

論文内容要旨

論文題目

Metagenomics-based studies on seasonal variations of microbial flora in the Ofunato Bay associated with environmental parameters

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要 旨

Marine microbes such as prokaryotes and small eukaryotes perform a number of essential roles in the environment, including nutrient recycling and energy production. Bacteria especially play important roles in recycling of organic matters and the transfer of nutrients and energy in the marine food web. So far, various findings have been reported about functions of bacteria in the above-mentioned material cycling in seawaters. However, traditional microbiological researches have been dependent on culture-based techniques for identification of microbes in environmental samples. It has been reported that only 0.1 – 1% of microbes in the environment are able to be cultured. Metagenomics is a genomic DNA-level characterization technique and this technique is now applicable for investigating the whole microbial communities, including unculturable microbes. Recent works have demonstrated that such metagenomic strategy is also applicable to small eukaryotes, including both autotrophic and heterotrophic organisms.

The Ofunato Bay is a semi-enclosed bay located at the center of the *Sanriku Rias* Coast facing on northeastern Pacific around Japan and well known for aquaculture production. The bay is receiving seasonal water currents from the Pacific Ocean such as cold Oyashio Current and Tsugaru Warm Current, and also freshwater inflow from the Sakari River. Such circumstances allow exchanges of water masses and discharge of nutrients which have possibly influences on microbial communities in the bay.

The objective of this study was to analyze both bacterial and eukaryotic picoplankton communities in the Ofunato Bay by using metagenomic approaches and clarify their relation with environmental parameters such as temperature and concentrations of nutrients. The results obtained indicated that the communities of bacteria and photosynthetic picoeukaryotes (PPEs) with $<3\ \mu\text{m}$ size in the Ofunato Bay changed seasonally along with the changes in chlorophyll *a* (chl-*a*) concentrations, which possibly reflect the abundances of large plankton. The details of the present

study including the above-mentioned results are shown below.

Seawater sampling locations and analytical procedures

About 8 L of seawaters in the Ofunato Bay were collected monthly in 2015 – 2017 with a Van Don water sampler from three sampling locations including KSt. 1 (the most inner area), KSt. 2 (the middle area) and KSt. 3 (the mouth of the bay), considering two sampling depths, 1 m (KSt. 1, KSt. 2 and KSt. 3) and 8 (KSt. 1) or 10 m (KSt. 2 and KSt. 3). KSt. 2 locates in the oyster aquaculture field. Environmental factors such as seawater temperature and dissolved oxygen (DO) were measured on site at each sampling station using a RINKO AAQ176 water quality profiler (JFE Advantech Co., Ltd.). The concentrations of inorganic nutrients such as dissolved inorganic nitrogen, phosphate and silicate were also determined along with the concentrations of chl-*a*.

Seawater samples collected as above were serially passed through 20-, 5-, 0.8- and 0.2- μ m pore size filters. Shotgun metagenomic sequencing was performed to determine nucleotide sequences of cells trapped on 0.2- and 0.8- μ m filters. Briefly, cells trapped on the filters were subjected to DNA extraction using the Power Water Isolation Kit (MO BIO Laboratories Inc.). The DNA libraries for shotgun sequencing were prepared using the Nextera XT DNA sample preparation Kit and MiSeq Reagent Kit V3 for 600 cycles (Illumina, Inc.). Finally, sequencing was carried out with an Illumina MiSeq sequencing system. The obtained raw reads were first joined by overlapping forward and reverse reads using the software FLASH and further processed using CLC Genomic Workbench for the trimming of low-quality reads <50 bp. After the quality-control pass, the entire datasets were compared to the NCBI NT database using BLASTn search. Taxonomic analysis was then performed using MEGAN software with LCA default parameters. Comparative analysis in MEGAN was also performed after normalizing counts.

Correlation analyses were carried out using the Pearson's product moment correlation coefficient test to determine the relationship between the abundances of PPEs and chl-*a* concentrations. Spearman's rank correlation (R_s) and NMDS analysis were performed to determine the relationships of the abundances of PPEs and the bacterial communities with environmental parameters, respectively.

Seasonal changes in the microbial communities in seawater samples trapped on 0.2- μ m filters from the Ofunato Bay

Shotgun metagenomic sequencing was employed to determine nucleotide sequences of cells trapped on 0.2- μ m filters from May 2016 to December 2017 in the Ofunato Bay and compared with those previously analyzed from January 2015 to April 2016 (Reza et al., 2018). The microbial communities analyzed were comprised of bacteria, eukaryotes, Archaea and viruses. Bacteria occupied the largest proportion at the two sampling depths in the three sampling stations. The relative abundances of eukaryotic cells were higher at 8 or 10 m depths than those at 1 m depth. In addition, Archaea tended to increase every year from October to February and sometimes additionally from September to February at both sampling depths. Small proportions of viruses were detected every year irrespective of the sampling stations and depths.

The bacterial communities were dominated by *Candidatus Pelagibacter* and *Planktomarina*, both belonging to Proteobacteria. *Ca. Pelagibacter* was the most abundant, followed by *Planktomarina* in 2015, whereas the proportion of *Ca. Pelagibacter* was low in many months in 2016 and 2017. Cold Oyashio Current is likely associated with such yearly changes in the proportion between *Ca. Pelagibacter* and *Planktomarina*, because seawater temperatures in 2015 were lower than those in 2016 and 2017 especially in March at 8 or 10 m depths. Another bacteria, *Synechococcus*, belonging to Cyanobacteria, showed a high seasonality and its relative abundance was positively correlated with seawater temperature at 8 or 10 m depth. Clustering analysis revealed that the bacterial communities in the Ofunato Bay were grouped in a season-dependent manner every year, although cluster-forming patterns were different from one year to another for the two depths at the three sampling stations.

The eukaryotic communities in cells trapped on 0.2- μ m filters were mainly occupied by prasinophyte algae, *Bathycoccus*, *Micromonas* and *Ostreococcus*, commonly known as PPEs. High relative abundances of PPEs were recorded from January to February and from June to July, while low relative abundances were observed from March to May and in September, irrespective of the three sampling stations and water depths in 2015. PPEs also showed seasonal variations in their relative abundances in 2016 and 2017, whereas *Micromonas* was higher in 2016 and 2017 than in 2015. Among the three PPE genera, *Ostreococcus* showed high abundances from June to July every year. In general, low abundances of PPEs were detected during high chl-*a* concentration period, indicating PPEs compete for nutrients against blooming diatoms. In addition, PPEs-associated viruses, commonly known as prasinoviruses, were detected with dynamic seasonal variations over the period of the three years from 2015 to 2017. Among the three PPE genera, the abundances of *Bathycoccus*-associated viruses and *Micromonas*-associated ones changed seasonally in a manner similar to their host cells. On the other hand, *Ostreococcus*-associated viruses showed seasonal fluctuation different from their host cell. Furthermore, virus infection periods were different among the three genera. Thus, seasonal changes in the abundances of Prasinophytes and their associated prasinoviruses showed complex virus-host relationship. Such seasonal changes in the abundances of viruses in association with host organisms of PPEs are the first evidence to my knowledge. Although the abundances of PPEs are thus thought to be influenced by their loss processes such as virus infection, it is likely that when larger phytoplankton are not dominant, PPEs contribute to an important part in the primary production in marine environments.

Seasonal changes of microbial communities in seawater samples trapped on 0.8- μ m filters from the Ofunato Bay in comparison with those of 0.2- μ m filters

The microbial communities in seawater samples trapped on 0.8 μ m filters in 2015 were dominated by bacteria at 1 m depth of the three sampling stations and at 8 m depth of KSt. 1, and by eukaryotes at 10 m depth of KSt. 2 and KSt. 3. In addition, Archaea was detected only marginally in the 0.8 μ m filters differently from those in 0.2 μ m filters from the same year. The former bacterial communities were dominated by *Synechococcus* and *Planktomarina*. *Candidatus Pelagibacter* was most abundant in 0.2 μ m filters, but marginal in 0.8 μ m filters. The relative abundances of *Synechococcus* in 0.8 μ m

filters were very low from March to May at both sampling depths. Meanwhile, *Synechococcus* showed a marked increase in June at both sampling depths. The bacterial communities during April and May were very complex and different from those in other months. Besides, several bacteria such as *Roseobacter*, *Polaribacter*, *Flavobacterium*, *Phaeobacter*, *Cytophaga* and *Arcobacter* showed seasonal fluctuation patterns similar to those of chl-*a* concentrations. Thus, it is predicted that these bacteria are associated with diatom blooms. NMDS analysis revealed that the bacterial communities during April and May formed a distinct cluster from those in other months and were significantly correlated with chl-*a* concentrations and DO, suggesting that diatom blooms possibly influence the bacterial communities during such blooming period.

The eukaryotic communities trapped on 0.8 μm filters in 2015 were mainly occupied with PPEs such as *Micromonas*, *Bathycoccus* and *Ostreococcus*. Among the three PPEs genera, *Micromonas* occupied the largest proportion, followed by *Bathycoccus* and *Ostreococcus*. However, the relative abundances of *Ostreococcus* were low from March to May and increased during June and July at both sampling depths, regardless of the three sampling stations. Obtained read numbers of PPEs were much more from 0.8 μm than 0.2 μm filters, indicating that PPEs were mainly trapped on 0.8 μm filters. However, seasonal variation patterns of the three genera were similar in both filters and showed negative correlations with chl-*a* concentrations. Besides, high relative abundances of oyster genus *Crassostrea* were observed in the eukaryotic communities from 0.8 μm filters in a specific manner to August at KSt. 2 in the mid of oyster aquaculture field, possibly corresponding to their spawning, whereas very low relative abundances of PPEs were observed during the same period at this location. These results indicate that PPEs are possible food candidates for oyster larvae. To confirm this hypothesis, a PPE strain *Prasinophyceae* sp. was isolated from seawaters of KSt. 2 in the Ofunato Bay and examined whether or not oyster larvae consume PPEs. As a result, it was found that cells of *Prasinophyceae* sp. were clearly ingested by oyster larvae. The relative abundances of PPEs were also influenced by virus infection. Prasinoviruses were detected in 0.8 μm filters and their peaks were detected in June for the three host organisms, *Micromonas*, *Ostreococcus* and *Bathycoccus*, as in the case of 0.2 μm filters. Furthermore, by being combined with data of seasonal variation profiling of environmental nutrients, it was suggested that PPEs could grow under low concentrations of nutrients in seawaters at both 1 m and 10 m depths in KSt. 2.

In conclusion, the present metagenomic-based study revealed that *Ca. Pelagibacter*, *Planktomarina* and *Synechococcus* were the major bacteria in the Ofunato Bay. Furthermore, *Micromonas*, *Bathycoccus* and *Ostreococcus* were the major small phytoplankton in the bay and possibly consumed by oyster larvae as food. In addition, this study revealed relationship among the relative abundances of bacteria and PPEs and chl-*a* concentrations. Seasonal changes in the microbial communities in the Ofunato Bay were reflected by environmental conditions. Nutrient rich Oyashio Current is possibly important for the exchange of seawater and supply of nutrients to primary producers and nutrient recyclers. Hence, the bay is a popular aquaculture area. Continuous bio-monitoring of microbial communities in the Ofunato Bay may provide baseline information and lead to a better understanding of marine ecosystem and associated economic activities.