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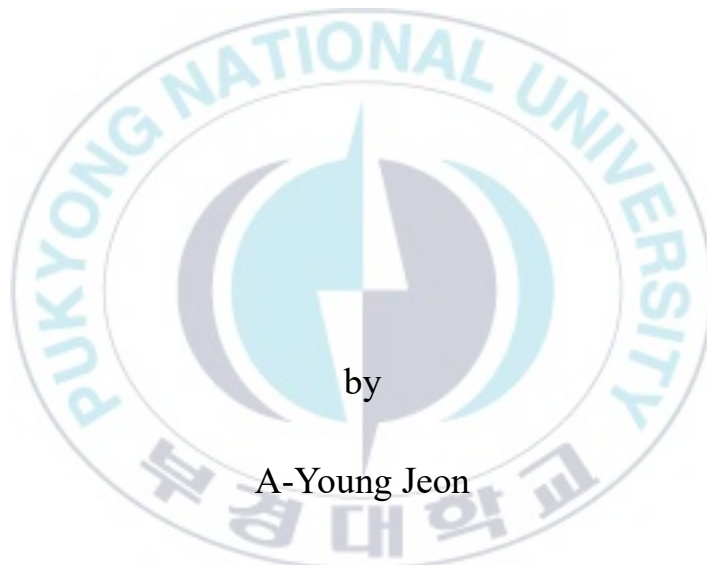
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Thesis for the Degree of Master of Science

Application of molecular biological
technique for the analysis of size-
fractionated phytoplankton communities



by

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February 21, 2020

Application of molecular biological technique for the analysis of size- fractionated phytoplankton communities

(크기분획된 식물플랑크톤의 군집구조 분석을 위한 분자생물학적 기술의 적용)

Advisor: Prof. Hyun-Woo Kim

by

A- Young Jeon

A thesis submitted in partial fulfillment of the requirements
for the degree of

Master of Engineering

in Department of Interdisciplinary Program of Biomedical Mechanical &
Electrical Engineering

The graduate School, Pukyong National University

February 2020

Application of molecular biological technique for the analysis of size-
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A dissertation

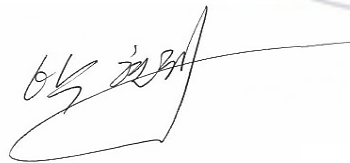
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TABLE OF CONTENTS

LIST OF FIGURES.....	ii
LIST OF TABLE.....	iii
ABSTRACT.....	iv
INTRODUCTION.....	1
MATERIALS AND METHODS.....	5
1. Sample collection and genomic DNA extraction.....	5
2. Quantitative analysis.....	8
3. Library construction and sequencing.....	9
4. Bioinformatic analysis.....	11
5. Statistical analysis.....	11
RESULTS.....	13
DISCUSSION.....	28
REFERENCES.....	36

LIST OF FIGURES

Fig. 1. Sampling site.....	7
Fig. 2. Total copy number of microorganism and phytoplankton in Jaran bay and Oryukdo.....	15
Fig. 3. Copy number by size fraction in Jaran bay and Oryukdo.....	16
Fig. 4. Seasonal changes in phytoplankton copy number.....	16
Fig. 5. Phytoplankton community structure at different month and size fraction	20
Fig. 6. Phylogenetic tree of phytoplankton OTU.....	21
Fig. 7. The size range of the main taxonomic group.....	25
Fig. 8. Heat map of phytoplankton OTU at different month and size fraction...	27

LIST OF TABLE

Table 1. Corrected proportion for each phylum 23



Application of molecular biological technique for the analysis of size-fractionated phytoplankton communities

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Abstract

Phytoplankton is responsible for 50% of global primary production through photosynthesis, playing a vital role in the carbon cycle. Therefore, the qualitative and quantitative of the phytoplankton community is essential to understand the biological processes reflecting the different environmental conditions. We here tested the reliability of the molecular biological tools, including qPCR and the high throughput sequencing (HTS), to know if those molecular tools can be an alternative to the laborious traditional observation in analyzing the phytoplankton community from the water samples. First, we conducted a quantitative PCR (qPCR) to quantify the microorganisms (16S universal primers) and phytoplankton (plastid 23S primers) from the water samples. We identified that the copy numbers of both the microorganism and the phytoplankton significantly different by locations and seasons. Second, we analyzed the size-fractionated phytoplankton community using the MiSeq platform to identify the size range of each phytoplankton taxon. We were able to know that the abundance of the phytoplankton taxa changes by time, and its traditional size fractionation was not precisely selective in differentiating phytoplankton taxa. From those results, we were able to know that molecular tools adopted in this study are promising to analyze the phytoplankton taxa for its low cost and labors. However, we also should know that copy numbers of each taxon obtained by qPCR is still far from the direct use for the estimation of its biomass. Therefore, further study should be conducted to transform the copy number by qPCR into the biomass of phytoplankton or microorganisms.

크기분획된 식물플랑크톤의 군집구조 분석을 위한 분자생물학적 기술의 적용

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요약

식물플랑크톤은 광합성을 통해서 전 세계 일차 생산의 50%를 담당하고 있으며, 탄소 순환에 중요한 역할을 하고있다. 그러므로 식물플랑크톤 군집에 대한 정성 및 정량 분석은 생물학적 과정을 이해하는 데 필수적이다. 우리는 물 샘플에 존재하는 식물플랑크톤 군집 분석에 있어서, 분자적인 도구가 기존방법의 대안이 될 수 있는지 알아보기 위해 quantitative PCR (qPCR) 과 High-throughput sequencing (HTS) 과 같은 분자생물학적인 방법의 신뢰성을 테스트 했다. 첫번째로, 우리는 물 샘플로부터 미생물과 식물플랑크톤을 정량하기 위해서 qPCR을 수행했다. 우리는 미생물과 식물플랑크톤 copy number가 지역과 계절에 따라서 유의한 차이가 있다는 것을 확인했다. 두번째로, 각 식물플랑크톤 분류군의 크기 범위를 확인하기 위해서 Miseq platform을 이용하여 크기분획된 식물플랑크톤 군집을 분석했다. 식물플랑크톤 분류군의 풍부도가 시간에 따라 변화한다는 것을 알수있었고, 기존의 크기분획이 정확하게 이루어지지 않았다는 것을 확인했다. 이러한 결과로부터 본 연구에서 이용된 분자적인 도구가 낮은 비용과 노동력으로 식물플랑크톤 분류군을 분석하는데 뛰어나다는 것을 확인했다. 하지만 qPCR로부터 얻어진 각 분류군의 copy number는 여전히 생물량 측정에 직접적으로 이용하기는 힘들다. 그러므로 qPCR에 의한 copy number를 미생물 또는 식물플랑크톤의 생물량으로 전환시키기 위한 추가연구가 필요하다.

INTRODUCTION

As the primary producer, phytoplankton are responsible for almost half of global primary production through photosynthesis (Chen et al. 2019). They contribute to carbon fixation by converting inorganic carbon in the atmosphere and seawater into organic carbon, playing an essential role in the global carbon cycle. For this reason, phytoplankton composition and biomass are important parameters for understanding the marine ecosystem (Fakioglu 2013, Schmidt 1999).

The cell size of phytoplankton varies in each taxon as well as its habitats or developmental stages (Sommer et al. 2017). However, the size range of each phylum is generally known; 20~140 μm in Bacillariophyta, 10~50 μm in Cryptophyta, or 3~7.5 μm in Haptophyta. The size of Chlorophyte is generally less than 6 μm , and the prokaryotic cyanobacteria were between 0.5 and 60 μm . The size structure of a phytoplankton community is an important biological factor that determines the magnitude and direction of energy flow (Bosak et al. 2012). When small phytoplankton dominates, the food web can be complicated resulting in greater energy and carbon losses, whereas the food web is relatively

simple and efficient transport of energy and carbon to higher trophic levels is performed during the large phytoplankton dominates (Pulina et al. 2018). Therefore, phytoplankton community analysis should be conducted considering the cell size for a better understanding of a marine ecosystem.

Traditional analyses of phytoplankton size structures are mainly dependent on either microscopic observation or different pigment analyses such as chlorophyll. But most of the studies were either a change in the ratio of phytoplankton (Gin et al. 2000, Park 2006) or a change in the concentration of chlorophyll (Biggs et al. 2019, Cole et al. 1986, Marañón et al. 2001) for each size according to changes in environmental factors. Recently, High-Throughput Sequencing (HTS) technique is used for community analysis of phytoplankton. For example, it has been widely used in diversity analysis, (Piredda et al. 2018, Le Bescot et al. 2016), community composition analysis (Mentes et al. 2017), red tide research (Kang et al. 2018, Grzebyk et al. 2017), correlation between phytoplankton and bacteria (Bunse et al. 2016, Marisol et al. 2018). However, a study on the size structure of phytoplankton using HTS is insufficient.

Microscopic methods and pigments analysis have been used for quantitative analysis of phytoplankton (Ediger et al. 2006, Qian et al. 2003,

Breton et al. 2000). However, the microscopic method requires considerable time for analysis by the specialists, and pigment analysis also needs a specialist with knowledge about the different pigments of the species (Schlüter et al. 2000, Irigoien et al. 2004). Quantitative PCR (qPCR) has been used to quantifying the target genes with relatively accurate way, which is mainly used for microbial studies (Kim et al. 2013). Since two primers showed extremely low cross-reactivity to other taxa and wide range of taxon coverage, we assumed that both 16S and plastid 23S universal primers can be used for the quantitative analysis of microbial and phytoplankton from the seawater samples. In fact, previous studies showed that qPCR can be used to obtain information about biomass and microbial community changes (Kim et al. 2013, Godhe et al. 2008). However, there may be an error in quantitative analysis using qPCR. When analyzing phytoplankton using 18S rRNA, there is a problem of variable copy numbers (Gong and Marchetti 2019) and cross-reactivity with eukaryotes. The problem of copy number variation still remains even when targeting the plastid 23S rRNA region with the high specificity of phytoplankton for reducing the cross-reactivity. The reason in that the copy number of plastid DNA varies depending on the developmental stage or species (Sakamoto and Takami 2018). Recently, metagenomic approach was adopted to estimate the number of phytoplankton cells (Gong and

Marchetti 2019). However, the amount of the phytoplankton genome sequence data is still limited mainly due to the high cost for genome sequencing and it is still far from its direct application for the quantitative analysis. Therefore, we here tested qPCR approach with two taxon-specific universal primers as an alternative to phytoplankton quantification.

To analyze the size-dependent phytoplankton communities, we conducted the HTS analysis and qPCR with water samples filtered by the different membrane pore sizes. MiSeq platforms was used with the library constructed by the amplicon of the plastid 23S primers for its low cross-reactivity and high taxon-specificity (Kang et al. 2018). The aim of this study is to analyze the size structure of phytoplankton using molecular biological methods and to confirm the size range of the main taxonomic group. We also compared the values between qPCR and total carbon amounts to reduce errors in measuring biomass by molecular biological methods.

MATERIALS AND METHODS

Sample collection and genomic DNA extraction

To analyze the phytoplankton community, Sampling was conducted once a month from April to June at Jaran bay and Oryukdo (Fig. 1). 3L of water samples were collected after measuring the temperature and salinity at three stations (Table 1). The collected waters were stored in the ice before they were filtered in the laboratory. In order to exclude the large zooplanktons, water samples were filtered by the sieve with 200 μm in mesh size. For the size fractionation, 500 ml of seawater was filtered (5.8 psi in pressure) through three membranes with different pore sizes (Polycarbonate Track Etch Membrane disk diatom 20 μm , Nuclepore Track Etch Membrane 2.0 μm , GN-6 Metrical® MCE Membrane Disc filter 0.45 μm). All the filtrations were triplicated. The filtered membranes were stored at -70°C until used for the DNA extraction. genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The membrane filters, Lysis buffer, and RNase A were put in a 2ml microtube and cut into the small pieces using sterilized scissors. After Homogenized by Tissue Laysers II (Qiagen,

Germany), the mixture were incubated for 2 hours at 65 °C. The extracted DNA was quantified by the ND-1000 nanodrop spectrometer (Thermo Scientific, USA) and stored at -70 °C until used for further analysis.



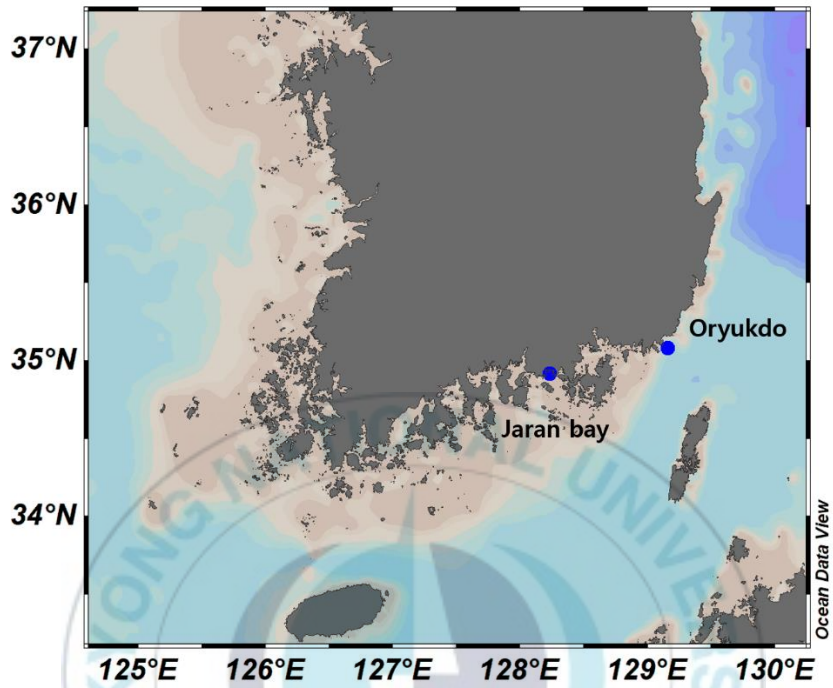


Fig. 1. Sampling sites

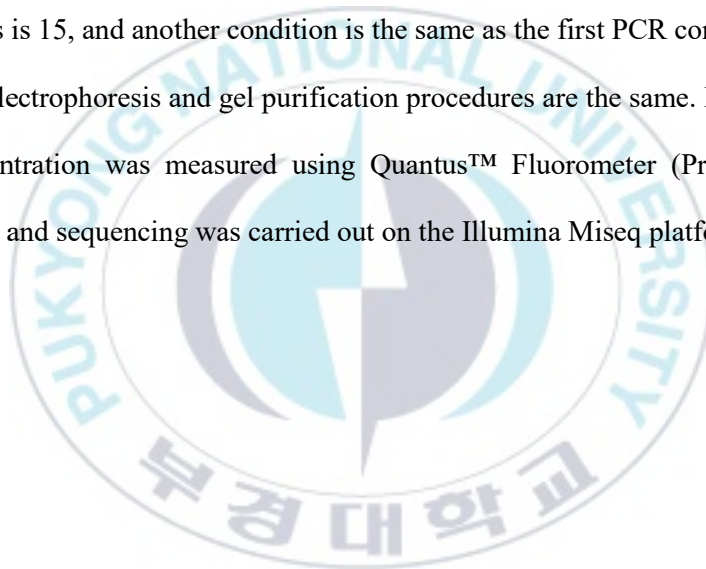
Quantitative analysis

For quantification of total microbes and phytoplankton, qPCR was performed using Mic Real-Time PCR System (Bio Molecular System, AUS). qPCR mixture (20 μ L) contained 4 μ L of the genomic DNA, 1 μ L of forward and reverse primers, 10 μ L of Luna®Universal qPCR Master Mix (NEB, USA), and 4 μ L of DNase/RNase-free water. 16S universal primers (Herlemann et al. 2011) and plastid 23S primers (Kang et al. 2018) was used for the total microorganism and phytoplankton, respectively. qPCR was performed under following conditions: initial denaturation at 94°C for 3min, followed by 35cycles at 94°C for 30s, 55°C for 30s, and 72°C for 30s, and a final extension at 72°C 3min. Serial dilution was performed using partial insulin-like growth factor 1 (Igf1) for the standard curve and the copy number was calculated by substituting Ct into the standard curve.

Library construction and sequencing

To assess the community structure of phytoplankton by size, Next Generation Sequencing (NGS) analysis was performed. Library preparation for NGS analysis was performed by attaching specialized adapters to both ends of the DNA fragments through PCR amplification. First PCR using the extracted DNA as a template was performed. PCR mixture is a total 20 μ L and consists of template, 1 μ L of 23S forward and reverse primers (10 pmol), 2 μ L of dNTP (each 2.5mM), 2 μ L of Ex Taq 10Xbuffer, 0.2 μ L of Ex Taq HS DNA polymerase (Takara, Japan), and DNase/RNase-free water. For regulation of concentration, the amount of template and the number of cycles were set differently according to the copy number of each sample, and the primers were used including the adaptor sequence. The first PCR was performed under the following condition: initial denaturation at 94°C for 3min, followed by 20-35cycles at 94°C for 30s, 55°C for 30s, and 72°C for 30s, and a final extension at 72°C 3min. After staining the first PCR product with Dyne Loading STAR (Dynebio, Korea), separated by electrophoresis on 1.5% agarose gel. The expected size of PCR products (471 - 478 bp) were cut from the gel and purified by pooling the triplet using an AccuPrep® PCR/Gel DNA

Purification Kit (Bioneer, Korea). The second PCR was performed in triplicate using purified products as a template. Second PCR mixture includes 4 μ L of template, 1 μ L of each index primer, 0.5 μ L of dNTP (each 10mM), 4 μ L of 5XPhusion HF Buffer (New England Biolabs, UK), 0.2 μ L of Phusion® High-Fidelity DNA Polymerase (New England Biolabs, UK), and 9.3 μ L of DNase/RNase-free water. The number of cycles is 15, and another condition is the same as the first PCR condition. The electrophoresis and gel purification procedures are the same. Library concentration was measured using Quantus™ Fluorometer (Promega, USA) and sequencing was carried out on the Illumina Miseq platform.



Bioinformatic analysis

Raw read sequences below QV30 and sequences shorter than 100 nucleotides were removed using CLC Genomic Workbench v.8.0 (CLC Bio, USA). Using Mothur software (v. 1.41.3), raw reads were merged, screened out those with low quality (maxal length = 440, minimal length=380, minimum overlap = 7, and mismatches = 0), and trimmed out the primer sequences (pdiffs = 0, rdiffs = 0). Sequences were clustered into operational taxonomic units (OTUs) with 98 % sequence identity using USEARCH (v. 8.1.1861) and each OTU was annotated using BLASTn. If the sequence identity is greater than 98%, we assign a species name. If the sequence identity is between 90% and 98%, top hit genus name was assigned. Finally, “Unknown” names were assigned for those with less than 90 % in sequence identity.

Statistical analysis

Statistical analyses were performed on the Statistical Package for

the Social Sciences (SPSS) version 22.0. Mann-Whitney U test was conducted to confirm that the difference of the average between samples was significant.

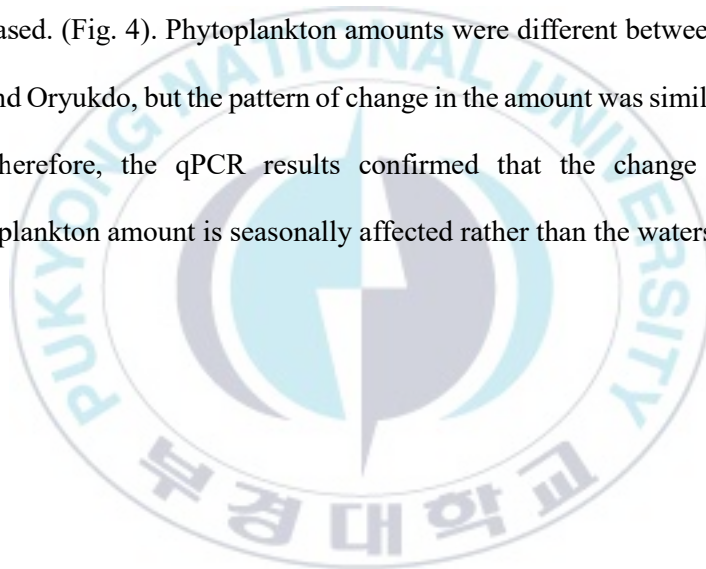


RESULTS

Quantitative analysis of microorganisms and phytoplankton

As a result of the quantitative analysis for phytoplankton and microorganisms, the average copy number of Jaran bay for total microorganisms is between 6811.82 ± 3237.54 and 38757 ± 438.71 (copy number/ 10^4). In phytoplankton, the values were between 790.33 ± 332.78 and 2922.25 ± 780.57 (copy number/ 10^4). In Oryukdo, the average copy number of microorganisms is between 9678.47 ± 2002.88 and 33946.99 ± 15427.05 (copy number/ 10^4). And, total phytoplankton copy number is between 434.05 ± 183.74 and 892.86 ± 68.83 (copy number/ 10^4). In Jaran bay, there was an average 10- fold difference between microbial and phytoplankton (Fig. 2A). On the other hand, it was more than a 30-fold difference in Oryukdo (Fig 2B). Overall, the pattern of the amount of microorganisms and phytoplankton were similar in Jaran bay and Oryukdo (Fig. 2). At size fraction in Jaran bay, there were 3, 5, and 13- fold differences between the amount of microorganisms and phytoplankton in micro, nano, pico size fraction, respectively (Fig. 3A).

In Oryukdo, the average difference of micro, nano, pico size fractions was 3, 12, 60-fold, respectively (Fig. 3B). The pattern of changes in microbial and phytoplankton amounts was similar in Jaran bay and Oryukdo (Fig. 3). When only the copy numbers of phytoplankton were analyzed, phytoplankton at nano-size fraction was higher in April, and pico size fraction increased in May. In June, the amount of nano-size fraction decreased. (Fig. 4). Phytoplankton amounts were different between Jaran bay and Oryukdo, but the pattern of change in the amount was similar (Fig. 4). Therefore, the qPCR results confirmed that the change in the phytoplankton amount is seasonally affected rather than the waters.



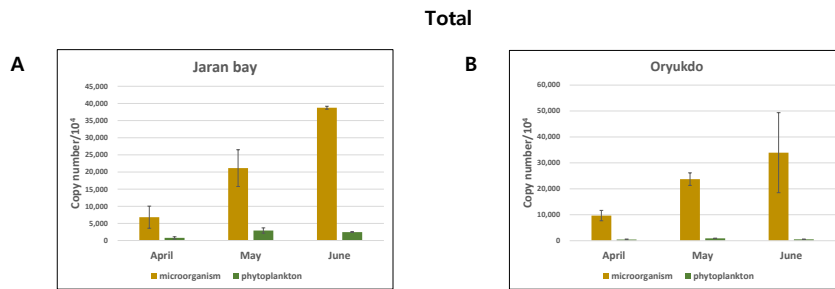


Fig. 2. Total copy number of microorganism and phytoplankton in Jaran bay and Oryukdo

(A) Total copy number of Jaran bay (B) Total copy number of Oryukdo. Each monthly, Mann-Whitney U test was conducted ($p > 0.05$).

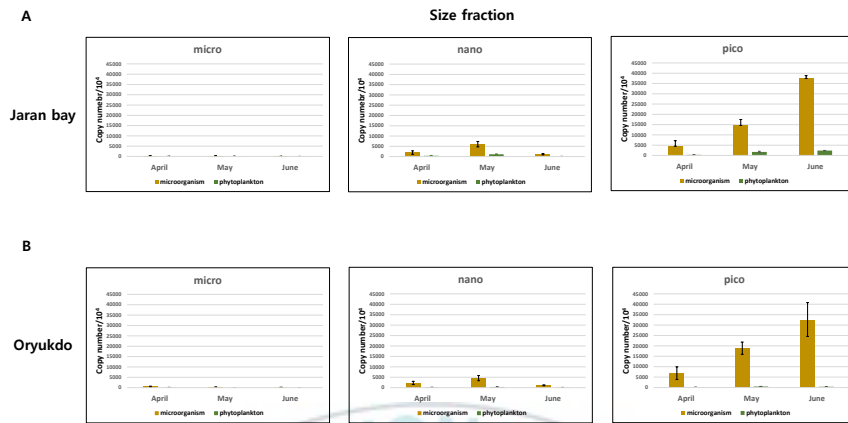


Fig. 3. Copy number by size-fractionated phytoplankton in Jaran bay and Oryukdo.

(A) Copy number of size-fractionated phytoplankton in Jaran bay (A) and in Oryukdo (B) Each monthly, Mann-Whitney U test was conducted ($p > 0.05$)

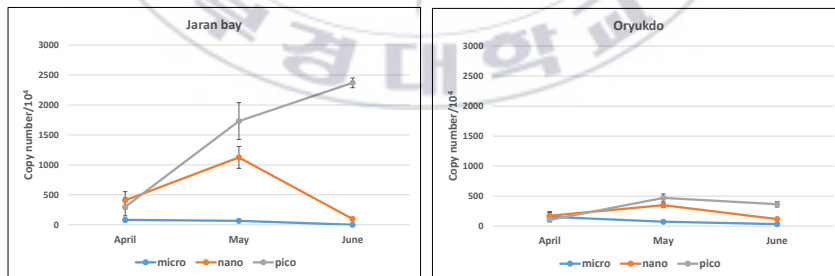


Fig. 4. Seasonal changes in phytoplankton copy number.

Phytoplankton community structure by size

We performed an NGS analysis to investigate the phytoplankton community structure of each fraction. Since seasonal differences were identified in the previous quantitative analysis, NGS was performed representatively in one station for each season. As a result of NGS, we obtained 193,327, 255,608 contigs from Jaran bay and Oryukdo, respectively. 242 OTUs in Jaran bay and 495 OTUs in Oryukdo were respectively generated at 98% as cut off sequence identity. Obtained OTUs were further classified into 20 phyla. The number of 'Unknown' OTUs with sequence identity less than 90% was 23 in Jaran bay and 58 in Oryukdo. Overall, the highest number of OTUs was identified as Bacillariophyta (198 OTUs) followed by Cyanobacteria (105 OTUs), Haptophyta (84 OTUs), Chlorophyta (75 OTUs), Ochrophyta (43 OTUs), Rhodophyta (27 OTUs), Miozoa (25 OTUs), Cryptophyta (23 OTUs). 65 OTUs (10%) were shared in all three fractions. 40 OTUs (6.2 %) were commonly identified between the micro- and nano- sized fractions, while 61 OTUs (9.4%) were between the nano- and pico-sized fractions. Finally, there were 5 OTUs (0.8%) shared between the micro- and pico-sized fractions.

The community change in the micro-sized fraction was high in Jaran bay but not in Oryukdo. In both Jaran bay and Oryukdo, the major dominant phylum in micro-sized fraction was Bacillariophyta and miozoa. In nano-sized fraction, Chlorophyta, Cryptophyta, Ochrophyta, and Haptophyta were dominated, while Chlorophyte and Cyanobacteria were abundant in the pico-sized fraction (Fig.5).

The Bacillariophyta and miozoa, which was abundant in the micro-sized fraction followed by in the nano size fraction. In Jaran bay, Bacillariophyta of micro-sized fraction was dominated by various species such as *Chaetoceros didymus*, *Lithodesmium undulatum* and *Rhizosolenia setihera*. On the other hand, in the nano size fraction, *Thalassiosira* dominated. In Miozoa, *Dinophysis acuta* predominantly in micro size and *Kryptoperidinium foliaceum* predominantly in nano size. Generally, Cryptophyta was dominated by *Teleaulax* and haptophyta by *Chrysochromulina parva* regardless of size. And Ochrophyta dominated by *Ochromonas* sp., *Mallomonas* sp., *Extocarpus* sp regardless of size. The dominant species of Chlorophyta were not different according to the size and were changing according to the season. In April and May, *Ostreococcus tauri* mainly appeared, and in June *Micromonas pusilla* appeared mainly. In June, Cyanobacteria accounted for a large proportion

of all size fractions, especially in the micro size fraction, more than Bacillariophyta or Miozoa. *Synechococcus* sp. is dominant in Cyanobacteria, and this species also accounts for a large proportion of Cyanobacteria in the micro size fraction.

In Oryukdo, the Bacillariophyta dominated by various species regardless of the season such as *Cerataulina daemon*, *Pseudo-nitzschia multiseriata*, *Actinocyclus* sp., and *Chaetoceros simplex*. And Ochrophyta dominated by *Heterosigma akashiwo* in all season. Except for bacillariophyta and Ochrophyta in Oryukdo, the dominant species in each phylum were not significantly different from Jaran Bay. However, unlike Jaran Bay, cyanobacteria did not account for a large proportion of the micro size fraction in June.

We constructed a phylogenetic tree using 234 OTUs (top 30 OTUs per sample). The highest number of OTUs was Bacillariophyta (84), followed by Haptophyta (41), Chlorophyta (31), and Cyanobacteria (26). We identified two unclassified OTUs, *Rosa* and *Broussonetia*, belonging to terrestrial plants. And Bacillariophyta OTUs were mixed with Miozoa OTUs.

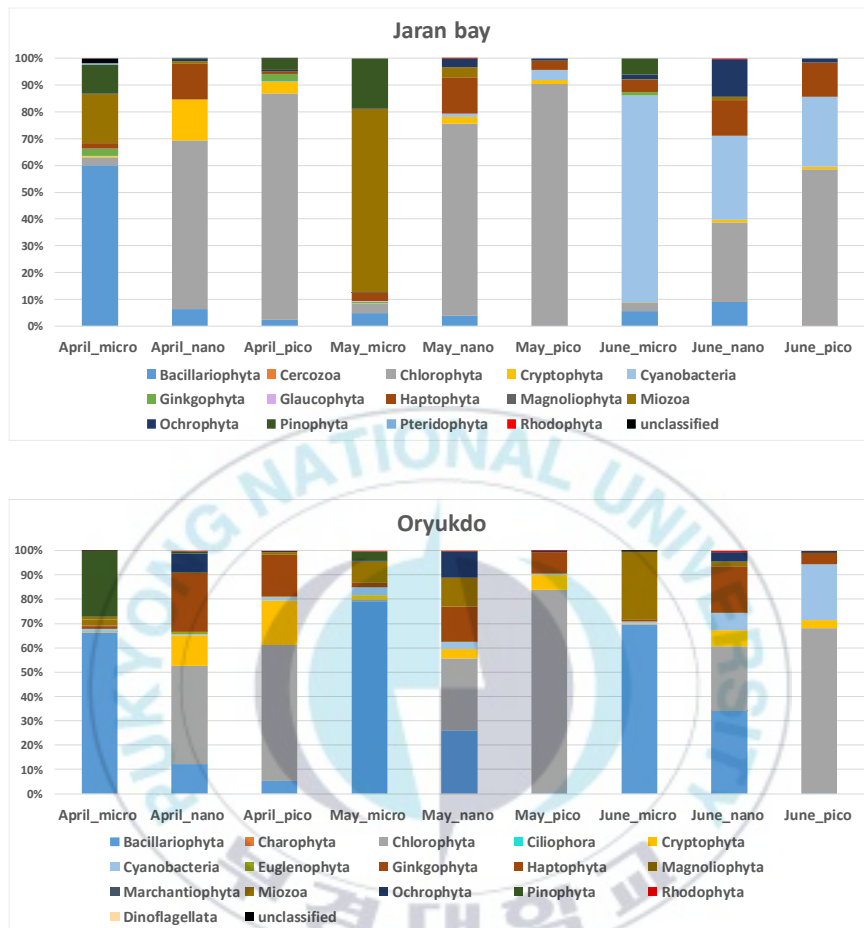


Fig. 5. Phytoplankton community structure at different month and size fraction.

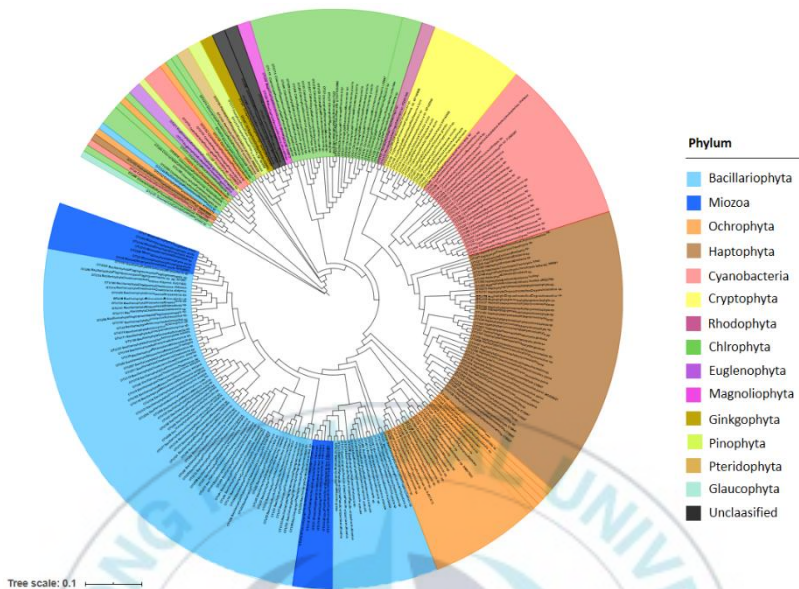


Fig. 6. Phylogenetic tree of phytoplankton OTU

The phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software (v. MEGA X) with the Minimum Evolution (ME) algorithm. Bootstrapping replications were 1,000.

Determination of phytoplankton size range

To identify the size of each phylum, we calculated the average proportion for each size. Since there was a difference in the NGS proportion and the copy number of each size, we calculated the total numbers of biomass. For example, Cyanobacteria accounted for the highest proportion of micro size fractions, but the copy number of the micro size fraction was 30 times less than the pico-sized fraction. Thus we multiplied the NGS proportion by the proportion of the copy number for each size (Table 2). Using this corrected proportion, only major taxonomic groups were analyzed (Fig. 7).

As a result, Bacillariophyta showed 9.17%, 5.22% and 0.22% of micro, nano and pico size fractions, respectively. The average proportion of Miozoa was 1.76%, 1.32% and 0.04% for micro, nano, and pico, respectively. Therefore, Bacillariophyta and Miozoa were separated into micro and nano size fractions. The average proportion of Ochrophyta was high in nano size fraction (1.93%). However, the pico size fraction also showed a higher proportion than other size fractions (0.47%). Cryptophyta (0.06%, 3.37%, 1.56% for micro, nano, pico, respectively) and Haptophyta (0.14%, 6.18%, 3.89% for micro, nano, pico, respectively)

also showed high average proportion in the nano and pico size fractions. Ochrophyta, Cryptophyta and Haptophyta separated into nano and pico size fraction. On the other hand, Chlorophyta (0.14%, 18.70% and 34.38% for micro, nano, pico, respectively) and Cyanobacteria (0.15%, 0.86%, 6.97% for micro, nano, pico, respectively) showed high average proportion in pico size fraction. Therefore, Chlorophyta and Cyanobacteria were separated in the smallest size. But compared to Cyanobacteria, Chlorophyta was also present in a significant proportion in nano size fraction. When arranged in order of size, Bacillariophyta and miozoa were the largest, followed by Ochrophyta, Cryptophyta and Haptophyta, Chlorophyta, and Cyanobacteria.

Table 1. Corrected proportion for each phylum.

	micro (200-20μm)							nano (20-2μm)							pico (2-0.45μm)						
	JR_April	JR_May	JR_June	OR_April	OR_May	OR_June	Average	JR_April	JR_May	JR_June	OR_April	OR_May	OR_June	Average	JR_April	JR_May	JR_June	OR_April	OR_May	OR_June	Average
Bacillariophyta	10.52	0.21	0.00	30.14	6.33	7.79	9.17	4.22	1.69	0.37	5.49	10.83	8.75	5.22	0.38	0.03	0.06	0.52	0.15	0.19	0.22
Cercozoa	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Charophyta	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chlorophyta	0.48	0.15	0.00	0.09	0.07	0.03	0.14	41.69	32.15	1.21	18.15	12.25	6.75	18.70	13.88	46.04	55.97	5.35	42.17	42.87	34.38
Ciliophora	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cryptophyta	0.06	0.01	0.00	0.16	0.13	0.03	0.06	10.12	1.07	0.04	5.54	1.71	1.71	3.37	0.71	0.83	0.94	1.74	3.05	2.10	1.56
Cyanobacteria	0.04	0.01	0.03	0.45	0.25	0.10	0.15	0.00	0.56	1.27	0.26	1.21	1.84	0.86	0.02	1.90	25.05	0.15	0.22	14.48	6.97
Dinoflagellata	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.01
Euglenophyta	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.45	0.01	0.04	0.08	0.00	0.00	0.00	0.02	0.00	0.00	0.00
Ginkgoophyta	0.51	0.02	0.00	0.51	0.00	0.00	0.17	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.45	0.00	0.00	0.00	0.00	0.00	0.08
Glaucophyta	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Haptophyta	0.36	0.13	0.00	0.13	0.15	0.10	0.14	8.80	6.03	0.55	10.85	5.99	4.84	6.18	0.15	1.78	12.29	1.64	4.51	3.00	3.89
Magnoliophyta	0.00	0.02	0.00	1.11	0.01	0.01	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Marchantiophyta	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Miozoa	3.20	2.97	0.00	0.60	0.68	3.08	1.76	0.62	1.67	0.05	0.06	4.99	0.52	1.32	0.04	0.03	0.00	0.11	0.03	0.02	0.04
Ochromophyta	0.02	0.01	0.00	0.00	0.02	0.02	0.01	0.64	1.44	0.57	3.50	4.41	0.99	1.93	0.05	0.37	1.56	0.06	0.29	0.47	0.47
Pinophyta	1.90	0.81	0.00	12.29	0.32	0.06	2.56	0.02	0.04	0.00	0.33	0.04	0.00	0.07	0.70	0.00	0.00	0.00	0.01	0.00	0.12
Pteridophyta	0.08	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rhodophyta	0.00	0.00	0.00	0.05	0.03	0.01	0.01	0.00	0.02	0.02	0.21	0.15	0.16	0.09	0.00	0.00	0.00	0.01	0.03	0.00	0.01
unclassified	0.32	0.00	0.00	0.01	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00

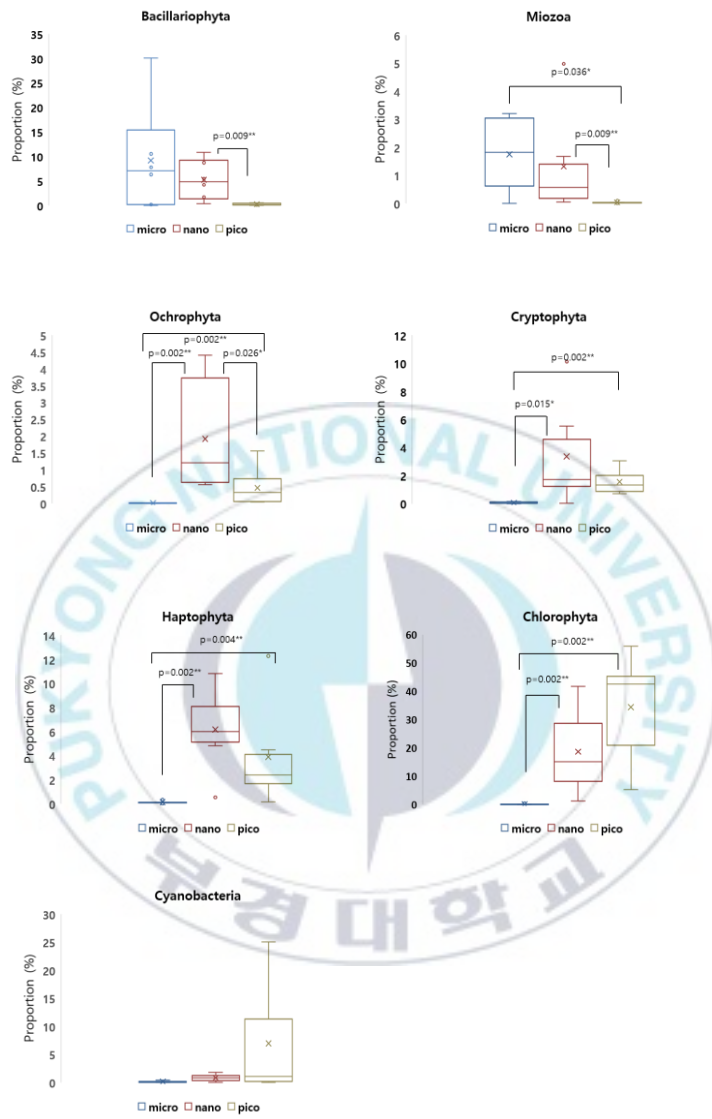


Fig. 7. The size range of the main taxonomic group.

Mann-Whitney U test was conducted (* : $p < 0.05$, ** : $p < 0.01$).

Heat map analysis

To confirm whether the community of each size is clearly divided, heat map analysis was conducted using OTUs obtained from the NGS results (Fig. 8). The size was classified into two clades and nine of the represented 18 OTUs were classified as micro size. The remaining nine OTUs were classified into nano- and pico-sized, respectively. In general, the micro size fraction was well distinguished from the other size fraction, and the species that could distinguish it were mainly *Ginkgo biloba* and *Pinus crassicornis* in April and May. In May and June, *Chaetoceros simplex* was the species that distinguished the micro size fraction from the other size fraction. The nano and pico-sized fraction were not clearly distinguished. The main reason for the not distinguished between the nano and pico-sized fraction was *Ostreococcus*, *Micromonas*, *Bathycoccus*, and *Chrysochromulina*.

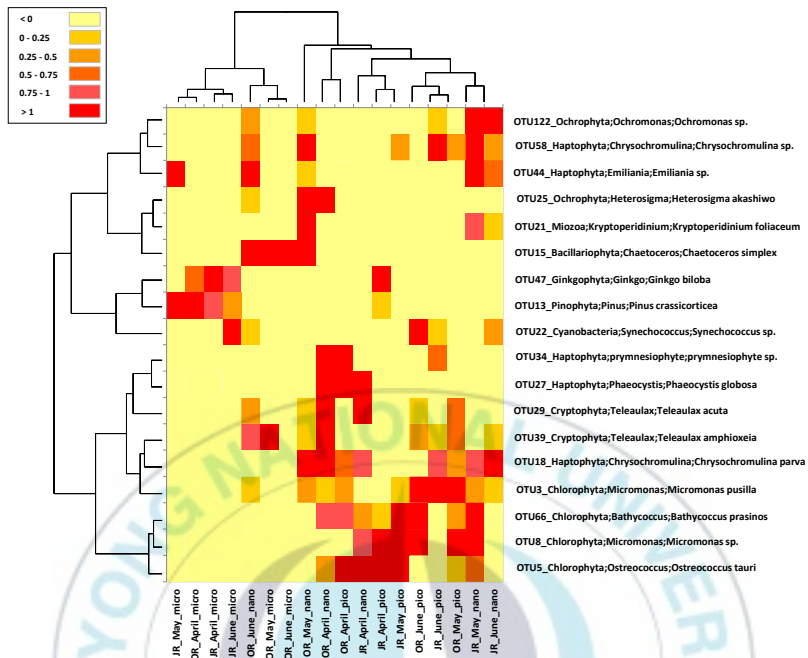


Fig. 8. Heat map of phytoplankton OTU at different month and size fraction.

JR : Jaran bay, OR : Oryukdo

DISCUSSION

In qPCR results, the difference in the amount between total microorganisms and total phytoplankton was lower in Jaran bay than in Oryukdo. This may be due to the growth of cyanobacteria in Jaran bay, based on NGS results. The amount of total microorganisms increased in Jaran bay and Oryukdo until June. According to a study conducted on Jaran bay in 2017, the growth of phytoplankton occurred after the growth of microorganisms (Yoon, 2018). And in the study of Yoon (2018), the amount of microorganisms increased until March. But in this study, the amount of microorganisms increased until June. So the pattern until March of the previous study coincided with the pattern of this study. Thus, phytoplankton bloom was delayed compared to 2017. In addition, in the results of the study conducted in the East Sea in 2017, the amount of microorganisms increased until April (Lee 2019). Thus, the phytoplankton bloom was delayed even in Oryukdo. This difference may be due to difference in water temperature because increase in water temperature is the main cause of phytoplankton bloom (Trombetta et al. 2019). In fact, water temperature in Jaran bay was on average 3°C lower than in 2017. Trombetta et al. (2019) said that although other factors (e.g. wind, salinity)

also affect phytoplankton blooms, water temperature has a significant effect on phytoplankton blooms in coastal waters. On the other hand, in Oryukdo, the water temperature was not much different from that of 2017 and the salinity was relatively low. Since there is a positive correlation between salinity and Chl a fluorescence, salinity may had an indirect effect on phytoplankton bloom, although not a direct effect (Trombetta et al. 2019).

According to the NGS results in this study, Bacillariophyta and Miozoa accounted for a large proportion of the micro and nano-sized fractions. These results were similar to previous studies that analyzed 28S rDNA (Elferink et al. 2017). Also similar to this study, There are studies that Cryptophyta and Haprophta belong to nano and pico size fraction (Lecointre and Le Guyader 2006, Mucko et al. 2018, Gran-Stadniczeňko et al. 2017). Previous studies showed that Chlorophyta and Cyanobacteria belong to the nano and pico-sized fractions (Pulina et al. 2018, Elferink et al. 2017). However, in this study, the proportion of cyanobacteria in the micro size fraction of Jaran bay was over 70%. Generally, the size of the Cyanobacteria cell is from 0.5 μm to 40 μm . Even the Cyanobacteria belonging to the micro size fraction were *Synechococcus* sp. with sizes from 0.8 μm to 1.5 μm . It can be assumed that the result of symbiosis.

Cocoid cyanobacteria in known to intracellular symbiosis in the diatom, and this Cyanobacteria symbiotic in the cytoplasm of the diatom (Janson 2002). Because we analyzed the chloroplast present in the cytoplasm, our result may include Cyanobacteria symbiotic to Bacillariophyta of micro sized-fraction.

Dominant taxon in all phylum did not differ by size. The general size ranges of *Rhizosolenia* (2.5-170 μm), *Chaetoceros* (10-50 μm) and *Thalassiosira* (4-32 μm), the representative dominant genus of Bacillariophyta, were in agreement with the results of this study. *Dinophysis acuta* (54-94 μm), the dominant genus of Miozoa, generally belongs to micro-sized fraction. But in this study, it also present nano-sized fraction. Dominant genus of Ochrophyta, Cryptophyta, and Haptophyta was *Ochromonas* (4-6,5 μm), *Teleaulax* (5-6.8 μm), and *Chrysochromulina* (3-13 μm), respectively. All of these genera are commonly known as nano-sized, but we also identified in the pico-sized fraction. In particular, in the case of haptophyte, the average proportion of nano and pico size fractions differs only 1.5 times. This indicating that there is a significant amount in the pico-sized fraction. *Ostreococcus* (0.8 μm) dominated in Chlorophyta and *Synechococcus* (0.8-1.5 μm) dominated in Cyanobacteria generally belonged to pico-sized fraction.

Cyanobacteria were identified in the pico-sized fraction in this study. On the other hand, Chlorophyta was more present in the nano-sized fraction than Cyanobacteria.

Ostreococcus, Micromonas, Bathycoccus, and Chrysochromulina, which caused the nano and pico-sized fractions to not be clearly divided, have generally sizes of $< 1 \mu\text{m}$, $< 2 \mu\text{m}$, $1-2 \mu\text{m}$ (pico-sized), and $3-13 \mu\text{m}$ (nano-sized), respectively. However, Ostreococcus, Micromonas, and Bathycoccus are also identified on the nano-sized fraction. And, Chrysochromulina is also identified in the pico-sized fraction. In addition, Ostreococcus can not be identified in June. Therefore, it is shown that the analysis should be done carefully according to the season and the development of phytoplankton.

Many OTUs belonged to more than one size fraction, and all phylum not show different species by size. Also, we found that some phylum distinguishes between size fractions well (Ochrophyta, Cryptophyta, Haptophyta), but some phylum overlap between size fractions (Bacillariophyta, Miozoa). This means that the size fractionation is not clear. This may be due to differences in life-history stages and the formation of chains or colonies (Elferink et al. 2017). The phytoplankton colonies can be up to several centimeters in size (Sommer et al. 2017). For

example, almost 20 species of Bacillariophyta have a size of less than 3 μm . However, since they generally form chains, they are likely to be found in filters with larger pore sizes (Vaulot et al. 2008). In particular, diatoms secrete large amounts of mucus and aggregate with each other (Kiørboe and Hansen 1993). Another reason is the vacuum pressure. Vacuum pressure should be kept below 10 psi to prevent the breakage of cells or pull cells through the filter (Baker 2000). Most previous studies that performed size fractionation of phytoplankton maintained vacuum pressures from 1.9 psi to 4.8 psi (Gin et al. 2000, Marañón et al. 2001, Varela et al. 2002, Ehnert and McRoy 2007, Moreno-Ostos et al. 2011). In this study, the vacuum pressure was maintained at 5.8 psi, which is higher than in previous studies. Thus, it may influence cell breakage or disruption of the chain. However, as *Ostreococcus* (0.8 μm) was found in a large amount in the nano-sized fraction, the vacuum pressure would not have much influence.

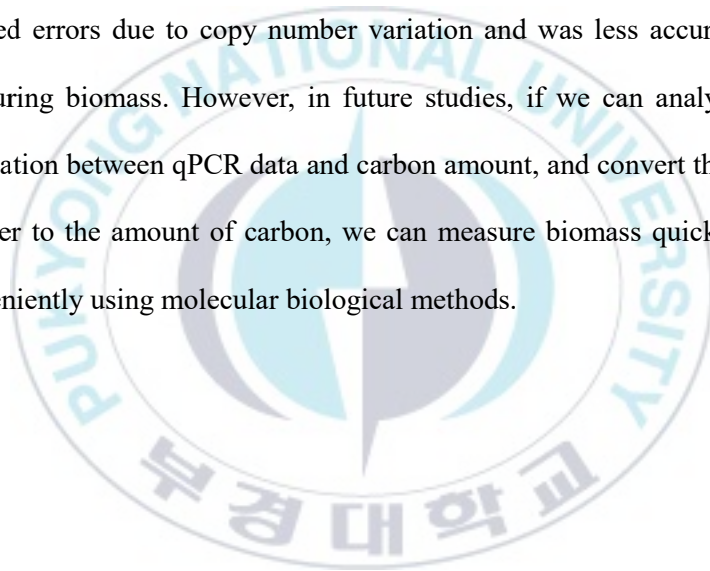
In this study, qPCR was used for the quantitative analysis of phytoplankton, but qPCR has errors because the copy number of plastid DNA varies by developmental stage and species (Sakamoto and Takami 2018). A recent study tried to measure the cell numbers with a high degree of accuracy based on the different copy numbers 18S V4-region gene from

the seven phytoplankton genomes (Gong and Marchetti 2019). For example, *Ostreococcus tauri* was estimated to have, on average, 3.4 copies of the 18S gene across 13 strains, which was similar to the previous study (Blanc-Mathieu et al. 2014). By contrast, they also found that an average of 160 18S genes were identified in the dinoflagellate, *Symbiodinium kawagutii*, which is typical character of dinoflagellates harboring large and repetitive genomes (Lin 2011, Wisecaver and Hackett 2011, Shoguchi et al. 2013, Lin et al. 2015). This result shows us that the phytoplankton cell numbers can be measured by molecular technique with high accuracy. However, it would take time for the practical application considering the high cost and time to obtain the full genome data of a variety of phytoplankton taxa

Carbon accounts for about 50% of the weight of organic matter in living organisms (Curl 1962), Carbon measurement is a relatively accurate method for measuring biomass. However, this carbon contains zooplankton and bacterial carbon, which makes it difficult to separate from phytoplankton carbon (Graff et al. 2012). Although there are some studies only measure the carbon of autotrophic organisms using a microscope and flow cytometry (Bosak et al. 2012, Taylor et al. 2015, Aytan et al. 2018), it is time-consuming. In contrast, qPCR is faster than

measuring carbon despite errors. Therefore, if we analyze the correlation between qPCR data and the amount of carbon and the data accumulate, we can measure biomass in a more convenient and accurate method. There was a difference between the qPCR results of this study and the carbon measurement results of previous studies. The copy number of pico-sized fraction was the highest proportion in this study, but the amount of carbon accounts for a high proportion of the micro size fraction (Bosak et al. 2012, Taylor et al. 2015, Aytan et al. 2018). Therefore, we simply estimate the conversion from copy number to the amount of carbon using reference. We used the data from study conducted in the coast of California (Taylor et al. 2015) and data from study conducted on the Black Sea in spring (Aytan et al. 2018). The average copy number of this study was 5.14%, 28.30% and 66.56% for micro, nano and pico-sized fraction, respectively. And, the average carbon amount was 51.94%, 40.48% and 7.58% for micro, nano and pico-sized fractions, respectively. As a result of the correction, the amount of the micro-sized fraction increased about 10 times and the amount of pico-sized fraction decreased about 10 times. This means that the amount of pico-sized fraction is overestimated in the qPCR result. But this value has a very large deviation, more research should be conducted by season, sea area and taxon.

In conclusion, we analyze the size structure of phytoplankton using NGS and qPCR. As a result, the size fractionation was not clear. Therefore, in order to analyze the size structure of phytoplankton, it is necessary to consider seasonal factors and the development of phytoplankton, and in particular, studies on *Ostreococcus*, *Micromonas*, *Bathycoccus* and *Chrysochromulina* are necessary. Quantitative analysis using qPCR showed errors due to copy number variation and was less accurate for measuring biomass. However, in future studies, if we can analyze the correlation between qPCR data and carbon amount, and convert the copy number to the amount of carbon, we can measure biomass quickly and conveniently using molecular biological methods.



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