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# Propidium Monoazide based selective iDNA monitoring method improves eDNA monitoring for harmful algal bloom *Alexandrium* species

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eDNA, also known as environmental DNA, has garnered significant attention due to its potential applications in various fields such as biodiversity assessment, species distribution monitoring, ecological interaction analysis, and quantitative analysis. However, the presence of non-selective DNA signals in eDNA samples poses challenges in accurately detecting species, assessing biodiversity, and conducting quantitative analysis. To address these limitations, this study developed a novel method for selectively detecting iDNA from specific species in eDNA samples. The method involved the application of PMA treatment to *Alexandrium* spp. effectively preventing the detection of non-selective exDNA signals. Additionally, by optimizing the filter size used in the sampling process, the researchers were able to selectively collect and analyze iDNA from species of interest, particularly *Alexandrium* spp. Furthermore, the study successfully demonstrated the selective collection and analysis of iDNA from *Alexandrium* spp. cysts present in the sediment layer, further strengthening the findings. The results indicated that the combined use of PMA treatment and filter size optimization significantly enhanced the selective detection capability of iDNA. The successful selective detection of iDNA from eDNA in the sediment layer highlights the practical applicability of the developed method. This study holds promise for advancing eDNA monitoring technology by providing a selective iDNA detection method utilizing PMA. Moreover, these findings lay the foundation for effectively utilizing iDNA in environmental conservation, monitoring, and ecological research.

## KEYWORDS

eDNA, iDNA, exDNA, *Alexandrium* spp., filter size optimization, selective detection, quantitative analysis

## 1 Introduction

Harmful algal blooms (HABs) are widespread, with severe implications for public health and aquatic ecosystems. They can significantly negatively impact fisheries, leading to reduced productivity and economic losses. *Alexandrium* spp. is a species known to cause HABs and is one of the organisms responsible for paralytic shellfish poisoning (PSP), a serious threat to human health when contaminated shellfish are consumed (Samsur et al., 2006). *Alexandrium* spp. produce saxitoxins (Whedon, 1936), which can accumulate in shellfish such as mussels, clams, and oysters and have harmful or fatal effects on humans or animals that consume them. Impacts of PSP outbreaks include poisoning and death from contaminated shellfish or fish, loss of wild and farmed seafood resources, damage to tourism and recreational activities, changes in ocean trophic structure, and deaths of marine mammals, fish, and seabirds (Anderson et al., 2012). This distribution of *Alexandrium* spp. has expanded to regional and global scales (Hallegraeff and Bolch, 1992).

*Alexandrium* spp. reproduce by both asexual and sexual reproductive cycles (Anderson, 1980), with trophozoites developing into blooms through cell division. Sexual reproduction results in the formation of swimming zygotes (planozygotes) by sexual induction, which develop into dormant spores in subsequent stages (Anderson, 1998). This sexual reproduction may contribute to maintaining genetic heterogeneity and maintaining and enhancing adaptability to the environment. Moreover, spores are more resistant to unfavorable environments compared to trophozoites and play an important role as a seed population for subsequent seasonal flowering the following year (Anderson and Wall, 1978). Therefore, it is very important to monitor the density distribution of cells and dormant spores in order to minimize PSP. However, it is difficult to identify microalgae by morphological features and dormant spores do not have species-specific morphological features that can be distinguished (Fukuyo, 1985). Furthermore, these methods require specialized expertise (Hopkins and Freckleton, 2002).

Environmental DNA (eDNA) refers to genetic material acquired from environmental samples, encompassing DNA originating from a wide range of sources including unicellular or small multicellular organisms, tissue particles (such as shed cells and feces), as well as gametes from multicellular organisms. Furthermore, eDNA also includes distinct fractions such as extracellular DNA (exDNA) and intracellular DNA (iDNA) (Pietramellara et al., 2009; Nagler et al., 2022). eDNA has emerged as a valuable tool for monitoring the presence and distribution of aquatic organisms (Lawson Handley, 2015). eDNA-based monitoring allows for qualitative detection using metabarcoding (Grzebyk et al., 2017) and quantitative analysis of single species using quantitative polymerase chain reaction (qPCR) with species-specific primers and probes (Salter et al., 2019). In comparison to conventional monitoring methods, eDNA monitoring offers advantages. Traditional monitoring relies on the ability to discern morphological characteristics, a task that can be challenging or even unfeasible for elusive organisms, such as microbes, which are difficult to identify. However, eDNA-based approaches overcome these limitations by relying solely on the organism's DNA for identification. Additionally, while conventional monitoring involves

time-consuming field sampling, sample sorting, and consulting identification guidebooks, eDNA monitoring offers a relatively easy and rapid process of water sampling and species identification. Quantifying biomass directly at the eDNA level in seawater samples is difficult (Günther et al., 2018). Presumably, this is because eDNA decays at different rates depending on environmental variables such as temperature, UV, pH, etc. (Strickler et al., 2015) and varies with organism distribution and habitat. Nevertheless, assessment of eDNA diversity and species can provide a more accurate approximation than traditional monitoring, and quantitative analysis of eDNA using qPCR is showing promise (Park et al., 2014; Valentini et al., 2016; Pont et al., 2023). While a cell is in a viable state, iDNA can remain relatively stable and contains the genetic information of that cell. On the other hand, exDNA is the DNA of an organism that has been released into the external environment. It can be derived from cellular decay, cellular effluents, feces, or dead organisms. Detection of exDNA is likely to overestimate microbial abundance by up to 55% (Carini et al., 2016) and detect organisms that have already died or moved on to other locations. If only iDNA can be selectively collected, quantitative changes in iDNA can indicate when a cell or organism is surviving or when cells are actively reproducing. Also, since iDNA is related to cell number, it can be correlated with cell density or biomass.

Propidium monoazide (PMA) is a photoreactive binding reagent (Nocker and Camper, 2006) that is impermeable to cell membranes. This means that PMA is permeable to the DNA of damaged or dead cells, but does not bind to the DNA of normally viable cells. Because of this property, PMA can be utilized to reduce false positives by binding to exDNA. PMA tends not to bind effectively to iDNA. This is because PMA is only permeable to the damaged cell membrane, whereas iDNA located inside the cell is more reliably protected. Therefore, the application of PMA to eDNA samples enables selective binding to exDNA, predominantly situated outside the cell. This approach facilitates the exclusive detection of iDNA, significantly enhancing the precision of eDNA monitoring. By detecting only DNA present within intact cells, this approach reduces potential false positives due to exDNA and enables sensitive detection of eDNA signals associated with microbial activity or the presence of biological species. In conclusion, PMA can reduce false positives in eDNA monitoring by selectively binding to exDNA as a photo-reactive DNA insertion agent. This can help minimize the influence of iDNA and obtain accurate biological information.

In this study, the applicability of PMA to cells and dormant spores of the marine harmful alga *Alexandrium* spp. was evaluated, and the feasibility of iDNA monitoring was confirmed by comparing live and dead cells at known concentrations. In addition, only iDNA was selectively collected from field samples by filtering seawater using pore size-specific filters, and the eDNA present in sediments was compared by the PMA-iDNA method. Thus, the PMA-iDNA monitoring method is expected to contribute to quantitative eDNA monitoring techniques by reducing the possibility of false positives. In addition, given the characteristics of iDNA, it is expected to provide a more accurate estimate of the presence or activity of living cells. The introduction of these PMA-iDNA monitoring techniques is expected to improve the reliability and usefulness of eDNA monitoring in various fields such as

environmental monitoring, ecological research, and aquatic life management.

## 2 Materials and methods

### 2.1 Target species and controls

The target species *Alexandrium catenella* (LIMS-PS-3427), *A. Pacificum* (LIMS-PS-2611), and *Gymnodinium impudicum*, (LIMS-PS-3373) were obtained from the Library of Marine Samples (LIMS, Korea) in May 2022. In addition, *Thalassionema* spp., *Skeletonema* spp., *Nitzschia* spp., and *Akashiwo sanguinea* were collected, isolated, and cultured under a microscope. In addition, the dominant species *Alexandrium tamarensense* was collected from Masan Bay, Korea in April 2023 and isolated and cultured. They were amplified using Euk-A (5'-AACCTGGTTGATCCTGCCAGT-3') and Euk-B (5'-GATCCTTCTGCAGGTTACCTAC-3') primers (Medlin et al, 1988). Species identification was made by sequencing, and all were used as controls for false positive tests. All organisms were cultured for at least 6 weeks in *f/2* medium under fluorescent lights with a 24 hr photoperiod of 3,000-10,000 Lux at a temperature of  $20 \pm 2^\circ\text{C}$ . *A. catenella* cells were cultured for 4 weeks at  $15^\circ\text{C}$  without *f/2* medium to induce cyst formation. *A. catenella* cells, cysts, and *A. tamarensense* cells were diluted to concentrations ranging from 10,000-1, 6,000-1, and 50,000-5 cells/mL, respectively, in order to create a standard curve for qPCR quantification. It's important to acknowledge that variations in cell size can lead to varying amounts of DNA when using DNA dilution methods (Behrenfeld et al., 2021). These standard curves provide a means to accurately determine the concentration of *A. catenella* cells, cysts, and *A. tamarensense* based on their corresponding Ct values, enabling precise quantitative analysis (Lee et al., 2017).

### 2.2 Cell disruption and PMA treatment

To selectively detect only iDNA using PMA, exDNA was generated using two methods: heat treatment of *A. catenella* cells (1,000 cells/mL) and cyst cultures (1,000 cells/mL) at  $60^\circ\text{C}$  for 30 min and exposure to 1% Triton x-100 (Junsei, Japan) for 10 min to selectively disrupt cell membranes. Dead cells were assumed to be exDNA and non-dead cells were assumed to be iDNA. The iDNA and exDNA were mixed at ratios of 100, 75, 50, 25, and 0% (Table 1) and the mixed DNA was defined as eDNA. Samples for each concentration were treated with 10 mM of PMAxx™ dye (Biotium, USA). The samples were incubated in the dark at room temperature for 10 min and then exposed to blue LED on ice for 30 min. Furthermore, to add credibility to, we utilized *A. tamarensense* at a concentration of 1,000 cells/mL. The sample was partitioned into two distinct groups representing 100% and 0% viable cell counts, achieved through treatment with Triton X-100. The evaluation was organized into the following experimental groups: A) PMA untreated, DNase untreated, B) PMA untreated, DNase treated, C) PMA treated, DNase treated, D) PMA treated, DNase untreated. The DNase treatment was administered, with a 5  $\mu\text{L}$  volume used instead of the manufacturer-recommended 1  $\mu\text{L}$ , as per their

TABLE 1 exDNA and iDNA mixing ratios for quantification of *A. catenella*, and *A. tamarensense* PMA-iDNA.

	100%	75%	50%	25%	0%
iDNA (cells/mL)	1000	750	500	250	0
exDNA (cells/mL)	0	250	500	750	1000

protocol, employing DNase I, RNase-free (Thermo Scientific, USA). Additionally, Group C involved treating with DNase first, followed by the application of PMA. Each sample was centrifuged at 13,000 x g for 5 min and extracted using the AccuPrep® Plant Genomic DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's instructions.

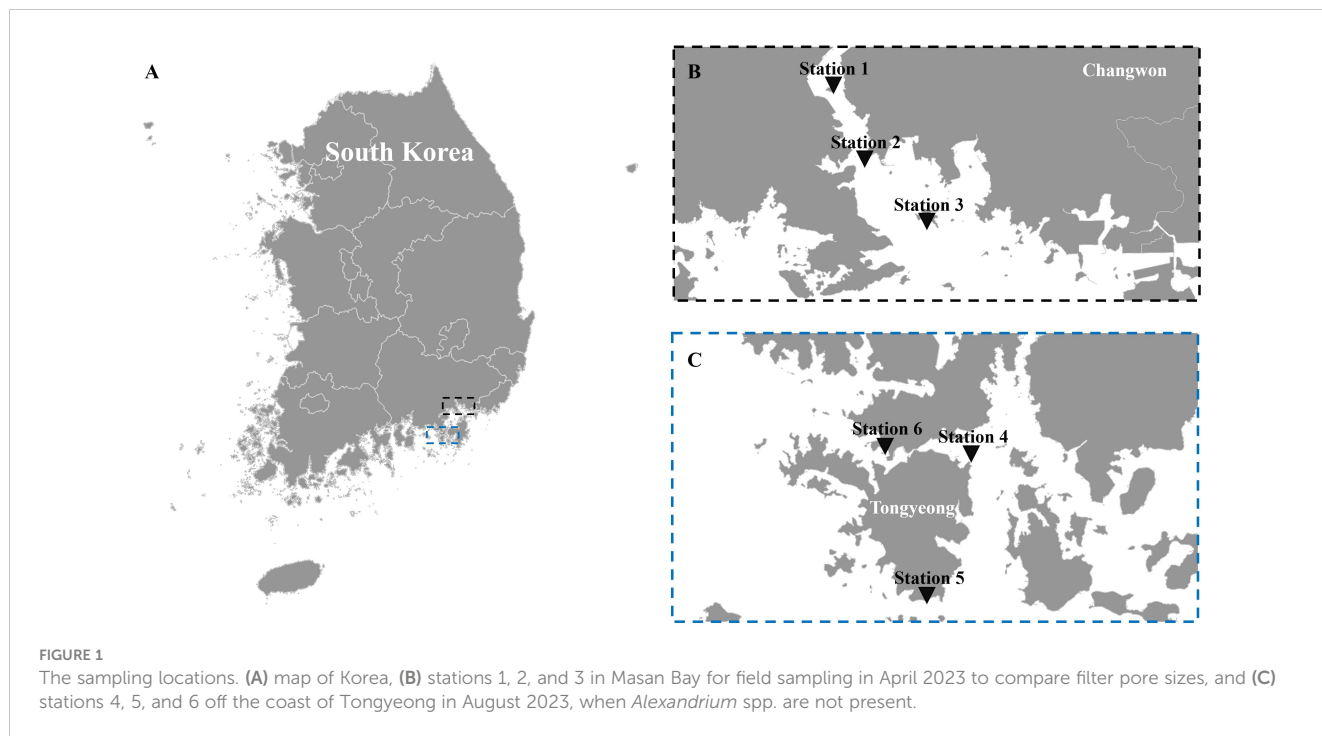
### 2.3 DNA extraction and primer design for quantitative analysis

Primer was designed to specifically detect the LSU region of the *A. catenella* 18s rRNA gene (DQ785887.1) from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/blast>) using Primer 3 (Whitehead Institute and Howard Hughes Medical Institute, MD) and Oligo Calc (Oligonucleotide Properties Calculator software). Multiple sequence alignments were performed using the NCBI, and data were visualized using NCBI MSA Viewer to confirm species specificity (Figure S1).

The designed primer sets Alx2 Forward (5'ATTTTCCTGCGGGGTGTGGA'3), and Alx2 Reverse (5'TCCGTGTTTCAAGACGGGGTCA'3) were used to adjust the qPCR template final concentration to 20  $\mu\text{L}$ . qPCR template was 10  $\mu\text{L}$  Prime Q-Master Mix with UDG (Genetbio, Korea), 7  $\mu\text{L}$  tertiary distilled water, 1  $\mu\text{L}$  Alx2 Forward, 1  $\mu\text{L}$  Alx2 Reverse, and 1  $\mu\text{L}$  DNA template. SYBR Green qPCR was performed using a CFX Opus 96 (BioRad, USA) at  $95^\circ\text{C}$  for 5 min with 35 cycles ( $95^\circ\text{C}$  for 30 sec,  $59^\circ\text{C}$  for 30 sec,  $72^\circ\text{C}$  for 30 sec), and all qPCR runs were performed in triplicate for reliability.

### 2.4 Field sampling methods and marine samples preparation

Masan Bay, a representative semi-enclosed sea in South Korea, is well known for its contaminated waters in the past. In April 2023, samples were collected at 3 Stations in Masan Bay (Figure 1). All filters used in the study have a diameter of 47 mm. The filter pore size was set to 30  $\mu\text{m}$  in order to selectively collect iDNA, considering the typical size range of *Alexandrium* spp. (30-40  $\mu\text{m}$ ) (Anderson et al., 2005). To compare PMA-iDNA monitoring, samples from the field were taken to the lab and subjected to comparison using filters commonly utilized in many eDNA studies, specifically 0.2, and 1.2  $\mu\text{m}$  filters (Lacoursière-Roussel et al., 2016). To selectively collect and compare iDNA, seawater filtration in the field was improvised using a filter with 30  $\mu\text{m}$  pores and a custom 3D-printed filter holder connected to a pump (Table 2; Figure 2). The filter was stored in 5 ml tubes on ice and



transported to the laboratory. In order to verify primer specificity, sampling was conducted in August 2023, a period when *Alexandrium* spp. were not anticipated to appear due to high water temperatures. Water samples were collected from the coastal waters of Tongyeong using a 0.2 μm filter to capture eDNA (Figure 1).

To fabricate the filter holder, 132D Design software (<http://www.123dapp.com/>) was utilized to design the model, and subsequently, the design file was converted using Cubicreator4 V4.4.0. (Cubicorn, Korea). 3D printing was performed on a Cubicorn Single Plus printer (Cubicorn, Korea) using PLA filament (3DBUYER, UK). The filter holder has an 800 μm pore-

size plate in the inlet for primary filtration of other suspended solids.

In addition, 4 L of seawater was collected at each vertex in sterilized collecting bottles, stored on ice, transported to the laboratory, and immediately filtered using filters with 0.2 and 1.2 μm pores until the filter clogged (Table 2). To collect sediment samples, a van Veen Grab with a surface area of 0.1 m<sup>2</sup> was used to collect sediment from each vertex. Sediment was transported to the laboratory on ice in sterile collection bags, stored at -4°C for 24 hr, and DNA was extracted. Water quality survey data (water temperature, pH, DO, turbidity, salinity, Chl-a) for Masan Bay in April 2023 were obtained from the Korea Marine Environment Management

**TABLE 2** Filters and seawater filtration volumes are used to collect eDNA (exDNA and iDNA) in the field.

Station	pore size (μm)	Filter type	Manufacturer	Amount of water filtered (mL)
1	0.2	Clellulose Nitrate	Whatman, UK	960
	1.2	Nylone	Millipore, USA	2,680
	30	Nylone	Millipore, USA	30,000
2	0.2	Clellulose Nitrate	Whatman, UK	1,020
	1.2	Nylone	Millipore, USA	3,000
	30	Nylone	Millipore, USA	30,000
3	0.2	Clellulose Nitrate	Whatman, UK	1,510
	1.2	Nylone	Millipore, USA	3,490
	30	Nylone	Millipore, USA	30,000
4	0.2	Clellulose Nitrate	Whatman, UK	1,300
5	0.2	Clellulose Nitrate	Whatman, UK	1,200
6	0.2	Clellulose Nitrate	Whatman, UK	1,550

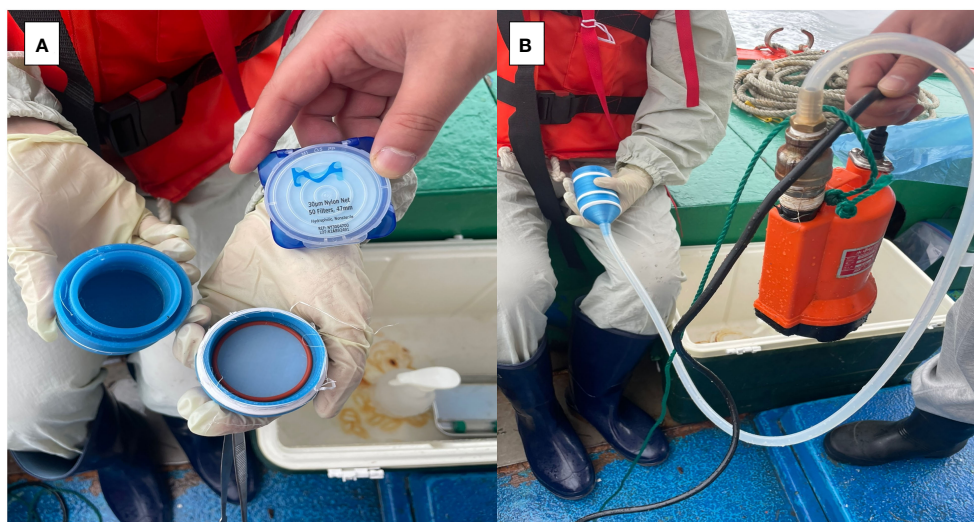


FIGURE 2

Filtration of eDNA using filters with 30um pore size in the field. (A) Filter holder fabricated with a 3D printer, (B) Filter holder connected to a pump.

Cooperation MEIS (Marine Environmental Information System) (KOEM, <https://www.meis.go.kr/mei/observe/port.do>) (Table S1). In addition, Suspended Solids affecting sample collection using filters are described in the Supplementary Material (Table S2). Seawater was collected from all sampling sites, fixed with Lugol's solution, and concentrated for analysis. A Sedgwick-Rafter chamber under a phase contrast microscope counting in triplicate, and data are shown in Supplementary Tables 3, 4. Qualitative analysis was performed at 400–1,000x magnification.

## 2.5 Filter and sediment eDNA extraction

The sample filter from the field and the filter treated in the lab were transferred to a 60 mm diameter Petri dish to which 3 mL of PBS was added. Then, it was shaken at 150 rpm for 2 hr in a shaker at 20°C. Using sterilized forceps and a scalpel, collect the filtered sample and divide it equally between two 1.5 mL tubes. The PMA-Untreated eDNA group was composed of one sample, while the PMA-Treated eDNA group consisted of another sample. The PMA-Untreated eDNA group was extracted by spin-down of 1.5 mL tubes at 13,000 RPM for 5 min, discarding the supernatant and using the AccuPrep<sup>®</sup> Plant Genomic DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's instructions. For PMA treatment, the PMA-treated eDNA group was centrifuged at 13,000 RPM for 5 min, the supernatant discarded, and the DNA was extracted as above after exposure to 400  $\mu$ L of PBS and 1  $\mu$ L of PMAxx<sup>™</sup> dye in blue light (450–490 nm) for 30 min.

To extract DNA from sediment samples, 20 g of sediment from each vertex was transferred to a 50 mL tube. Subsequently, 10 mL of PBS was added, and the mixture was centrifuged at 2,000 RPM for 20 min. The supernatant was carefully transferred to a new tube and further centrifuged at 13,000 RPM for 5 min. Following this step, the supernatant was discarded, and 800  $\mu$ L of PBS was added to the sediment pellet. The resulting mixture was divided equally, with half

of it transferred to one 1.5 mL tube for the PMA Untreated eDNA group and the other half transferred to another 1.5 mL tube for the PMA Treated eDNA group. DNA extraction was then performed on both groups following the same procedure as described above.

## 2.6 Statistical analysis

The correlation between cell concentration and Cycle of Threshold (Ct) value was determined using a standard curve. Statistical analysis comparing the results PMA Untreated eDNA group and PMA Treated eDNA group was performed using a t-test after checking for normal distribution. All statistical analyses and graphs were visualized using R Studio and Excel.

## 3 Results

Species specificity testing was conducted using primers specifically designed to detect only *Alexandrium* spp. The tested species included *Alexandrium catenella*, *Alexandrium pacificum*, and *Alexandrium tamarense*. Additionally, the Dinoflagellata *Gymnodinium impudicum* and *Akashiwo sanguinea*, as well as the Diatoms *Thalassionema* species, *Skeletonema costatum*, and *Nitzschia* spp., which coexist in the same Marine region, were included in the testing (Table 3). The results of the species specificity test for the primer indicate that no amplification was observed in any of the species tested, except for *Alexandrium* spp. This confirms the specific detection of *Alexandrium* spp. by the Alx2 primer set, as evidenced by the positive amplification observed exclusively in the tested *Alexandrium* spp. samples. Morphological analysis using traditional microscopy showed that *Alexandrium* spp. were the priority species at Stations 1, 2, and 3 in Masan Bay in April 2023 (Table S3), and *Alexandrium* spp. were not detected at Stations 4, 5, and 6 in August 2023 (Table S4).

TABLE 3 PCR results for control and target species in primer validation.

Species	Origin	Positive/Negative
<i>Alexandrium catenella</i>	LIMS	+
<i>Alexandrium pacificum</i>	LIMS	+
<i>Alexandrium tamarense</i>	Field	+
<i>Gymnodinium impudicum</i>	LIMS	-
<i>Akashiwo sanguinea</i>	Field	-
<i>Thalassionema</i> spp.	Field	-
<i>Skeletonema costatum</i>	Field	-
<i>Nitzschia</i> spp.	Field	-

In August 2023, the eDNA detection results for stations 4, 5, and 6 showed a significant contrast between the untreated and treated PMA groups. Specifically, the untreated PMA group showed a Ct value indicating DNA amplification, while the PMA-treated group showed no Ct value, suggesting that DNA amplification was successfully inhibited. This indicates that the Alx2 primer set specifically detects *Alexandrium* spp. and that exDNA is a potential source of error in eDNA monitoring (Figure 3).

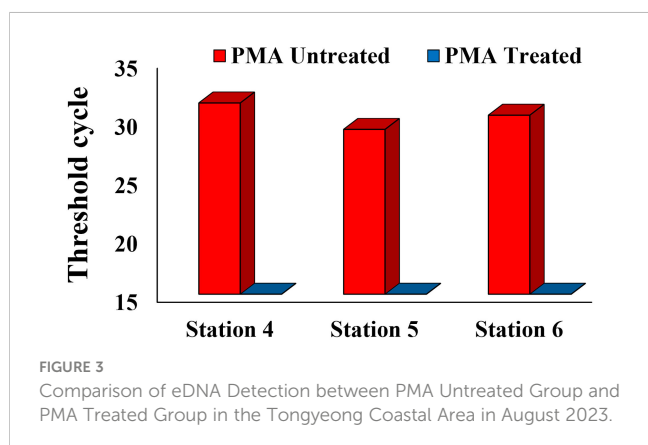
Quantitative analysis is significantly facilitated by the utilization of standard curves. In this study, we formulated standard curves to establish a correlation between the concentration of *A. catenella* cells, cysts, and *A. tamarense* cells and their corresponding Ct values (Figure 4). The generation of Standard curves using the dilution series of *A. catenella* cells and cysts yielded high R<sup>2</sup> values of 0.9963 and 0.9965, which are close to 1, while *A. tamarense* cells also showed a value of 0.9919, close to 1. These R<sup>2</sup> values indicate a strong correlation between the concentrations of *A. catenella* cells and cysts and their corresponding Ct values. The high R<sup>2</sup> values suggest that the standard curves are highly reliable and accurate in quantifying the target species. Finally, to obtain log cell counts for field samples, the standard curve for cells of *A. catenella* and *A. tamarense* (Figure 4D) showed an R<sup>2</sup> value of 0.9911.

A standard curve enables the mathematical modeling of the relationship between concentration and Ct value, providing a means to accurately estimate the concentration in various samples. This makes standard curves valuable for comparative

analysis across different samples or experimental conditions. By analyzing multiple samples on a consistent basis, it becomes possible to compare relative concentration differences or determine statistical variances between groups. These capabilities make standard curves an important tool in quantifying and comparing target concentrations in a study.

To assess the effectiveness of the PMA-iDNA monitoring method, laboratory-cultured *A. catenella* cells and cysts were divided into two groups: PMA Untreated and PMA Treated. This division allowed for a comparison of the performance of the monitoring method with and without the use of PMA. Furthermore, the slopes of the standard curves for Cells and Cysts presented in Figures 4A, B were used to convert the Ct values into logarithmic values. The results of PMA treatment for each percentage of the sample heat-treated at 60°C for 30 min (Figure 5) showed no significant difference between PMA Untreated and PMA Treated groups at 100% for both cells and cysts. However, as the percentage of exDNA increased, the Log cells abundance also decreased, indicating that PMA selectively detects only iDNA. However, even in the PMA Untreated group, the Log value decreased as the percentage of exDNA increased. It is worth noting that the heat treatment method used could potentially affect the eDNA.

Triton X-100 (1%), a non-ionic surfactant, was used to selectively disrupt cells without causing DNA damage; this method showed no decrease in log values for the PMA Untreated group, contrast the heat-treated method, for both cells and cysts, and both cells and cysts showed P-values above 0.05 at 100% concentration. Similarly, the log cell abundance of the PMA-treated group decreased as the percentage of exDNA increased (Figure 6). The results obtained from the study suggest that Triton X-100 (1%) effectively disrupts cells without causing eDNA damage. The findings also confirm the ability of PMA treatment to selectively detect iDNA. Notably, no significant differences were observed between the PMA Untreated and PMA Treated groups at a concentration of 100% for both cells and cysts, regardless of PMA treatment. However, as the percentage decreased, the Log cells abundance decreased, indicating that PMA selectively detects iDNA. However, both cells and cysts showed a log cell count in the 0% group. The findings of this study have the potential to make a significant contribution to the enhancement and standardization of eDNA quantification methods utilizing qPCR. The accurate analysis of iDNA and exDNA through the application of PMA treatment shows promise for advancing eDNA monitoring techniques. By effectively differentiating between iDNA and exDNA, researchers can improve the accuracy and reliability of eDNA-based assessments, thereby enhancing our understanding of ecosystems and facilitating more robust environmental monitoring and management strategies. Furthermore, to add credibility to the above experiment, the results of each of the four groups treated with DNase and PMA showed that log cell abundance remained consistent in Group A regardless of the percentage of viable cells at 100% and 0%. In contrast, Groups B, C, and D showed higher values of log cell abundance at 100% viable cells and lower values at 0% (Figure 7).



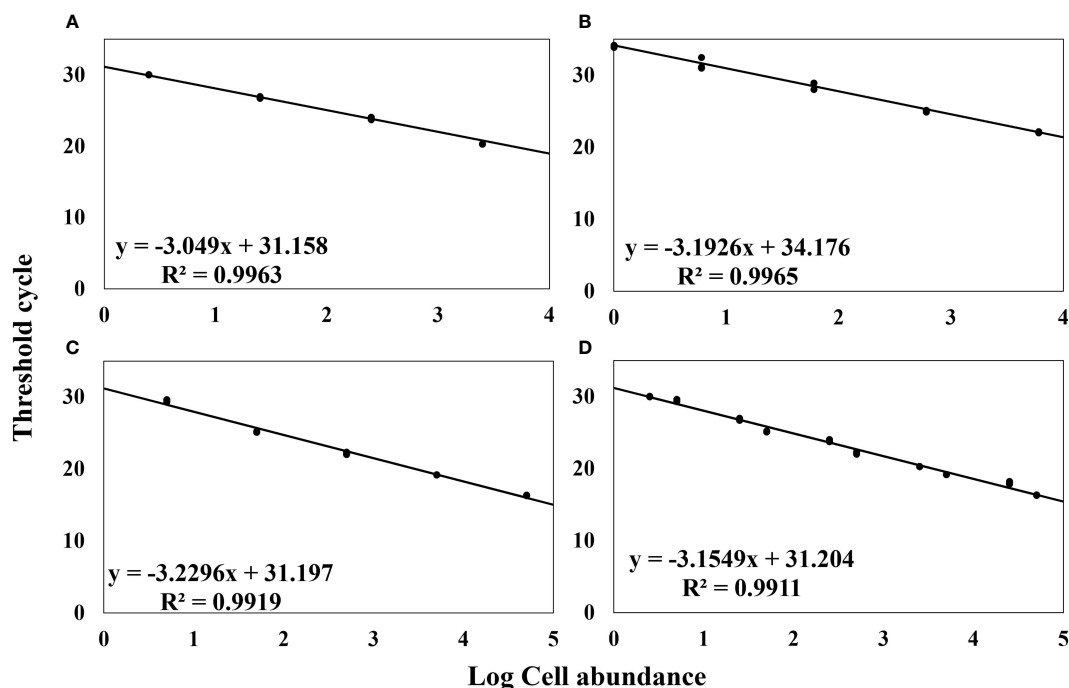


FIGURE 4 Standard Curve of Cell Concentration (log) and Ct Values. (A) *Alexandrium catenella* Cells Concentration, (B) *Alexandrium catenella* Cysts Concentration, (C) *Alexandrium tamarense* Cells Concentration, (D) *Alexandrium catenella* and *Alexandrium tamarense* Cells Concentration.

PMA-treated and untreated groups were compared with pore size filters of 0.2 μm and 1.2 μm (Turner et al., 2014; Kumar et al., 2022), which are commonly used pore sizes in conventional eDNA sampling methods, as well as a relatively large filter pore of 30 μm, in order to selectively collect only iDNA from field sampling (Figure 8). In the absence of PMA treatment, the log cell abundance of *Alexandrium* spp. at all vertices was observed to be in the order of 1.2 μm, 0.2 μm, and 30 μm. However, upon treating the filter samples with PMA and extracting DNA, it was found that the abundance order shifted to 30 μm, 1.2 μm, and 0.2 μm. This observation suggests that the smaller pore size filter successfully captures a wide range of eDNA types, including both exDNA and

iDNA. Pore size filters of 30 μm selectively capture iDNA and relatively large microorganisms larger than 30 μm, while allowing exDNA and other microorganisms to pass through. As a consequence, the yield of exDNA is reduced, but the filter effectively increases the yield of iDNA. The results presented highlight the distinction between conventional eDNA sampling methods utilizing small pore sizes and iDNA selective collection methods employing filters with relatively large pores and PMA treatment. By selectively collecting only iDNA through PMA processing, errors in eDNA analysis, such as quantitative overestimation and the inability to differentiate between living and dead organisms, can be minimized. The combination of

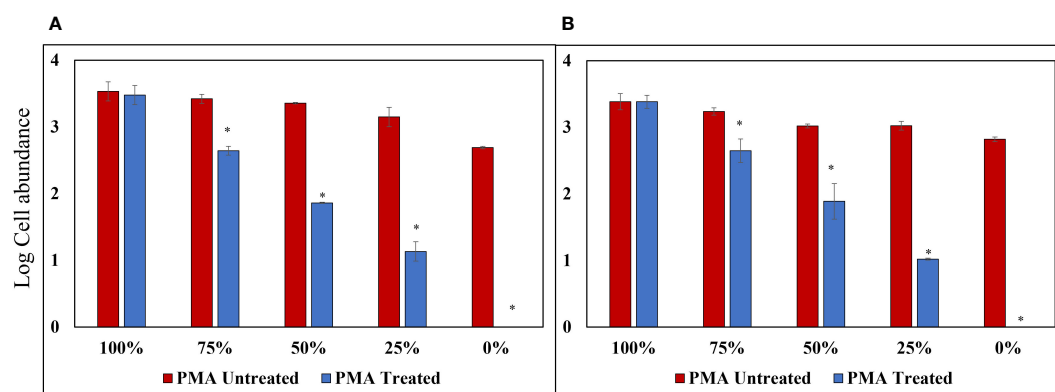


FIGURE 5 PMA Treatment Comparison for Samples Heat-Treated at 60°C for 30 min. (A) *Alexandrium catenella* cells (1,000 cells/mL). (B) *Alexandrium catenella* cysts (1,000 cells/mL). \*P<0.05.

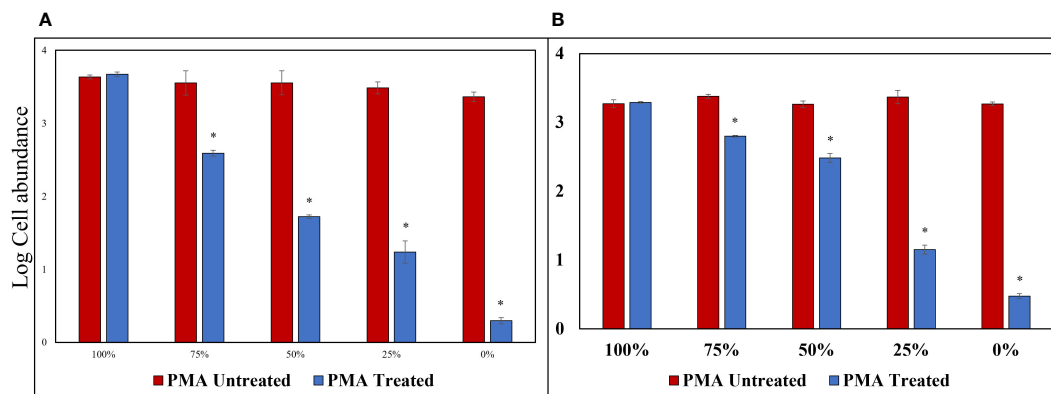


FIGURE 6  
PMA Treatment Comparison for Samples Treated with Triton X-100 (1%). (A) *Alexandrium catenella* cells (1,000 cells/mL). (B) *Alexandrium catenella* cysts (1,000 cells/mL). \*P<0.05.

PMA treatment and an appropriate filter size can indeed be a useful strategy for effectively collecting iDNA during field sampling. However, it is important to note that the size of the DNA can result in variations in the relative amount of iDNA generated by the method used in this study, particularly for certain microbial species, cells of higher organisms, and small microorganisms. This consideration should be taken into account when conducting comparative studies of iDNA from different organisms in the environment.

It is suggested that sediment layers have the ability to store DNA for long periods of time in biological monitoring (Corinaldesi et al., 2008). Deposits act as reservoirs for exDNA and iDNA, with exDNA predominantly representing the majority rather than iDNA (Corinaldesi et al., 2005). Therefore, the method can distinguish between living and dead organisms and has the capability to detect not only organisms that currently exist but also those that existed in the past. Given the low abundance of

*Alexandrium* spp. at lower depths (Martin et al., 2005), it can be inferred that the DNA detected in the sediment layer originated from cysts or that exDNA was present within the sediment layer. In April 2023, the PMA untreated in Masan Bay sediments exhibited a high abundance of *Alexandrium* spp., whereas the treated samples showed a low abundance (Figure 9). In marine sediments, eDNA can originate from living cells or organisms, as well as natural or anthropogenic processes (Dell'Anno and Danovaro, 2005). Therefore, DNA extraction from sediment samples cannot exclude DNA derived from deceased organisms. Nevertheless, by selectively detecting only iDNA, the PMA-treated method appears to overcome the limitations from a sediment biomonitoring perspective. The application of selective iDNA targeted analysis can potentially prove valuable in biomonitoring studies, aiding in the differentiation between living and deceased organisms (Corinaldesi et al., 2018).

## 4 Discussion

*Alexandrium* spp. are recognized as the main contributor to red tide blooms in various marine regions worldwide (Shikata et al., 2020). These organisms are also significant harmful algae, particularly in coastal or shelf waters, capable of disrupting marine ecosystems (Anderson et al., 2012). Red tide events, which can have adverse ecological and economic impacts, are often linked to *Alexandrium* spp. and can be influenced by environmental factors such as oceanic conditions, temperature, and nutrient levels (Min and Kim, 2023). As a result, monitoring *Alexandrium* spp. in the environment becomes essential to understanding the effects of human activities on marine ecosystems. For this purpose, targeted primers were developed. 3 *Alexandrium* species were cultivated in the laboratory for a minimum of 6 weeks, with an additional species from the same group used as a control for comparison. Significantly, no amplification was observed in species other than *Alexandrium* spp., confirming the specificity of this primer design. Such precision in primer design has the potential to enhance environmental monitoring and early warning systems. This

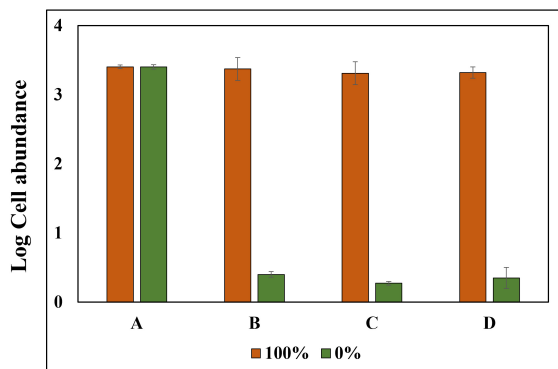


FIGURE 7  
Experimental Setup and Treatment Categories for PMA Verification using *Alexandrium tamarensis*. Experiments were performed involving *Alexandrium tamarensis* with live cell ratios of 100% and 0%. The samples were systematically divided into four distinct treatment groups: (A) Untreated with PMA, Untreated with DNase. (B) Untreated with PMA, Treated with DNase. (C) Treated with PMA, Treated with DNase. (D) Treated with PMA, Untreated with DNase.



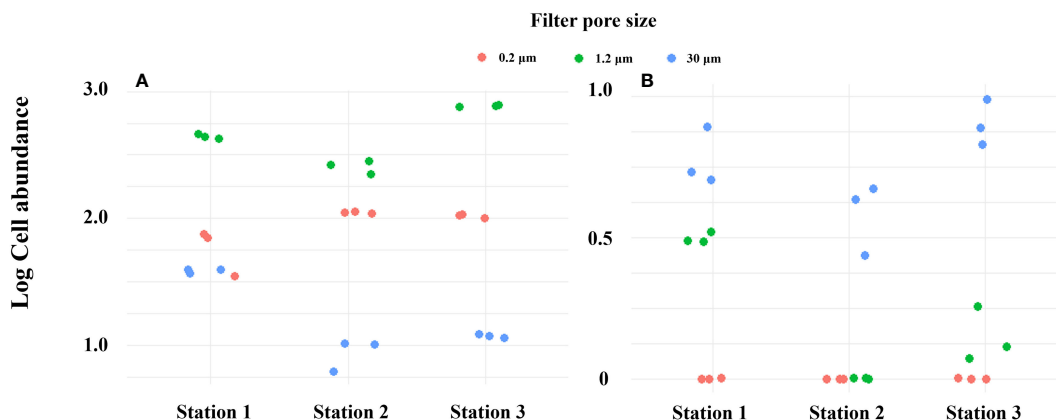


FIGURE 8 DNA yield by filter pore size in Masan Bay in April 2023. (A) PMA Untreated group. (B) PMA Treated group.

primer configuration, tailored specifically for *Alexandrium* spp., demonstrates the promising potential for practical application in environmental assessment and protection.

In this study, for quantitative analysis using eDNA in sediment layers and seawater, standard curves were generated to investigate the relationship between *A. catenella* cells, cysts, and *A. tamarense* concentrations and Ct values. This analysis confirmed the feasibility of modeling the relationship between concentrations and Ct values, allowing us to estimate concentrations in other samples. In addition, a high  $R^2$  value indicates that the standard curve fits the data well and contributes to the reliability and accuracy of the measurement results.

If protected from physical degradation, exDNA can persist for years (Agnelli et al., 2007). While iDNA is protected from external degradation factors in the environment and has a longer half-life, internal degradation factors associated with cell death can result in DNA fragmentation (Hotchkiss et al., 2009). In conclusion, the findings suggest that different forms of DNA, including extracellular eDNA, damaged intracellular eDNA, and intact intracellular eDNA, may undergo distinct degradation processes

(Hirohara et al., 2021). These processes can be influenced by various factors such as environmental conditions, enzymatic activity, and the physiological state of the cells or organisms. Understanding these separate degradation processes is crucial for interpreting eDNA data accurately and assessing its persistence and reliability as a biomonitoring tool. Therefore, it is considered more reliable to selectively detect iDNA in seawater than to detect total eDNA. By specifically targeting and analyzing iDNA, the focus is shifted to intact cell-derived DNA, which provides a clearer indication of the presence and abundance of living organisms. This approach helps to minimize potential errors and biases associated with the detection of degraded or extracellular DNA, contributing to a more accurate assessment of biodiversity, ecological dynamics, and environmental monitoring in marine ecosystems.

The results presented in Figure 5 demonstrate that the heat treatment method leads to the absence of log cell values in both the cell and cyst categories within the PMA 0% treatment group. Additionally, there is a gradual decrease in log cell counts for eDNA (iDNA, exDNA) in both cell and cyst categories in the absence of PMA treatment. These observations strongly suggest

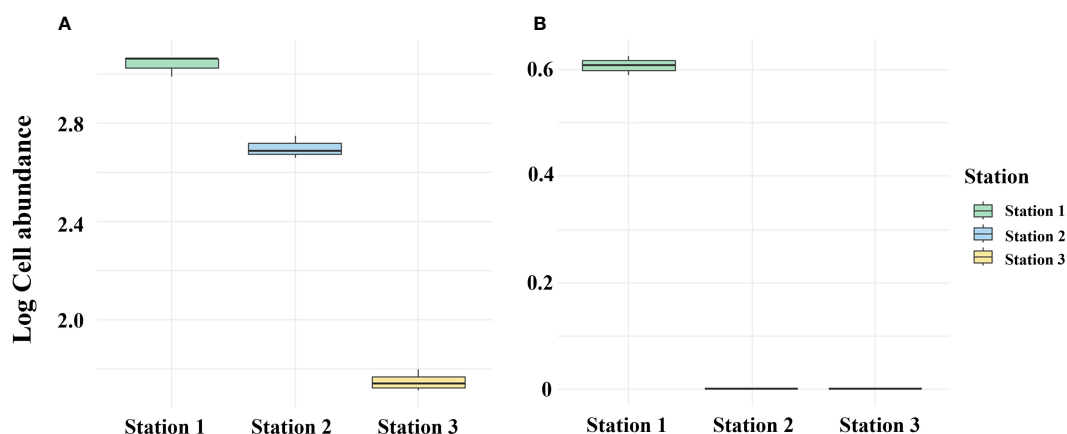


FIGURE 9 Comparison between PMA-treated and untreated eDNA groups of April 2023 Masan Bay sediment. (A) PMA Untreated group. (B) PMA Treated group.

that the heat treatment method affects both iDNA and exDNA. However, the PMA-iDNA experiment, which generates randomized exDNA, highlights that only sufficient iDNA is detected. Contrastingly, Triton X-100 treatment did not impact the log cell count of eDNA (iDNA, exDNA) in the no PMA group, offering a distinct approach from the heat treatment. Nevertheless, DNA amplification occurred in the 0% PMA treatment group, implying that Triton X-100 might not completely degrade all iDNA. Furthermore, the higher log cell count observed in cysts in the PMA-treated group, as opposed to cells, aligns with the well-established understanding that cyst membranes are more resilient than cells. In summary, the heat treatment method's effect primarily pertained to exDNA without detecting iDNA in the 0% live cell group. On the other hand, the Triton X-100 treatment method identified iDNA while not affecting exDNA within the same group. Both methodologies collectively highlight PMA's exclusive covalent binding to exDNA, enabling the selective detection of iDNA while excluding exDNA. The potential of iDNA-PMA monitoring is further supported by the insights presented in [Figure 7](#). In this fig, the results of the four groups subjected to DNase and PMA treatment lend credibility to the aforementioned experiments. Specifically, the outcomes demonstrate that log cell abundance remained stable in Group A regardless of the percentage of viable cells, spanning from 100% down to 0%. In contrast, Groups B, C, and D exhibited higher log cell abundance values at 100% viable cells, which progressively decreased to lower values at 0% ([Figure 7](#)). This reinforcement underscores the reliability and effectiveness of the iDNA-PMA monitoring approach.

In our study, we observed that the eDNA yields were high when using relatively small pore sizes of 0.2 and 1.2  $\mu\text{m}$  filters in seawater. However, when it came to the selective detection of iDNA treated with PMA, which is designed to target DNA from living organisms, the yields with 0.2 and 1.2  $\mu\text{m}$  filters were lower compared to the 30  $\mu\text{m}$  filter. This suggests that employing a combination of PMA treatment and a larger pore size filter, such as 30  $\mu\text{m}$ , may be a useful strategy for effectively collecting iDNA during field sampling. By selectively capturing iDNA while minimizing the capture of other particles and exDNA, this approach can enhance the accuracy and reliability of iDNA analysis, providing valuable insights into the presence and activity of living organisms in aquatic environments. In August 2023, eDNA detection at stations 4, 5, and 6 exhibited a notable contrast between untreated and PMA-treated groups ([Figure 3](#)). exDNA is generated from cell breakdown post-mortem or active shedding by organisms in the environment ([Ibanez de Aldecoa et al., 2017](#)). Particularly, exDNA introduced into soil or water displays significant resistance to rapid degradation, persisting for hours to days ([Nielsen et al., 2007](#)). Therefore, DNA amplification in the PMA-untreated group likely indicates exDNA detection, while the absence of amplification in the PMA-treated group implies specificity of the Alx2 primer set for *Alexandrium* spp. These outcomes enable us to anticipate errors in eDNA monitoring due to the specificity of primers and the persistence of exDNA.

*Alexandrium* spp. cysts exist in the sediment layer depending on environmental conditions and give rise to HABs ([Dale, 1983](#)). The eDNA derived from these cysts serves as a crucial indicator for detecting the distribution and occurrence of *Alexandrium* spp. in

the ocean. However, DNA extraction from sediments carries the risk of overestimation or false positives. To tackle these challenges, sediment samples can be treated with PMA to assess the potential of environmental monitoring for *Alexandrium* spp. cysts. PMA-treated eDNA samples selectively detect only iDNA. Consequently, the separation of PMA-treated eDNA samples into exDNA and iDNA improves the accuracy of eDNA detection.

While eDNA monitoring serves various purposes in environmental monitoring, such as biodiversity surveys and species detection, it has limitations in quantitative detection and epidemiological surveys. However, PMA-iDNA monitoring selectively detects intact DNA and can overcome these limitations. However, in the future, there is a need to improve the capability of PMA-iDNA to investigate a wide range of biological groups by optimizing the target species' iDNA size and the filter size used. It is essential to understand the variations in iDNA quantity that may arise based on the DNA size of specific microbial species, higher organisms, cells, and tiny microorganisms. This understanding will serve as the foundation for accurate iDNA monitoring of diverse biological groups.

## 5 Conclusion

To the best of our knowledge, the detection of iDNA in Seawater and sediments using PMA treatment and filter size optimization for improved selective detection of iDNA has been pioneered in this study. A method has been developed to prevent non-selective detection of eDNA through PMA treatment and minimize potential errors in eDNA sampling by optimizing the filter size. This approach reduces the risk of quantitative overestimation in eDNA studies and enhances the accuracy of species identification. The method is not only applicable to the analysis of *Alexandrium* spp. eDNA but also holds promise for the assessment of other harmful algal blooms (HABs) or phytoplankton, as well as for higher organism PMA-iDNA monitoring assays. Furthermore, it can serve as a rapid and reliable approach to mitigate errors in eDNA analysis.

## Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

## Author contributions

K-WY: Conceptualization, Data curation, Investigation, Methodology, Writing- original draft. H-SS: Data curation, Formal analysis, Investigation, Writing- review & editing. M-JS: Formal analysis, Investigation, Data curation, Writing- review & editing. MC-K: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing- review & editing, Writing- original draft.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## References

- Agnelli, A., Ascher, J., Corti, G., Ceccherini, M. T., Pietramellara, G., and Nannipieri, P. (2007). Purification and isotopic signatures ( $\Delta 13\text{ C}$ ,  $\Delta 15\text{ N}$ ,  $\Delta 14\text{ C}$ ) of soil extracellular DNA. *Biol. Fertility Soils* 44, 353–361. doi: 10.1007/s00374-007-0213-y
- Anderson, D. M. (1980). Effects of temperature conditioning on development and germination of gonyaulax tamarensis (Dinophyceae) hypnozygotes 1. *J. Phycol.* 16, 166–172. doi: 10.1111/j.1529-8817.1980.tb03013.x
- Anderson, D. M. (1998). Physiology and bloom dynamics of toxic alexandrium species, with emphasis on life cycle transitions. *Nato Asi Ser. G Ecol. Sci.* 41, 29–48.
- Anderson, D. M., Alpermann, T. J., Cembella, A. D., Collos, Y., Masseret, E., and Montresor, M. (2012). The globally distributed genus alexandrium: multifaceted roles in marine ecosystems and impacts on human health. *Harmful Algae* 14, 10–35. doi: 10.1016/j.hal.2011.10.012
- Anderson, D. M., Kulis, D. M., Keafer, B. A., Gribble, K. E., Marin, R., and Scholin, C. A. (2005). Identification and enumeration of alexandrium spp. from the gulf of maine using molecular probes. *Deep Sea Res. Part II: Topical Stud. Oceanography* 52 (19), 2467–2490. doi: 10.1016/j.dsr2.2005.06.015
- Anderson, D. M., and Wall, D. (1978). Potential importance of benthic cysts of Gonyaulax Tamarensis and G. excavata in initiating toxic dinoflagellate blooms 1, 2, 3. *J. Phycol.* 14, 224–234.
- Behrenfeld, M. J., O'Malley, R., Boss, E., Karp-Boss, L., and Mundt, C. (2021). Phytoplankton biodiversity and the inverted paradox. *ISME Commun.* 1 (1), 52. doi: 10.1038/s43705-021-00056-6
- Carini, P., Marsden, P. J., Leff, J. W., Morgan, E. E., Strickland, M. S., and Fierer, N. (2016). Relic DNA is abundant in soil and obscures estimates of soil microbial diversity. *Nat. Microbiol.* 2 (3), 1–6. doi: 10.1038/nmicrbiol.2016.242
- Corinaldesi, C., Beolchini, F., and Dell'Anno, A. (2008). Damage and degradation rates of extracellular DNA in marine sediments: implications for the preservation of gene sequences. *Mol. Ecol.* 17 (17), 3939–3951. doi: 10.1111/j.1365-294X.2008.03880.x
- Corinaldesi, C., Danovaro, R., and Dell'Anno, A. (2005). Simultaneous recovery of extracellular and intracellular DNA suitable for molecular studies from marine sediments. *Appl. Environ. Microbiol.* 71 (1), 46–50. doi: 10.1128/AEM.71.1.46-50.2005
- Corinaldesi, C., Tangherlini, M., Manea, E., and Dell'Anno, A. (2018). Extracellular DNA as a genetic recorder of microbial diversity in benthic deep-sea ecosystems. *Sci. Rep.* 8 (1), 1–9. doi: 10.1038/s41598-018-20302-7
- Dale, B. (1983). "Dinoflagellate resting cysts: benthic plankton," in *Survival strategies of the algae*, 69–136.
- Dell'Anno, A., and Danovaro, R. (2005). Extracellular DNA plays a key role in deep-sea ecosystem functioning. *Science* 309 (5744), 2179. doi: 10.1126/science.1117475
- Fukuyo, Y. (1985). Morphology of protogonyaulax tamarensis (Lebour) taylor and protogonyaulax catenella (Whedon and kofoid) taylor from Japanese coastal waters. *Bull. Mar. Sci.* 37 (2), 529–537.
- Grzebyk, D., Audic, S., Lasserre, B., Abadie, E., de Vargas, C., and Bec, B. (2017). Insights into the Harmful Algal Flora in Northwestern Mediterranean Coastal Lagoons Revealed by Pyrosequencing Metabarcodes of the 28S rRNA Gene. *Harmful Algae* 68, 1–16. doi: 10.1016/j.hal.2017.06.003
- Günther, B., Kneibelsberger, T., Neumann, H., Laakmann, S., and Martínez Arbizu, P. (2018). Metabarcoding of marine environmental DNA based on mitochondrial and nuclear genes. *Sci. Rep.* 8 (1), 14822. doi: 10.1038/s41598-018-32917-x
- Hallegraeff, G. M., and Bolch, C. J. (1992). Transport of diatom and dinoflagellate resting spores in ships' Ballast water: implications for plankton biogeography and aquaculture. *J. Plankton Res.* 14 (8), 1067–1084. doi: 10.1093/plankt/14.8.1067
- Hirohara, T., Tsuru, K., Miyagawa, K., Paine, R. T. R., and Yamanaka, H. (2021). The application of PMA (Propidium monoazide) to different target sequence lengths of zebrafish eDNA: A new approach aimed toward improving environmental DNA ecology and biological surveillance. *Front. Ecol. Evol.* 9, 632973. doi: 10.3389/fevo.2021.632973
- Hopkins, G. W., and Freckleton, R. P. (2002). Declines in the numbers of amateur and professional taxonomists: implications for conservation. *Anim. Conserv.* 5, 245–249.
- Hotchkiss, R. S., Strasser, A., McDunn, J. E., and Swanson, P. E. (2009). Cell death. *New Engl. J. Med.* 361 (16), 1570–1583. doi: 10.1056/NEJMr0901217
- Ibanez de Aldecoa, A. L., Zafra, O., and González-Pastor, JoséE. (2017). Mechanisms and regulation of extracellular DNA release and its biological roles in microbial communities. *Front. Microbiol.* 8, 1390. doi: 10.3389/fmicb.2017.01390
- Kumar, G., Farrell, E., Reaume, A. M., Eble, J. A., and Gaither, M. R. (2022). One size does not fit all: tuning eDNA protocols for high- and low-turbidity water sampling. *Environ. DNA* 4 (1), 167–180. doi: 10.1002/edn3.235
- Lacoursière-Roussel, A., Rosabal, M., and Bernatchez, L. (2016). Estimating Fish Abundance and Biomass from eDNA Concentrations: Variability among Capture Methods and Environmental Conditions. *Mol. Ecol. Resour.* 16 (6), 1401–1414. doi: 10.1111/1755-0998.12522
- Lawson Handley, L. (2015). How will the 'molecular revolution' contribute to biological recording? *Biol. J. Linn. Soc.* 115 (3), 750–766. doi: 10.1111/bij.12516
- Lee, S. Y., Jeong, H. J., Seong, K. A., Lim, A. S., Kim, J. H., Lee, K. H., et al. (2017). Improved real-time PCR method for quantification of the abundance of all known ribotypes of the ichthyotoxic dinoflagellate cochlodinium polykrikoides by comparing 4 different preparation methods. *Harmful Algae* 63, 23–31. doi: 10.1016/j.hal.2017.01.006
- Martin, J. L., Page, F. H., Hanke, A., Strain, P. M., and LeGresley, M. M. (2005). Alexandrium fundyense vertical distribution patterns during 1982, 2001 and 2002 in the offshore bay of fundy, eastern Canada. *Deep Sea Res. Part II: Topical Stud. Oceanography* 52 (19), 2569–2592. doi: 10.1016/j.dsr2.2005.06.010
- Medlin, L., Elwood, H. J., Stickel, S., and Sogin, M. L. (1988). The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71 (2), 491–499. doi: 10.1016/0378-1119(88)90066-2
- MEIS (Marine Environmental Information System). *Marine environment observation & Survey*. Available at: <https://www.meis.go.kr/mei/observe/port.do>.
- Min, J., and Kim, K. Y. (2023). Seasonal change and subniche dynamics of three alexandrium species in the korea strait. *Harmful Algae* 125, 102420. doi: 10.1016/j.hal.2023.102420.
- Nagler, M., Podmirseg, S. M., Ascher-Jenull, J., Sint, D., and Traugott, M. (2022). Why eDNA fractions need consideration in biomonitoring. *Mol. Ecol. Resour.* 22 (7), 2458–2705. doi: 10.1111/1755-0998.13658.
- Nielsen, K. M., Johnsen, PålJ., Bensasson, D., and Daffonchio, D. (2007). Release and persistence of extracellular DNA in the environment. *Environ. Biosafety Res.* 6 (1-2), 37–53. doi: 10.1051/ebr:2007031

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1257343/full#supplementary-material>

- Nocker, A., and Camper, A. K. (2006). Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. *Appl. Environ. Microbiol.* 72 (3), 1997–2004. doi: 10.1128/AEM.72.3.1997-2004.2006
- Park, B. S., Wang, P., Kim, J. H., Kim, J.-H., Gobler, C. J., and Han, M.-S. (2014). Resolving the Intra-Specific Succession within *Cochlodinium Polykrikoides* Populations in Southern Korean Coastal Waters Via use of Quantitative PCR Assays. *Harmful Algae* 37, 133–141. doi: 10.1016/j.hal.2014.04.019
- Pietramellara, G., Ascher, J., Borgogni, F., Ceccherini, M. T., Guerri, G., and Nannipieri, P. (2009). Extracellular DNA in soil and sediment: fate and ecological relevance. *Biol. Fertility Soils* 45, 219–235. doi: 10.1007/s00374-008-0345-8
- Pont, D., Meulenbroek, P., Bammer, V., Dejean, T., Erős, T., Jean, P., et al. (2023). Quantitative Monitoring of Diverse Fish Communities on a Large Scale Combining eDNA Metabarcoding and qPCR. *Mol. Ecol. Resour.* 23 (2), 396–409. doi: 10.1111/1755-0998.13715
- Salter, I., Joensen, M., Kristiansen, R., Steingrund, P., and Vestergaard, P. (2019). Environmental DNA concentrations are correlated with regional biomass of atlantic cod in oceanic waters. *Commun. Biol.* 2 (1), 461. doi: 10.1038/s42003-019-0696-8
- Samsur, M., Yamaguchi, Y., Sagara, T., Takatani, T., Arakawa, O., and Noguchi, T. (2006). Accumulation and depuration profiles of PSP toxins in the short-necked clam *tapes japonica* fed with the toxic dinoflagellate alexandrium catenella. *Toxicon* 48 (3), 323–330. doi: 10.1016/j.toxicon.2006.06.002
- Shikata, T., Taniguchi, E., Sakamoto, S., Kitatsuji, S., Yamasaki, Y., Yoshida, M., et al. (2020). Phylogeny, growth and toxicity of the noxious red-tide dinoflagellate alexandrium leei in Japan. *Regional Stud. Mar. Sci.* 36, 101265. doi: 10.1016/j.rsma.2020.101265
- Strickler, K. M., Fremier, A. K., and Goldberg, C. S. (2015). Quantifying Effects of UV-B, Temperature, and pH on eDNA Degradation in Aquatic Microcosms. *Biol. Conserv.* 183, 85–92. doi: 10.1016/j.biocon.2014.11.038
- Turner, C. R., Barnes, M. A., Xu, C. C. Y., Jones, S. E., Jerde, C. L., and Lodge, D. M. (2014). Particle size distribution and optimal capture of aqueous microbial eDNA. *Methods Ecol. Evol.* 5 (7), 676–684. doi: 10.1111/2041-210X.12206
- Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., et al. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Mol. Ecol.* 25 (4), 929–942. doi: 10.1111/mec.13428
- Whedon, W. F. (1936). Dinoflagellates of the san francisco region. I. on the skeletal morphology of two new species, gonyaulax catenella and G. Acatenella. *Univ. Calif. Publ. Zool.* 41, 25–34.