DOCTORAL THESIS
（要約）

Mechanisms Controlling Bacterial Community Structure in Coastal Marine Environments

(沿岸域における微生物群集構造の形成メカニズム)

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Dedicated to my beloved family,

their sacrifices are admirable,

their inspirations are the sources of my ability.

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ABSTRACT

Bacteria are widely distributed in aquatic and terrestrial environments and their community structure is one of the most fundamental information to understand their roles in the environments, evolutionary processes and characteristics of the environments they live. At any environments, multiple factors including physicochemical and biological ones are involved in the formation of specific community structures. The objective of this thesis was to clarify mechanisms controlling bacterial community structure in the environment. For this investigation, several strategies were taken. Firstly, research was conducted by combining three approaches, namely, field surveys, DNA sequence analyses by using newly developed bioinformatics approach, and laboratory culture experiments. This is because each approach has advantages and disadvantages. The combination of them should give us new information. Secondly, for the field survey, community structural analyses were conducted at two closely located stations in the coastal area, because the subtle difference in environmental conditions may show the similarity and dissimilarity of the community structures. If there is any particular OTU that appears only one of them, it indicates the presence of a strain uniquely adapted to the environment, and also it may lead to the formation of unique community structures. Thirdly, specific attention was given not to all phylogenetic groups, but to three major classes, Flavobacteriia, Alphaproteobacteria, and Gammaproteobacteria. This is because it is practically impossible to cover whole phylogenetic groups for investigation, and these three have been reported as dominant ones in marine environments. It is assumed that members

belonging to these three phylogenetic groups respond to the environments in different ways. So, different mechanisms may actually control their structures and biomass.

The field surveys were conducted for five times at two closely located stations, the port side and sea side in Oarai, Ibaraki Prefecture from March 2013 to July 2014. In order to see the differences between particle-associated (PA) bacteria and free-living (FL) bacteria, seawater samples were filtrated through 3.0 μm and 0.22 μm filters, followed by DNA extraction and sequencing of 16S rRNA gene by using 454 GS Junior sequencer. The followings were clarified. First, the classes Flavobacteriia, Alphaproteobacteria, Gammaproteobacteria contributed about 78 to 98% of the relative abundance in the port side station while about 62 to 92% of the relative abundance in the sea side station, and there was no apparent difference between the two stations. Top 25 most abundant OTUs contributed about 24 to 73% of the relative abundance in different samples at port side and about 12 to 56% of the relative abundance at sea side station; 23 of these most abundant OTUs belonged to these three major classes. Second, differences appeared at family or genus level between the two stations. In addition, there was some unique OTU, most of which belong to Gammaproteobacteria. This means that although the three classes steadily appear in the environments, phylogenetic groups or OTUs belonging to them may dynamically change depending on the environmental conditions, especially those in Gammaproteobacteria. Third, there was no apparent difference between PA and FL bacterial community structures, suggesting that there might be rather frequent exchanges of bacterial cells between the two fractions in the area investigated. Fourth, species richness was similar among the three major classes whereas the evenness of Flavobacteriia was significantly higher than those of Gammaproteobacteria and Alphaproteobacteria, indicating that members

belonging to Flavobacteriia have different way or mechanism to distribute or adapt to the environments. Because information on the bacterial community structures in marine environments in western Pacific or Asian area is quite limited, these results are one of the very few attempts conducted by using NGS. In order to further clarify the difference, habitability was analyzed for OTUs appeared to the environments.

Habitability is defined as the ability of any organism to inhabit different environments. The recently developed database, the MetaMetaDB (http://mmdb.aori.u-tokyo.ac.jp/) enables us to check habitability, or from which environments any 16S rRNA sequence in question has been so far obtained and deposited. Analyses at different phylogenetic levels from 97% (species) and 85% (order) level of identity were conducted. The results showed the followings. First, at stations with the lower salinity (salinity 0.5 to 5.0; riverine and estuarine stations), sequences with "freshwater-groundwater", "human" and "wastewater" habitabilities dominated, while at the stations with higher salinity (salinity 32 to 35; Oarai coastal, Kuroshio Current stations), most sequences were "marine". Second, among three classes, the members of Flavobacteriia were abundantly present in both more saline and less saline stations, while those of Alphaproteobacteria and Gammaproteobacteria mostly in more saline stations. This indicates that members in Flavobacteriia are more diverse and distributed to both freshwater and seawater environments, while those in other two classes are more specified to marine environments. The diverged groups may be constantly present in varieties of environments, although the biomass may not be too high. Because the information of habitability is only available through MetaMetaDB and this site is still not yet widely used, this is the first knowledge about the habitability of bacteria in coastal environments.

Habitability shows that members of the class Flavobacteriia are widely adapted to various environments, indicating they are reactive to the environmental changes and more stable. This may at least partly explain the high evenness of Flavobacteriia in coastal environments. Although habitability shows that Flavobacteriia is possibly different from other two classes, there may be other factors controlling their biomass and community structures in the environments. In addition, the difference between Alphaproteobacteria and Gammaproteobacteria was not clear from the habitability analyses. As various types of organic materials, in terms of molecular size, element composition and quantity are present in seawater, it is assumed that members in three classes show different utilization pattern of such materials in the sea. In order to answer this question, several seawater culture experiments were conducted with the amendments of different monomeric and polymeric organic substances. Seawater samples were taken from Oarai coast or from off-shore Kuroshio Current, north station (NBD) and south station (SBD) of the Pacific, filtrated and incubated after addition of 6 different organic materials. The community structures were followed during the course of incubation. The results showed the followings.

First, the patterns of increases were different among three classes. Second, some members of the class Gammaproteobacteria, more specifically the genera *Vibrio* and *Alteromonas* generally reacted quickly and used the low molecular weight substances and proliferated massively. *Pseudoalteromonas* was involved in degradation of high molecular weight substances like starch. Because the biomass of Gammaproteobacteria in the environments is generally about a half of those of other classes, it is suggested that members of Gammaproteobacteria may be susceptible to top-down control such as predations and viral lysis. Third, members of Alphaproteobacteria showed a similar

tendency in utilizing monomers, but become abundant at the end in some cases especially in the polymer treated samples, indicating the presence of polymer degraders in this class. Finally, the relative abundances of members of Flavobacteriia were maintained at a minimum level but showed a steady growth. Some of them seem to be polymer degraders as well. Although similar culture experiments had been conducted, this research is the most extensive one with the combination of different sets of organic matter and also community structure analyses by using NGS.

In conclusion, although apparent community structures, especially the relative abundance of Flavobacteriia, Alphaproteobacteria, and Gammaproteobacteria were rather consistent in two closely located coastal environments, the constituent phylogenetic groups in each show much more variations depending on location and time. By combining field survey, habitability analyses by using MetaMetaDB and culture experiments with different organic materials, the different mechanism to maintain their biomass and community structures were clarified. In short, Gammaproteobacteria is characterized as the fast grower, monomer utilizer and higher sensitivity to changes. Compared with Gammaproteobacteria, members in Alphaproteobacteria grow slowly and some of them are polymer degraders that make them possible for maintaining their biomass and wide distribution. Flavobacteriia is characterized as slow grower and polymer utilizer. Further investigation on distribution pattern, physiology, phylogeny and elaborate experiments may further clarify the characteristics of each group and help understand the mechanism of community structure formation more clearly in future.

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LIST OF ACRONYMS AND ABBREVIATIONS

Acronyms/ Abbreviations		Elaborations
AFM	:	Atomic Force Microscopy
AORI	:	Atmosphere and Ocean Research Institute
BSA	:	Bovine Serum Albumin
CCA	:	Canonical Correspondence Analysis
DAPI	:	4',6-Diamidino-2-Phenylindole
DDBJ	:	DNA Data Bank of Japan
DFAA	:	Dissolved Free Amino Acid
DOC	:	Dissolved Organic Carbon
DOM	:	Dissolved Organic Matters
DRA	:	DDBJ Sequence Read Archive
EPS	:	Extracellular Polymeric Substances
FL	:	Free-living
GA	:	Glutamic Acid
HMW	:	High Molecular Weight
JAMSTEC	:	Japan Agency for Marine-Earth Science and Technology
LMW	:	Low Molecular Weight
NMDS	:	Non-Metric Multidimensional Scaling
Meta-MetaDB	:	Meta-Metagenomic DataBase

LIST OF ACRONYMS AND ABBREVIATIONS (CONT'D)

Acronyms/ Abbreviations		Elaborations
MHIs	:	Microbial Habitability Indices
MID	:	Multiplex Identifier
NAGA	:	N-Acetyl Glucosamine
NASA		National Aeronautics and Space Administration
NGS	:	Next Generation Sequencing
ODV	:	Ocean Data View
OTU	:	Operational Taxonomic Unit
PA	:	Particle-associated
PAHs	:	Polycyclic Aromatic Hydrocarbons
PBS	:	Phosphate-Buffered Saline
PCR	:	Polymerase Chain Reaction
POC	:	Particulate Organic Carbon
POM	:	Particulate Organic Matter
RCA	:	*Roseobacter* Clade-Affiliated
RDA	:	Redundancy Analysis
RDP	:	Ribosomal Database Project
SIMPER	:	Similarity percentage analysis
SST	:	Sea Surface Temperature

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CHAPTER 1:

General Introduction

1.1 Microorganisms, microbial communities, and diversity

In general, because of their extraordinary adaptation ability and diversity, microorganisms distribute almost everywhere on the earth surface environments including those where other groups of organisms cannot live (Oren 2009, Sunagawa et al. 2015). Microorganisms are the most numerous and diverse organisms on the planet and doing almost all biological functions (Kirchman 2012). In natural microbial habitats, any microbial population may interact with other populations of living organisms and form assemblages called microbial communities. Those processes are controlled by the availability of resources (such as food) and environmental conditions (temperature, pH, oxygen content, and so on) (Madigan et al. 2009). Because of competition for the resources and fluctuations in environmental conditions, some microbes may become obligate inhabitants to a particular environment while some acquire adaptability to diverse habitats and/or environmental conditions. They respond to the environmental changes either for short term or long term basis. To evaluate the short-term responses of microbes, we usually consider their gene expression pattern, and for a long term, their community structure. All the ecosystems of the earth are under dynamic and constant changes due to living organisms, and in some cases, totally controlled by the microorganisms because any other living organisms cannot thrive and/or only microbes have the ability to change them. So, studying the microbial communities, their contributions to the ecosystem and their diversity is always a matter of interest for not only microbiologists but also the ecologists or those who are investigating on natural processes on the planet.

Microbial diversity has been a focused part of microbiology, including a broad range of variability of all types of microorganisms such as Bacteria and Archaea (the prokaryotes), algae, fungi, protozoa, viruses, and so many others. The diversity of

microorganisms is still an unexplored area, even the right order of magnitude is unknown and in fact, an issue of controversy (Finlay and Esteban 2004, Hedlund and Staley 2004, Whitfield 2005, Pedros-Alio 2006). Although several attempts have been made, estimations of actual species numbers are thought to be inexplicable at any scale in any environment (Curtis et al. 2002). But, it is necessary to explore the community structure and the diversity of microorganisms in different ecosystems and observe their variability for time and space scale to select specific model of global change, explore potential genes for medicine and biotechnology, and to understand the evolutionary process (Baldauf 2003, Pedros-Alio 2006).

Among different members in the microbial world, prokaryotes represent the greatest biomass with high phylogenetic and metabolic diversifications. They are grouped into two domains of life, Bacteria, and Archaea. The former is more diversified and has more biomass than the latter. Moreover, the former distributes much widely and are sensitive to environmental changes than the latter. Some members in Archaea are known as extremophiles (Madigan et al. 2009). So, in this research, community structures of bacteria are mostly considered.

The community structure of bacteria is one of the most fundamental information to understand the microbial processes in the environments and also characteristics of environments, because of the following reasons. First, the community structure is a sum of different species, which possesses a unique set of genes. Once we know the community structure and whole genome of each species in it, we will be able to know what kind of functional genes are present in the environment in question. This enables us to know their functions in nature at the genetic level. Second, the apparent community structures are results of the interactions between microorganisms and the environment. In addition, this reflects long-term evolutionary processes and also short-

term ecological processes. Therefore, to know the community structure is the first step of knowing microbial processes and also to know the characteristics of environments.

1.2 Utilization of molecular techniques in aquatic environmental microbiology and the concept of "majorities" and "minorities"

The analyses of bacterial community structures had been hampered by methodological limitations, mainly due to the difficulty to cultivate prokaryotic cells. Recent developments of molecular techniques, however, considerably overcome this problem by directly obtaining the genetic information without cultivation (DeLong et al. 1993, Giovannoni et al. 1995, Acinas et al. 1997, Hiorns et al. 1997). Furthermore, the introduction of the sequencing techniques, especially the next generation sequencing (NGS) made it possible to obtain by far more sequence data within a short period of time. Previous techniques such as fingerprinting or gene cloning methods give us typically up to one hundred of the operational taxonomic unit (OTU), whereas, NGSs up to several to a hundred thousands of reads in one sample. This made it possible to show the presences of few species of "majorities" and a huge number of species present as "minorities" (Sogin et al. 2006). The latter usually appear once (singleton) or twice (doubleton) in one sample (Figure 1-1).

As apparent individual numbers are controlled by the relative balance between the growth and death, the "majorities" may have high growth rate and/or high protective mechanisms against predation or death. Even if a particular group has high growth rates, they cannot maintain high individual numbers if they don't have efficient protective mechanisms against predators. The "minorities" may be those with low growth rate and certain protective mechanisms, or those with high growth rate and with low protective mechanisms. The actual maintenance mechanism is, however, not clear. The concept of these "majorities" and the "minorities" lead to the following questions: In what kinds of

environments, how are "minorities" distributed? What controls their distribution? Whether the "minorities" are always staying as minor or can they turn into the major? If so, what control such transitions? What controls the relative proportion of the major and minor fractions? These questions are important to clarify to assess how bacterial community structures are formed. In order to answer these questions, intensive field investigations using NGS analyses are required. In addition, information on the isolates, especially whole genome sequence data are quite helpful to discuss the possible factors. For this reason, the present research was focused on Bacteria, especially some major classes, the Flavobacteriia, Alphaproteobacteria and Gammaproteobacteria, that have been investigated intensively.

1.3 The majorities and minorities (major groups and minor of bacteria)

At present, so far 50 to 100 phyla are known in different natural environments and only a few are seen in many different environments while others show fluctuations depending on the types of habitats (Kirchman 2012). In surface seawater, the "majorities" included Alphaproteobacteria and Gammaproteobacteria of the phylum Proteobacteria (Fuhrman & Davis 1997, Lopez-Garcia et al. 2001, DeLong et al. 2006, Pham et al. 2008, Barberan and Casamayor 2010), and the Flavobacteriia of the phylum Bacteroidetes (Glöckner et al. 1999, Kirchman 2002, Amaral-Zettler et al. 2010, Barberan and Casamayor 2010, Kirchman 2012), are generally ubiquitous, while the "minorities" tend to vary with spatiotemporal and environmental changes.

In freshwater, the class Betaproteobacteria is the most abundant followed by the Gammaproteobacteria and Alphaproteobacteria (Hiorns et al. 1997, Glöckner et al. 1999, Kirchman et al. 2005, Kirchman 2012). In seawater, the classes Alphaproteobacteria (mostly SAR11), (Fuhrman and Davis 1997, Lopez-Garcia et al. 2001, DeLong et al. 2006, Pham et al. 2008, Gilbert et al. 2012) and Gammaproteobacteria are widely

distributed, while the class Betaproteobacteria is relatively less abundant (Kirchman 2012). The second most abundant is the phylum Bacteroidetes, dominant in both freshwater and saltwater (Glöckner et al. 1999, Kirchman 2002, Amaral-Zettler et al. 2010, Kirchman 2012); and within the phylum, the class Flavobacteriia is reported to be abundant in the oceans, while the Sphingobacteriia in lakes (Barberan and Casamayor 2010, Kirchman 2012). In the coastal marine environments Bacteroidetes usually cover between 10 to 30% of the total bacterioplankton counts (Alonso-Saez and Gasol, 2007).

Further lower pahylogenetic level analyses of these three major classes, the Alphaproteobacteria, Gammaproteobacteria and Flavobacteriia of the coastal seawater environments also showed dominancy by some typical groups. For example, within the class Alphaproteobacteria, the SAR11 clade (Giovannoni et al. 1990, Field et al. 1997, Morris et al. 2002, DeLong et al. 2006, Gilbert et al. 2012, Giovannoni and Vergin 2012, Kirchman 2012, Sunagawa et al. 2015) and the marine Rhodobacterales are widespread, abundant, and metabolically versatile groups in oceanic surface environment especially the the members of marine *Roseobacter* clade (Dang et al. 2008, Gilbert et al. 2012, Fu et al. 2013). Within the class Gammaproteobacteria, the clade SAR86 of the order Oceanospirillales is known as the most abundant uncultivated constituent of bacterial assemblages at the ocean surface (Dupont et al. 2012, Giovannoni and Vergin 2012, Sunagawa et al. 2015); the SAR92 clade (Sting et al. 2007, Giovannoni and Vergin 2012) as well as the order Vibrionales and Pseudomonadales (Gilbert et al. 2012). While in the class Flavobacteriia, the order Flavobacteriales is mostly dominant (Gilbert et al. 2012) and the pyrotags NS2b, NS4 and NS5 of this order usually appear with high frequencies (Korlević et al. 2015); the members of the genus *Flavobacterium* of the same order are also distributed in soil and freshwater habitats (Bergey et al. 1923). In

the North Atlantic Ocean, the genus *Polaribacter* was found to be the most abundant (Gomez-Pereira et al. 2010).

Based on this knowledge, this research was focused on three major groups, i.e., Alphaproteobacteria, Gammaproteobacteria, and Flavobacteria. These three groups share a considerable part of populations in coastal seawater, and there have been many isolates together with information of genetics, physiology, and phylogeny. Therefore, the question is how distributions of these three groups are fluctuating depending on environmental conditions and how minor ones belonging to these three groups are behaving in the coastal environments. Moreover, we do not have enough knowledge about the richness and evenness of these three major classes as well as what determine their degree of abundance and biodiversity, especially in case of coastal marine habitats. These types of information can be possible to obtain by the intensive investigations emphasizing to the lower phylogenetic levels.

1.4 Coastal environment

The coastal environments are fluctuating habitats because of the influences of terrestrial, freshwater and oceanic conditions. Some areas are also affected by anthropogenic activities. Organic materials, nutrients, pollutants and microorganism may be brought into coastal environments depending on the geographical characteristics, season, local weather, currents and so on. Being a dynamic environment, the communities of the coastal environments required a higher level of adaptability as the community structure of the coastal bacteria is formed as a result of complex interactions between many inner and outer factors. Bacterial members are mainly increased as results of growth and inflow from the surrounding freshwaters, estuarine and marine waters. The growth rate of the coastal bacteria is mainly influenced by different physical and chemical factors, availability of nutrients and intra and/or interspecies

interactions. On the other hand, bacterial members of the coastal areas are declined by death, grazing and viral lysis; and due to outflow to the oceanic environments because of the wind and oceanic currents (Figure 1-2).

In the seawater as well as on the ocean floor, microorganisms are widely distributed and they influence the physical, chemical, geological and biological conditions of those habitats (ZoBell 1946). Like in other ecosystems, bacteria share the largest biomass among all living organisms here. Hence, it is essential to understand their functions in order to clarify how marine ecosystems are functioning and maintained. Different surveys conducted on diversified habitats of marine environments suggested that bacteria are widespread with complex community structures including many unknown members. So among different major habitats of the earth, studying the geographic distributions, community structure and diversity of the bacteria in marine ecosystem is of special attention to the microbial ecologists. Although, lots of works are yet to do but we do know the "majorities" of the bacterial community of the marine habitats.

The diversity of any environment is assessed considering two main features such as the richness, considered the total number of species/phylotypes in a community and, the evenness, considered the total number of species as well as the number of individuals in each species. The uniformity in the presence of "minorities" will ultimately determine the evenness as well as the degree of biodiversity of the marine ecosystem while dominating by the "majorities" will make it uneven. So, the bacterial diversity of the coastal marine ecosystem will be changed by the relative proportion between major and minor groups.

1.5 Factors influencing community structures

Unlike other ecosystems, bacterial community structure and diversity of the coastal marine environments is modified by many environmental conditions (Ruan et al. 2006,

Gilbert et al. 2009, Gilbert et al. 2012); influences by the environmental changes as well as by the surrounding habitats. The influencing factors can broadly be divided into physical e.g. temperature, light, pressure, water current, tide, solar radiation etc.; chemical e.g. salinity, organic matter, inorganic matter, pH etc.; and biological e.g. density, growth, interaction with other microbes, competition, predation, symbiosis, relative growth and death rates etc. Among these influencing factors, temperature (physical); salinity, nutrients, oxygen concentration, pollution, etc. (chemical); and predation, competition, plankton bloom (biological) were reported as more influencing to the bacterial diversity of the coastal marine environments (Fuhrman et. al. 2006, Andersson et al. 2010, Gilbert et al. 2010, Du et al. 2013). However, many of these important influencing factors are more or less dependent on the following aspects.

1.5.1 Spatiotemporal fluctuations

The community composition of bacteria is usually modified by the seasonal changes and biogeographical distributions (Treusch et al. 2009). It is well documented that the coastal bacterial community composition is usually changed seasonally (Andersson et al. 2010, Gilbert et al. 2012, Du et al. 2013). By assessing the changes in bacterial community structure due to the seasonal changes, we are able to identify the microbial sensitivity to different conditions i.e. which group/gene responds to what situation or environmental changes. Moreover, as specific bacteria possess a particular set of genes, examination of bacterial community structures together with environmental parameters offers information to understand what kinds of functions are being performed by them to that environment. Previous investigations conducted to evaluate the changes in bacterial lifestyle and community structure due to the seasonal influences were mainly concentrated to the major phylogenetic levels, mostly because of the methodological limitations. Now a day, invention of molecular techniques and

utilization in the environmental microbiology made it possible to look at the finer scale, as it is expected that the seasonal influences will be much more prominent to the lower taxonomic levels (Mary et al, 2006). Although the community structures of major groups at large spatiotemporal scales in coastal seawater have been documented, analyses of minor groups have seldom been accomplished (Du et al. 2013). So, sampling at two closely located areas in different seasons and intensive observations of the bacterial community structure at the finer scale, giving more emphasis one the minor groups, may enable us to have a clearer concept regarding the formation of bacterial community in an environment which is lacking in previous reports mostly due to methodological limitations possible to address now (Giovannoni et al. 1995, Acinas et al. 1997, Hiorns et al. 1997, DeLong et al. 1999).

Another example of advantages of finer scale intensive observations is the identification of some unique groups. Bacteria respond to environmental changes by changing their gene expression patterns over a short period of time and over a long period, some of them adapt to that environment. So, some bacteria are evolutionary well adapted to a particular environment which made them unique/ location specific inhabitants of a particular environment while some have the adaptability to diverse habitats and/or environmental conditions and thus, bacterial community structure can be different even at two closely located points. Local scale similarity analysis showed that there is a unique association between different groups and between bacteria and environment (Ruan et al. 2006). If two closely located areas are sampled repeatedly at different seasons and the bacterial community structure is investigated intensively, especially considering the lower phylogenetic levels, it is expected that the sites will share majority bacterial populations while some taxa will appear in one of the two sites only, or consistent differences in the minor groups at each site may appear. Here, the

groups found in one of the two closely located areas in most of the samplings were termed as "unique groups." These unique groups may be regarded as potential indicators of subtle differences in environmental changes. Alternatively, the presence of such groups may indicate certain environmental conditions that have been overlooked by common oceanographic analytical methods.

Fuhrman et al. (2006) showed that bacterial composition is highly repeatable over season and their diversity is predictable considering the spatiotemporal changes in the environmental factors. They found that the OTUs were mostly from among the members of the *Alteromonas* (Gammaproteobacteria), Bacteroidetes, Alphaproteobacteria (including members of the SAR11 and SAR116 clusters), and Verrucomicrobia. In a study in the coastal waters of the South China Sea, Du et al. (2013) reported that the spring and summer samples were predominated by the Alphaproteobacteria, followed by Cyanobacteria and Gammaproteobacteria. In coastal waters, another study showed that the Sphingobacteria-Flavobacteria of the phylum Bacteroidetes dominated numerically in spring and early summer while the Alphaproteobacteria from late summer to winter (Mary et al. 2006). The SAR11 and SAR116 clade of Alphaproteobacteria, and SAR86 of Gammaproteobacteria exhibited stronger increases at the ocean surface during summer periods (Morris et al. 2005, Treusch et al. 2009). These overall findings proved that the members of the class Alphaproteobacteria, Gammaproteobacteria and Flavobacteriia showed seasonal dominancy although the patterns may vary according to the spatiotemporal conditions.

1.5.2 Influences by the adjacent habitats

Introduction and mixing of the exogenous bacterial groups from the adjacent terrestrial and riverine as well as from the estuarine and marine habitats influence the bacterial community structure of the coastal environment (Crump et al. 1999). The

coastal waters receive a substantial amount of bacterial and nutritional supply from terrestrial sources (Danovaro and Pusceddu 2007), which result higher diversification and production. The coastal environments are usually subjected to pollutions by the surrounding habitats and human activities. There is evidence that less pollution and better water quality i.e. better ecological conditions usually influence the bacterial diversity positively (Halliday et al. 2014); and the higher level of anthropogenic activities (sewage disposal and port activity) influence negatively in coastal environments (Cury et al. 2011).

We are not certain about the relative proportion of bacterial populations from the surrounding habitats especially, how much from the non-marine sources. Studying the spatiotemporal changes in coastal bacterial community structure emphasized mostly on the bacterial response to the variability in space and time. These studies were overlooked or totally ignored the probable introduction of bacterial populations from other habitats. Because, it is difficult to assess the origin or sources of bacteria by the commonly applied physiological or genetical approaches. This important aspect can now be possible to evaluate by studying the adaptability or "habitability" (please see below) of the available bacterial phylotypes, which can ultimately be used to assess the influences by the surrounding habitats to the bacterial community structure of the coastal environments.

1.5.3 Organic matter

1.5.3.1 Particulate organic matter (POM)

In the aquatic environments, the production, processing, and utilizations of the particle organic matter (POM) is a major concern to the microbial ecologists (Simon et al. 2002). The heterotrophic bacterial groups are known to play the major role in remineralization or breaking down the particles to make the nutrients available for reuse in the pelagic

waters (Cotner and Biddanda 2002) and to convert the particle organic carbon (POC) to dissolved organic carbon (DOC) (Smith et al. 1992, Martinez et al. 1996). These concepts divided the aquatic bacteria into two fractions, the particle-associated (PA) groups, those remained attached to the particles to process it and the free-living (FL) groups, those remain free in the waters and used up the nutrients dissolved and available for them (Foreman and Covert, 2003).

1.5.3.2 Dissolved organic matter (DOM)

Bacterial metabolic activities are much more diverse than those of eukaryotes in aquatic environments and thus, various chemical compounds especially dissolved organic compounds are utilized solely by bacteria (Figure 1-3). Bacterial cells are composed of various elements, but carbon (C), nitrogen (N), and phosphorus (P) share the most. Bacterial cells require these elements for their growth and survival; especially the C and N. Heterotrophic bacteria require organic compounds as their source of carbon and energy. Different types of bacteria utilize different types of amino acids, fatty acids, organic acids, sugars, nitrogen bases, aromatic compounds, and other organic compounds (Madigan et al. 2009).

The dissolved organic matter (DOM) in seawater are viewed as a mixture of two different fractions based on the size: low molecular weight (<1 kDa) and high molecular weight (HMW, $\geqslant$1 kDa) (Sosa et al. 2015). The low molecular weight substances can also be termed as the "monomeric substances or monomers", and the high molecular weight substances as "polymeric substances or polymers". The polymers have to be hydrolyzed to monomers prior to being taken up by the microbes. The monomers can be quickly used and sustain high bacterial biomass, whereas polymers are slowly degraded by the certain group(s) with less increase of biomass because they have to synthesize degrading enzymes. Considering the utilization of the nutrients, the bacterial

populations may be broadly divided into two major groups (Stuart and Robert, 2003) (a) the polymer degraders and (b) the monomer utilizers. The latter group does not have certain degradation enzymes and thus, act as scavengers, or take up "spilled" degraded materials. The community structure should be formed at least as result interactions between these two groups.

The members of the class Alphaproteobacteria were reported to be involved in utilization of LMW organic substances (Cottrell and Kirchman 2000, Malmstrom et al. 2004, Elifantz et al. 2005), while the phylum Bacteroidetes in HMW organic substances (Cottrell and Kirchman 2000, Elifantz et al. 2005, Fuhrman and Hagstrom 2008, Fernandez-Gomez et al. 2013). From a study conducted in Delaware Bay, Cottrell and Kirchman (2000) reported that utilization of organic matter varied among the major phylogenetic groups, including LMW DOM. Cytophaga-like (Bacteroidetes) took up the chitin and protein (polymers) as well as N-Acetylglucosamine (NAGA, monomer), and Alphaproteobacteria preferred amino acids (monomers) to proteins. The members of Beta and Gamma-proteobacteria were noted as the bacterial group consuming less chitin and NAGA, as well as less protein and amino acids. The members belonging to class Alphaproteobacteria, typically SAR11 seem to prefer monomers including amino acids and sugars (Morris et al. 2002). It was also reported that utilization of organic materials, both monomers, and polymers, differed according to the salinity (Elifantz et al. 2005). They reported that, for monomers such as in glucose assimilation, Actinobacteria contributed mostly in freshwater, while the Alphaproteobacteria in saline water. But, for extracellular polymeric substances (EPS) assimilation, various bacterial groups contributed, i.e., Actinobacteria and Betaproteobacteria in the freshwater, whereas Cytophaga-like Bacteria (Bacteroidetes) as well as Alpha- and Gammaproteobacteria in the relatively higher saline waters (Elifantz et al. 2005).

These polymer degrading groups can be distinguished from the monomer utilizers by conducting culture experiments using different doses of some candidate chemicals, comprising of both low and high molecular weight substances. Therefore, the combination of general physiological information available from the literature, genetic information, and culture experiments may show us how organic compounds may possibly control the community structure in the sea.

1.6 Particle-associated (PA) and free-living (FL) bacterial community

The community composition and diversity of PA and FL bacteria have been comparing in various environments and reported they are different (Crespo et al. 2013, Mohit et al. 2014). Generally, it was observed that the particle-associated groups have enhanced rates of cell-specific activities compared to the bacteria of the surroundings (Kirchman 1993). Because, the composition of the organic materials in the suspended particles and those dissolved in waters is different, it is also expected that the seasonal influence will be different between the PA and the FL fractions of bacteria (Allgaier et al. 2006, Ghiglione et al. 2007, Rösel et al. 2012). However, most of the previous studies were conducted in either offshore saline water or in freshwater, relatively less in the coastal brackish water. The coastal environment is relatively fluctuating in terms of salinity and a mixing environment included suspended particle from both freshwater as well as marine water, should have an influence on these two fractions, unlike the other stable environments. Thus, it is also necessary to differentiate these two groups for better assessment of the spatiotemporal variations and influences by the adjacent habitats in bacterial diversity and community structure of the coastal environments.

1.7 Bacterial habitability

A primary question in environmental microbiology is how bacteria adapt to diversified environments and expand or stay in their possible habitats. Although the

community structure at one particular space and time offers an important clue to understand those process, sole genetic data offers no meaningful information. If the information on the habitability, which is defined as the ability to inhabit in different environments**,** is available for each OTU or phylogenetic group in coastal environments, we may be able to infer whether the OTU or group may be indigenous one or brought from other environments. For example, the habitability of one particular OTU turns out to be terrestrial, the OUT is assumed to be originated from terrestrial environment and temporally staying or surviving in the coastal zone. The idea on the habitability has never been addressed in previous community structure analyses because there was no suitable way to infer habitability of prokaryotes or any living organisms based on genetic information. The recently developed database, MetaMetaDB (Yang and Iwasaki 2014) (http://mmdb.aori.u-tokyo.ac.jp/) has made it possible to check the habitability of prokaryotes. It contains data set of 16S rRNA sequences derived from 454 platforms in DDBJ Sequence Read Archive (DRA) and offers environmental categories that indicate in what kind of environments each sequence is obtained and recorded. It is noteworthy that the lack of a record doesn't mean the absence of the corresponding sequence, and that there may be some biases of habitable environments depending on the relative amount of deposited sequence data. Nevertheless, this can be a new tool to infer habitability of prokaryotic organisms in natural environments. Hiraoka et al. (2015) checked the habitabilities of two soil prokaryotic communities by MetaMetaDB and showed that the soil affected by the tsunami in 2011 tend to contain more sequences of marine habitat compared with the one unaffected. It is assumed that this approach may be particularly useful for coastal microbial populations because they may originate from various sources.

1.8 Objectives and outlines of this study

The study was contacted with the aim to clarify the mechanism controlling bacterial community structure in the coastal marine environments. Following research questions were raised.

1. How does the bacterial community structure fluctuate due to spatiotemporal fluctuations?
2. To what extent do the adjacent environments influence the coastal community?
3. How do the bacteria respond to different organic matter?

So, the objectives of this study were as follows-

1. To appraise the spatiotemporal fluctuations in bacterial abundance and community structure of the coastal marine environments with special emphasis on three major classes, the Flavobacteriia, Alphaproteobacteria and Gammaproteobacteria.
2. To evaluate the mechanisms adopted by these three major classes to maintain their populations and assess the similarity between two closely located sampling stations and between PA and FL fractions.
3. To identify any predominant and unique members, and evaluate the biodiversity (species richness and evenness) of the bacterial community giving emphasis on these three major classes.
4. To assess the formation of community structure and possible influences by the adjacent habitats to the bacterial community composition of the coastal marine environments in Japan.
5. To evaluate bacterial response to different organic matter for the assessment of their life-style and nutritional preferences.

The following hypotheses were made for this investigation.

1. Three major phylogenetic groups, Flavobacteriia, Alphaproteobacteria, and Gammaproteobacteria have different adaptation mechanisms by which each group is continuously present in marine environments.
2. The differences in their adaptation mechanisms may appear by repeated samplings and community structure analyses at two closely located sampling stations in coastal environments.
3. Some OTUs belonging to these three phylogenetic groups appear consistently, whereas some minor groups appear at a limited time and/or space. Some of the OTUs/ groups are appeared as unique to certain environment or condition.
4. The community structure formation processes and possible influences by the adjacent habitats may be partly explained by the habitability analyses.
5. Certain phylogenetic groups tend to associate with particles and use polymeric substances in the sea, while other as the free-living state and prefer mostly the monomeric dissolved matter. Such characters can be confirmed by laboratory culture experiments with the addition of different organic monomeric and polymeric compounds.

In order to verify these hypotheses, following investigations were made, which were described into 5 chapters including General Introduction (Chapter 1) and Discussion (Chapter 5). Chapter 2 was for the hypotheses 1~3. The fluctuation patterns of bacterial community structures in coastal marine environments were clarified with special emphasis on the three major phylogenetic groups, i.e., Flavobacteriia, Alphaproteobacteria and Gammaproteobacteria. Considering local and seasonal fluctuations, 5 times seawater samplings were conducted at two closely located coastal stations, the port side and the sea side, of Oarai, Ibaraki, Japan. Bacterial samples were

separated into the particle-associated (PA) and free-living (FL) fractions to see the possible differences. Prokaryotic community structures were analyzed by next generation sequencing (454 GS Junior). Some indices such as species richness or diversity index were calculated for comparisons among the three major phylogenetic groups.

Chapter 3 was to prove the 4th hypothesis and the "habitability" of OTUs obtained in the previous chapter was examined. Habitability is defined as the ability to inhabit in different environments. Recently developed database, MetaMetaDB (Yang and Iwasaki 2014) (http://mmdb.aori.u-tokyo.ac.jp/) is the database that systematically combines 16S rRNA sequences data from 46 different database. MetaMetaDB has made it possible to search in which environments a particular 16S rRNA sequence is present. Series of sampling points were targeted from river, brackish, coastal port and shore, and offshore environments.

Chapter 4 was for the 5th hypothesis. It was expected that different organic material stimulate the growth of different phylogenetic group. Also, monomers and their comparable polymers also show different growth patterns because not all microorganisms possess degradation enzymes. Laboratory experiments were conducted with seawater collected from coastal environment (Oarai, Ibaraki Prefecture) or offshore environment (Kuroshio Current area, North station-NBD and South station-SBD of the Pacific). As monomers, glucose, glutamic acid and N-acetylglucosamine (NAGA), and as polymers, starch, Bovine Serum Albumin (BSA), and chitin were added prior to incubation experiment.

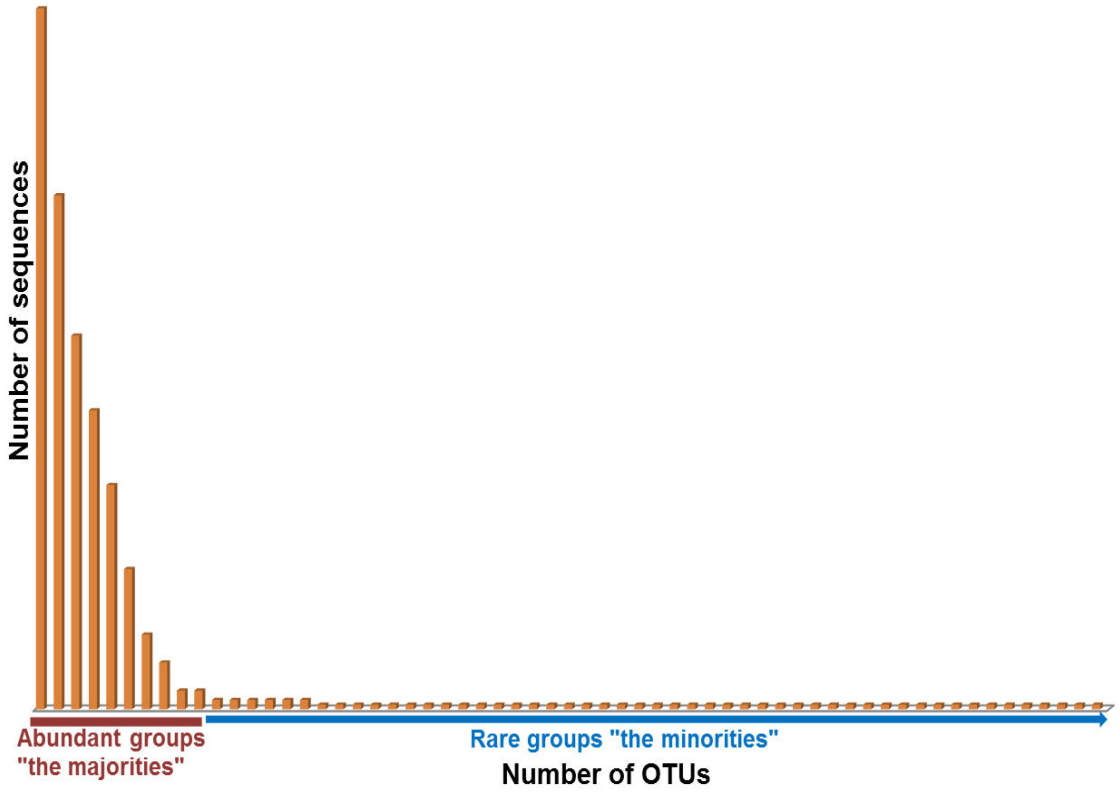

Figure 1-1. A typical taxa-abundance curve (Curtis et al. 2002) of prokaryotes, sowing the existence of abundant groups or "the majorities" with a huge diversity or a long tail of rare groups or "the minorities" in the environment.

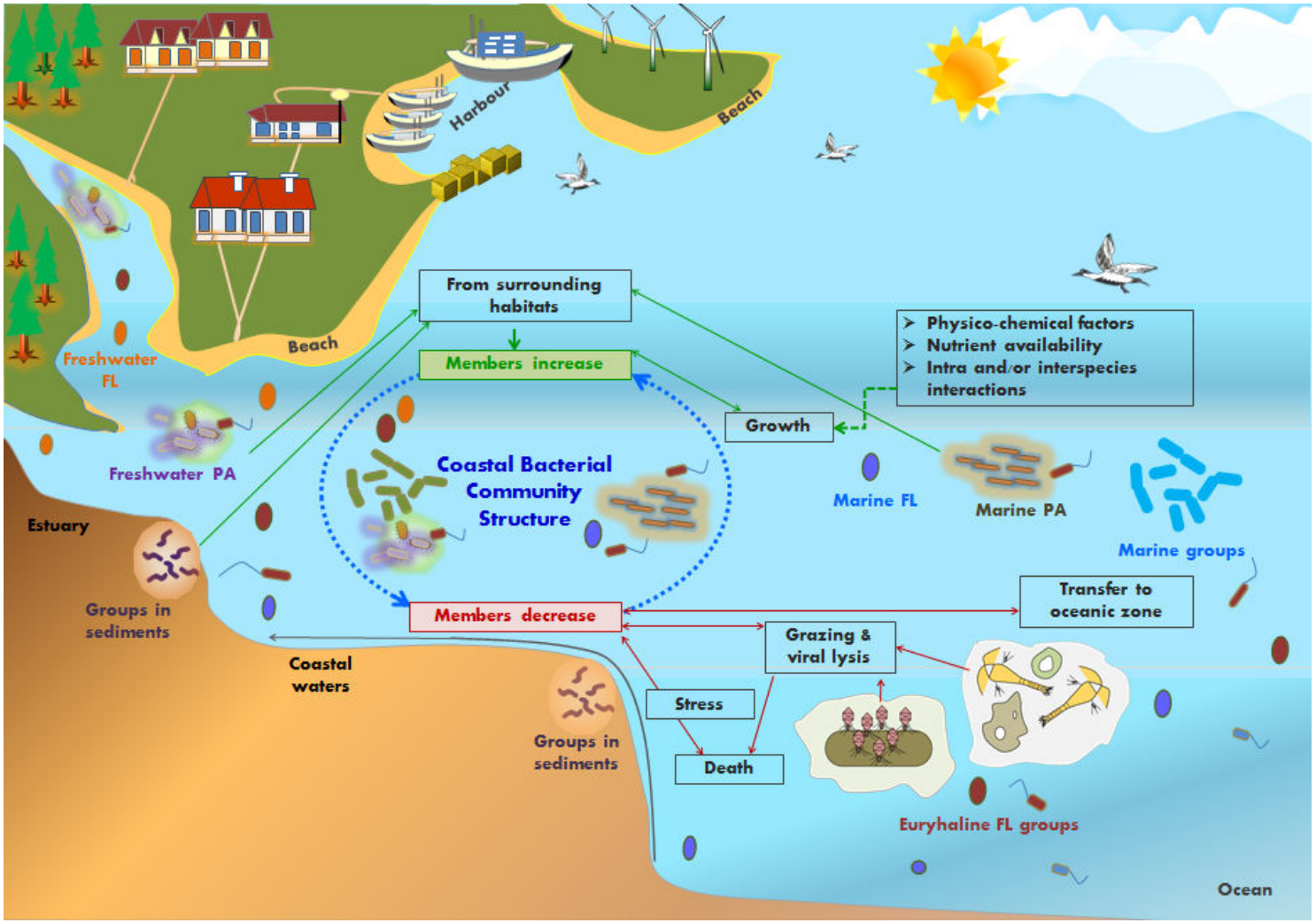

Figure 1-2. Schematic diagram showing the formation of bacterial community structure in coastal marine environments.

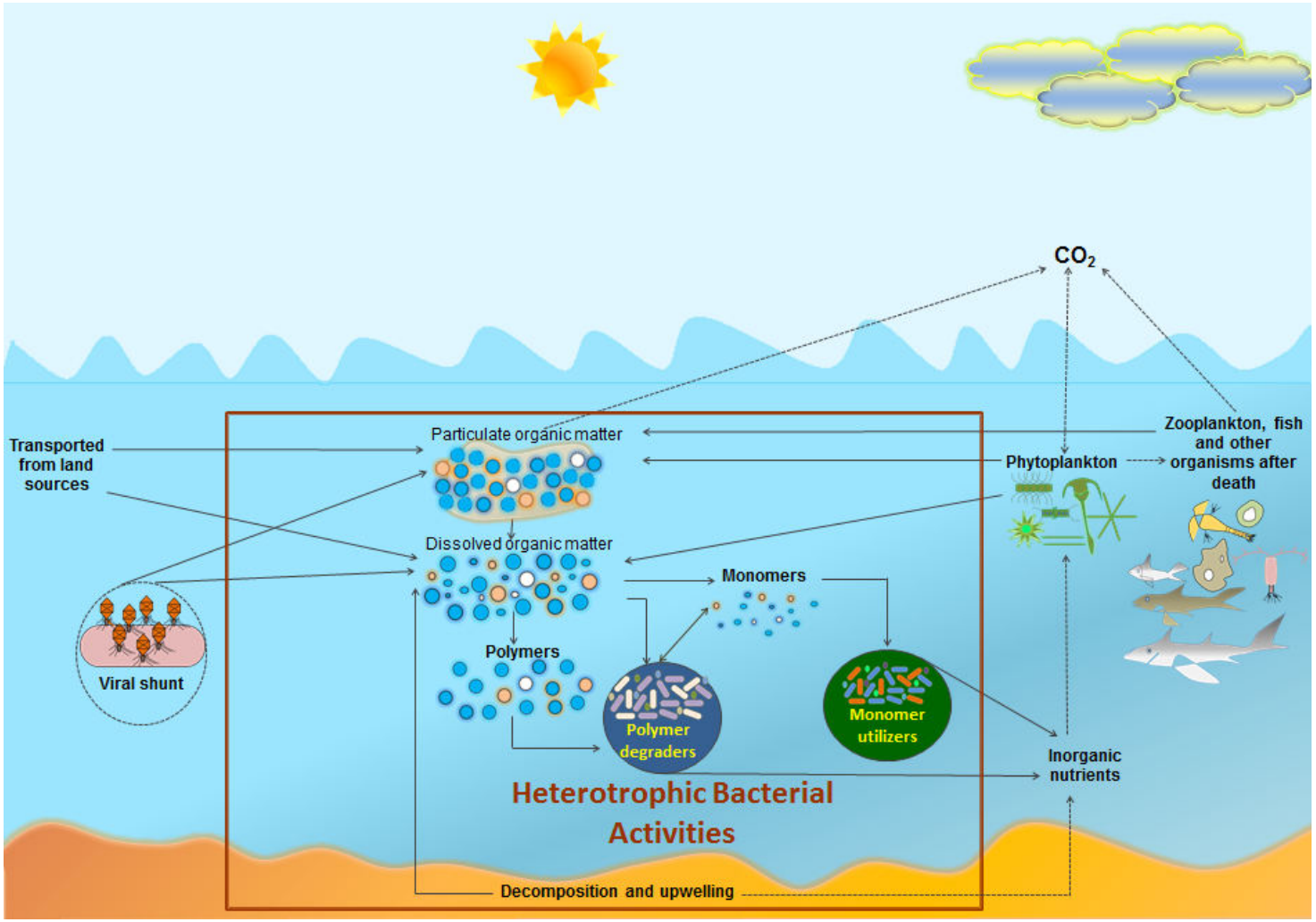

Figure 1-3. Schematic diagram showing the sources and flow of nutrients and utilization by different bacterial populations in the coastal environments.

CHAPTER 2:

Spatiotemporal Fluctuations of Bacterial Community Structure

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CHAPTER 3:

Habitability Analysis of Bacteria

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CHAPTER 4:

Bacterial Response to Organic Matter

Page 142 to 199 is removed due to copyright issue

CHAPTER 5:

General Discussion, Summary and Conclusion

5.1 Aims and major findings

The community structures of bacteria are formed depending on various factors, i.e., physicochemical ones such as temperature, light, pressure, water current, tide, solar radiation, salinity, quantity and quality of inorganic and organic compounds, pH and biological ones such as density, interaction among microorganism and with higher organisms, competition, predation, symbiosis, relative growth and death rates, and so on. Although it is thus complicated, community structures are basic information when understanding microbial roles and also environmental characteristics.

The aims of this investigation were to evaluate the mechanisms controlling bacterial community structure in the coastal habitat, one of the most fluctuating marine environments. Specific attention was given to three major phylogenetic groups, Flavobacteriia, Alphaproteobacteria and Gammaproteobacteria, and samplings were repeated at two closely located points in Oarai coastal area. Furthermore, habitability and utilization pattern of different organic materials were investigated. Bacteria were chosen because, compared with Archaea, much more information on the community structure, phylogeny, physiology and functions is available and suitable for this research.

The major findings of different studies included in this thesis can be summarized as follows-

1. At phylum level, Bacteroidetes and Proteobacteria and at class level Flavobacteriia, Alphaproteobacteria, and Gammaproteobacteria were consistently dominant, contributing about 62 to 98% of the total populations (Figure 2-5).
2. Average contributions of Flavobacteriia and Alphaproteobacteria to the total were similar, about 27 to 35%, while that of Gammaproteobacteria was about 15 to 17% (Figure 2-5). Average numbers of OTUs of Flavobacteriia were higher than those of Gammaproteobacteria while average numbers of genera, families, and orders of Flavobacteriia were lower than those of Gammaproteobacteria (Figure

2-13). The species richness of Flavobacteriia, Alphaproteobacteria, and Gammaproteobacteria were similar, while evenness of Flavobacteriia was significantly higher than those of two classes (Figure 2-12).

3. The apparent difference of the community structures between the two stations became more apparent at lower phylogenetic levels (Figures 2-5, 2-7 to 2-8 and 2-10). Some locations specific OTUs or genera appeared at one of the two stations and most of them are members of the Gammaproteobacteria (Table 2-7). Some condition-specific unique groups were also identified (Table 2-8). Except in some cases, there were no consistent differences between PA and FL fractions (Figure 2-5 and 2-22) although, the percentage of overlapping OTUs between the fractions was about 13.2% in the port side and about 16.5% in the sea side station (Figure 2-23).
4. Habitability analyses of seasonally obtained phylotypes, at both the 97% (species) and 85% (order) level of identity showed that most of the phylotypes are assigned to "marine", indicating the identified bacterial groups are mostly distributed to marine environments and their distribution is driven by the salinity. At 85% (order) level of identity, OTUs from the non-marine sources contributed about 24.6% to 58.5% of the total at the port side station and about 17.4% to 41.2% of the total at the sea side station, indicating the port side station is subjected more influences by the freshwater, terrestrial and anthropogenic activities.
5. Habitability analyses also showed that the members belonging to Flavobacteriia were composed of multiple groups assigned to different environments (Figure 3-2 and Table 3-5 to 3-9). Alpha- and Gammaproteobacteria did not clearly indicate the co-occurrence of members of different habitabilities. MetaMetaDB did not show any distinct difference between the PA and FL fractions.

6. Culture experiments showed that members in Gammaproteobacteria always react quickly and utilize the monomeric substances (Figure 4-14 to 4-18, 4-20 to 4-21, 4-23 to 4-26 and Table 4-4). Some of the members in Gammaproteobacteria may also involve in degradations of certain polymeric substances (Figure 4-24 to 4-26). Members in Alphaproteobacteria show similar tendency utilizing monomeric substances but react at slower mode than the members in Gammaproteobacteria (Figure 4-14 to 4-16, 4-20, 4-24 and 4-26). However, the tendency varied according to the type of organic substances. Members in Flavobacteriia showed a minimum but steady growth without any quick response like the members in Alphaproteobacteria and Gammaproteobacteria and maintained relatively higher abundance in the polymer treated tanks (Figure 4-24).

5.2 Spatiotemporal fluctuations of bacterial community structure

Since the introduction of NGS for the microbial community structure analyses in aquatic environments (Sogin et al. 2006), relatively few investigations were conducted in western Pacific and Asian area (Du et al. 2013). This research is one of the few such works. The results showed that Alphaproteobacteria, Gammaproteobacteria, and Flavobacteriia constantly shared a major part of the communities, regardless of the sampling points and time. This is consistent with former works in other environments (Treusch et al. 2009, Anderson et al. 2010, Gilbert et al. 2012). However, if the community structures are analyzed at the lower phylogenetic level, this is not always the case. This research shows that even at two very closely related environments, the community structures can differ considerably. In addition, the appearance of "unique groups", which was found in one of the two areas in most of the samplings or at a particular sampling period, show the presence of sensitive groups that can be regarded as potential indicators of subtle differences in environmental changes (Tables 2-7 and 2-8). Some genera such as *Hellea* and *Nerida* were identified from Mediterranean Sea

(Alain et al. 2008, Lekunberri et al. 2014). Although extensive comparative works are required, the presence of such a rare or unique group may indicate some characteristics of the environment, and help clarify how unique community structures are formed (Lee et al. 2006, Peeler et al. 2006).

Among the three classes, Flavobacteriia, Alphaproteobacteria, and Gammaproteobacteria, Flavobacteriia showed higher numbers of OTUs and also significantly higher evenness. This indicates that Flavobacteriia is composed of many OTUs with relatively constant and few individual numbers. Although further works are necessary, this may be consistent with the followings. First, there may not be fast growers in the class Flavobacteriia, and if any, those are quite minor. Second, each OTU may be utilizing organic matter supplied to the environments rather steadily. Third, the members may have relatively high tolerance to environmental change and/or defense mechanisms against predators. This makes them possible to expand their distribution and maintain their populations even if the condition is not perfectly favorable for them. In contrast, Alphaproteobacteria and Gammaproteobacteria may include fast grower that are rather sensitive to environmental conditions.

5.3. Habitability analysis of bacteria

In order to verify the possibilities stated above and assess the possible adaptation and influences by the adjacent habitats, the habitability analyses were conducted for bacterial populations in Oarai coastal areas, Naka River, and offshore environments by using the MetaMetaDB (Yang and Iwasaki, 2014). To my knowledge, this research is the first case to apply the data in coastal environments. Although simple sequence data doesn't offer any information on their distribution or origin, this database enables us to find out the habitats of a particular sequence by consulting multiple 16S rRNA sequence databases. The results show that sequences from lower saline stations were dominated by "freshwater and groundwater", "human", or "wastewater" while those from higher

saline stations were dominated by "marine" (Figure 3-8). These results indicate that the port side station is subjected to more influences by the freshwater, terrestrial and anthropogenic port activities. The sea side station is subjected to more influences by the offshore oceanic waters, which may result higher salinity compare to port side (Table 2-1). So, the salinity may determine the relative proportion between the marine and non-marine OTUs of any coastal water. These types of evaluation to assess the possible influences by the adjacent habitats have never been conducted before.

The MetaMetaDB also indicate that although the class Flavobacteriia were present at all the stations (Figure 3-2), members with different habitabilities are coexisting (Table 3-5 to 3-9). A considerable percentage of phylotypes were assigned to "sediments-soil", "wastewaters", "plants-roots" and "fish" at 85% level of identification. This suggests that the members of class Flavobacteriia are specifically present and maintained in particular locations. The present result is consistent with the previous studies showing the wide distribution of class Flavobacteriia in both freshwater and marine habitats (Glockner et al. 1999, Kirchman 2002, Amaral-Zettler et al. 2010, Kirchman 2012). It may also partly explain the reasons of higher evenness of Flavobacteriia compare to others. Alphaproteobacteria and Gammaproteobacteria, however, did not clearly indicate the presence of populations of different habitabilities.

Because MetaMetaDB is still a newly developed database, some precautions are necessary for the interpretation. First, it depends on the data previously deposited to related database. So, it is expected that data from extreme environments may be much less compared with environments that are easily accessible. Therefore, the lack of apparent habitat information does not necessarily indicate the absence of the particular OTU in the habitat. Second, the quality and reliability of MetaMetaDB depends on the primary data deposited. Ambiguous description of original data leads to the less reliable examinations. With all these possible drawbacks, however, it is evident that

MetaMetaDB offers new information on the habitability, which is otherwise not available from simple sequence data.

5. 4. Bacterial response to organic matter

Among various physicochemical and biological factors controlling bacterial growth (Madigan et al. 2009), the limiting factor is generally the availability of organic matter. It is expected that depending on the quantity and quality of organic matter, different bacterial group utilize them preferentially and increase their biomass (Kirchman 2008, Nagata 2008). The present culture experiments were conducted with the addition of three different sets of monomers and polymers. In addition, change of bacterial community structures was continuously monitored by NGS for several days. Although there have been similar culture experiments (e.g. Cottrell and Kirchman 2000, Elifantz et al. 2005), this works should be most extensive one, making it possible to compare the utilization patterns among different organic compounds and different bacterial phylogenetic groups simultaneously.

The ranges of utilizable organic compounds have been examined by other methods. Firstly, when new species or taxonomic groups are first described, the utilization ability of different organic compounds is usually considered. Therefore, general utilization patterns are accumulating for each phylogenetic group. Secondly, recent whole genome information shows the presence of genes for certain metabolic pathways that indicate the utilization of particular compounds. These two methods, however, show potential ability and do not offer data whether those cells actually utilized them in the environments. The third method is to combine micro-autoradiography and FISH (Cottrell and Kirchman 2000). In short, a radiolabelled compound is added to sample seawater, incubated and then filtered. Cells are transferred to the glass slide, probed with fluorescent oligonucleotide and coated with an autoradiographic film emulsion. This method enables the researcher to identify the cells taken up the added radiolabelled

compound at single cell level under the microscopy. However, the only broad phylogenetic grouping is possible because of the limited specificity range of probes for FISH. In addition, there is a possibility that slow utilizers may be below the detection level. Among them, the culture experiment enables us to assess the influences by some controlling factors on the coastal community structure such as bacterial response to the qualitative and quantitative supply of organic matter, growth rate, competition etc. It is, however, noteworthy that the culture cannot completely mimic the natural condition. For instance, before the incubation, seawater samples were filtrated to remove the predators, typically flagellates to see the growth of each phylogenetic group. Viruses are still in the medium. Also, some cautions are needed for the case of polymer utilization. From the apparent growth pattern, it is not completely clear which groups are polymer degraders and which are monomer utilizers. Nevertheless, marked differences in the growth pattern among the members in Flavobacteriia, Alphaproteobacteria, and Gammaproteobacteria strongly indicate differences in their responses to organic compounds.

5.5. Future research

Although present investigation offered some new information on how community structures are formed in marine environments, there still remain many questions and works. In order to further progress the research in this field, followings will be required. First, it is still needed to clarify the microbial community structure in various environments using the latest techniques. Our knowledge in western Pacific and Asian areas is especially limited. There may be unknown or unique groups of microorganisms in such environments. Statistical and bioinformatics analyses of the data together with environmental parameters will show more details how the community structures are controlled by the environment. Second, it is already evident that major phylogenetic groups are present everywhere in marine environments. However, there seem to be

some minor groups that are unique to small scale environments. Integrated knowledge of such minor groups around the world may clarify the unique characteristics of those groups and also the environments. Third, although recent molecular techniques are quite a powerful tool, there are limitations of those techniques. Approach for culturing target microorganism for taxonomical, genetic and physiological analyses will be critical to evaluate the data obtained by molecular techniques. Finally, culture experiments that mimic environmental conditions should be a very effective approach, especially for observing the effect of particular environmental effects or looking at particular biological processes. In any case, it will be important to obtain all the possible data using various newly available techniques for extensive comparative works.

5. 5. Conclusion

In order to clarify how microbial communities are formed and maintained in aquatic environments, investigations with special reference to three classes, Flavobacteriia, Alphaproteobacteria, Gammaproteobacteria were conducted. By combining three approaches, i.e., field observations of community structures of bacteria at two closely located coastal environments, habitability analysis and laboratory culture experiments with different monomers and polymers were taken into considerations for comparative works among these three phylogenetic groups. The three groups were Flavobacteriia, Alphaproteobacteria, Gammaproteobacteria appeared constantly as the three most dominant classes, whereas much more variations were noted at the family or genus level assessments. The distribution of each family or genus is generally variable in terms of space and time, and there are some groups unique to the local environmental condition. The communities in PA and FL did not show clear differences. Flavobacteriia is characterized by high evenness and diversification into many OTUs specific to environments. They are probably polymer degraders. Gammaproteobacteria are generally quickly responding mostly to monomers as well as polymers. Compared

with members in Flavobacteriia, those in Alphaproteobacteria usually react to environmental change quickly. However, compared with Gammaproteobacteria, there are more polymer degraders in Alphaproteobacteria and steadily distribute in the environment.

So, like the other marine habitat coastal environments are predominated by the members of the class Flavobacteriia, Alphaproteobacteria, and Gammaproteobacteria. The similarities between the fractions (PA and FL) indicating the exchange of populations between them is rather quick in the coastal waters compare to other habitats. The members of the Flavobacteriia are relatively stable, probably has higher adaptability and protection against adverse situations, which make them more even. Their preferences to some polymers indicate they may prefer the particle-associated life style. In contrast, the members of the Gammaproteobacteria are more sensitive, fluctuating and probably has less protective mechanisms, which make them uneven and less abundant compare to the Flavobacteriia and Alphaproteobacteria, although, they are efficient utilizers of nutrients and fast growers. Their preferences especially to the monomers indicate they may prefer free-living state. In Alphaproteobacteria, the members are medium growers; more stable compare to the Gammaproteobacteria but less compare to the Flavobacteriia.

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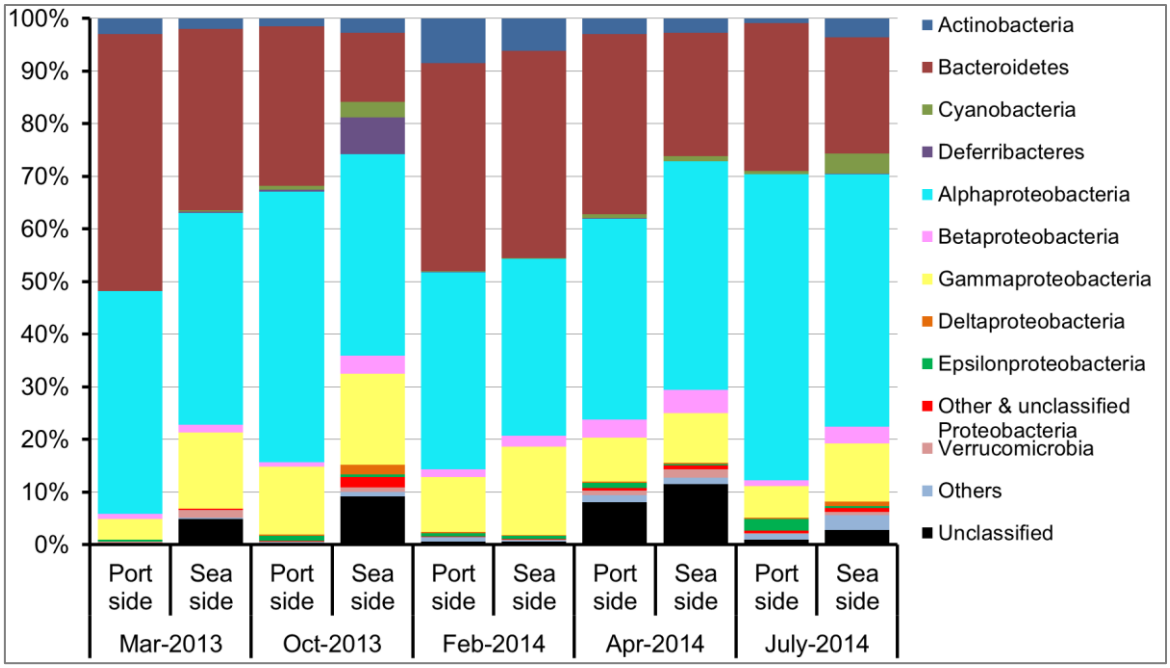
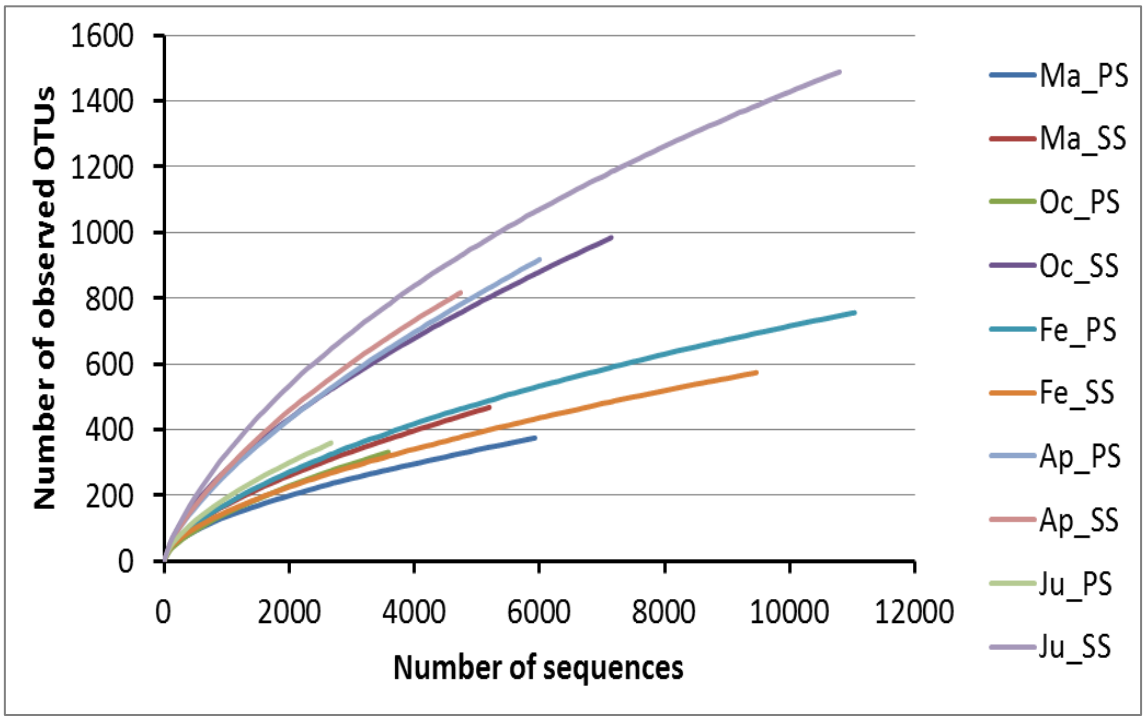
Appendices

Appendix 1. Total bacterial community structure of the port side and sea side stations.



For total community, seawater samples were filtered directly through 0.2 µm pore sized Sterivex filter units. The obtained samples were processed and sequenced as the same methods described in Chapter 2. The groups "Others" referred to the sum of those phyla did not individually contributed 1% of the sequences in at least one sample and "Unclassified" were the unidentified/unknown members.

Appendix 2. Rarefaction curves of the total bacterial community structure showing the number of observed OTUs at 0.03 cut-off levels for different samples.



For total community, seawater samples were filtered directly through 0.2 µm pore sized Sterivex filter units. The first and the second part of the sample IDs' is expressing the sampling periods (Ma=March, Oc= October, Fe= February, Ap= April and Ju= July), and the sampling stations (PS= port side, SS= sea side) respectively.

Appendix 3. ANOVA: single factor test for the species richness (Chao index) among the three major classes, the Flavobacteriia, Alphaproteobacteria, and Gammaproteobacteria.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Flavobacteriia	20	8092.769	404.6384	15265.61
Alphaproteobacteria	20	6697.278	334.8639	11487.84
Gammaproteobacteria	20	6244.826	312.2413	31687.47

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	92783.35	2	46391.67	2.381466	0.101552	3.158843
Within Groups	1110377	57	19480.3			
Total	1203161	59				

Appendix 4. ANOVA: single factor test for the richness-evenness (inv Simpson index) among the three major classes, the Flavobacteriia, Alphaproteobacteria, and Gammaproteobacteria.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Flavobacteriia	20	544.5443	27.22721	297.9005
Alphaproteobacteria	20	244.6542	12.23271	142.3619
Gammaproteobacteria	20	343.2469	17.16235	398.0108

ANOVA

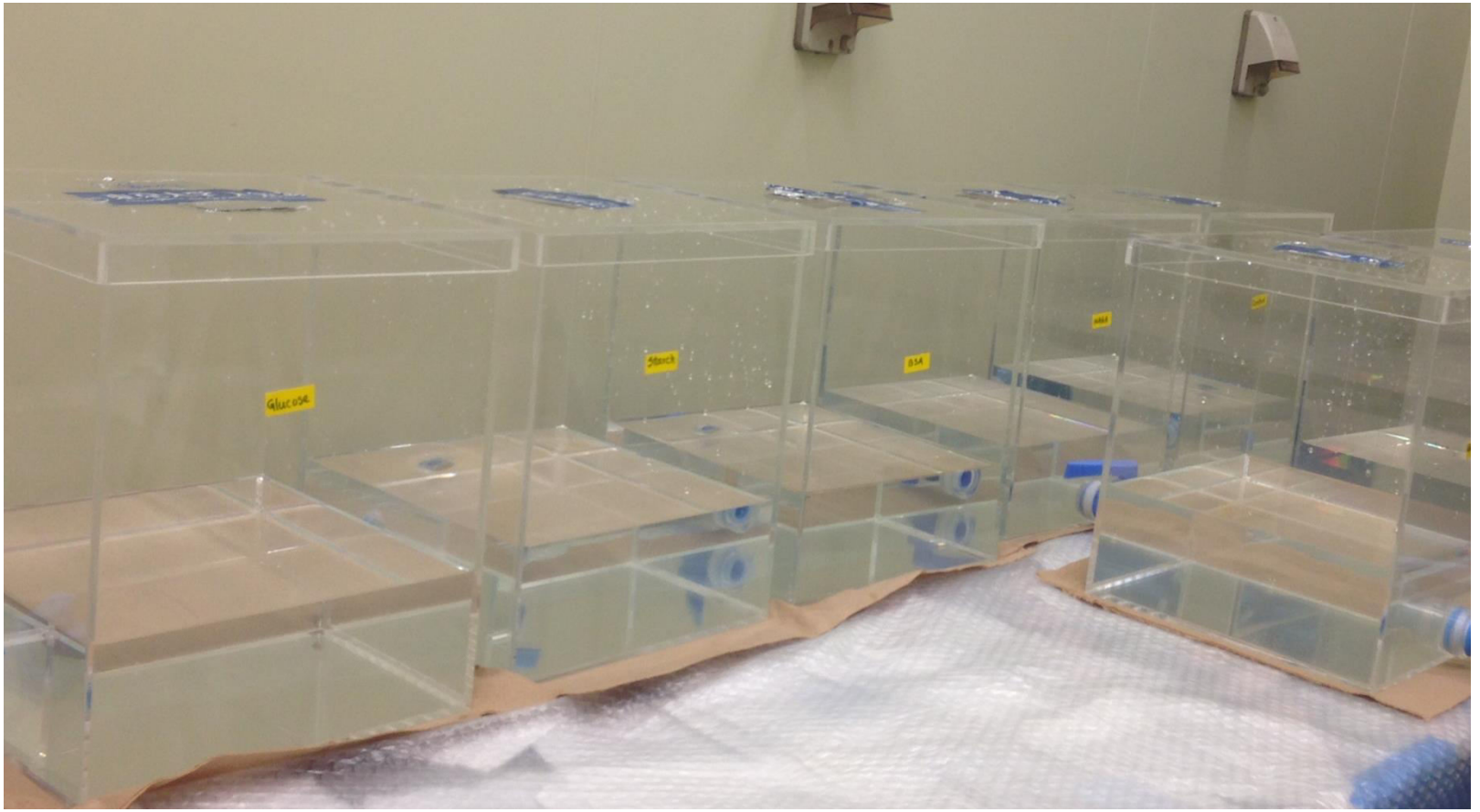
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2336.254	2	1168.127	4.180475	0.020224	3.158843
Within Groups	15927.19	57	279.4244			
Total	18263.44	59				

Appendix 5. Photos of the tanks used for culture experiments.

A: empty

E

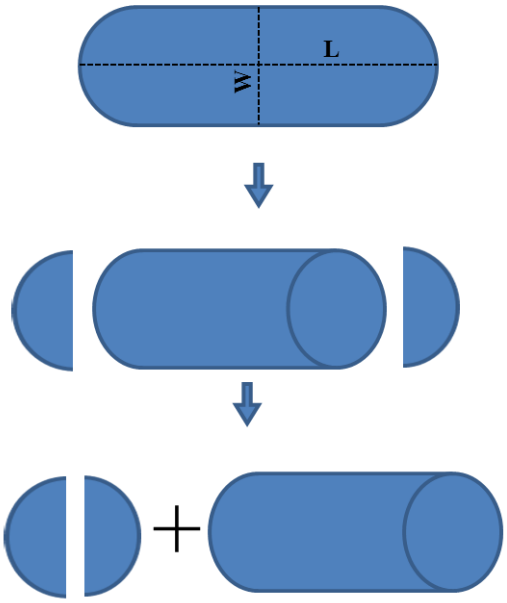
Appendix 5. (continued)



B: with seawater

F

Appendix 6. Calculation of the cell volume. The length and width was measured by using Atomic Force Microscopy (AFM).

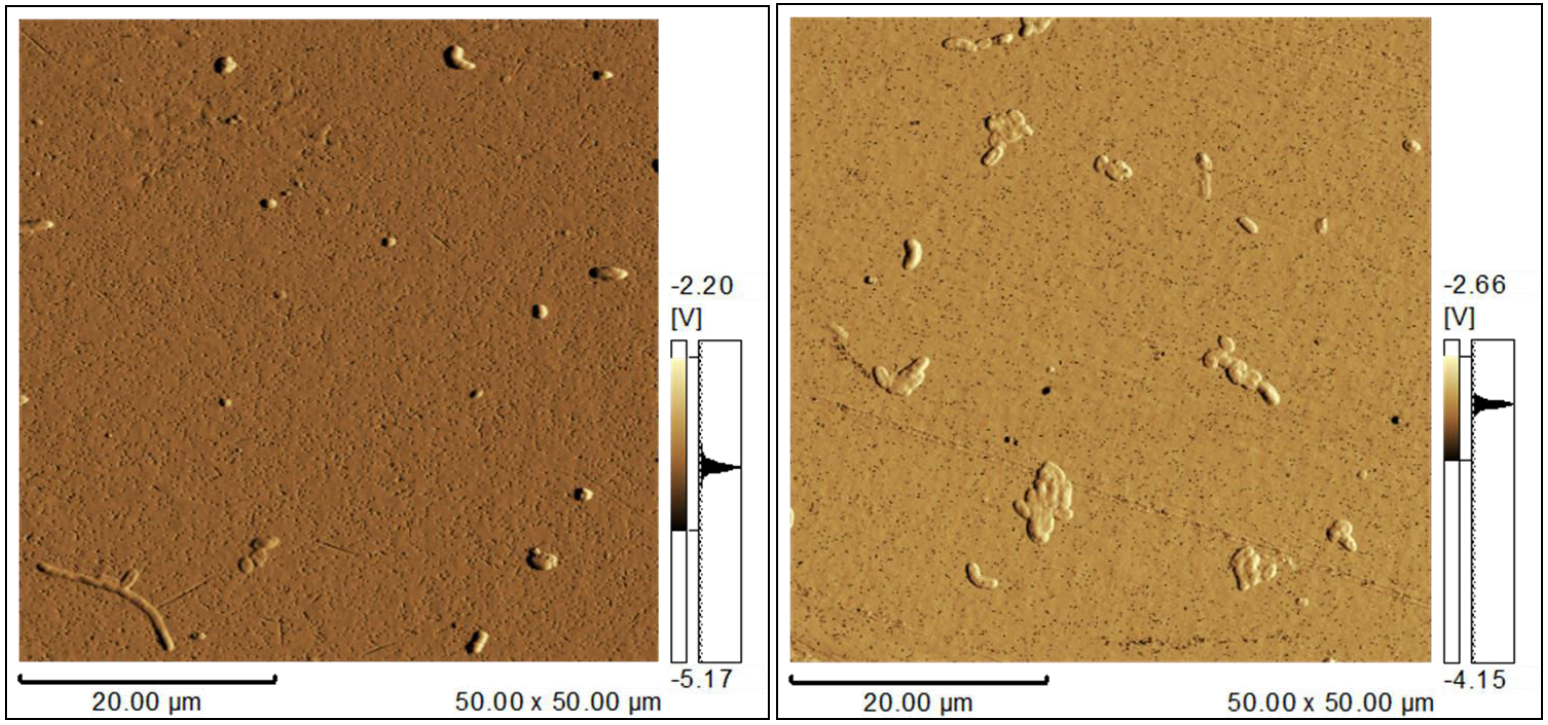


Volume of a circle = $4/3\pi r^3$ + Volume of a cylinder = $\pi r^2 H$

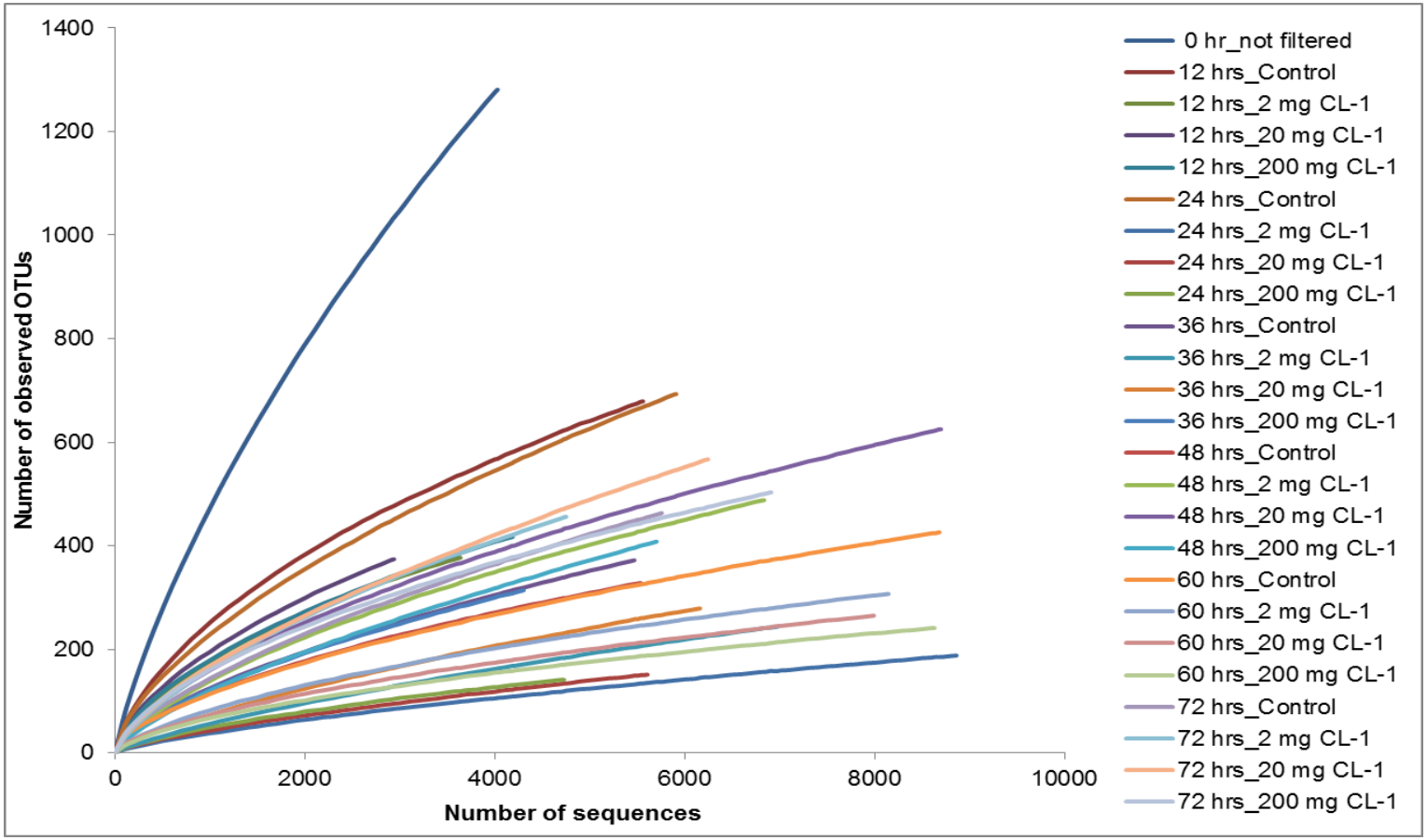
Cell volume = $\{4 \times \pi \times (W/2)^3/3\} + \{\pi \times (W/2)^2 \times (L-W)\}$

Here, $\pi = 3.14$, W = cell width, L= cell length, r = radius = W/2, H = L-W

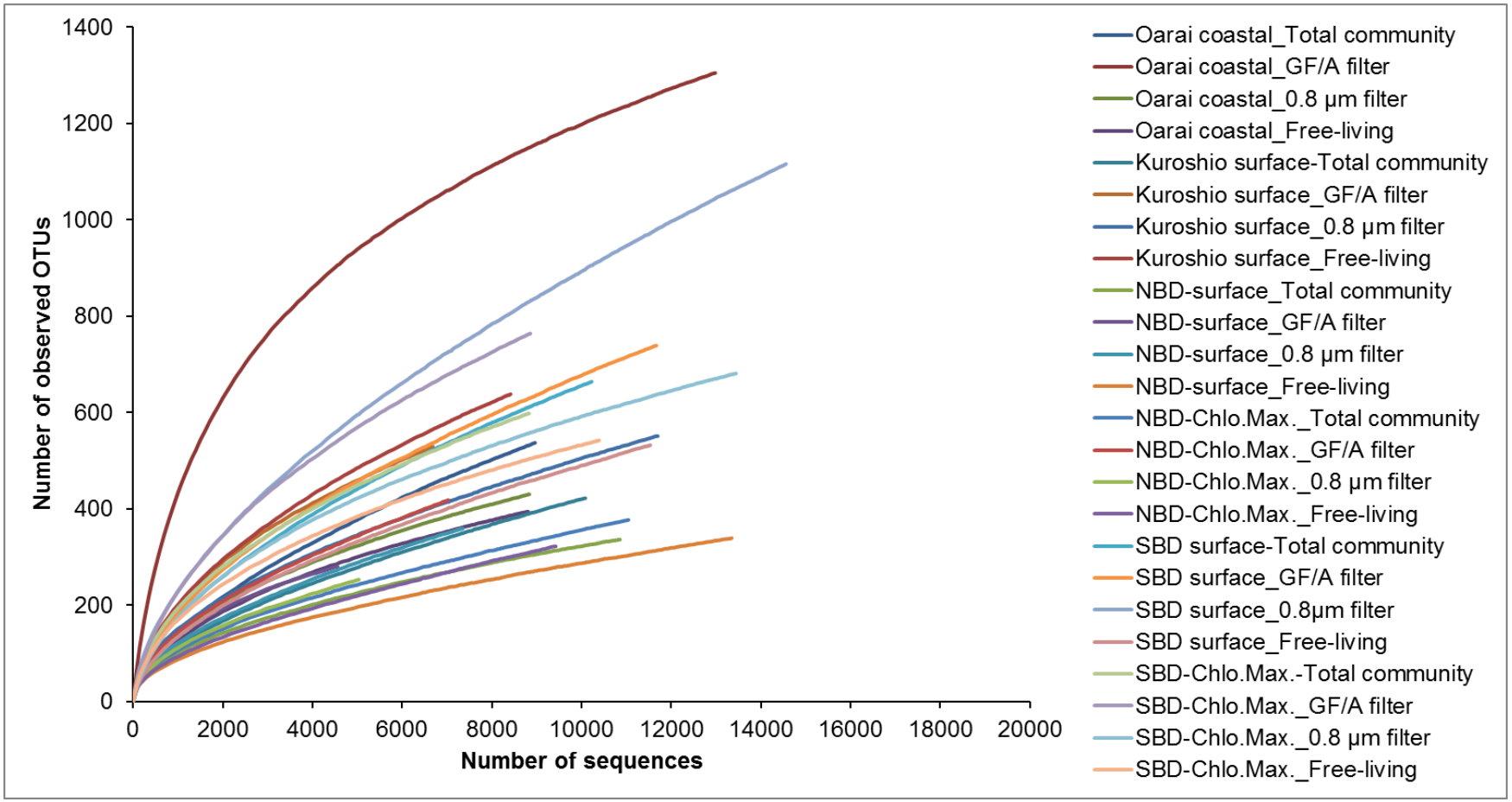
Appendix 7. Bacterial cells imaged by using atomic force microscopy (AFM) A. control and B. glutamic acid treated tanks after 36 hours of treatments.



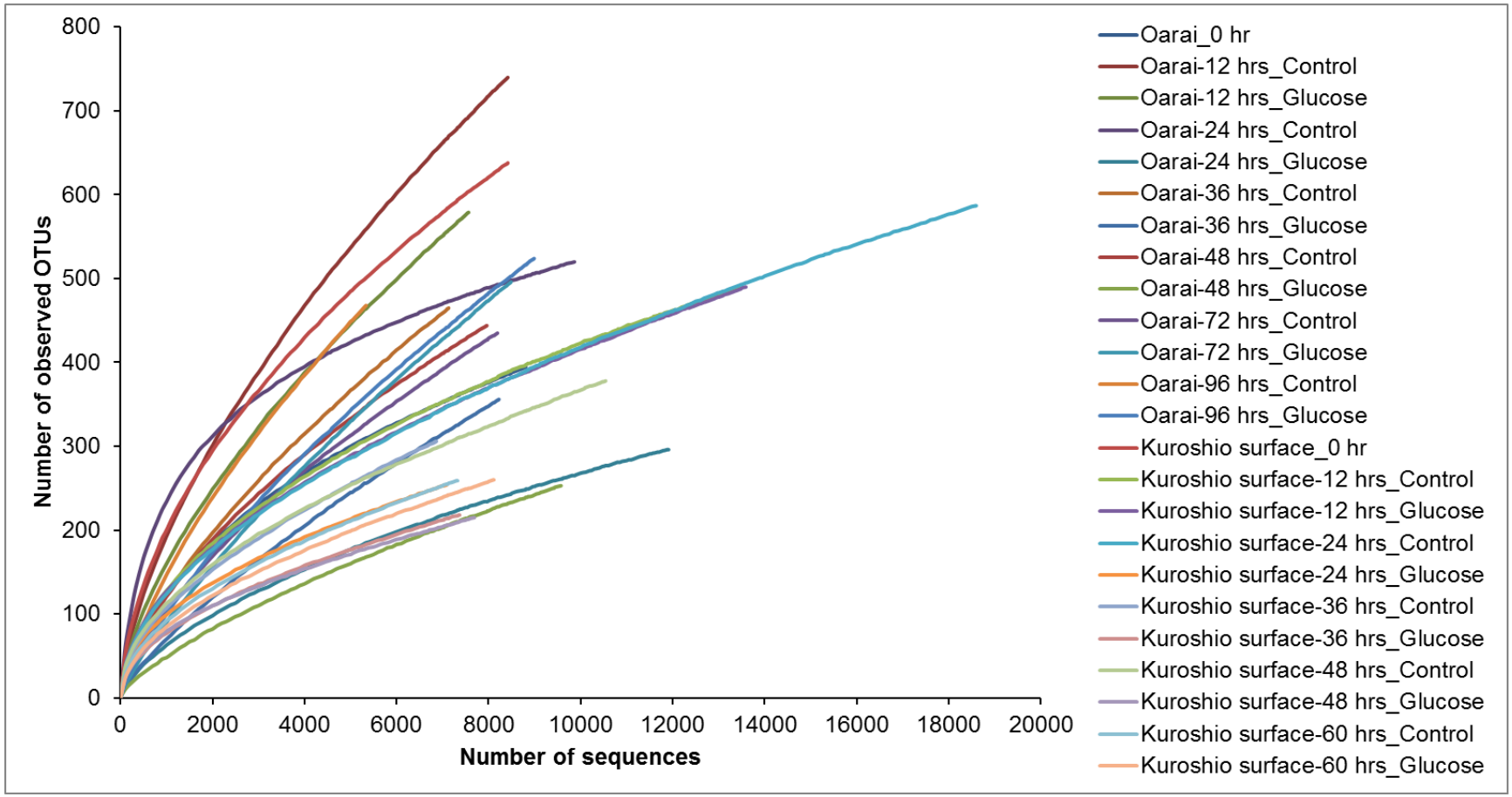
Appendix 8. Rarefaction curves of the set 1 culture experiment showing the number of observed OTUs at 0.03 cut-off levels.



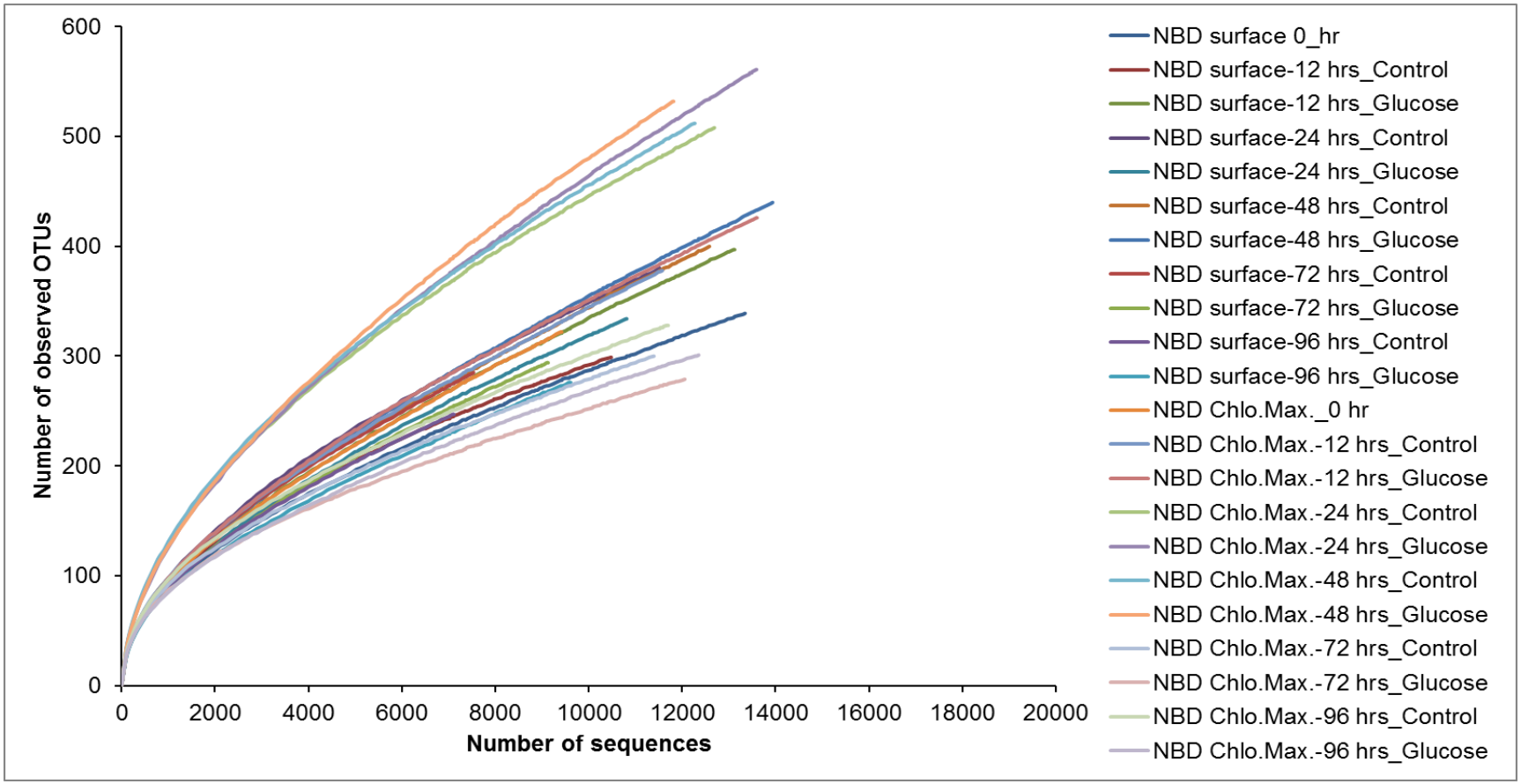
Appendix 9. Rarefaction curves of the total, particle-associated and free-living community structures of water samples from different locations used in the set 2 culture experiments showing the number of observed OTUs at 0.03 cut-off levels.



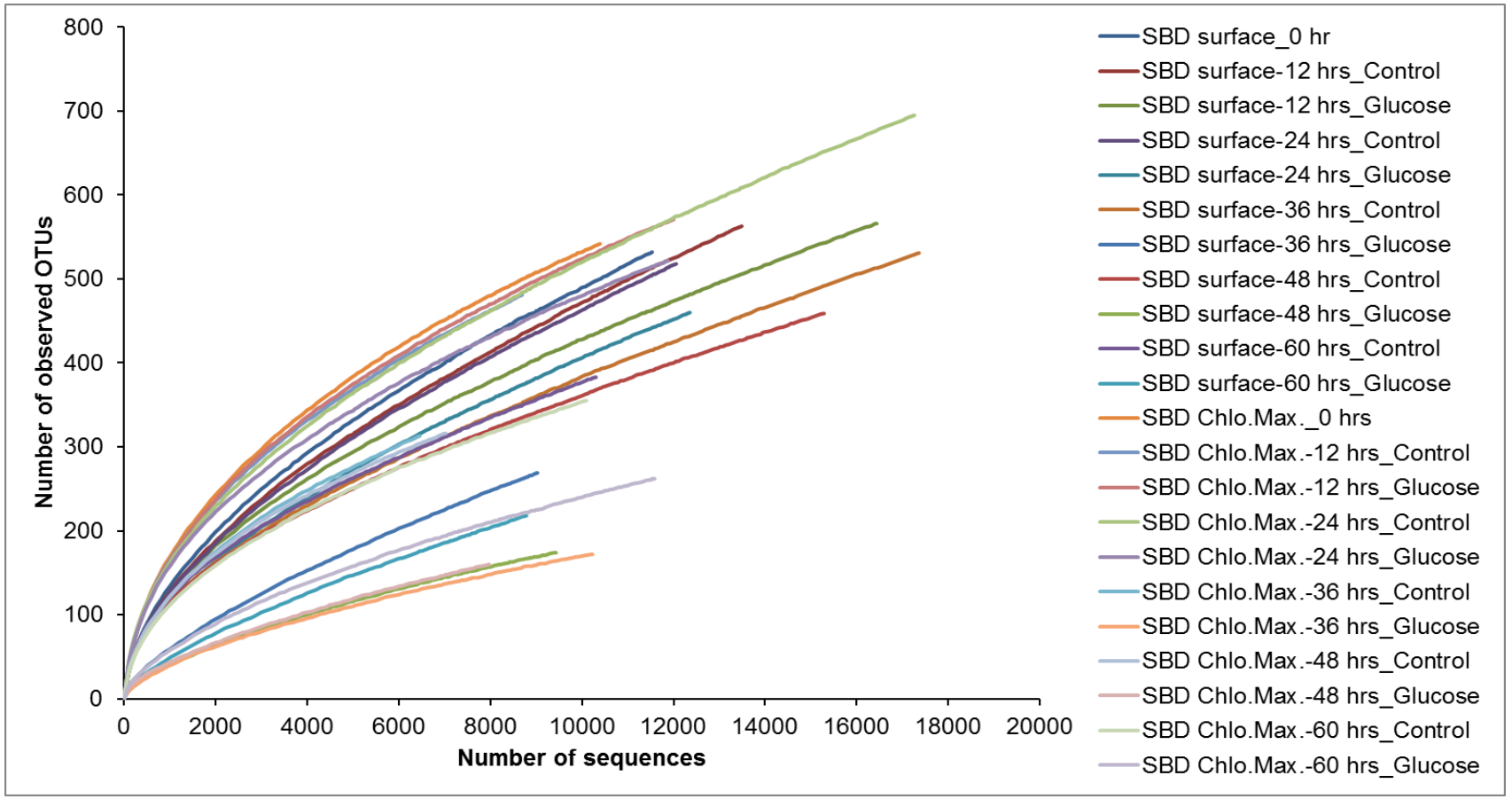
Appendix 10. Rarefaction curves of the Oarai coastal and Kuroshio Current water samples used the set 2 culture experiments showing the number of observed OTUs at 0.03 cut-off levels.



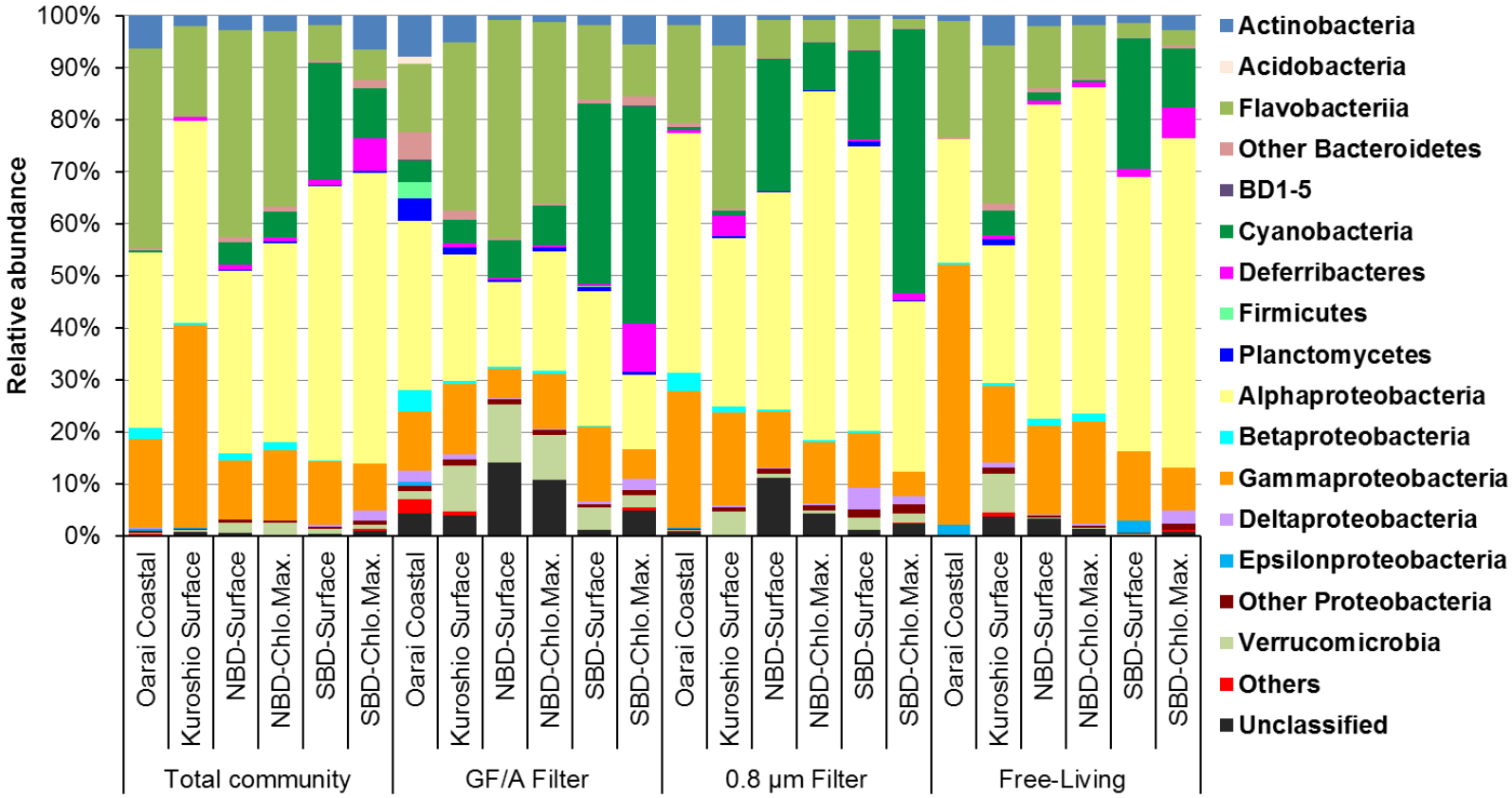
Appendix 11. Rarefaction curves for the seawater samples of NBD used in the set 2 culture experiments showing the number of observed OTUs at 0.03 cut-off levels.



Appendix 12. Rarefaction curves for the seawater samples of SBD used in the set 2 culture experiments showing the number of observed OTUs at 0.03 cut-off levels.

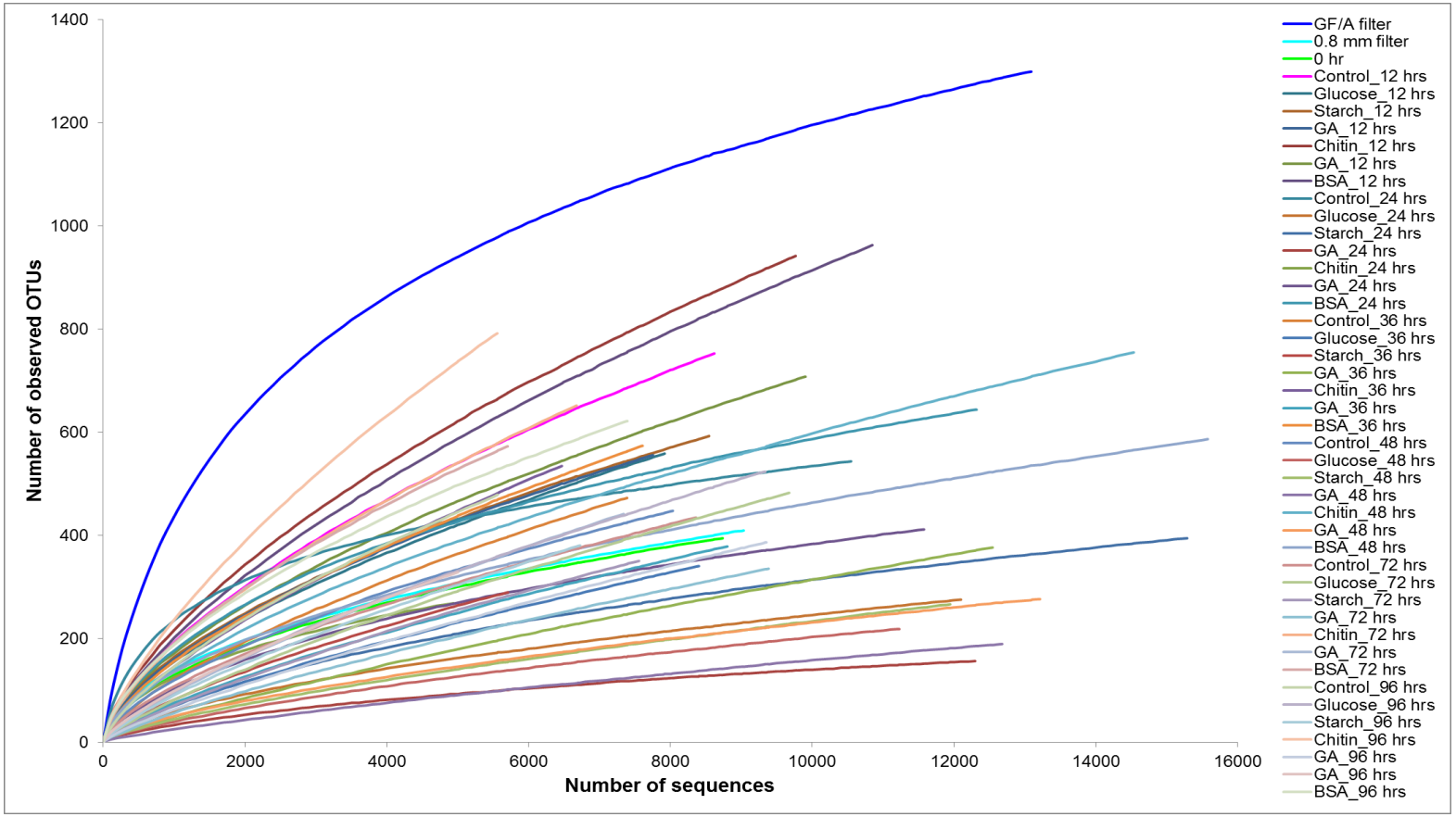


Appendix 13. Pretreatment bacterial community structures of different fractions of seawater samples used in the set 2 culture experiments.



Total, obtained after direct filtration of the water with 0.22 µm pore sized Sterivex filter; particle-associated, obtained after filtration with both 1.6 µm pore sized GF/A and 0.8 µm pore sized membrane filters; and free-living, obtained after filtration of the pre-filtered (removed particle-associated groups as mentioned above) water with 0.22 µm pore sized Sterivex filter

Appendix 14. Rarefaction curves of the set 3 culture experiment showing indicating the number of observed OTUs at 0.03 cut-off levels.



Culture experiment was conducted by treating the filtered seawater with some monomers e.g. glucose, N-acetyle glucosamine (NAGA) and glutamic acid (GA) and, polymers e.g. starch, chitin and bovine serum albumin (BSA) considering 20 mg of carbon per liter according to the Table 4-3.

O

Appendix 15. Categories of the microbial habitats obtained after combining different similar types of microbial habitats classified in the MetaMetaDB.

Categories of habitats used	Classification of microbial habitats in the MetaMetaDB
Fish	fish
Freshwater-groundwater	freshwater, groundwater, hot springs
Human	human, human_gut, human_lung, human_oral, human_skin
Marine	aquatic, beach_sand, coral, hydrothermal_vent, hypersaline_lake, marine, marine_sediment
Oil production facilities	oil_production _facility
Plants-roots	phyllosphere, rhizosphere, root
Sediments -soil	fossil, sediment, soil
Wastewaters	wastewater
Others	ant_fungus_garden, bioreactors, bioreactor_ sludge, bovine_gut, compost, epibiont, food, food_fermentation, gut, ice, mouse_gut , pig