Development of a high-sensitivity qPCR method of harmful *Dinophysis* spp. using synthetic oligonucleotides

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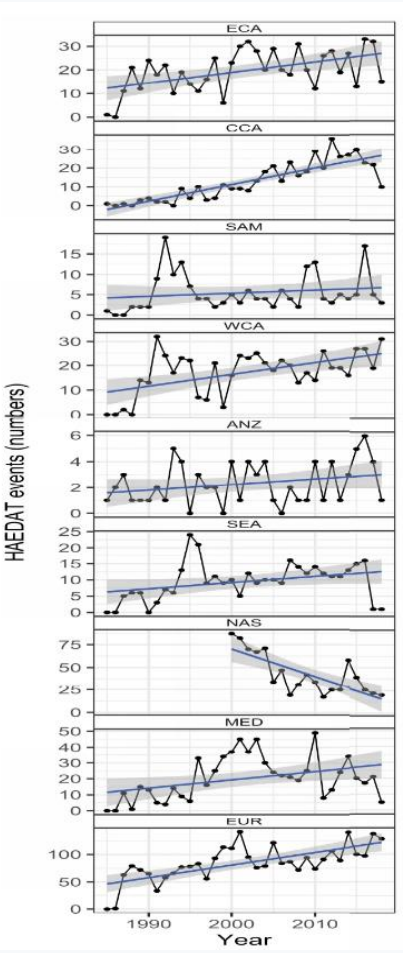

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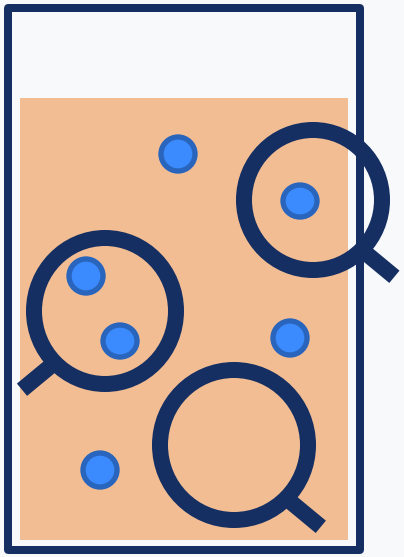
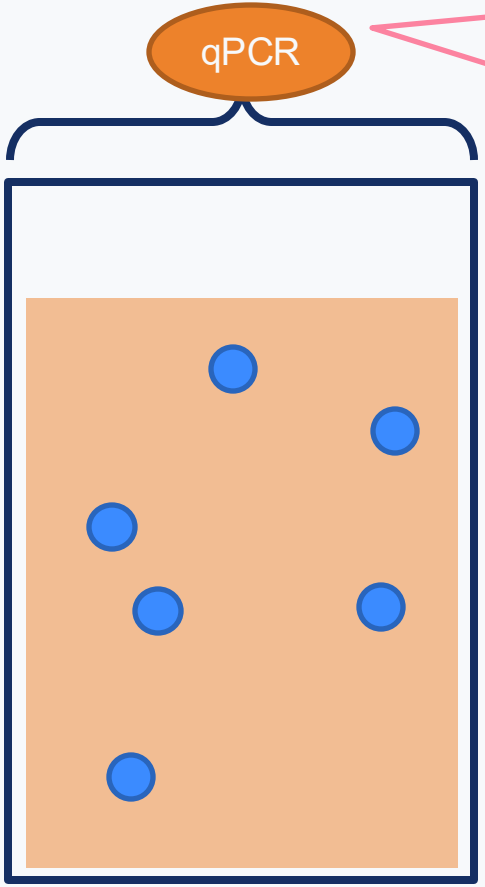


- **Harmful algal blooms (HABs)** are the rapid growth of toxic algae that threaten marine ecosystems, fisheries industry, and even human health.
- Both globally and in Korea, **incidents of shellfish poisoning** have sharply increased, as toxins are accumulated in bivalves that feed on harmful algae.
- When **people consume these contaminated shellfish**, they can suffer from different types of poisoning.
- **Early detection of HAB-forming organisms is essential**, but microscopic methods lack the sensitivity to detect low-abundance cells.

01 Introduction



become one of the most preferred methods for **detecting HABs**

requires **standard curve** which is typically generated using genomic DNA from cultured cells of target organisms.

***Dinophysis* spp.**
: HABs species
: notoriously difficult to culture

- This highlights the **limitations of DNA-based standard curves** in qPCR.
- Recent studies in bacterial detection have successfully used **synthetic oligonucleotide-based standard curves** as an alternative to live cell-based standards (Han et al., 2023).

01 Introduction

Objective of this study

1) To develop a qPCR detection method for unculturable *Dinophysis* spp., using synthetic oligonucleotide DNA as a standard.
2) To establish a technique that allows for accurate quantification of *Dinophysis* without the need for cultured cells.
3) To propose a sensitive and practical molecular detection strategy for the early monitoring of harmful algal blooms.

02 Materials and Methods

- Primer design
- *In-silico* validation
- Genomic DNA qPCR
- Specificity Test

- Oligonucleotide synthesis and copy number calculation
- qPCR assay using oligonucleotide DNA
- Estimated of LSU rDNA copy number per Dinophysis cell

- Spike test
- Field eDNA test

02 Materials and Methods

1) Primer set & *in-silico* test

Primer	Sequences (5'→3')	Product length	TA(℃)
Forward	AAG CAA GCG GGA GCA AGT TT	136 bp	65
Reverse	GCA GAA GGT TAT GCT CAT CG		

* Patent in preparation

- A species-specific primer set targeting LSU rDNA was designed.
- For *in-silico* test,
  - LSU rDNA sequences from 30 strains of 14 *Dinophysis* species (GenBank, NCBI) were used.
  - BLAST analysis confirmed no significant similarity to non-target organisms.

02 Materials and Methods

2) EvaGreen based qPCR condition

Dinophysis strain isolation YD-KNU

Genomic DNA extraction

qPCR based on genomic DNA

- **PCR condition**
  - **Initial denaturation :** 98 ℃ for 3 min
  - **Cycling (x40) :** 98 ℃ for 10 sec, 65 ℃ for 10 sec
  - **Melting curve :** from 65 ℃ to 95 ℃, increasing by 0.5 ℃ every step

    Hold 5 sec at each step, the measure fluorescence

02 Materials and Methods

3) Specificity test

- Phytoplankton cultures of 14 dinoflagellate species, 1 diatom species and 1 raphidophyte species were obtained.
- PCR reactions were performed using these cultures to evaluate the specificity of the designed primer set.

Class	Species		Strain name	Class	Species		Strain name
Dinophyceae	*Dinophysis*	*acumina*	YD-KNU	Dinophyceae	*Heterocpasa*	*triquetra*	HT
	Akashiwo	*sanguinea*	HY-A-24-08		*Prorocentrum*	*koreanum*	Pr-2306Sp-B6
	Alexandrium	*catenella*	HY-A-24-11			*minimum*	PM
		insuetum	HY-A-24-05		*Scripciella*	sp.	HY-A-24-04, HY-A-24-10
		pacificum	HY-A-23-008		*Thecadinium*	*kofoidii*	TK-2102PS-02
	Amphidinium	*massartii*	Am-1904UD-09		*Tripos*	*muelleri*	HY-A-24-06
	Amphidinium	*thermaeum*	Am-1904UD-13	Rhapidophyceae	*Heterosigma*	*akashiwo*	HY-A-24-12
	Heterocapsa	*horiguchii*	HH-2003PS-01	Bacillophyceae	*Pseudo-nitzschia*	*pungens*	GN03D708G4
	Heterocpasa	*pseudotriquetra*	HP-1803PS-01, HP-1804HD-01				

02 Materials and Methods

4) Oligonucleotide synthesis

- Oligo information (LSU synthetic oligonucleotide DNA169bp)

5’ TGTGGTGTGTCTTACCTAGTGGGTCATTGTGGGTTTATGCATTAAGATAAATCAAAGCAAGC
GGGAGCAAGTTTACGAGTTTGTGAATGTGTGTTGTTATGCTTGTGCGTAAGCTTTAGTGTTTGT
CTGGCTGCAGGGCTGTCCATCCTCAAGCTTTGCTGTGGTTGCGATGAGCATAACCTTCTGCAC
AGATCTT 3'

Forward Primer

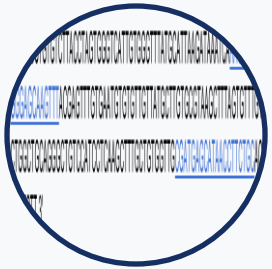
Reverse Primer

Forward: 0.3 nmole, 60830.91 MW 196 bp
Reverse: 0.3 nmole, 60140.73 MW 196 bp

- Copy number calculation

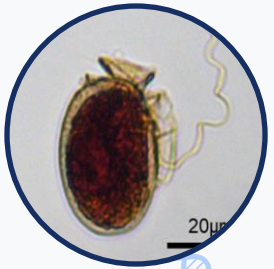
$$\text{Copynumbers} = \frac{\text{Oligo amount (ng)} \times \text{Avogardro's number } (6.022 \times 10^{23})}{\text{Oligo length (bp)} \times 1 \times 10^{9} \times 660}$$

$$\text{ng} = \text{nmole} \times \text{MW}$$



Oligonucleotide synthesis

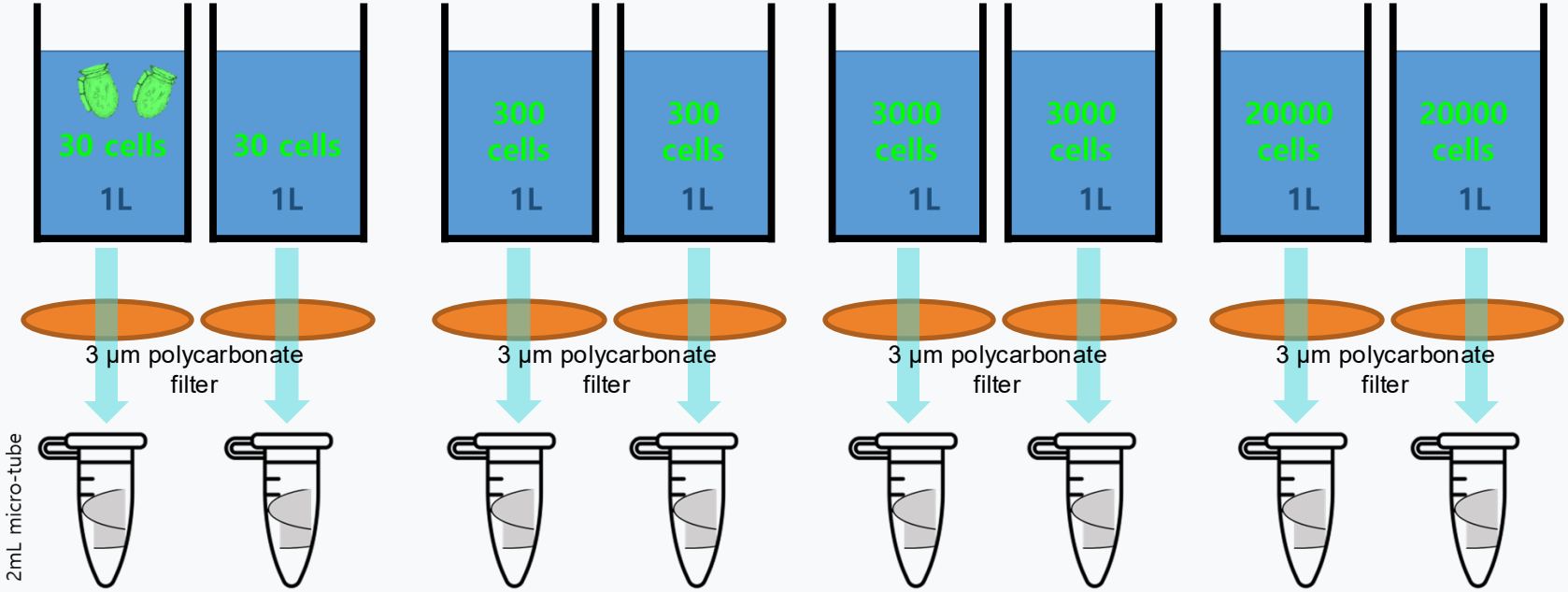
Perform a qPCR



Copy number per cell (*Dinophysis*)

02 Materials and Methods

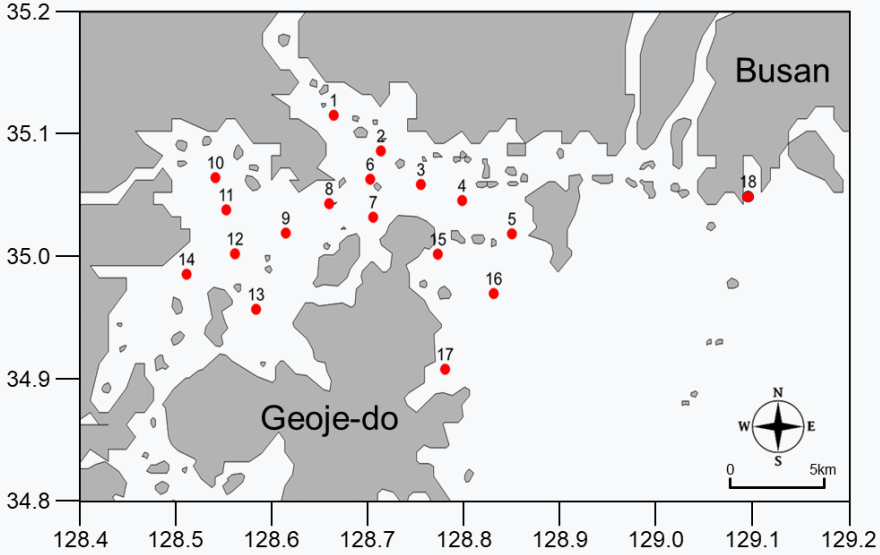
5) Spike test

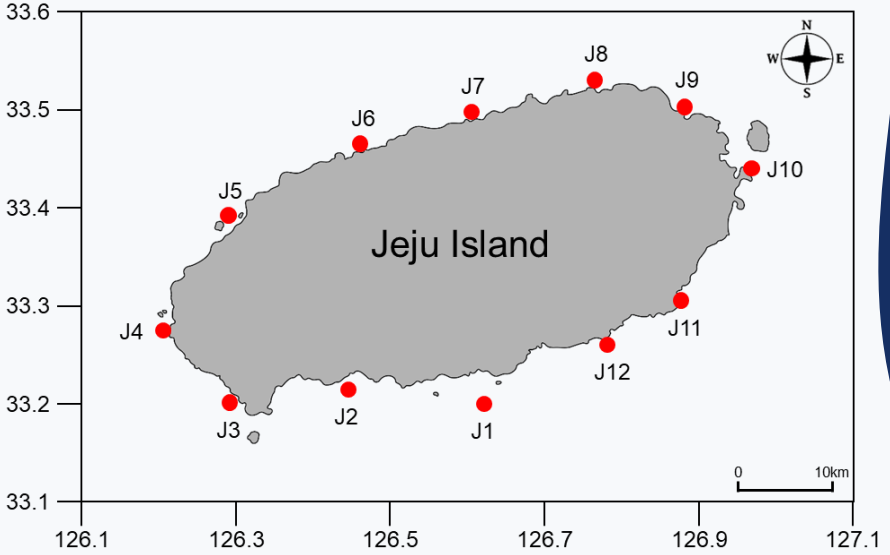


- *D. acuminata* cells were spiked into 1 L of *Dinophysis*-free seawater at four concentrations (30, 300, 3,000, 20,000 cells/L).
- EvaGreen-based qPCR assay was applied to verify the accuracy and field applicability of the qPCR method.

02 Materials and Methods

6) Field application

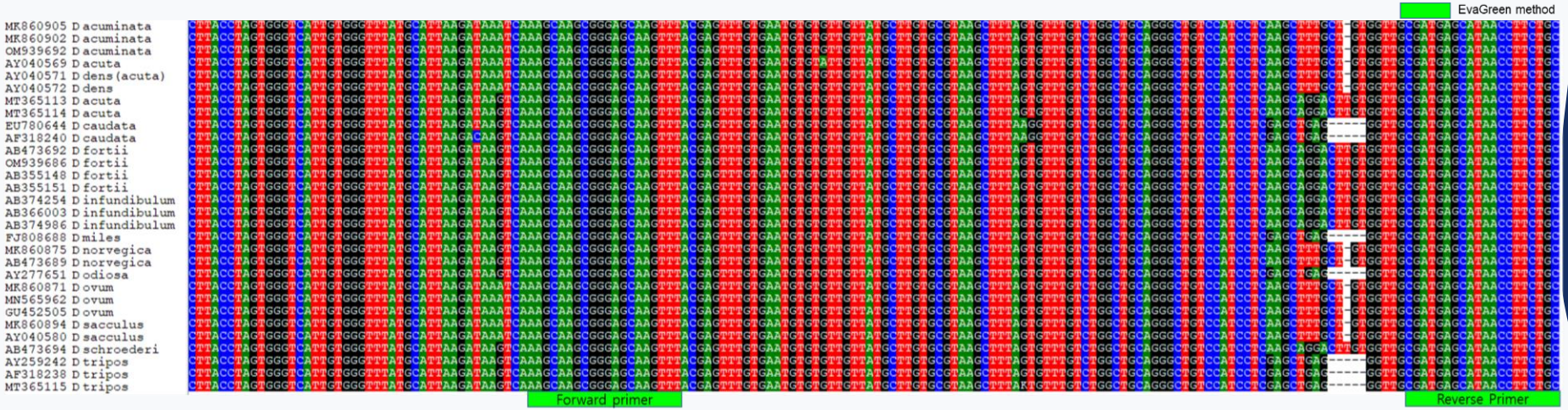




- **Sampling stations:** Southern coast of Korea and coastal waters off Jeju Island
- **Sampling period:** 2022–2023
- **500 mL surface seawater** was filtered using **3 μm polycarbonate membrane filters**

03 Results and discussions

1) *in-silico* test



- Designed primers **matched** only *Dinophysis* sequences via BLAST.
- The primer binding regions are highly conserved among *Dinophysis* species.

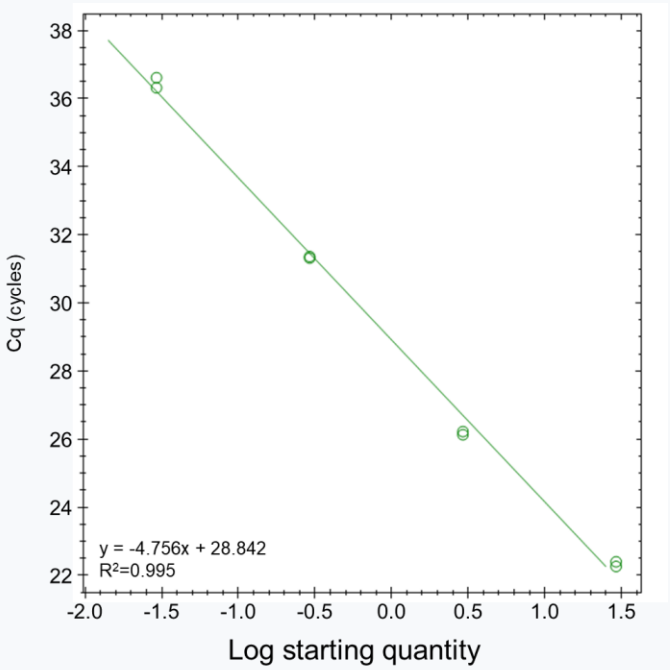
03 Results and discussions

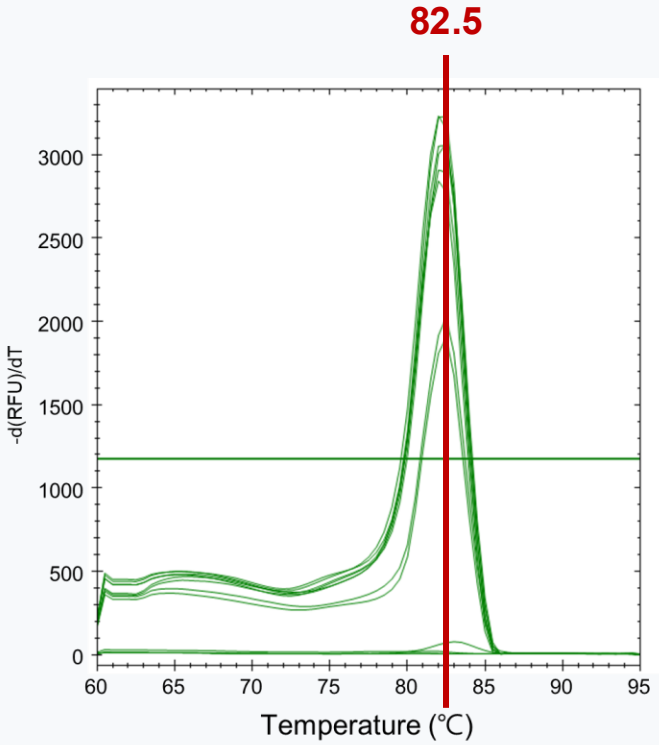
2) Specificity test

Class	Species		Strain name	qPCR result
Dinophyceae	***Dinophysis***	***acumina***	**YD-KNU**	**Pos**
	Akashiwo	*sanguinea*	HY-A-24-08	n.d
	Alexandrium	*catenella*	HY-A-24-11	n.d
		insuetum	HY-A-24-05	n.d
		pacificum	HY-A-23-008	n.d
	Amphidinium	*massartii*	Am-1904UD-09	n.d
	Amphidinium	*thermaeum*	Am-1904UD-13	n.d
	Heterocapsa	*horiguchii*	HH-2003PS-01	n.d
	Heterocpasa	*pseudotriquetra*	HP-1803PS-01, HP-1804HD-01	n.d
		triquetra	HT	n.d
	Prorocentrum	*koreanum*	Pr-2306Sp-B6	n.d
		minimum	PM	n.d
	Scripciella	sp.	HY-A-24-04, HY-A-24-10	n.d
	Thecadinium	*kofoidii*	TK-2102PS-02	n.d
	Tripos	*muelleri*	HY-A-24-06	n.d
Rhapidophyceae	*Heterosigma*	*akashiwo*	HY-A-24-12	n.d
Bacillophyceae	*Pseudo-nitzschia*	*pungens*	GN03D708G4	n.d

03 Results and discussions

3) qPCR condition screening

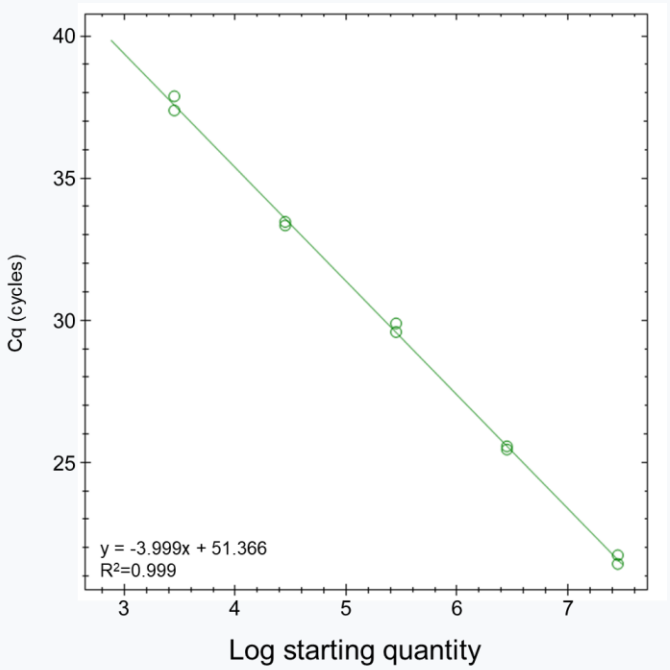


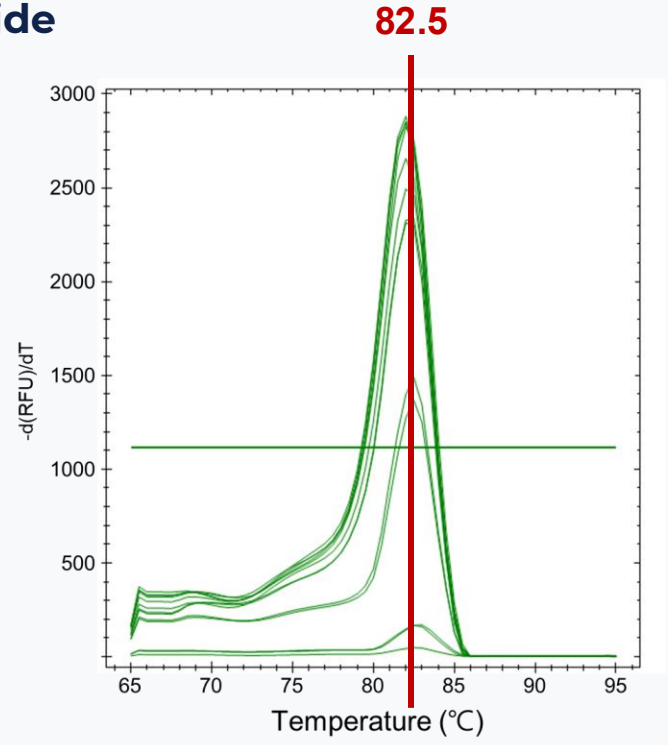


- Standard curve: $R^2 = 0.995$
- Melting peak : 82.5 °C, amplification was specific.
- Validated qPCR conditions optimized for detecting *Dinophysis* DNA.

03 Results and discussions

4) Standard based on synthetic oligonucleotide

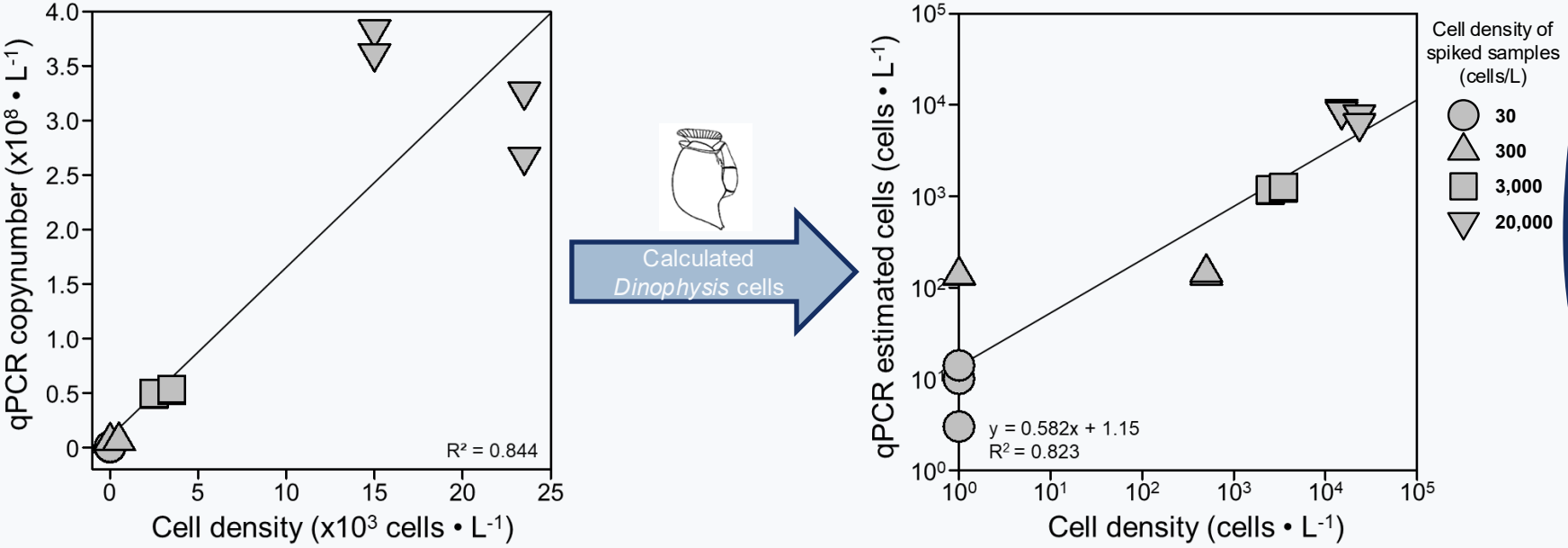




- Standard curve: $R^2 = 0.999$
- Melting peak : 82.5 °C, amplification was specific.
- Synthetic oligo standard is reliable for quantifying *Dinophysis* DNA using qPCR method.

03 Results and discussions

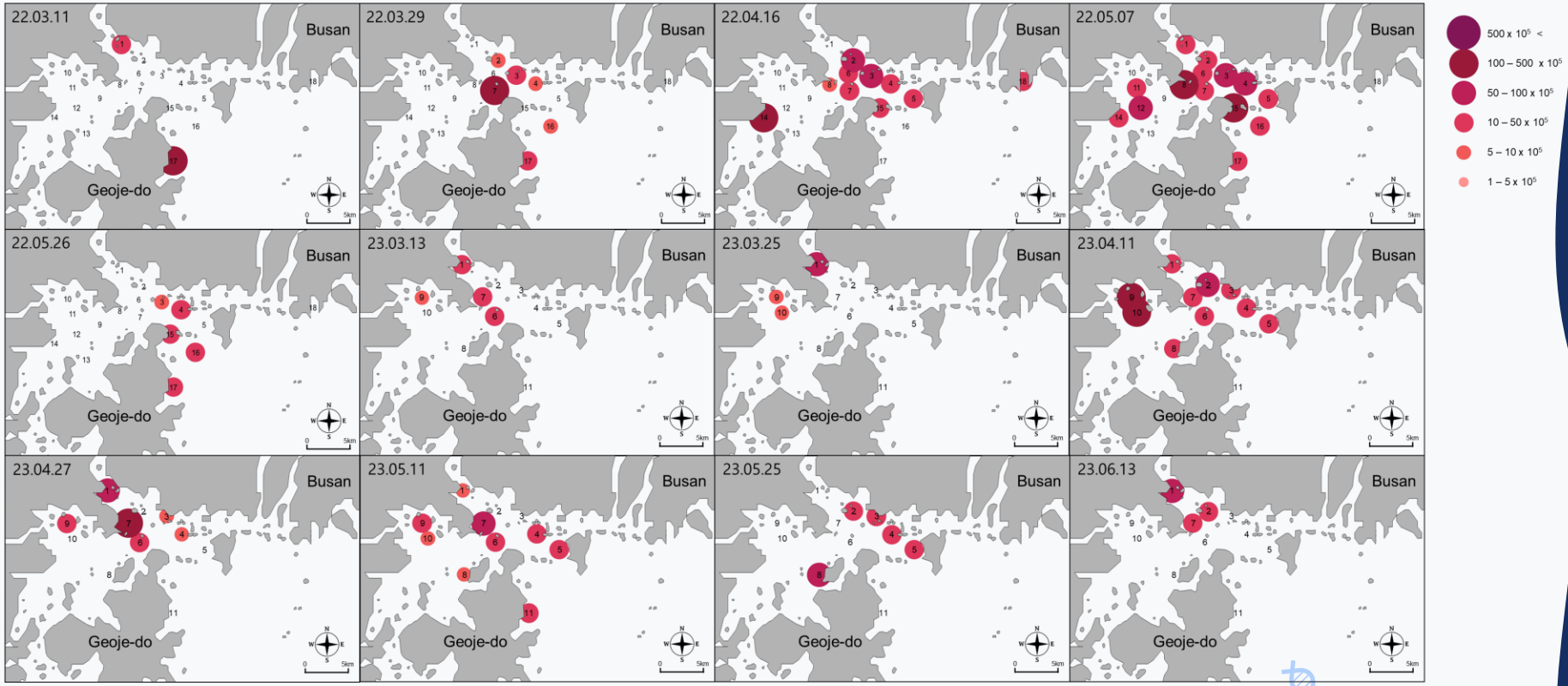
5) Spike test



- The qPCR results showed a strong linear correlation between the copy number and cell density.
- Cells estimated by qPCR based on the copy number per cell also showed a similar trend to the cell density.
- Importantly, qPCR enabled detection even in low-density samples where *Dinophysis* cells were not detectable under the microscope.

03 Results and discussions

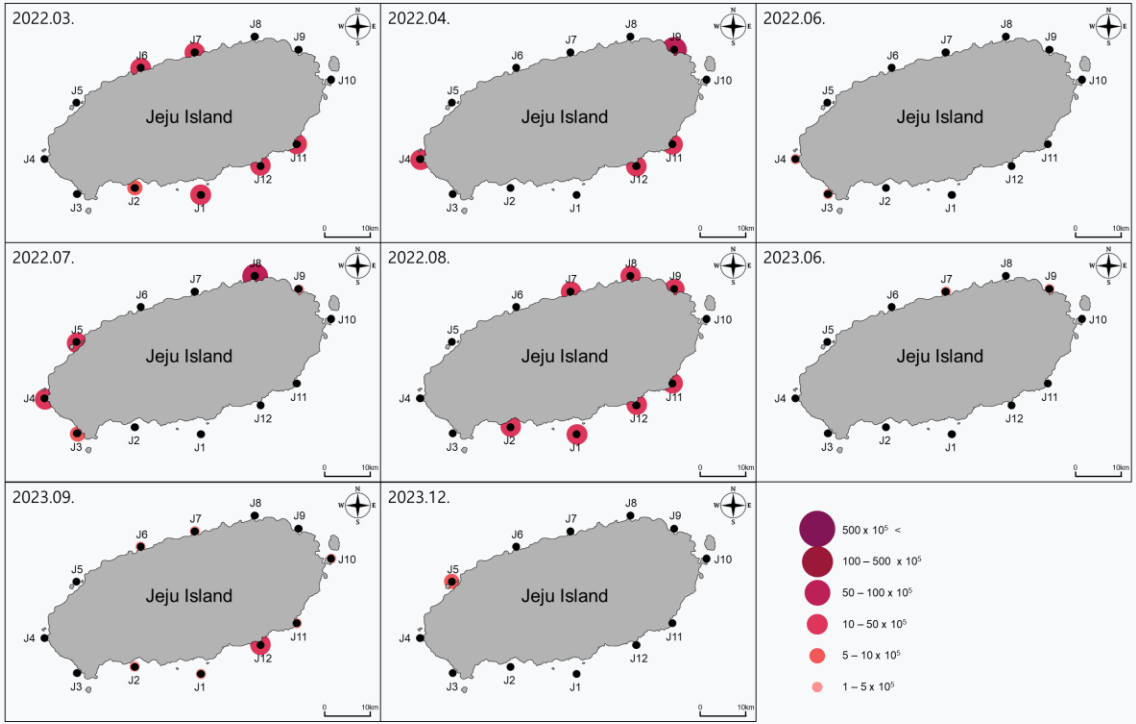
6) Field application (Jinhae bay)



- *Dinophysis* spp. showed higher abundances during the spring seasons in both 2022 and 2023.

03 Results and discussions

6) Field application (Jeju coast)



- *Dinophysis* spp. was detected from March to August in 2022, with the exception of June.
- In 2023, *Dinophysis* appeared in small quantities, mostly in June, September, and December.

04 Summary and Conclusion

- We developed an EvaGreen-based qPCR detection method targeting the LSU rDNA of *Dinophysis* spp. using a synthetic oligonucleotide standard, without the need for cultured cells.
- The designed primers showed specificity in both in-silico test and specificity tests, amplifying only *Dinophysis* DNA.
- The method showed high sensitivity and accuracy, with a strong correlation between qPCR results and microscopic counts in spike experiments.
- In field applications, *Dinophysis* was successfully detected in natural seawater samples.
- This method enabled the effective analysis of field samples, demonstrating its applicability for environmental monitoring.
- Furthermore, this study highlights the potential for detecting HAB-forming species,
- even those that are non-culturable, by using only DNA sequence information.

Thank You