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**Seasonal changes in the communities of photosynthetic picoeukaryotes in Ofunato Bay
as revealed by shotgun metagenomic sequencing**

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Running title: Picoeukaryotic communities in Ofunato Bay

ABSTRACT

Small photosynthetic eukaryotes play important roles in oceanic food webs in coastal regions. We investigated seasonal changes in the communities of photosynthetic picoeukaryotes (PPEs) of the class Mamiellophyceae, including the genera *Bathycoccus*, *Micromonas* and *Ostreococcus*, in Ofunato Bay, which is located in northeastern Japan and faces the Pacific Ocean. The abundances of PPEs were assessed over a period of one year in 2015 at three sampling stations, KSt. 1 (innermost bay area), KSt. 2 (middle bay area) and KSt. 3 (bay entrance area) at depths of 1 m (KSt. 1, KSt. 2 and KSt. 3), 8 m (KSt. 1) or 10 m (KSt. 2 and KSt. 3) by employing MiSeq shotgun metagenomic sequencing. The total abundances of *Bathycoccus*, *Ostreococcus* and *Micromonas* were in the ranges of 42-49%, 35-49% and 13-17%, respectively. Considering all assayed sampling stations and depths, seasonal changes revealed high abundances of PPEs during the winter and summer and low abundances during late winter to early spring and late summer to early autumn. *Bathycoccus* was most abundant in the winter, and *Ostreococcus* showed a high abundance during the summer. Another genus, *Micromonas*, was relatively low in abundance throughout the study period. Taken together with previously suggested blooming periods of phytoplankton, as revealed by chlorophyll *a* concentrations in Ofunato Bay during spring and late autumn, these results for PPEs suggest that greater phytoplankton blooming has a negative influence on the seasonal occurrences of PPEs in the bay.

Keywords

Photosynthetic picoeukaryotes

MiSeq

Ofunato Bay

Spring bloom

1. Introduction

Small photosynthetic eukaryotes that are $<3\ \mu\text{m}$ in size are important contributors to the global primary production for carbon and mineral cycles (Li, 1994; Vaulot et al., 2008). Among them, photosynthetic picoeukaryotes (PPEs) are the major oceanic primary producers (Dong et al. 2016). PPEs may contribute significantly to the carbon flux through the microbial web in coastal systems (Worden et al., 2004; Bec et al., 2005), especially when larger phytoplankton (nanoplankton and microplankton) are not blooming (Vargas et al., 2007). The three genera of PPEs, *Bathycoccus*, *Micromonas*, and *Ostreococcus*, all belong to the order Mamiellales in the class Mamiellophyceae under the phylum Chlorophyta (Not et al., 2004; Zhu et al., 2005; Worden, 2006). Traditionally, morphological features have been employed for the identification and classification of picoeukaryotes (Jing et al., 2010). However, these features alone are not sufficient to clearly distinguish species or groups of picoeukaryotes due to their tiny sizes and cryptic morphological characteristics (Sims et al., 2002). Metagenomic approaches based on shotgun sequencing have provided new insights into the structure of the picoeukaryote communities in various ecosystems. However, until recently, metagenomics have not been applied to eukaryotic populations. The first reason is that filtered samples that are used in general for metagenomic studies are dominated by prokaryotic species (Piganeau et al., 2008). However, recent works have demonstrated that this strategy is applicable to small eukaryotes, including both autotrophic (Cuvelier et al., 2010; Lepere et al., 2011; Monier et al., 2012) and heterotrophic organisms (Heywood et al., 2011).

Ofunato Bay, which faces the Pacific Ocean and is located in the northeastern Iwate Prefecture of Japan, is famous for having one of the highest biodiversity of marine organisms in the world. It is a deep coastal bay located at the center of the Sanriku *Rias Coast* and is considered to be an economically and environmentally important asset. In fact, the bay has

Buoy-and-Rope type culture facilities for scallops and oysters. Because of the importance of the bay, we have investigated basin-scale seasonal changes in the community of marine free-living bacterioplankton in Ofunato Bay (Reza et al., 2018a, b). However, marine ecosystem consists of various organisms that include not only bacterioplankton but also phytoplankton, such as picoplankton, nanoplankton and microplankton, which are considered to play important roles in the primary productivity.

The objective of this study was to investigate the seasonal changes in the community of picoeukaryotes in Ofunato Bay by employing metagenomic sequencing. This investigation contributes to a better understanding of seasonal changes in the presence of PPEs in the bay and thus provides a baseline for a comprehensive understanding of the microbial communities in the bay and its primary productivities.

2. Materials and methods

2.1. Sample collection

Sample collection was performed according to Reza et al. (2018a). Briefly, seawater samples were collected monthly from three stations (KSt. 1, KSt. 2 and KSt. 3) in Ofunato Bay over a period of one year in 2015. KSt. 1 is located near the Sakari River (the most inner area of the bay), KSt. 2 is located near an oyster aquaculture area (the middle area of the bay), and KSt. 3 is located at the mouth of the bay, adjacent to the Pacific Ocean. All three sampling stations comprehensively cover the total area of Ofunato Bay. For the metagenomic study, approximately 8 L of seawater was taken from each sampling station at 1 (KSt. 1, KSt. 2 and KSt. 3), 8 (KSt. 1) or 10 m (KSt. 2 and KSt. 3) depths. Basic water quality parameters were measured by a water quality profiler (RINKO AAQ176, JFE Advanced Co. Ltd., Nishinomiya, Japan).

2.2. Isolation of DNA

The seawater samples were serially passed through 20-, 5-, 0.8- and 0.22- μ m filters using a peristaltic pump. The fraction trapped at 0.22- μ m filters, including small-sized microbes, was used for DNA extraction. Total DNA was extracted using the PowerWater Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA), according to the manufacturer's instructions. The DNA concentrations were quantified with a Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, California, USA) and read with a Qubit Fluorometer (Invitrogen). The DNA libraries for shotgun sequencing were prepared using the Nextera XT DNA sample preparation Kit and MiSeq Reagent Kit V3 (Illumina, Inc., San Diego, CA). The quality and sizes of the products were assessed with a Bioanalyzer 2100 using a DNA 7500 chip. Finally, sequencing was carried out with an Illumina MiSeq sequencing system. The whole genome shotgun raw reads have been deposited into the DDBJ Sequence Read Archive under the accession number DRA005744.

2.3. Sequencing analysis

For the shotgun sequencing analysis, the obtained raw reads were processed using CLC Genomic Workbench (version 8) for the trimming of low-quality scores with <50 bp. After the quality-control pass, the entire datasets were compared to the NCBI NT database using BLASTn and were analyzed using the MEGAN software (Version 5.10.3) with a maximum expected value of $1e-10$. The abundances of PPEs were assessed by counting the number of reads from the MEGAN output.

2.4. Statistical analysis

Correlation analyses were carried out using the Pearson's product-moment correlation coefficient test to determine the relationship between the abundances of PPEs and chlorophyll

a concentrations on the basis of monthly datasets for samples from the three sampling stations at two depths.

3. Results

3.1. Hydrographic conditions

During the study period, the water temperature at a depth of 1 m ranged from 5.54°C (March at KSt. 3) to 25.31°C (August at KSt. 2), and at the depths of 8 and 10 m, the water temperature ranged from 4.66°C (March at 10 m on KSt. 3) to 20.11°C (September at 8 m on KSt. 1). The water temperature at KSt. 3 (bay entrance) was lower than those at KSt. 2 (middle bay area) and KSt. 1 (innermost bay area) irrespective of the depth. Thus, it seems that the temperature of the seawater in the bay was influenced in the winter (from January to April) by the cold *Oyashio Current*, whereas from May to November, it was influenced by the warm *Kuroshio Current*. Similar changes in seawater temperature have been previously reported by Ogata et al. (1982) and Yamada et al. (2017). It is notable that the warm *Tsugaru Current* coexists with the *Oyashio* and *Kuroshio Currents* in this area (Hanawa and Mitsudera, 1987).

Salinity showed little seasonal change and ranged from 20.76 (September at 1 m on KSt. 2) to 33.88 (February at 1 m on KSt. 2) (Fig. 1). The lower salinity values were attributable to heavy rainfalls that occurred during this period according to the local atmospheric data from Ofunato City (data not shown). Salinity was more constant at KSt. 3 than at KSt. 1 and KSt. 2 and was little affected by the heavy rainfalls.

3.2. Chlorophyll *a* concentrations

Chlorophyll *a* (chl-*a*) concentrations also showed seasonal changes for all sampling stations and depths (Fig. 2). The mean concentrations of total chl-*a* ranged from 0.37 to 20.32 µg/L,

with the highest concentrations in March at 8 m depth on KSt. 1 and the lowest in February at 1m depth on KSt. 2. Generally the concentrations of chl-*a* remained low from late autumn (December) to early winter (January and February), increased in early spring (March to May) but suddenly decreased in June irrespective of the sampling stations and depths. These results are consistent with the observation by Yamada et al. (2017) in the Ofunato Bay. Furthermore, high concentrations of chl-*a* were also detected during early autumn (October and November), especially at 1m depth for the three sampling stations.

3.3. Abundances of PPEs

Considering all the sampling stations and depths, the highest number of the total reads for PPEs were detected from KSt. 2 at a depth of 10 m (n=97971); the lowest number was from KSt. 1 at a depth of 1 m (n=42080) (Fig. 3). The total read number from KSt. 3 at a depth of 1 m was almost double that from KSt. 1 and KSt. 2 at the same depth.

The relative abundances of the three genera of PPEs, *Bathycoccus*, *Ostreococcus* and *Micromonas*, all of which belong to the order Mamiellales in the class Mamiellophyceae under the phylum of Chlorophyta, had ranges of 42-49%, 35-49% and 13-17%, respectively, irrespective of the three sampling stations or depth (Table 1). Thus, the relative abundances of *Bathycoccus* and *Ostreococcus* were comparable and 2- to 3-fold higher than that of *Micromonas*. The highest abundance was recorded for *Bathycoccus* from KSt. 1 at a depth of 1 m and for *Ostreococcus* from KSt. 1 at a depth of 8 m, whereas the lowest abundance with *Micromonas* was recorded for KSt. 1 at a depth of 8 m.

3.3. Seasonal changes in the communities of picoeukaryotes

The investigation for seasonal changes determined in this study revealed high abundances of PPEs in winter from December to February and in early summer from June to July, while low

abundances were observed in late spring from March to May and in late summer from September to November, irrespective of the three sampling stations and water depths tested (Fig. 4). The abundances of PPEs were remarkably low in late summer. When the three genera were compared, *Bathycoccus* was the most abundant in winter, especially in February, with 5,000-8,000 reads, which was assayed at a depth of 1 m at the three sampling stations and at a depth of 8 m at KSt. 1. *Bathycoccus* dominated the PPE community during February, followed by *Ostreococcus* and *Micromonas*. In comparison, *Ostreococcus* was found to be most abundant from June to July, irrespective of the three sampling stations or water depth. The abundance of *Ostreococcus* tended to shift upward in June at the depth of 1 m, while it peaked in July at the depths of 8 and 10 m. Namely, a noticeable rise in the abundance of *Ostreococcus* was observed in July for all the stations, especially at depths of 8 or 10 m, exhibiting abundances from 8,000 to 20,000 reads irrespective of the three sampling stations. Another genus, *Micromonas*, showed relatively low abundances throughout the study period, showing variable peaks in the abundance among all stations and depths. Thus, blooms in the PPE communities were found in two seasons over the course of the year. These seasonal changes were attributable almost exclusively to the abundance of *Bathycoccus* in the winter, irrespective of the three sampling stations, and partly in summer especially at a depth of 1 m for the three stations. In contrast, the bloom of PPEs in the summer was attributable to the increased abundance of *Ostreococcus*, especially at the depths of 8 and 10 m for all the three stations.

4. Discussion

According to the collected temperature data (Fig. 1), cold *Oyashio Current* waters likely entered the bay from January to April and were influenced by the warm *Kuroshio Current* waters from May to November, although the effect of the warm *Tsugaru Current* likely needs

to be considered (Hanawa and Mitsudera, 1987). The seasonal changes in the observed abundances of the three genera of PPEs, *Bathycoccus*, *Ostreococcus* and *Micromonas*, were possibly associated with changes in seawater temperature but apparently not with those in salinity.

During the early spring, very low abundances of PPEs were observed in Ofunato Bay (Fig. 4). Collado-Fabbri et al. (2011) reported that the abundances of PPEs were low during spring for the Chile upwelling ecosystem. Gonzalez et al. (2007) reported that large chain-forming diatoms are very productive in spring near this area. One possibility for this phenomenon is that picophytoplankton are weak competitors compared with microphytoplankton, such as diatoms (Litchman et al., 2007). Countway and Caron (2006) found that the peak in the abundance of diatoms in April and that of *Ostreococcus* were among the lowest detected during the peak of the diatom bloom. Diatoms tend to have significantly higher maximum nutrient uptake rates compared to other groups (Litchman et al., 2006). Thus, diatoms are good nutrient competitors. It has also been reported by Larsen et al. (2004) and Sherr et al. (2005) that when larger phytoplankton dominates an area, picophytoplankton abundances are low. In our study, the trend for seasonal changes in the abundances of *Ostreococcus* was upward from June to July at depths of 8 and 10 m compared with those at a depth of 1 m. Countway and Caron (2006) observed that the abundances of *Ostreococcus* were higher at a depth of 30 m than those at a depth of 5 m. In our study, *Micromonas* did not dominate the PPE communities in Ofunato Bay, which contrasts with what has previously been reported for the coastal waters of the Northern Hemisphere (Not et al., 2005, 2008).

During the late summer to autumn, we observed low abundances of PPEs in Ofunato Bay. On the other hand, high concentrations of chl-*a* were observed during these periods, especially at 1 m depth of the three sampling stations (Supplementary Fig. S1). There was a significant negative correlation existed between the abundances of picoeukaryotes and chl-*a*

concentrations on 1m depth at KSt. 1 ($r = -0.66$, $P < 0.05$) and KSt. 3 ($r = -0.58$, $P < 0.05$), (Supplementary Table S1). This relationship was particularly true for *Bathycoccus* which showed a significant negative relationship with chl-*a* concentrations on 1m depth at KSt. 1 ($r = -0.74$, $P < 0.01$) and KSt. 3 ($r = -0.64$, $P < 0.05$) (Supplementary Table S2). Although no data are available on the autumn bloom for other microbes in Ofunato Bay, high concentrations of chlorophyll *a* (chl-*a*) have been reported in May and April, suggesting the high abundances of microplankton and nanoplankton in the bay (Yamada et al., 2017). Yamada et al. (2017) also observed that chl-*a* concentrations were also high in July and August in Ofunato Bay, which again indicates a high abundance of nanoplankton and microplankton. Autumn blooms are less well understood, although their occurrence has been widely documented (Raymont, 1963; Hardy, 1970; Ross, 1977; Steele and Henderson, 1978; Lalli and Parsons, 1997; Herring, 2002). The increased vertical mixing and the breakdown of stratification in autumn associated with other environmental factors may have an influence on the abundances of eukaryotic picoplankton in Ofunato Bay.

According to Itoh et al. (2006) and Okumura et al. (2015), the spring blooms were attributable to the increased abundances of diatoms, and the autumn bloom resulted from an increased abundance of dinoflagellates in Kesenuma Bay, Miyagi Prefecture, Japan, which is located south of Ofunato Bay. Kamiyama et al. (2009) reported that the oyster spawning season in Ishinomaki Bay, another bay in the Miyagi Prefecture, is concentrated in mid-summer, producing many planktonic oyster larvae in late July through August. Picoplankton-sized particles (0.5 to 3 μm) constitute a large percentage of the food ingested by small larvae of the Eastern oyster *Crassostrea virginica* (Baldwin and Newell, 1995). Ofunato Bay also provides aquaculture grounds for the giant Pacific oyster *Crassostrea gigas* and the Yezo giant scallop *Patinopecten yessoensis*. These planktonic larvae may ingest a large proportion of picoeukaryotes in Ofunato Bay, which not yet been investigated.

In conclusion, the abundances of the three assayed genera of PPEs, *Bathycoccus*, *Ostreococcus* and *Micromonas*, all of which belong to the order Mamiellales in the class Mamiellophyceae under the phylum of Chlorophyta, showed seasonal changes in Ofunato Bay, with higher abundances during both the winter and summer seasons, irrespective of the three sampling stations and sampling depth at 1, 8 or 10 m. However, the reason(s) for these seasonal changes are not yet fully understood.

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Figure Legends

Fig. 1. Seasonal variations of temperature and salinity in 2015 at depths of 1 (KSt. 1, KSt. 2 and KSt. 3), 8 (KSt. 1) or 10 m (KSt. 2 and KSt. 3) at the three sampling stations, KSt. 1 (innermost area), KSt. 2 (middle area) and KSt. 3 (bay entrance area) in Ofunato Bay.

Fig. 2. Seasonal variations of chlorophyll *a* (chl-*a*) concentration in 2015 at depths of 1 (KSt. 1, KSt. 2 and KSt. 3), 8 (KSt. 1) or 10 m (KSt. 2 and KSt. 3) at the three sampling stations, KSt. 1 (innermost area), KSt. 2 (middle area) and KSt. 3 (bay entrance area) in Ofunato Bay.

Fig. 3. Total read numbers of PPEs at depths of 1 (KSt. 1, KSt. 2 and KSt. 3), 8 (KSt. 1) or 10 m (KSt. 2 and KSt. 3) at the three sampling stations, KSt. 1 (innermost area), KSt. 2 (middle area) and KSt. 3 (bay entrance area) in Ofunato Bay in 2015.

Fig. 4. Seasonal changes in the read numbers for the three PPEs at depths of 1 (KSt. 1, KSt. 2 and KSt. 3), 8 (KSt. 1) or 10 m (KSt. 2 and KSt. 3) at the three sampling stations, KSt. 1 (innermost area), KSt. 2 (middle area) and KSt. 3 (bay entrance area) in Ofunato Bay in 2015.

Figure 1

Figure 2

Figure 3

KSt. 1-1 m

KSt. 1-8 m

KSt. 2-1 m

KSt. 2-10 m

KSt. 3-1 m

KSt. 3-10 m

— Bathycoccus — Ostreococcus — Micromonas

— Bathycoccus — Ostreococcus — Micromonas

Figure 4