36.62° N, 126.35° E; Gunsan, 35.93° N, 126.52° E), during the spring and summer 2016. Furthermore, we used ddPCR to quantify the abundance of *C. polykrikoides* from samples collected from nearshore and offshore waters of the southern coast of Korea during cruises of the National Institute of Fisheries Science (NIFS) research vessel *Tamgu 20* in August of 2016 and 2017. Seawater samples (l L) were filtered through a 0.45 μm cellulose acetate (CA) filter (Advantech Inc. Tokyo, Japan) and stored at -80°C until gDNA was prepared to estimate the specific copies per cell by ddPCR.

In 2016, *C. polykrikoides* occurrence was determined by inverted microcopy on Lugol-preserved samples that were prepared by netting, whereas in 2017, Lugol-preserved Niskin bottle samples (1 L) were concentrated by 1/10 using the settling and siphoning method and quantified through a microscope using the Sedgwick-Rafter counting chamber.

Overall procedure

Fig. 1 presents a flow chart describing the overall procedure for identifying and quantifying *C. polykrikoi-*

des before blooming occurs using ddPCR. In brief, *C. polykrikoides* was isolated on a 0.45 μm CA filter to remove seawater, and field seawater samples were also filtered in the same way under low vacuum (<100 mm Hg). gDNA of *C. polykrikoides* was extracted from the filter using two simple steps: first, gDNA was beaded with Zirconia beads in lysis buffer, then neutralized after 30 min boiling in order to minimize loss of DNA from viable cells. gDNA in the homogenized supernatant was analyzed as the template for each PCR. Approximately 1 h of homogenization was needed to process 24 samples at once, a more consistent and faster rate than the affinity-column-based methods commonly used by commercial DNA prep kits for plants or soil (Qiagen, Hilden, Germany). A glass-fiber filter (GFF; Millipore, Billerica, MA, USA) was also used to collect cells to compare the efficiency of filter media, but we found that GFFs yielded very low amounts of gDNA and the copy number for each *C. polykrikoides* fluctuated greatly because of the affinity of GFF to negatively-charged nucleic acids.

DNA preparation

The initial concentrations of *C. polykrikoides* were established using an autopipette to deliver a predetermined volume of culture with a known cell density to the bottles containing the waters collected from the different locations. gDNA used for determining the copy number per cell of *C. polykrikoides* was extracted via CA filters. Field seawater samples were also filtered for the same purpose under relatively low vacuum (<100 mm Hg).

gDNA both from the samples with known cell abundances and the field samples were extracted in two steps: gDNA was first extracted by lysing cells on a filter with five 2 mm Zirconia beads (Watson Co., Tokyo, Japan) in a lysis buffer (25 mM NaOH and 2 mM EDTA), and then via boiling in a ThermoMixer C (Eppendorf AG, Hamburg, Germany) for 30 min. After neutralization through the addition of an equal volume of 40 mM Tris-HCl (pH 5.5), 5 μL of gDNA supernatant solution was used as the template for each PCR.

PCR and droplet digital PCR

A *C. polykrikoides* specific primer (C.poly ITS-F and C.poly ITS-R) was designed using the ITS sequences, the specificity of which was validated using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and also against regular PCR purified gDNA from *C. polykrikoides* and other dinoflagellate cultures (C.poly ITS-F, 5′-GACGCAGCGAAGTGTGATAA-3′ and C.poly ITS-R, 5′-CAACGCCTTGACAAACAAGA-3′).

Ribosomal DNA, which is widely used in molecular phylogenic studies, has highly conserved sequences that are organized as a repeated multigene family separated by an ITS in the genome. Sequences of Dinophyceae ITS (located between 18S and 28S rDNA) were obtained from the NCBI nucleotide bank (https://www.ncbi.nlm.nih.gov/nuccore/) and aligned with the ITS sequences of *C. polykrikoides* to identify variable sequence regions that could be used to construct *C. polykrikoides*-specific primers. The specificity of the ITS primers for *C. polykrikoides* was validated using the NCBI Primer-BLAST tool against known NCBI DNA sequences (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) as well as regular PCRs against gDNA from *C. fulvescens*, *Alexandrium* spp., *Ulva ohnoi* (green alga), *Pseudo-nitzschia* sp. (diatom), and *Dinophysis* spp.; no cross-reactivity was observed (Supplementary Fig. S1).

Thermal cycling consisted of 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 20 s, and extension at 72°C for 20 s in a T100 Thermal Cycler (Bio-Rad Lab Inc., Hercules, CA, USA); in total 20 μL of PCR reaction was used, along with 10 μL of 2× prime Taq premix (Genet Bio, Daejeon, Korea) and 1 μL of 2 pmol of each primer (forward and reverse) with 0.1 μg DNA templates.

A QX200 Droplet Digital PCR (ddPCR) system (Bio-Rad Lab Inc., USA) was used to quantify the absolute copy numbers per *C. polykrikoides*. We followed the manufacturer's recommended protocols for droplet generation and for reading the droplets after running the same PCR conditions. Each ddPCR reaction was performed in a final volume of 20 μL containing 10 μL of EvaGreen Supermix (Bio-Rad Lab Inc., Munich, Germany), 1 μL of forward and reverse primer (2 pmol each), and 5 μL of the gDNA solution. Following PCR amplification, the droplets were read by QX200 Droplet Reader and QuantaSoft software ver. 1.7.4 (Bio-Rad Lab Inc., USA), which counts the number of positive- or negative-drops used to calculate the absolute quantification of the target DNA. All chemicals used for the ddPCR assay were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) or Bio-Rad (Bio-Rad Lab Inc., USA), unless otherwise specified.

Data analysis

Data were presented as the mean ± standard error. One-way analysis of variance (ANOVA) followed by Duncan's multiple comparison tests, or a Student-Newman-Keuls test for multiple comparisons in Minitab 14 (Minit-

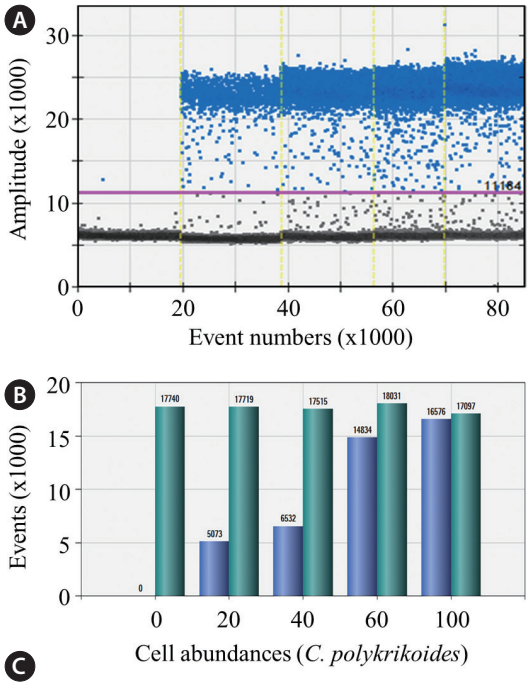
ab Inc., State College, PA, USA), were used to compare the number of copies per *C. polykrikoides* cell between the cell abundances, sample locations, and ddPCR estimates and microscopic cell counts.

RESULTS AND DISCUSSION

We applied the advanced quantifying method using ddPCR with *C. polykrikoides*-specific primers. To determine the genomic copy number per single *C. polykrikoides* cell, ddPCR was performed on 5 µL of a gDNA soup prepared from samples containing a known abundance (Fig. 2). Data with over 10,000 drops in total, along with a threshold attained by adding 5,000 to a basal amplitude for eliminating false positives, were included in estimations of the specific copy number per single *C. polykrikoides* (Fig. 2A & B). Total copies of *C. polykrikoides* were highly linearly correlated with cell abundances (r^2 = 0.930, n = 56), and no significant differences in the average number of copies per cell were detected between the cell abundances (p = 0.609). A single *C. polykrikoides* produced an average of 157 ± 16 copies of the target DNA fragments (Fig. 2C).

Estimates of cell abundances established via ddPCR were validated by comparing the results with direct microscopic cell counts (Fig. 3A). Although ddPCR estimates were slightly higher than microscopic cell counting, the differences were not significant (p = 0.221, n = 3). Moreover, the specific copy number per single cell measured by ddPCR did not differ among the various seawater samples collected from different locations (p = 0.395, n = 3) (Fig. 3B). Taken together, these results indicate that, in addition to detecting relatively low abundances of *C. polykrikoides*, the ddPCR method has a higher sensitivity and is a more cost and time effective tool for monitoring HAB species. As such, the use of ddPCR represents a more efficient approach to evaluating field samples from marine environments that contain complex organic and inorganic mixtures.

Following verification of the ddPCR method as appropriate for quantifying *C. polykrikoides* in seawater samples, we applied this method to samples collected near and offshore of the southern coast of Korea in late August 2016 and 2017. In August of 2016, *C. polykrikoides* was detected in samples taken from 8 of 25 stations, with densities exceeding 100 cells L^{-1} at only one station (400-17) (Fig. 4). To test the detection of *C. polykrikoides* by ddPCR at the eight stations, Lugol's fixed samples that were prepared by netting at the same time as sampling was un-



Known cell numbers	Total copies	Copies cell^{-1}
23	2,924	133
35	6,479	186
46	5,201	113
55	9,543	175
85	10,963	126

Fig. 2. Performing droplet digital polymerase chain reaction (ddPCR) with genomic DNA (gDNA) of *Cochlodinium polykrikoides* to determine the specific copy numbers of target DNA sequences with the internal transcribed spacer-specific primer. (A) Total and positive signaled droplets were visualized after running ddPCR, and data with over 10,000 droplets in total were included in the calculation of the copy number. Calculation of the specific copy number was based on the threshold by adding 5,000 at the basal negative amplitude in (B). (C) To acquire the genomic copy number per single *C. polykrikoides* cell, the ddPCR was performed from various known cell numbers.

Table 1. Comparison of estimates of *Cochlodinium polykrikoides* cell abundance in Korean coastal waters in August 2017 by microscopic observation and ddPCR assay

Location	Sampling date	Abundance (cells L^{-1})	
		Microscopic observation	ddPCR assay
Mokpo	Aug 7	0	7
Yeosu	Aug 8	0	0
Goheung	Aug 8	0	7
Tongyong	Aug 8	0	0
Masan	Aug 9	320 ± 238[a]	193

Locations are indicated as solid squares in Fig. 4.
ddPCR, droplet digital polymerase chain reaction.
[a]Mean ± standard deviation (n = 3).

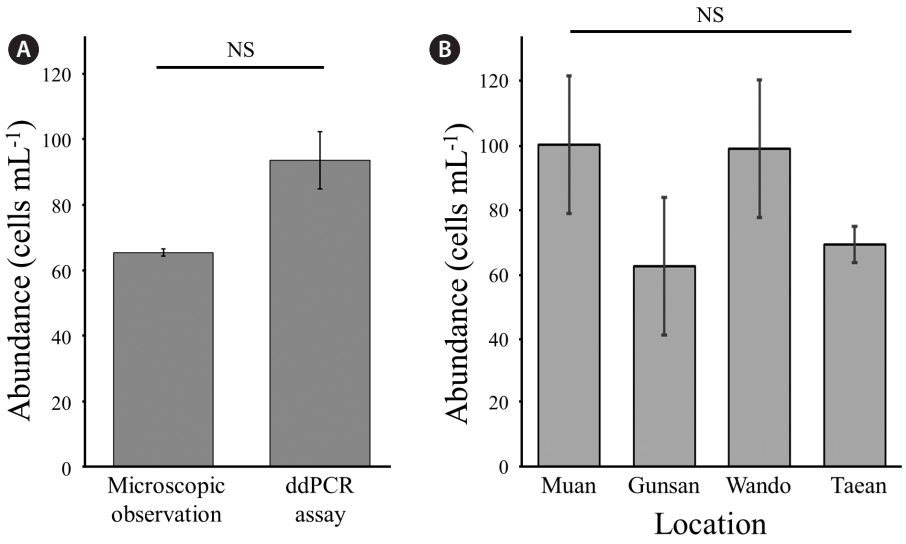


Fig. 3. Comparison of *Cochlodinium polykrikoides* abundance as estimated by droplet digital polymerase chain reaction (ddPCR) and microscopic cell counts (A), and between seawater samples collected at different locations (B), which contained prepared genomic DNA (gDNA) (approximately 100 cells). NS, not significant at $p > 0.05$.

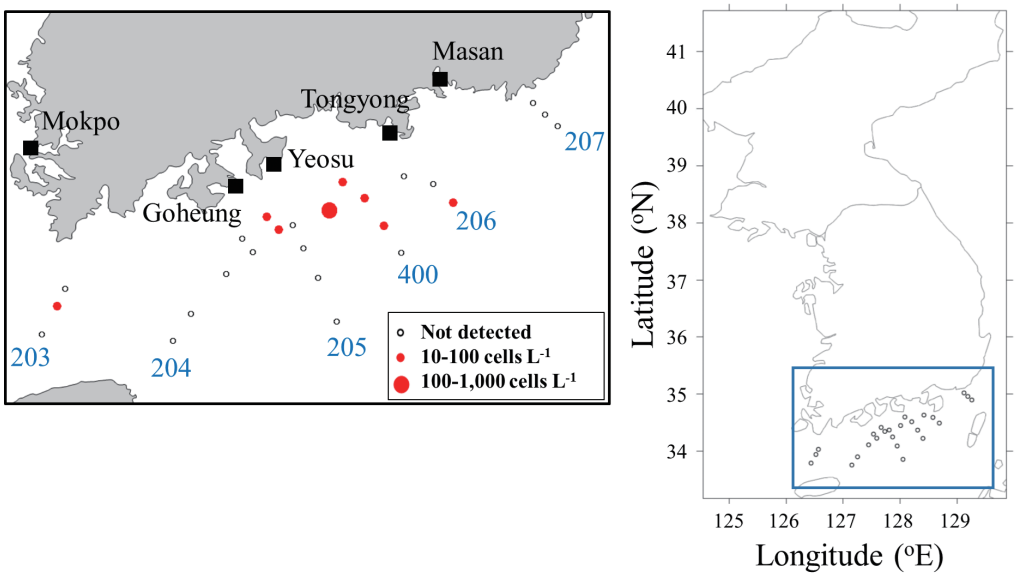


Fig. 4. Map of sampling stations and cell abundance of *Cochlodinium polykrikoides* estimated by droplet digital polymerase chain reaction (ddPCR) off the southern coast of Korea in late August 2016.

dertaken were observed through a microscope, with *C. polykrikoides* identified at only three stations. In August 2017, seawater samples for quantitative evaluation by both microscopic cell counts and ddPCR assays were collected at five separate locations in Korean coastal waters (Table 1). The abundance of *C. polykrikoides* was counted only in samples taken from Masan, for which an average of 320 cells L^{-1} was established by microscopic observation and 193 cells L^{-1} by ddPCR assay. No *C. polykrikoides* cells were observed under the microscope in samples collected from the other four locations, whereas ddPCR detected *C. polykrikoides* at two locations, Mokpo and Goheung. These results suggest that ddPCR can be used to estimate the abundance of target species even at very low cell numbers.

There are several possible reasons for the discrepancy between the two methods of quantifying abundance, including the relatively low densities of *C. polykrikoides* that occurred during the summer of both 2016 and 2017, which may have been insufficient for visual detection of *C. polykrikoides* cells. Since the 1980s, *C. polykrikoides* blooms have reappeared annually, but no blooming events were reported along the coasts of Korea in either 2016 or 2017. In addition, an unarmored chain-forming *Cochlodinium* that exhibited a wide range of morphological variability was found to easily rupture and pass through the net mesh, which confounded *C. polykrikoides* identification. Moreover, identifying cysts through microscopic observation is not always reliable because cysts are often difficult to recognize (Kim et al. 2007), and as such discerning cysts through a microscope may be an inadequate approach for identifying species in the early stages of bloom formation.

PCR technologies, regular PCR, qPCR, and ddPCR are highly sensitive tools for analyzing the occurrence and amount of target DNA with specific primers. qPCR visualizes a relative amount of target DNA by comparing their threshold cycles (*Ct* values) directly, or by comparing the *Ct* values with the *Ct* values of control or reference groups. Regular PCR and qPCR are less effective because of the presence of PCR inhibitors in seawater samples, however, which may result in inaccurate estimates of target DNA. Natural seawater samples contain PCR inhibitors like salts, pigments, EPSs, humic acids, and other substances (Wilson 1997). In contrast, ddPCR is an advanced technology for accurately quantifying target DNA fragments. One key advantage of ddPCR is that, unlike qPCR, it can tolerate the presence of PCR inhibitors in samples while maintaining reproducibility and consistency. Moreover, identifying gDNA copies via standard curves or references is not required for ddPCR assays (Sanders et al. 2011, Devonshire et al. 2015, Scollo et al. 2016).

Cochlodinium polykrikoides blooms have become more frequent in recent decades as results of global warming and increasing eutrophication, and consequently, their impacts on fisheries and aquaculture industries have intensified worldwide (Kudela and Gobler 2012, Lee et al. 2013, Park et al. 2013). Daily monitoring, including the rapid and precise identification and quantification of red-tide causing dinoflagellate species, is needed to predict when and where these blooms occur. To address this need, we present a new advanced tool, ddPCR, for efficiently quantifying the absolute abundance of *C. polykrikoides* in seawater samples.

CONCLUSION

The copy number of ITS fragments per cell is unique to each species and thus can be used to estimate the abundance of *C. polykrikoides* even in complex environments. In this study, we developed a novel procedure for preparing gDNA and performing ddPCR to quantify the abundance of red-tide-causing *C. polykrikoides*. Some modifications can be made to improve the accuracy and sensitivity of monitoring *C. polykrikoides* abundance with our ddPCR protocol; for instance, specific RNA-target primers can be used to exclude possible gDNA contamination from non-living *C. polykrikoides* cells, and more than one specific primer set or multiple markers for one species can be applied to improve the accuracy of estimates, although this would require more detailed genome and transcriptome information about a target species. How methods for the early detection and monitoring of invasive and harmful organisms in marine ecosystems—including but not limited to *C. polykrikoides*—can be improved will be the focus of future research.

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SUPPLEMENTARY MATERIAL

Supplementary Fig. S1. Validation of *Cochlodinium polykrikoides*-specific primer. (A) NCBI Primer-BLAST tool demonstrated no cross-reactivity with other species against *C. polykrikoides*-specific primers. (B) Electrophoresis after performing regular polymerase chain reaction (PCR) with *C. polykrikoides*-specific primers exhibited no PCR amplification product against gDNA of other non-target species (https://www.e-algae.org).

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