Study on Aquatic Coastal Ecosystem Management in Tropical and Temperate Areas Using Microbiological Function Measurement Approaches

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Abstract

Coastal zone management involves managing coastal areas to balance environmental, economic, human health, and activities. Throughout the world, coastal zone has experienced environmental degradation and pollution. This problem is more insidious and difficult to solve because coastal zones were historically seen as having an essentially infinite capacity to dilute waste from human activities. Estuaries and coastal waters are also suffering from increasing eutrophication and turbidity, harmful algae bloom, fisheries collapse, and decreasing of biodiversity. At the same-time coastal waters are increasingly polluted and impacted by hydrocarbons from low-level, chronic oil spills as well as occasional and often catastrophic oil spills. Some of these coastal waters are also showing signs of impacts by climate change. Nevertheless, the management had an effective performance, but far from a success. Continuous research on coastal environmental changes by intensive anthropogenic activities is still needed to bridge the gaps between science and management for sustainable coastal ecosystem development.

This study proposed aquatic coastal ecosystem management in tropical and temperate zone by using microbiological function measurement approaches which support the various functions of coastal zone. I will conduct detailed investigative research on each function to collect information that contributes to coastal ecosystem management and use the obtained results to propose comprehensive management of coastal areas and lead to sustainable maintenance of coastal areas. This analytical approach supports interdisciplinary aspects for coastal planning, and indicates that the use of suitable model

of microbiological functions are useful for discriminating environmental potential among sites, but they are inadequate as predictors for long-term sustainability.

The mangrove forest has established the most important coastal ecosystem in the tropical coastal area. Mangroves are recognized as highly productive ecosystems that provide organic matter and shelters to adjacent coastal ecosystems. However, mangrove habitats were often nutrient limited, particularly for combined nitrogen, while nitrogen fixation may be an important nitrogen input to these ecosystems because of its potential to provide nitrogen in usable form to plants. The qualitative and quantitative understanding of nitrogen cycle in the forest and ecological function evaluated by microbial community structure. So, microbial function measurement is important for maintenance and management of mangrove forest. For tropical coastal zone, present study was focusing on the determination of nitrogen cycle in rhizospheric sediment in the Republic of the Union of Myanmar mangrove forest.

The microbial diversity, functional genes abundance of nitrogen-fixation (*nifH*) and hydrocarbon degradation bacteria (*alkB*) genes in mangrove sediments were studied by using molecular assessment technique. The nitrogen fixation activity in mangrove sediments were measured by nitrogenease enzyme activities and functional gene (*nifH*) abundance. Next-generation sequencing (NGS) was used to determine the microbial diversities in mangrove sediments. Quantitative real-time PCR (qPCR) analysis was used to examine the abundance of *nifH* and *alkB* genes in the sediments. The highest nitrogen fixation rate, *nifH* and *alkB* genes copy number were observed in Site 3. According to

Pearson's correlation analysis revealed that nitrogen fixation rate was strongly correlated with the abundance of *nifH* gene in all sites. And the abundance of *nifH* and *alkB* genes in sediment depth profile were correlated among three sites. NGS statistical data analyses were applied to evaluate species richness, the number of OTUs, and the difference occurring between the communities from the four different depths in each site. Microbial diversities in sediments depth profile of Site 2 and 1 had similar structure but Site 3 was different. The highest microbial ß- diversity index was shown in Site 3, followed by Site 1 and Site 2. The microbial community in each site showed higher diversity in shallow depth and that is decreased with depth. Bacterial phylum Proteobacteria, such as Deltaproteobacteria, Gammapreoteobacteria, Alphaproteobacteria and Betaproterobacteria were more prevalent in all sites. The bacterial composition shifted according to the depth and the affiliation of some groups suggests wide-scale participation in key nutrient cycles. These results are linked to investigate the functional microbial communities which involved in key processes of nitrogen cycle in mangrove sediments. This study demonstrates the current state of knowledges in microbial diversity of mangrove sediment and illustrates the important contributions of these microorganisms make the productivity of mangrove ecosystems.

With growing needs of humans' daily life, more and more crude oil has been explored and exploited worldwide. However, releases of crude oil, due to spills from ship tankers, offshore platforms, and drilling wells, are now threatening the marine and coastal ecosystems. Temperate conditions impose many challenges for oil spill response where

is low temperatures and extended periods of darkness in the winter. In the temperate zone, the study focused on investigating the seasonal changes of crude oil degradation efficiencies in simulated petroleum-polluted seawater. Seawater samples were collected from two stations in Tokyo Bay seasonally, and were made into petroleum-polluted seawater by adding the mixture of n-alkanes in fixed concentration in the laboratory. A 28 days of bioremediation incubation experiment was carried out with and without additional nutrients supplement in each season, and the remain hydrocarbons during incubations were measured by GC/MS. *alkB* gene, the functional gene responsible for alkane degradation, was also recorded throughout the incubation. The results showed that bioremediation efficiency of petroleum hydrocarbon was the highest in summer, followed by spring, autumn, and winter respectively. Alkanes with as much as 34 carbon atoms can be significantly degraded within 28 days of incubation in summer, and in contrast, no obvious degradation can be recognized in winter regardless of the number of carbon atoms. N-alkanes degradation efficiency was generally associated with, but not necessarily correlated to the copy number of *alkB* gene. This study provides an integrated insight into the effect of temperature on the bioremediation of petroleum hydrocarbons in different molecular weights.

This study is in the tropical coastal ecosystems (nitrogen cycle in mangrove forest) and the temperate coastal ecosystem (biostimulation in oil spill) by microbiological function measurement. It showed that the structure and activity of microbial communities that are greatly depending on the difference of environmental factors and changes.

Because of the microbial community structure and this functions play an important role in the maintenance of coastal ecosystem. These study results clearly demonstrate the management options that necessary for sustainable use of coastal ecosystems. And the research methods will contribute to future coastal ecosystem assessment. However, in the supplement of global exploratory research, it is necessary to carry out continuous research and analysis by evaluation. So, these facts will be supported by the management and sustainable development of the coastal ecosystem.

In the near future, a lot of microbiological function measurement will be replaced by more large-scale for environmental management. And this would absolutely contribute to assessing the conservation of regional scale base on tropical and temperate areas. Further research on management valuation in selected sites is needed to complete this global scale explorative research. In sum, we should learn more about microbiological function measurements, it is apparent that ignoring these benefits is detrimental to coastal management and planning.

Keywords: Mangrove, Nitrogenase, *nifH* gene, Proteobacteria, Crenarchaeota, Bioremediation, Biostimulation, Petroleum pollution, hydrocarbon degradation, *alkB* gene

Contents

Abbreviations

alkB genes	hydrocarbon degradation functional genes
ARA	acetylene reduction assay
CK	control blank without nutrients treatment
DAPI	4',6' diamino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DO	dissolved oxygen
EC	electrical conductivity
EDTA	ethylene diamine tetra-acetic acid
FAO	food and agriculture organization
FID	flame ionization detector
GC	gas chromatography
GC/MS	gas chromatography mass spectrometry
n-alkane	normal straight – chain compound (Aliphatic hydrocarbon)
NGS	next generation sequencing
NT	nutrients treatment
nifH gene	nitrogenase reductase subunit H
n-hexane	saturated aliphatic hydrocarbon
qPCR	quantitative polymerase chain reaction
TPH	total petroleum hydrocarbon

Chapter 1: General introduction

1.1. Management and development of marine and coastal environment

1.1.1. Ecosystem in tropical coastal regions

In tropical regions, so many varieties of habitats composed with mangrove, seagrass, coral, salt marsh, seaweed forest, etc. And many species are including marine mammals to microorganisms and that were interacted with each other as well a variety of habitat types. In the coast of the tropical region is indented by a number of rivers which form estuaries at their confluence with the sea. The estuary is an integral part of the coastal environment. They are outfall region of the river, making transitional one between the fluvial and marine environs. They are also effective nutrient traps and provide a vital source of natural resources to human and are used for commercial, industrial and recreational purposes. Sometime these areas were partially filled with effluents by the municipal sewage, industrial wastes and ballast water and oil film from boat and ship activities (Barbier *et al*. 2011). The complex estuarine ecosystems comprise of mangroves, mud flat, sand dune and salt marsh. Base on the tidal regime, salinity structure, sedimentation process and monsoon effects are strongly influence on the ecosystem of estuarine and coastal regions.

In estuarine and coastal region, one kind of unique ecosystem is mangrove habitat which is a significant feature in the tropical region because they have only appeared in tropical belt within 30˚ North and South Latitude. About 60 % of mangroves forests are found in South East Asia region, however, unfortunately most are seriously degraded

(Islam and Wahab 2005). Approximately 55 species were recorded in South East Asia countries, among them 34 species were found in the Republic of the Union of Myanmar. Some of the mangrove forests is grown in salt influence channel and coastal edge but some are found in freshwater influencing river-estuary. Mangrove ecosystem is slightly different with the soil structure, tidal regime and local climatic condition. It has utilized that ecosystem as spawning, feeding and nursery ground.

Seagrass is also found in the tropical region mostly in Indo-Pacific region. They occurred in the intertidal and mid-tidal zones of shallow and sheltered localities of the sea, gulf, bays, backwater, and lagoon. About 60 species were recorded in the world. Among them, the estuarine and coastal region of Thailand, Malaysia and Indonesia are more abundant than other areas (Barbier *et al.* 2011). Seagrass soil is three times carbon sequestration than mangrove soil. Estuarine and marine microbes are available in seagrass plants and soil which are the influence on the soil nutrients, soil quality, water quality and carbon keeping (Desonie 2007).

Coral reefs form the most dynamic ecosystem providing shelter and nourishment to the thousands of marine flora and fauna. So many forms of coral reef types (atoll, fringing, and barrier) are recorded in the tropical region. So many crevasse and reef type are form as a shelter for fish and other crustacean and some behave as nursery grounds. Most of the microbes were found in the coral body as symbiotic system mainly supporting to coral health and nourishment.

In the estuarine and coastal areas grown salt marsh plants which ecosystem behave

as the unique habitat for freshwater species and marine species. Saltmarsh is also found in the tropical coastal region especially situated in coastal line and estuarine which are comprised with muddy substrate. Most saltmarsh communities prefer somewhat muddy areas that gradually slope towards the sea and river-estuarine with the marked tidal effect of complete submergence during high tide and successive exposure during low tide. The substrate of the saltmarsh is always wet and composes of dead plant's parts. Salt marsh soils are enrichments in nitrogen and carbon contents.

1.1.2. Ecosystem in temperate coastal regions

In geography, temperate of the earth is in the middle latitudes, which span between the tropics and the polar regions. These zones generally have wider temperature ranges throughout the year and more distinct seasonal changes compared to tropical climates, where such variation is often small. In some climate classification, the temperate zone often divided into several smaller climate zones, based on monthly temperature, the coldest month, and rainfall (Bailey 1983). These include humid subtropical climate, mediterranean climate, oceanic and continental climate. The vast majority of the world's population resides in temperate zones, especially in the northern hemisphere due to its greater mass of land.

Temperate marine ecosystems include a wide array of habitats. There are estuarine, marsh, seagrass and kelp beds, rocky coastlines, sandy, muddy and cobble shores, the deep sea and the open ocean. Temperate seas are plentiful in life and have played a central role in sources of food for humans. Changes to atmospheric and oceanographic condition,

including increased temperature, ocean acidification, sea level rise and altered ocean currents, which may affect on the temperate marine ecosystem.

The richest temperate flora (some 24,000 taxa) is found in southern Africa (Richardson *et al.* 2005). The significant feature of the temperate ecosystem is Kelp forests which exist worldwide throughout the temperate to the polar coastal ocean. Physically formed by brown macro-algae, kelp forests provide a unique, three-dimensional habitat for marine organisms (Steneck *et al*. 2002). Bacteria living in kelp forest contribute to the important process which is utilizing kelp-derived substances to form the basis for the food web and make kelp primary production available to large organisms.

1.1.3. Impact of various sectors on the coastal environment

The coastal environments are a hazard by various threats in related to specific human activities as results from poorly managed activities (Figure 1.1).

Land use and human populations

60 % of the world's human population live in the coastal area, within about 100 kilometers of the shore. About 3.5 billion people rely heavily on coastal and marine ecosystems (Nicholls and Small 2002). This proportion is expected to increase, along with growing urbanization, industrialization, and transportation, putting even greater pressure on the living and non-living resources of the coastal area (e.g. off-shore airports, wind-energy parks, land reclamation, etc.).

Coastal industries and constructions

Industrial development has altered, disturbed, and destroyed coastal ecosystems. Main industrial activities affecting on the coastal areas that included metal smelting and processing, chemical, petrochemical (oil and gas storage and refining), shipbuilding, power plants (coal, oil gas, nuclear energy), and food processing (including fish). The construction of artificial islands is now well developed in Japan and Southern North Sea, for instance in the Netherlands for the installation of a future airport. Changes to the shoreline have been extensive in recent decades. Especially, threats from rising sea levels and sinking landmasses have required new strategies to be developed (Barbier *et al.* 2011).

Dredging and dumping at sea

Dredging mainly causes physical disturbance and may result in the redistribution of contamination (eg. detritus and organic pollutants) through release from the sediment. The bulk of material eligible for dumping at sea comes from dredging operations from navigation channels, material removed in coastal engineering projects, beach nourishment, reclamation and coastal marsh preservation. It can contribute to eutrophication in naturally nutrient-rich coastal waters.

Groundwater discharge into the coastal zone

Groundwater flows directly into the ocean where a coastal aquifer connected to the sea. Furthermore, artesian aquifers can extend for considerable distances from shore, underneath the continental shelf (Lindeboom 2002). In some cases, these deeper aquifers may have fractures or other breaches in the overlying confining layers, allowing

groundwater to flow into the sea.

Recreation and tourism

Coastal areas provide recreation opportunities for local people and for tourists (Lim and McAleer 2005). Tourism cause pressures on coastal ecosystems by an excessive influx of visitors. Most often, tourism will disturb and threaten local populations and wildlife and their habitats, which attracted them to the area in the first instance.

Coastal hazards

The coastlines of many countries face high risks of damage from certain types of natural disasters. Along with many densely populated coastlines, the risks of natural disasters are being increased by population growth and unmanaged development projects, including residential urban development (Lindeboom 2002). Tsunamis are quite a different phenomenon and that are associated with subsea earth movements. However, their speed and height can cause extensive coastal destruction (Nicholls and Small 2002).

Threats to biodiversity

The composition and structure of the fauna, flora, and habitats of coastal seas have been changing at an unusual rate in the last few decades. Due to changes in the global climate, invasive species and an increase in human activities. The Convention on Biological Diversity (1992) to refer, there are many causes to losses of marine biodiversity, especially in the coastal waters of industrialized countries (Secretariat *et al.* 1992). Poor fisheries management and another important aspect of the detrimental exploitation of marine living resources are due to the lack of an integrated approach to coastal zone

management, and there are leading to impoverished functioning.

1.2. Management and sustainable development of mangrove ecosystem

1.2.1. Important of nitrogen fixation in mangrove ecosystem

Mangrove represents unique and ecologically important coastal habitats throughout the tropical and subtropical which is occupying around 180,000 km^2 around the world. Chapman (1940), Davis (1940) and MacNae (1969) defined mangrove as a general term applied to plants which live in muddy, loose, wet soils in tropical tidal waters. Mangroves are trees or shrubs that grow between the high-water mark of spring tides and a level close to but above mean a level. They are circum-tropical on sheltered shores and often grow along the banks of rivers as far inland as the tide penetrates. Mangrove swamps supply both organic and inorganic material to adjacent coastal waters and related biotopes (Kathiresan and Bingham 2001). Despite their high production rates, mangrove habitats are often nutrient limited, particularly for combined nitrogen (Ryther and Dunstan 1971). Nitrogen fixation has a physiologically and phylogenetically diverse microbial function, and it is considered the major source of combined nitrogen input in mangrove forest (Zhang *et al.* 2008) (Figure 1.2). High rates of nitrogen fixation are associated with mangrove bark, decaying mangrove leaves, pneumatophores roots, and mangrove rhizosphere soil (Zuberer and Silver 1978, 1979), as assessed by nitrogenase activity-based methods.

1.2.2. Nitrogen fixation functional gene (*nifH* gene)

Nitrogen-fixing bacteria play the vital role in the biological conversion of dinitrogen (N_2) gas into ammonia (NH_3). *nifH* is a highly conserved functional gene for nitrogen-fixing microorganisms (Coelho *et al.* 2009; Liu *et al.* 2012; Man-Aharonovich *et al.* 2007) and it's a useful marker in molecular studies. Because it can provide evidence for potential nitrogen fixation in diverse terrestrial and marine environments (Coelho *et al*. 2009). *nifH* gene has sufficient variation to detect shifts in the community structure of nitrogen-fixers in ecosystems under varying physicochemical characteristics and soil types (Bagwell *et al.* 2002; Pereira e-Silva *et al*. 2011), as each habitat selects compatible different groups of nitrogen-fixing organisms (Zehr *et al*. 2003).

1.2.3. Environmental factors affecting on nitrogen fixation rate

Nitrogen fixation may be limited by tropical environmental stressful conditions, such as high temperatures, drought and soil acidity. Mangrove ecosystems are clearly affected by a gradient of flooding frequency (Dini-Andreote *et al.* 2016), which is higher at the initial stages of succession. In particular salinity, as well as the initial nutrient input from marine origin, are likely to be the major determinants of the soil microbial assembly processes during salt marsh development. Reported by Buchan *et al*. (2003), the presence of marsh vegetation (mangrove) has also been suggested to constitute a strong driver of the microbial communities, which profit from the nutrients exuded by the plant roots and also act on the organic matter decomposition processes. Especially those associated with soil pH and other environmental variables also influence microbial distribution, such as

regional climate (Bhattacharya and Mahajan 2003; Maila *et al*. 2006), soil type, and physicochemical characteristics (Hamamura *et al*. 2006) and vegetation (Joner *et al*. 2001), Oxygen, electrolytic conductivity, and carbon, nitrogen, and sulphur contents have been reported to influence nitrogen fixation in soils (Hsu and Buckley 2009).

1.2.4. Management and development of mangrove ecosystem

Mangroves ecosystem is supporting wildlife and fisheries resources, supplying the range of commercial products and providing the number of ecological services (such as sewage wastewater treatment, and coastal protection). Saenger (2002) said that the mangrove ecosystems play an important role in not only for maintaining water quality in estuaries but also for protecting the shoreline from storm damage and erosion. Despite a greatly increased awareness of the environmental, and socio-economic importance of mangroves, that is continuous to be destroyed in many parts of the world. Due to urbanization, pollution, and aquaculture developments. So, a multidisciplinary approach towards their management is essential.

Reported by FAO (1994), mangrove ecosystem quantifiable targets that serve to focus management efforts and measure performance. Awareness about the ecological importance and threats to mangroves will be increased among the public, policy-makers, environmental officials and managers, NGOs, the scientific community, and international lending and development institutions, foundations and other funding organizations (Kingston 1995).

1.3. Coastal oil spill pollution and cleaning-up

1.3.1. Historical background of oil spill in Tokyo Bay

In Japan, the Nakhodka oil spill was occurred in 1997 which is the worst oil spill accident. On July 2, 1997, about 1500 m^3 of crude oil was released to the sea surface from the Japanese tanker Diamond Grace. After one day, the surface oil spread wide, covering the center of Tokyo Bay about 10 km × 12 km. In fact, on March 18, 2014, a collision accident occurred between a Korean ship and a Panama ship, heavy oil of the spill greatly damaged on marine products in Tokyo Bay (Asahi Shimbun 2014). Tokyo bay has expressed concern over potential environmental hazards from chemical oil absorbents. However, many ships in the cleanup operation have dumped chemical absorbents in the bay to neutralize of the oil slick. Although, these chemicals will affect fish and other elements of Tokyo Bay environment. Mostly, Tokyo Bay's shoreline is occupied by docks and heavy industrial complexes such as refineries and chemical plants but the bay is still a source of food. Therefore, a real-time forecasting model for oil spill spreading supports needed for response and clean-up operations (Figure 1.3).

1.3.2. Effects of oil pollution on the coastal marine environment

The marine environment is a dynamic and various network of habitats and species, interwoven by complex physical and ecological processes that interact with humans and their activities at many levels (IPIECA-IOGP 2015). Coastal industrial production and the shipping routes are the main source of oil pollution. According to the IPIECA-IOGP report (2015), mineral oils (i.e. petroleum) derive from plant material and animals that

originated millions of years ago and have been modified over time by heat and pressure underground. In many locations, these underground reservoirs of oil are connected to the surface by geological features such as faults or salt domes, and in some areas natural seeps occur through the seabed. Oil spill in the marine environment can be highly toxic to the marine microbial communities. However, a part of these communities resists to this type of pollution and are able to metabolize the pollutant.

1.3.3. Effects of oil pollution on socio-economic condition

Oil spills can happen on land or in water, at any time of the day or night, and in any weather conditions. When oils enter to the oceans from seeps, urban runoff or a spill, they become subject to a variety of processes collectively termed “weathering” (Prince *et al*. 2003). Almost all oils float, allowing the smallest molecules to evaporate (Betancourt *et al*. 2007) which are either photochemically degraded (Hamilton. 2003) or washed from the atmosphere in rain and then biodegraded (Arzayus *et al*. 2001). Especially, the development of oil exploration in polar and deep-water environments brings a number of challenges to oil spill response and science. In cold water ecosystems tend to be longer-lived and slower-growing than those from warmer climates and waters, and the rate of many biological processes is relatively slow. It is often assumed that recovery from oil spills will take longer, but this depends on many factors (Camus and Smit 2018).

1.3.4. Prevention and control of oil spill (emphasize on bioremediation)

Preventing spills is the best way to protect our health and the environment from exposure to oil. Bioremediation is a potentially important option for dealing with marine

oil spills. Bioremediation for marine oil spills can be approached in two different ways depending on the case (Figure 1.3). This includes bioaugmentation which involves the introducing of oil-degrading microorganisms to the affected site, and biostimulation which involves the adding of supplemental nutrients to the affected site to aid the existing oil-degrading microorganisms (Mahjoubi *et al*. 2018). Moreover, bioremediation is a new technology but the continuous research and investigating of the practicality and efficiency of this process are still needed (Prince. 2010).

1.3.5. Hydrocarbon degradation functional gene (*alkB* gene)

In marine system, the various chemical and microbiological aspects of petroleum oil and alkane biodegradation have been relatively well studied but there is a general lack of knowledge concerning the diversity or abundance of the functional genes involved. AlkB hydroxylases can only accommodate certain alkane chain-lengths, due to restrictions in the enzyme active site. Recombinant hosts for alkane hydroxylases can provide functional rubredoxin and rubredoxin reductase to evaluate the activity of novel *alkB* genes. Only 10 – 20 % of the normal hydroxylase activity was necessary for the recombinants to survive on alkanes. Testing *alkB* genes in these hosts allow for a mechanism to select for a certain alkane substrate range in cloned *alkB* genes and provides a better understanding of what residues are involved in substrate binding. In the 21st century, alkane degradation studies have been conducted on petroleum-degrading bacteria in molecular biology (Maeng *et al*. 1996; Rehm and Reiff 1981). Researches were carried out using molecular biological techniques such as PCR (Beilen *et al*. 2002; Smits *et al*. 1999; Vomberg and

Klinner 2000).

1.3.6. Environmental factors affecting bioremediation rate (emphasize on temperature)

Bioremediation is a complex process, whose quantitative and qualitative aspects depend on the nature and amount of the pollutant present, the ambient and seasonal environmental conditions, and the composition of the indigenous microbial community. Many contaminated sites are characterized by poor nutrient concentrations (i.e., low levels of nitrogen and phosphorus), elevated or low temperatures, and a diverse range of co-contaminants, such as heavy metals, which can influence the process of bioremediation by inhibiting the growth of the pollutant-degrading microorganisms. Indeed, bioremediation treatments are often designed to overcome these limitations which may contribute to environmental persistence. Especially, the temperature has a considerable effect on the ability of microorganisms to degrade hydrocarbons. At high temperatures, solubility, bioavailability, hydrocarbons distribution and diffusion rate increase, which promote the microbial biodegradation ability and enhance the biodegradation rate. On the other hand, very high temperature decreases oxygen solubility and limits the aerobic microbial biodegradation (Margesin and Schinner 2001). Of particular importance are pH, redox potential, the supply of oxygen, moisture, inorganic nutrients, cation-exchange capacity, pollutant bioavailability, and soil porosity (Atlas *et al*. 1981).

1.4. Objectives of this study

Problem addressed

The key environmental problems were facing the coastal and marine areas, which were related to habitat conversion and destruction. Coastal areas are heavily populated and exploited. There require intensive management and infrastructure to sustain coastal ecological systems.

Objectives and Hypothesis

The main objective of this study is to describe the microbiological function measurement of aquatic coastal zone management associated with environmental dynamics and microbial community interactions (Figure 1.5). Because some microbiological function-based approaches combined with the conservation system assessment would be a good solution to this, and large-scale investigation would also help to gain a better understanding of the aquatic coastal zone management plan. The microbiological function approaches based on site-study in tropical and laboratory test in temperate coastal regions, that have been able to restore the ecological processes of a healthy coastal environment and, as such, will reinstate the full beneficial functions of the aquatic coastal ecosystem. In the current research was studied by the two sides, 1; focusing on-site study of mangrove conservation and 2; focusing on laboratory test of oil spills bioremediation. For site study identified in tropical mangrove forest in the Republic of the Union of Myanmar and incubation test identified in temperate seawater of Tokyo Bay in Japan (Figure 1.4). Firstly, I want to check nutrient flow then I want to check how

microbial community responds to environmental pollution. However, to date only site study has used culture-independent molecular methods to examine the responsible microbial function of *nifH* and *alkB* genes in mangrove sediments, and in incubation test, the abundance of *alkB* genes were examined in polluted seawater by microcosm culture, a region where natural seeps are sources of widespread petroleum hydrocarbons. It was hypothesized that (i) mangrove sediment was acting as the major substrate for bacterial growth in mangrove ecosystem and the diversity of microorganisms also many functions including the biogeochemical cycling in the natural environment which as key drivers of nutrient flow and the degradation of many carbon sources, (ii) the microbial diversity in mangrove sediment would check how the microbial community may responsible for environmental pollution, and (iii) the abundance of certain *alkB* gene types may reflect the levels of measured hydrocarbon not only in sediments but also in seawater, and therefore this assay could be used as a complementary tool for monitoring petroleum inputs into global ocean.

Thesis structure

This research consists of four chapters full, and each chapter is as follows (Figure 1.4).

In chapter 1, I described the general introduction of this research and past research, tasks and objectives.

In chapter 2, I determined the assessment of nitrogen cycle in mangrove forest rhizospheric sediments of the Republic of the Union of Myanmar using by microbial

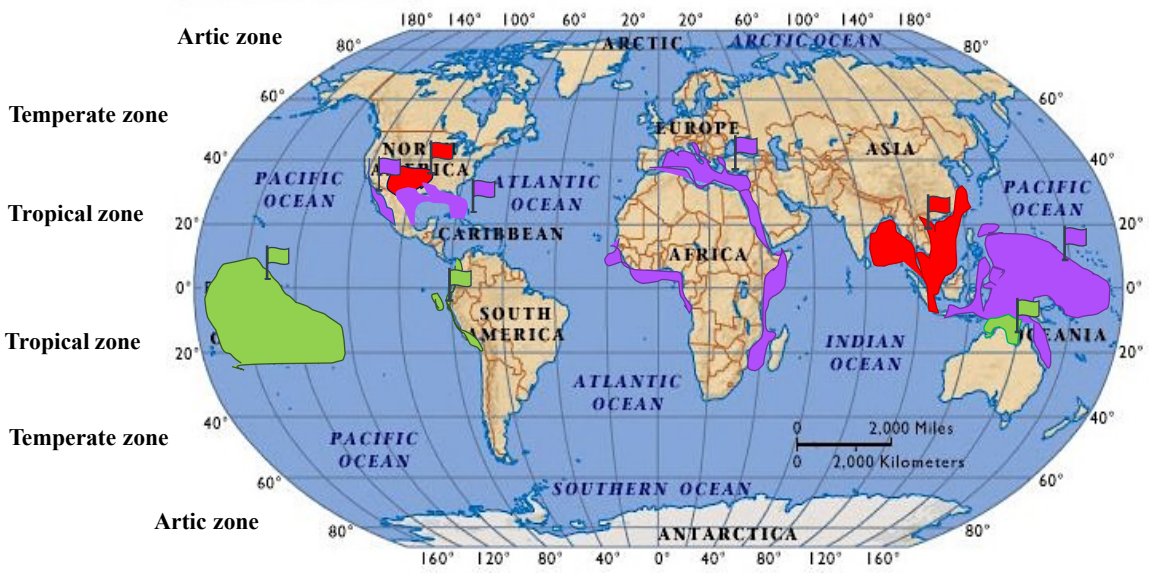
activities and diversities. Then, I studied the microbial diversity in mangrove sediments. To assess alkane biodegradation potential, the diversity and distribution of hydrocarbon degradation bacteria (*alkB*) genes were examined in sediments of mangrove forest.

In chapter 3, I investigated seasonal changes of crude oil degradation efficiencies in simulated petroleum-polluted seawater collected from Tokyo Bay. I demonstrated degradation dynamics of individual hydrocarbons in different carbon number and connected seasonal petroleum degradation to the shifts of potential functional genes of alkane-degrading bacteria.

In chapter 4, I discussed the results which obtained in this research, then a summary and discusses future prospects.

Expected outcomes and goals

This research can provide scientific advice on matters related to the utilization and management of marine resources in given regions. There would be achieved; 1. Improve the understanding of coastal ecosystems and the human influences on them. 2. It might assist the world climatic condition and control of coastal pollution, and managed the eutrophication appearance in the coastal region. 3. Foster coastal ecosystem management solutions through sustained community engagement, and improve operations and structure in coastal zone management. This work would gain further insight into our understanding of coastal zone management, and provide a basis for focusing on maintaining the health and stability of the coastal regions both environmentally and economically, that will continue into the long-term future (Figure 1.5).



http://www.conservation.org/Marine/map.htm

The most biologically valuable areas

The most threatened marine areas

The most critical areas for marine conservation

Figure 1.1. Map and description of marine areas (Modified based on map from http://www.conservation.org/Marine/map.htm)

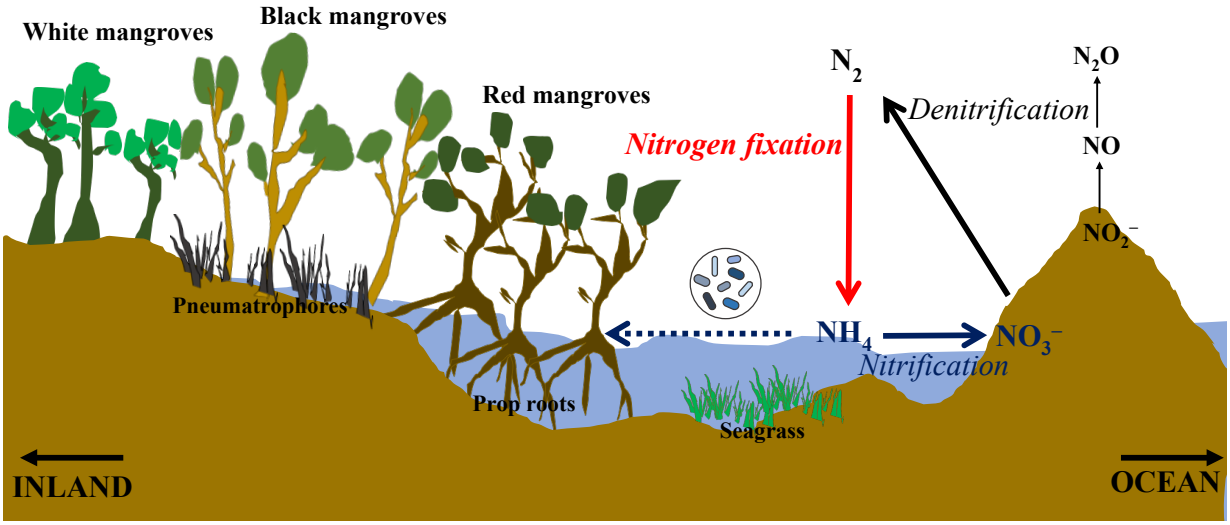


Figure 1.2. Microbial activities in nitrogen cycle of mangrove forest (Modified based on from https://www.pinterest.jp/sasi15sm/mangrove-infographic/?lp=true)

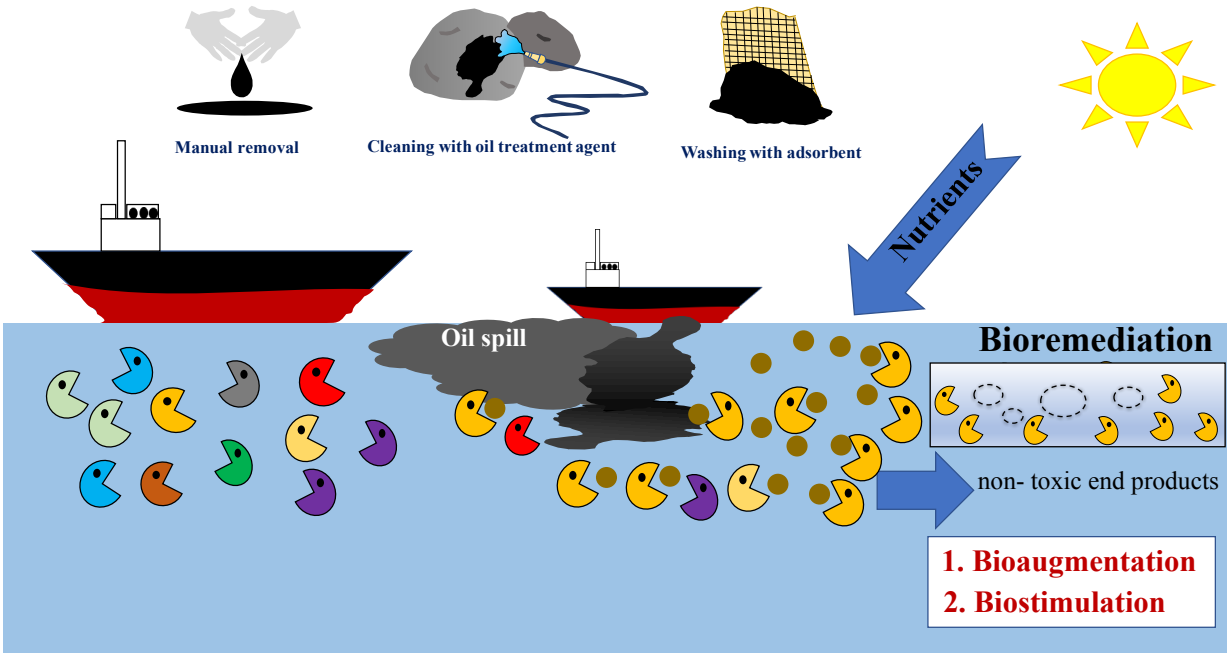


Figure 1.3. Oil spill removal in simulated petroleum-polluted seawater by bioremediation.

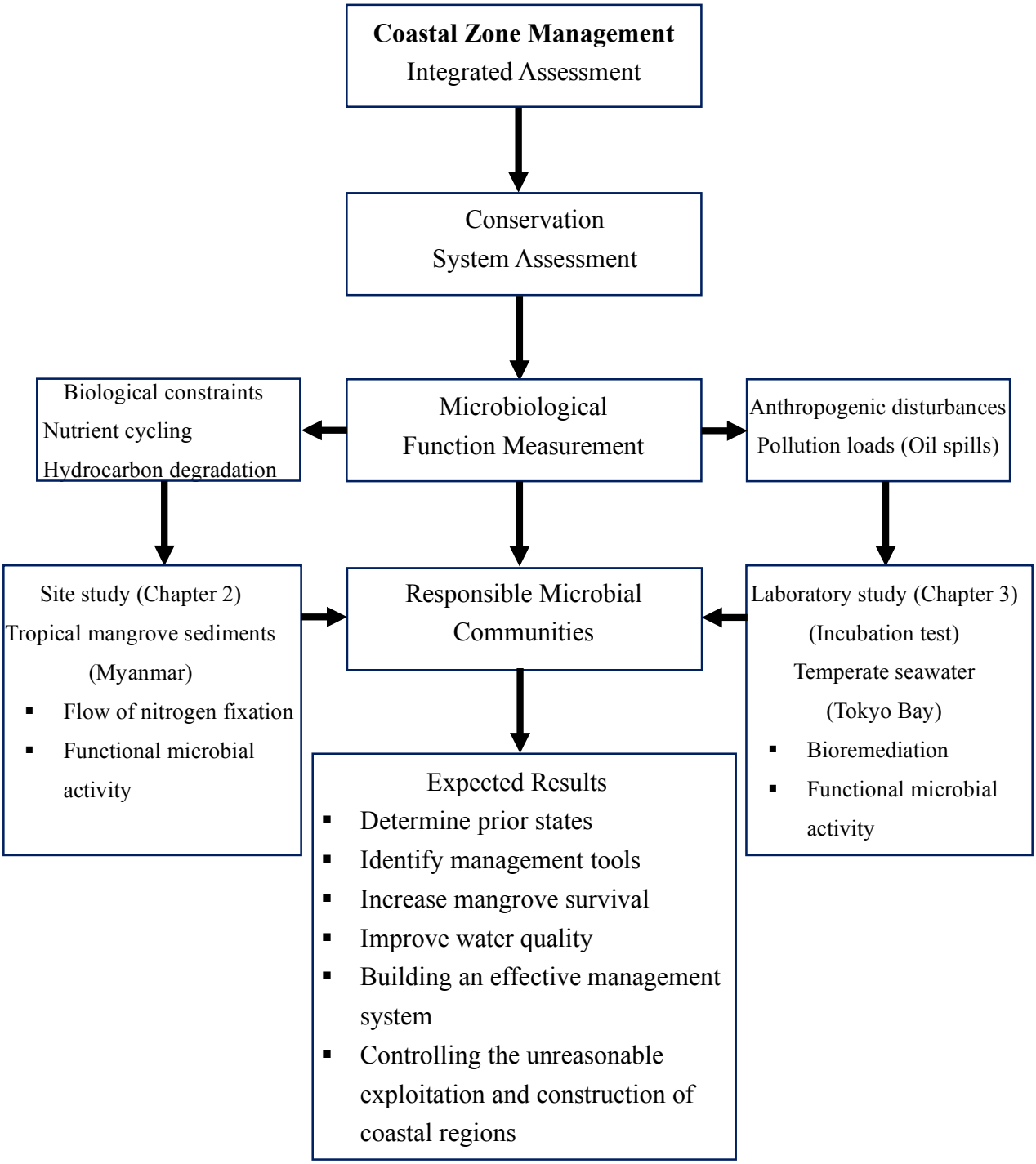


Figure 1.4. Schematic representation of my study showing the conservation of coastal ecosystems under the regional based measurement of microbiological functions that use to be investigated prior to plan sustainable of aquatic coastal zone management.

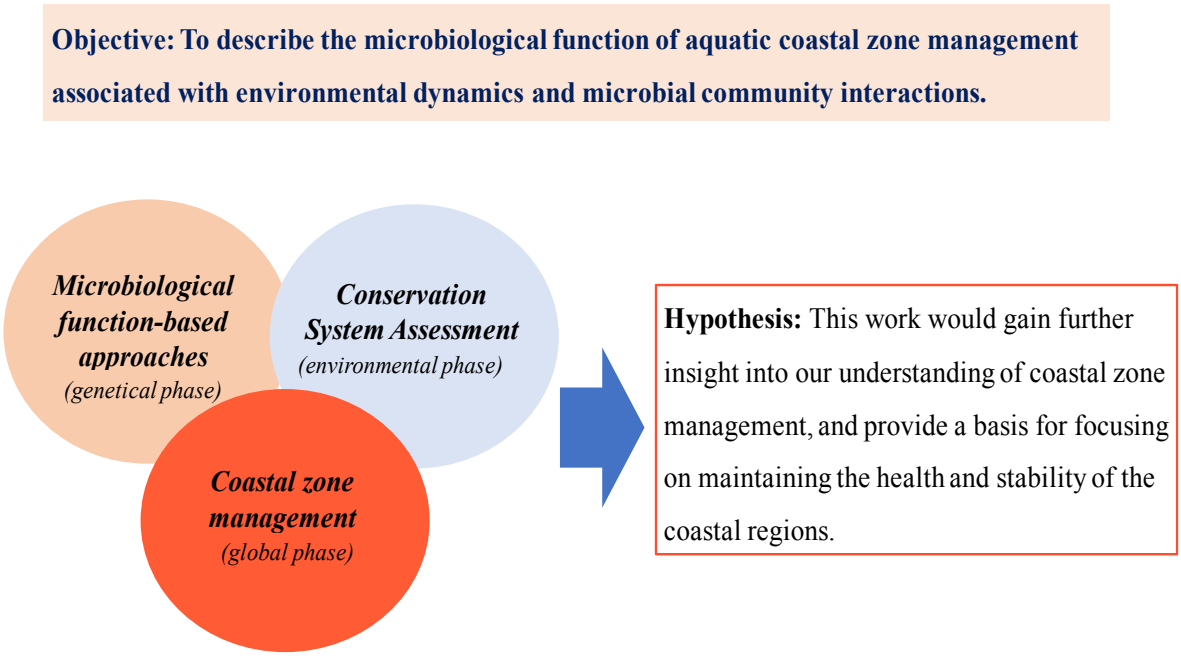


Figure 1.5. General schematic diagram represents the correlation of objective, approach and hypothesis.

Chapter 2: Management in tropical coastal area by assessment of microbiological potential for the nitrogen cycle in mangrove ecosystem

2.1. Introduction

The Republic of the Union of Myanmar has a long coastal region which strength approximately 2,300 km^2 from the mouth of Naff River to Pakchan River, and includes the Bay of Bengal and the Andaman Sea. For the tropical area, sediment samples were collected from Shwe Thaung Yan mangrove forest in Myanmar. Because mangroves are widely distributed in Earth's tropical and subtropical regions, covering approximately 60 to 75 % of global coastlines. Mangroves are recognized as highly productive ecosystems that provide organic matter and shelters to adjacent coastal ecosystems. The availability of nitrogen limits primary production in many terrestrial and marine ecosystems. Many previous researches showed that mangrove habitats were often nutrient limited, particularly for combined nitrogen, while nitrogen fixation may be an important nitrogen input term to these ecosystems because of its potential to provide nitrogen in usable form to plants (Zuberer and Silver 1978, 1979). In Raymond *et al.* (2004) research, for organisms to fix nitrogen, either in association with plants or not, the presence and activity of the nitrogenase enzyme complex is required. *nifH* gene is a highly conserved functional gene for nitrogen-fixing microorganisms and has been thoroughly studied (Coelho *et al.* 2009; Man-Aharonovich *et al.* 2007). In regard to oil pollution, mangroves are especially affected by the oil hydrocarbons (Lewis 1983). In the sea, the oil spilled may converge a

large part of coastal environment. If the oil reaches a mangrove forest, it may persist by tidal action and there due to the limited wash resulting from the vegetation density and in anaerobic sediments microbial decomposition (Lewis 1983). In the marine environment, coastal sediments are the natural sink of hydrocarbons (Board *et al.* 2003) and the microbes with the ability to degrade hydrocarbons. There are isolated from both contaminated and non-contaminated sites in the environment. Only is a widely accepted method for site cleanup.

In this study, I determined the assessment of nitrogen cycle in rhizospheric sediment of Myanmar mangrove forest using by microbial activities and diversities. Then, I studied microbial diversity and functional genes abundance of nitrogen-fixation *nifH* gene and hydrocarbon degradation *alkB* gene in mangrove forest. The study proposes the understanding of microbe-nutrient-plant relationship that functions as a mechanism to recycle and conserve nutrients in the mangrove ecosystem. And to assess alkane biodegradation potential, the abundance and distribution of hydrocarbon degradation bacteria (*alkB*) genes were examined in sediments of mangrove forest.

2.2. Materials and methods

2.2.1. Study site

Mangroves sediment samples collected from the Republic of the Union of Myanmar. It can be subdivided into three main areas, namely Rakhine Coast, Ayeyarwady Delta and Tanintharyi Coast. For this study, I already selected the Shwe Thaung Yan coastal region

mangrove forest sediments. Shwe Thaung Yan coastal and adjacent areas involved in Rakhine Coastal area which is situated in the Bay of Bengal. Site 1 (17º 04.222' N 94º 28.913' E) is located by the mouth of Magyi tidal creek and dominance mangrove specie by *Bugueria gymnorrhiza* spp. Site 2 (17º 04.324'N 94º 27.917'E) is located by the middle of tidal creek and dominance mangrove specie by *Ceriops tagal* spp. Site 3 (17º 04.701'N 94º 28.141'E) is located by the marine park (special maintained area) and dominance mangrove specie by *Rhizophora mucronata* spp. Shwe Thaung Yan mangrove forest has two channels one is mainstream Magyi tidal creek and another one is U-To tidal creek. All study sites are along the Magyi tidal creek in Shwe Thaung Yan, the Republic of the Union of Myanmar (Figure 2.1).

2.2.2. Sampling

For physicochemical and microbial analysis, approximately 200 g of soil samples were collected from each of the three locations at low tide. Subsamples were collected at the rhizosphere region aseptically using by spade and triplicate from four different depth i.e, 1) 0 - 5 cm; 2) 5 - 10 cm; 3) 10 - 15 cm; 4) 15 - 20 cm at three sites. Soil samples were always taken close to the roots of the trees (Figure 2.2). All samples were collected into a plastic zipper bag and immediately transferred into an icebox transported to Japan for analyses.

2.2.3. *In situ* measurement of physicochemical parameters in mangrove forest

Temperature, redox potential, and pH of the sediment samples were measured *in situ* using the thermometer, electric digital pH and conductivity meter (Horiba). Each

electrode was calibrated with pH 4 and 7 buffers before using. Pore-water salinity was measured by reflectometer. In addition, the soil samples were analyzed for their relative sediment structure because of microbe and vegetation types of mangrove are dependent on the soil types (e.g. muddy or sandy loam or silt-mud type).

2.2.4. Procedure for measuring soil nitrogenase activity (Acetylene Reduction Assay)

Nitrogen fixation rates were measured by the acetylene reduction technique (Capone and Montoya 2001) based on nitrogenase enzyme activity. Acetylene reduction assay (ARA) is a commonly used assay and is widely accepted as an effective and reproducible, instantaneous nitrogen fixation rate measurement. Previous to incubation 100 mL glass vials purge with Argon gas put into the flushing headspace vials (to create an anaerobic environment).

About 10 mL wet sediment sample was extruded from each layer sediment sample bag using 5 mL sterile syringes (with the top end of the syringe removed) put into the vials and sealed with an open cap over a Teflon - silicone septa. 10 % of the atmosphere of each vial containing 10 mL soil was substituted with gaseous acetylene (10 %) from a commercial cylinder inject to each vial (0.1 atm, 10 % C_2H_2 by volume) through the Teflon-silicone septa. The vials were shaken by hand in order to avoid heterogeneities within the gas phase. Samples were incubated in the dark at ambient water temperature (24^{o}C) for 24 h. From each incubation assay, 1 mL gas sample was taken from the vials at every 6 hours interval (specifically 0, 6, 12,18 and 24) measuring by gas chromatograph. The ethylene concentration was determined by a GC-14B gas chromatograph with FID

detector (Shimadzu, Japan). Representative retention times in minutes are methane, 0.8; acetylene, 5.6; and ethylene, 7.8. 1 mL of gas sample from each vial was injected into GC-14B and Sincarbon ST (50/80 mesh) packed column with 3 m and 3 mm a stainless-steel column (column temperature 60°C). Temperature at both the injector and detector was 210°C while oven temperature was kept at 160°C. The carrier gases flow rates were 60, 50 and 50 mL/min of helium (He), hydrogen (H_2) and oxygen (O_2) respectively (Table 2.1). The chromatograms were used to integrate the areas of the curves of acetylene (C_2H_2) and ethylene (C_2H_4) to estimate C_2H_4 production (Holguin *et al.* 1992, 2001). The acetylene reduction rates were calculated based on dry weight of sediments (samples were oven-dried at 60°C for 48 h). Ethylene peak heights were measured and related to calibrations made with standard C_2H_2 (0.976%) and C_2H_4 (0.965%) concentration. The acetylene reduction rates were converted to rates of nitrogen fixation using the theoretical factor of three acetylene molecules equivalent to one nitrogen molecule (Turner and Gibson, 1980).

2.2.5. DNA extraction from sediment samples

Total DNA was extracted from each sample using the ISOIL for Beads Beating DNA extraction kit (Nippon Gene). DNA extraction protocol was based on described procedures for the extraction of total DNA from soil samples. Briefly, the sediment slurry (wet weight, 0.5 g) was placed in a beads tube and suspended in 950 µL of Lysis solution BB, 50 µL of Lysis solution 20S was added. The sample was placed horizontally on a 6000 - rpm shaker at 5 m/s for 40 s. The 600 µL supernatant was decanted into a 2 mL

clean tube after centrifugation for 1 min at 12,000 g rpm and 25˚C. The sediment pellet was again suspended in 400 µL of purification solution and the crude extract was extracted with an equal volume of chloroform (containing 4% [vol/vol] isoamyl alcohol to minimize foaming) and centrifuged at 12,000 g for 15 min at 25˚C. 800 µL of aqueous phase (upper layer) was transferred to 2 mL clean tube, and the DNA was precipitated by adding 800 µL precipitation solution. The DNA was left to precipitate by centrifugation at 20,000 g and 4˚C for 15 min. The DNA was again suspended in 1 mL of wash solution for 10 min at 4˚C and 20,000 g centrifuged. The DNA was pelleted by centrifugation at 16,000 g for 30 min at 20˚C. The DNA pellet was rinsed with 1 mL of 70 % (vol/vol) ethanol and 2 µL of Ethachinmate solution was centrifuged by 20,000 g for 5 min at 4˚C. The DNA was resuspended in 50 µL of TE buffer (pH 8.0) and diluted 1:10 to facilitate PCR amplification. After extraction, the quantity, integrity, and quality of the DNA obtained were checked by 1 % (wt/vol) agarose gel electrophoresis, followed by staining in ethidium bromide and visualization in UV light. The extracted DNA concentration was further quantified by NanoDrop spectrophotometer analysis (Thermo Scientific) in triplicate. All DNA extracts were stored at -30˚C.

2.2.6. PCR amplification of 16S rRNA, *nifH* and *alkB* genes

16S rRNA, *nifH* and *alkB* genes from genomic DNA were amplified by 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') for 16S rDNA (Lane 1991), PolF (5'- TGCGAYCCSAARGCBGACTC–3') and PolR (5'-ATSGCCATCATYTCRCCGGA-3') for *nifH* gene (Poly *et al*. 2001a), alkB-F (5'-

AAYACIGCICAYGARCTIGGICAYAA-3') and alkB-R (5'-G C R T G R T G R T C I G ARTGICGYTG-3') for *alkB* gene (Bell *et al.* 2011), respectively (Table 2.2). Amplification products of expected sizes are (1500 bp and 360 bp). All PCRs (final volume of 20 µL) contained 2 µL of 10× TAKARA Ex Taq Buffer (Mg^{2+}plus), 1.6 µL (2.5 mM) of each deoxynucleoside triphosphate, 0.5 µL of each primer, 0.1 µL of Ex Taq polymerase (5 U/µL), 1 µL of DNA template (3-30 ng/µL), and deionized water up to 14.3 µL. PCR cycling conditions was performed in a Veriti™ thermal cycler (Applied Biosystems) starting with 4 min at 95˚C, followed by 35 cycles of 95˚C for 1 min, annealing at 50˚C for 16S rDNA and *nifH* at 58˚C, *alkB* at 58˚C for 1 min, at 72˚C (the temperature was decreased by 0.5˚C every cycle until the touchdown temperature of 58˚C was reached) for 1min, and primer extension at 72˚C for 1 min, followed by final incubation for 7 min at 72˚C, at fastest ramp rate (4˚C/s) from the denaturation to the annealing step. Negative controls without DNA were run in all amplifications. PCR products were checked by TAKARA pHY marker (20%) and 1% (wt/vol) agarose gel electrophoresis (50 V, 1 h), followed by staining in Redsafe™ Nucleic Acid Stain Solution and visualization in UV light.

2.2.7. Validation and optimization for real-time PCR (qPCR) assay

For targeting 16S rRNA, *nifH* and *alkB* genes were amplified by three primer sets for my research (Table 2.3). In each primer set, the validation and optimization part were included the adjustment of primer concentration in real-time PCR reaction mixture and the adjustment of annealing temperature in real-time PCR assay. Because of the ensuring

of optimum assay efficiency and ensuring of optimum primer specificity. For targeting *nifH* gene and *alkB* gene were choose the same PCR amplification primer sets were used to check the different optimized conditions. These primer sets were shown the higher *nifH* and *alkB* genes copy detection at suitable optimize condition. Finally, I was chosen to use in further real-time PCR analysis.

2.2.8. Real-time PCR amplification of 16S rDNA, *nifH* and *alkB* genes in mangrove sediments

The abundance of nitrogen fixers and alkane degradation bacteria were quantified by quantitative PCR (qPCR) targeting the *nifH* and *alkB* genes. The copy numbers of *nifH*, *alkB,* and 16S rRNA genes in all samples were determined in triplicate on three times diluted samples. For determination of differences between the sediments depth profile of nitrogen fixation microbe community, I made the standard from constructed *nifH* gene clone library with samples collected at Hasaki brackish water sediment, which is the similar environmental sediment for mangrove forest. I used the primer PolF and PolR (Poly *et al.* 2001a) for PCR amplification (Veriti 96-Well Thermal Cycler, Applied Biosystems). PCR products from four replicate reactions for sample were pooled and subjected to 2 % agarose gel electrophoresis (50 V, 1 h). Bands of expected sizes were excised and purified using a NucleoSpin® Gel and PCR Clean-up Gel Extraction Kit according to the manufacturer's instructions. PCR products were dissolved in 20 μL of NE buffer. Purified DNA was then cloned using a TOPO-TA Cloning Kit (Invitrogen) and Escherichia coli DH5α™ Competent Cells (TAKARA BIO INC., Japan) according to the

manufacturer's instructions. Colony hybridization to identify positive *nifH* inserts in clones was performed as a template for PCR using the M13 primers to confirm inserts. The real-time PCR (qPCR) standards for 16S rRNA gene and *alkB* were taken from the Republic of the Union of Myanmar mangrove sediment samples.

The reactions were performed in an ABI Prism 7500 Cycler (Applied Biosystems, Germany) equipped with ABI 7500 software (ver. 1.4.1). In volumes of (20 µL) for SYBR Green detection was containing 10 µL of Power SYBR green master mix (Applied Biosystems), 0.4 µL of ROX Reference Dye (50×conc.), primers (all for each at optimum concentration, varies from 0.2 to 0.25 µM), 2 µL of extracted DNA (DNA extracts from 3:10 dilutions of crude extracts were used and exhibited no PCR inhibition), and distilled water. In volumes of (20 µL) for TaqMan detection was containing 10 µL of THUNDERBIRDTM Probe qPCR Mix (TOYOBO), 0.4 µL of ROX Reference Dye (50×conc.), 0.25 µM of TaqMan Probe, primers (all for each at optimum concentration, varies from 0.2 to 0.25 µM), 1 µL of extracted DNA, and distilled water. All of the reaction mixtures were performed in 96-well real-time PCR plates (Applied Biosystems) tingly sealed with optical tape (Applied Biosystems). Real-time PCR cycling conditions for SYBR Green detection of the amplifications were performed with one initial denaturation step at 95˚C for 30 s, followed by 45 amplification cycles of 95˚C for 5 s, annealing temperature for 34 s and an extension of 30 s at 72˚C. Thermocycling conditions for TaqMan detection were performed with one initial denaturation step at 95˚C for 1 min, followed by 45 amplification cycles of 95˚C for 15 s, primer annealing

temperature for 45 s. The specificity of the amplification products was confirmed by melting curve analysis, and the expected sizes of the amplified fragments were checked in a 1.5% agarose gel stained with ethidium bromide. Standard curves were obtained by using serial dilutions (10^7 to 10^1) of known copies of PCR fragments for *nifH* and 16S rRNA genes and (10^8 to 10^2) for *alkB* gene. Threshold values obtained from sample amplification were interpolated in the standard curve determining the number of genes found per gram of mangrove sediment.

2.2.9. DNA extraction, metagenomic library construction and analysis of next generation sequencing (NGS)

Metagenomic DNA was extracted from 0.5 g of each sediment sample using ISOIL for Beads Beating DNA extraction kit (Nippon Gene), in accordance with the manufacturers' protocol. DNA quality and quantity were assessed using the NanoDrop (Thermo Fischer Scientific, USA) spectrophotometer and the Qubit (Thermo Fischer Scientific, USA) fluorometer. For the DNA sequencing library construction, we used Illumina MiSeq® DNA sample Amplicon library preparation protocol. Libraries were sequenced using the Illumina MiSeq 2 × 250-bp paired-end technology. Pre-assembly quality control (QC) was performed on the Illumina MiSeq raw reads using the Trimmomatic ver. 0.0.13.2 (Bolger *et al*. 2014). QC includes read trimming, removal of ambiguous bases, removal of short reads, removal of duplicates, removal of foreign vectors, filtering out low-quality reads and removal of sequencing adapters.

2.2.10. Statistical analysis

Normally distributed data were tested for significant differences with the independent t-test. Analysis of variance of real-time PCR data (one-way ANOVA, multiple range tests) was performed using the SPSS package (ABI.SPSS. version 25). Pearson's moment correlation analysis was used to find out whether there were some environmental factors affecting the distributions of *nifH* and *alkB* genes in mangrove sediments. Finally, correlation analysis of microbial community structures with environmental factors, nitrogen fixation rate and nitrogen fixation functional gene was conducted using Microsoft Excel program with Pearson's moment correlation.

2.3. Results

2.3.1. *In situ* measurement of physicochemical parameters in mangrove forest

The physicochemical properties of three sites of sediment samples were shown in Table 2.4. The pH of all sites was less than 7.0, indicating that the soil samples were slightly acidic. Electrical conductivity (EC) contents were 1.750 µS/cm (Site 1), 1.725 µS/cm (Site 2) and 1.675 µS/cm (Site 3), respectively. Seawater and sediment porewater salinity at Site 1 (*B. gymnorrhiza* spp.) was much higher than Site 2 (*R. mucronata* spp.) and Site 3 (*C. tagal* spp.). Seawater and soil salinity ranged from 31 ‰ to 35 ‰. The sediment temperature measured at three depths (5 cm, 10 cm, and 15 cm) ranged from 27.9 ˚C to 29.2 ˚C. Three distinct sites, comprising three bulk sediment types, namely clay - loam (Site 1), mud - clay (Site 2), and mud - clay - loam (Site 3) rhizosphere soils.

This indicated that the rhizosphere soil samples were substantially different from each other.

2.3.2. Nitrogenase activity in mangrove sediments

Reduction of C_2H_2 to C_2H_4 by nitrogenase of mangrove sediment samples were examined with respect to a wide variety of characteristics, and the striking similarities between nitrogen fixation and C_2H_2 reduction were reported in these experiments. It follows that, for calibration of the relationship between acetylene reduction and nitrogen fixation, the acetylene concentration should be adjusted to give equimolar concentrations of acetylene and nitrogen in solution. To confirm the occurrence of nitrogen fixation in the samples of mangrove rhizosphere sediments with high nitrogen intake, I additionally examined the acetylene-reducing activities in four-layer samples from each site. In this assay, each layer sample was divided into two: one was analyzed aerobic condition and the other was an anaerobic condition. By comparing these two types of sample, I also evaluated the effect of both conditions on the nitrogen-fixing activity. In general, the anaerobic condition of C_2H_2 reduction activities was low and aerobic condition sediments generally exhibited high. All the samples of aerobic condition were showed acetylene-reducing activities (ranging from 0.14 to 2.36 nmol of C_2H_4/g (dry sediment)/h), but under the anaerobic condition, it was 0.05 to 0.88 (nmol of C_2H_4/g (dry sediment)/h). Aerobic activities showed 2.8 to 2.7 times higher than the values of the corresponding anaerobic condition (Tables 2.5, 2.6, 2.7). Rates of ethylene production showed a similar pattern for two sites (1 and 2) with relatively from 0 to 24 h during incubation. The highest rates

observed were 2.36 and 1.10 (nmol of C_2H_4/g (dry sediment)/h) for surface layer (0 - 5 cm) and the second layer (5 - 10 cm) in Site 3. There was no statistically significant difference in nitrogenase activity between all sediment samples during the incubation (R_2= 0.878 (Adjusted R_2= 0.857). The highest nitrogen fixation rate was occurred in Site 3 under the two conditions (aerobic and anaerobic) (Figure 2.5; A and B), followed by Site 1 (Figure 2.3; A and B), and Site 2 (Figure 2.4; A and B). In contrast, the activities in the deepest sediment layer (15 - 20 cm) of all sites were very low. The differences of nitrogen fixation rates in aerobic and anaerobic conditions were statistically significant (Multiple Regression: $p < 0.001$, $n = 4$). Aerobic and anaerobic rates were highly significantly different, and the acetylene-reduction rates were correlated positively with the time during the incubation periods. The nitrogen fixation rates were estimated the theoretical reduction ratio C_2H_2:N_2 = 3:1 was used.

2.3.3. Real-time PCR (qPCR) assay validation and optimization

The adjustment of primer concentration for real-time PCR reaction mixture (to confirm the optimum efficiency) and annealing temperature (to confirm the optimum specificity in primer) was included in the sector of the real-time PCR assay validation and optimization. I have empirically tested the melting temperature of all of the primers via end-point PCR. I have tested the melting temperature of the all primer sets in a similar manner, and the gradient was 58 to 60°C. Since most TaqMan probes were designed to target 16S rDNA, I relied on the sequences of the primers for specificity. To examine possible cross-reactivity of the primers, I initially examined the formation of PCR

products after 35 cycles in three-step PCRs. And then, assay reactions were performed using a matrix of concentrations of forward and reverse primers to seek the primer concentrations yielding minimal CT values and consequently the highest amplification efficiencies. After finding the optimum primer concentration and annealing temperature, the optimum condition for each primer set (Table 2.3) has been confirmed and analyzed for the copy numbers of *nifH*, *alkB* and 16S rRNA genes in all samples.

2.3.4. Abundance of 16S rRNA genes in mangrove sediment

The highest copies number of 16S rRNA gene were obtained from Site 3 (ranged from 1.31×10^9 to 3.63×10^9 copies /wet gram of sediment) while Site 2 (ranged from 2.88×10^8 to 7.45×10^8 copies/wet gram of sediment) and Site 1 (ranged from 1.79×10^8 to 5.64×10^8 copies per wet gram of sediment) respectively. Especially, the high copies number were found at surface layer (0 - 5 cm: 3.63×10^9 copies/wet gram of sediment, 7.45×10^8 copies/wet gram of sediment) in Site 3 and Site 2, only Site 1 was found 5.64×10^8 copies/wet gram of sediment in 5-10 cm. Except 5 - 10 cm in Site 1, in all layers of rhizosphere sediment samples for three sites kept stable throughout the sediment layers with gradually decrease with depth, which is similar to the vertical distribution of 16S rRNA gene abundance of mangrove forest. In Shwe Thaung Yan mangrove forest, the 16S rRNA gene of sediment depth profile at the mangrove maintenance area in Site 3 was occurred the highest gene copy number among the three sites (Figure 2.6).

2.3.5. Abundance of *nifH* gene in mangrove sediments

For comparison and a clear understanding of the abundance and distribution of *nifH* genes in disparate mangrove systems based on real-time PCR (qPCR) analysis. *nifH* genes copy numbers were detected and quantified across all site samples. The efficiency value for amplification was 99.26 %, and logarithmic regression curves reached a value of 0.98 (R^2). The amplification specificity was found to be robust, with single peaks being detected during melting curve analysis. The qPCR results (efficiency 97.32 %; $R^2 = 0.997$) showed a heterogeneous distribution of the *nifH* gene among three sample sites (Figure 2.7). *nifH* gene copy numbers ranged from 1.23×10^5 to 5.55×10^6 copies/wet gram of sediment at surface layers in Site 1, Site 2 which were lower *nifH* gene abundances than Site 3. *nifH* gene abundances increased at 5 - 10 cm among all sites (9.90×10^5 to 7.40×10^6 copies/wet gram of sediment) but decreased at 15 - 20 cm (1.64×10^5 to 2.31×10^6 copies/wet gram of sediment). In addition, the high *nifH* gene abundances showed at surface layer (0 - 5 cm) in Site 2, but at 5 - 10 cm in Site 1 and 3. All sites were shown as significantly higher copy number in surface layers than lower layers ($p < 0.05$, $n = 3$).

2.3.6. Abundance of *alkB* gene in mangrove sediments

alkB gene was detected and quantified across all site samples. The efficiency value for amplification of this target gene was 100 %, and logarithmic regression curves reached a value of 0.98 (R^2). The amplification specificity was found to be robust, with single peaks being detected during melting curve analysis. The qPCR results (efficiency 98.32 %; $R^2 = 0.985$) showed a heterogeneous distribution of the *alkB* gene among three sites

(Figure 2.9). The *alkB* copy number in Site 1, it was ranging from 2.15 x 10^5 to 1.01 x 10^6 (copies/wet gram of sediment). The abundance of Site 2 ranged from 3.06 x 10^5 to 1.27 x 10^6 (copies/wet gram of sediment). The high copy numbers were identified in Site 3 ranged from 1.26 x 10^6 - 4.23 x 10^6 (copies/wet gram of sediment). The lowest value was observed at surface layer (0-5 cm) in Site 1 and the highest at surface layer (0-5 cm) in Site 3, respectively. A number of copies of the *alkB* gene varied from 3.06×10^5 to 1.27×10^6 copies/gram wet sediment in Site 2 (middle of cheek) sediments, and 2.15×10^5 to 1.01×10^6 copies/ wet gram of sediment in Site 1 (mouth of tidal cheek) (Figure 2.8).

2.3.7. Microbial community structures in mangrove sediments

In this study, the description of bacterial communities using a culture-independent approach enable an overview of the groups inhabiting each depth of mangrove. Classification of 16S rDNA sequences recovered from next generation sequencing (NGS) showed a dramatic shift in the microbial population by depth (0-5, 5-10, 10-15, 15-20 cm) per each of three sites. Illumina sequencing generated a total of 1.867 million raw read sequences from 12 samples (Site 1, 2, 3), with an average length of 300 bp. After trimming, a total of 1.631 million sequences were obtained: 0.557 million reads for Site 1, 0.588 million reads for Site 2, 0.484 million reads for Site 3, respectively. Statistical analyses were applied to evaluate species richness, the number of OTUs, Chao1, Shannon, samples PD and the difference occurring between the communities from the four different depths (Table 2.8). Microbial diversity concerns both taxon richness and evenness. The OTUs were defined at a cut-off of 97 % sequence similarity, a commonly recognized level

for comparative analysis of 16S rRNA gene (Orcutt *et al.* 2009). Confirming the results provided by NGS analysis, differences between fours depths (0-5, 5-10, 10-15, 15-20 cm) within the three sites were detected by the richness of species estimator. In addition, the illumine sequencing showed that 0-5 cm depth of Site 1 and 2 were presented higher diversity as shown by Shannon index (10.8 and 11.0), followed by 15-20 cm depth of Site 2 (10.5), 5-10 cm depth of Site 1 (10.3) and 0-5 cm depth of Site 3 (10.3), and the lowest (9.6) in 10-15 cm depth of Site 3, respectively. The rarefaction curves trend showed that microbial diversities in surface mangrove sediments were higher than in deeper layers (Figure 2.9). Despite the rarefaction curves have shown differences between depths, the curve did not achieve the plateau, revealed that this result should be viewed as an underestimation of the total diversity due the number of sequences sampled.

The obtained sequences were classified using the RDP database, and the dominant phylum in all samples was Proteobacteria with 88% of total sequences (Figure 2.10). The phyla that presented the low differences between Site 2 (42 phyla), Site 1 (40 phyla) and Site 3 (38 phyla). In this study, totally 50 phyla were investigated. Bacterial phylum Proteobacteria (39.3 %) dominated in all samples, followed by Chloroflexi (14.8 %), Acidobacteria (3.7 %), Planctomycetes (3.7 %), Bacteroidetes (3.0 %), Caldithrix (2.3 %), Actinobacteria (1.8 %), Firmicutes (1.8 %), Spirochaetes (1.6 %), Nitrospirae (1.4 %), and Gemmatimonadetes (1.4 %) (unclassified sequences were not taken into account). For three phyla of Archaea, Crenarchaeota (8.5 %) was dominant in three sites followed by Euryarchaeota (1.9 %) and Parvarchaeota (0.2 %). Moreover, phylum Proteobacteria

in Site 3 was more abundant than in other two sites. Between the four sediment layers in Site 1, bacterial phylum Proteobacteria dominated, followed by Chloroflexi, Crenarchaeota (Archaea), Planctomycetes, and Bacteroidetes. The highest abundance of Cyanobacteria and Crenarchaeota were found only 0-5 cm and 15-20 cm of site 1. Whereas in Site 2, bacterial phylum Proteobacteria dominated, followed by Chloroflexi, Crenarchaeota (Archaea), Planctomycetes, Euryarchaeota (Archaea) and Bacteroidetes (unclassified sequences were not taken into account). At Site 3, also Proteobacteria was dominated in four sediment layers and followed with Chloroflexi and Acidobacteria, Planctomycetes, Actinobacteria, Bacteroidetes and Gammatimonadetes (Figure 2.10). The high abundance of phylum Acidobacteria was found in all layers of Site 3. In addition, the Site 3 phyla dominant pattern is similar between the four layers.

Generally, phylum Proteobacteria of three classes (Gammaproteobacteria, Deltaproteobacteria, Alphaproteobacteria) contributed to the most abundant classes in all study sites. Therefore, another class Betaproteobacteria only detected in surface layer for Site 1, except surface layer in Site 2 and all layer in Site 3. The class Anaerollneae (Phylum: Chloroflexi) can be observed within the range of 6.43 % to 10.16 %. However, Dehalococcoidetes (Phylum: Chloroflexi) present mostly in study Site 1 and 2 while almost no detection in study Site 3. Based on the results, Thaumarchaeota (Phylum: Crenarchaeota) followed the same pattern as Dehalococcoidetes (Phylum: Chloroflexi).

A more detailed analysis of the data based on phyla at the class level revealed that when comparing Site 1, 2 and 3. From 50 phyla were collected the 97 classes in all

sediment samples. All the results, Phylum Proteobacteria of class Deltaproteobacteria was the most dominated class in 12 layers of all site (range from 22.48 % to 29.74 %) (Figure 2.11). And the lowest one could be found in classes Thaumarchaeota and Methanobacteria (Phyla: Crenarchaeota and Euryarchaeota). It was observed an enrichment of the Proteobacteria classes Deltaproteobacteria and Gammaproteobacteria, followed by Phylum Chloroflexi of classes Dehalococcoidetes and Anaerollneae, and Phylum Euryarchaeota of class Thermoplasmata in Site 1. In Site 2, except surface layer (0-5 cm) was found similar dominance pattern with Site 1. In Site 3, an enrichment of Proteobacteria classes Deltaproteobacteria, Gammaproteobacteria, Alphaproteobacteria, followed by the Chloroflexi of class Anaerollneae were identified in four sediment layers.

In the principal coordinate analysis (PCoA) using the weighted UniFrac metrics (Figure 2.12). UniFrac, measures the phylogenetic distance between sets of taxa in a phylogenetic tree as the fraction of the branch length of the tree that leads to descendants from either one environment or the other, but not both. This technique can be used for the overall comparison for significant differences among the bacterial communities by finding clusters of samples and ordination techniques that will reflect the similarity of the biological communities. My comparisons are made by taking sequences from the same dataset, one should expect to find a single cluster if the number of sequences is representative of the overall community. PCoA was adopted to evaluate whether the taxonomic and phylogenetic relationships among the twelve samples have a significantly spatial dynamic. In Figure 2.12, the grouping of sediment depth profile in all sites

confirmed their high similarity in both microbial diversity and abundance. The sediment depth profile of all sites grouped tightly, and the three sites could be separated into two lineages; one included Site 1 and 2 (both were mangrove forest along the tidal cheek), the other included Site 3 (mangrove park). However, the divisions between Site 3 and surface layer of other two sites were more distinct and separated by PC2. The results implied that they were populated with phylogenetic closely-related organisms, but the abundances of microbes were different. For result ordinations, the top community abundance of the surface layer from Site 2 was closer to Site 3. As shown by analysis, PCoA results obviously showed that the bacterial communities in surface layer had a high similarity.

2.4. Discussion

2.4.1. Nitrogen fixation rate in mangrove sediment

The physicochemical indicators in seawater and sediment (pH, EC, and salinity) were similar to those of each site, with the exception of the extreme salinity at Site 1 (mouth of the channel). And then, the relatively high salinity was found at Site 3. Possibly, circulation of water is poor, and high air temperature (29.2˚C) leading to higher salinity at Site 3. However, acetylene reduction assay (ARA) showed that nitrogen fixation was taking place at all sites. Site 1 had low N_2-fixing activity, probably a consequence of extremely high salinity in the sediment, and the sediment structure was clay loam type. So far, Site 1 and Site 2 showed the similar pattern of N_2-fixing activity and these sites sediment also appeared similar physicochemical characteristics. The highest nitrogen-

fixing activity was detected at Site 3, perhaps because of the relatively high concentration of mud loam soil and sediment salinity was lower than other two sites. Although, the best predictor for the activity of nitrogen fixation in all sites sediment pH was around 6.1. On the other hand, one of the strong effects is pollution. Site 1 and 2 were located on the tidal cheek and it is very near to the local village. On the other hand, Site 3 was located in the park and it is far from local village. Some reports (Dias *et al.* 2010; Holguin *et al*. 2001) were suggested a correlation between nitrogen pollution and low nitrogen fixation activities in mangrove forests. Also, Mohammadi *et al.* (2012) reported that the environment factors such as temperature, pH, nutrient availability and soil condition had a significant difference in nitrogen fixation activities. Zuberer and Silver (1978) recommenced, nitrogenase activity was associated with many different components of the mangrove ecosystem. These included sediments, mangrove root systems, mangrove leaf litter, and litter from macro-algae and seagrasses, as well as low activity were found in fresh and healthy mangrove leave. Evidence for nitrogen fixation has been confined chiefly to the sediments, with highest rates found within the surface layer (0-5 cm) and mostly in aerobic condition. Expressed in terms of ammonia nitrogen fixed, rates in this layer ranged from 0.22 to 1.18 nitrogen fixation rate (nM/g (dry weight)/h). The mangrove sediment habitat can occur nitrogen fixation because the main reason is the oxygen gradient, thus including the anaerobic conditions, the presence of carbonaceous and other nutrients (Andreote *et al.* 2012; Li *et al.* 2011). All of these results suggested that nitrogen fixation in sediments would be used as an indicator of the health of a

mangrove forests ecosystem in the tropical environment. Acetylene reduction activity (ARA), which indicated nitrogen fixation was found within the well-oxygenated sediment layer, suggesting the possible occurrence of bacterial populations within the microenvironment of mangrove sediments under other factors. So, I have sufficient evidence to conclude with reasonable assurance that the acetylene reduction activity in the sediments was related to their environmental characteristics.

2.4.2. The relationship between nitrogen fixation rate, nitrogen fixation functional gene (*nifH*) and environmental factors in mangrove sediments

In this study, I measured the nitrogen fixation rate in Shwe Thaung Yan mangrove forest. And the correlations between the individual environmental factors variables and microbial abundance were also analyzed. Although, geographical distance and environmental factors variables are the two main factors that drive microbial distribution patterns in sediments (Martiny *et al.* 2011). Bouvier and Giorgio (2002), Gao *et al.* (2016) were found that salinity has been proven to be a vital environmental factor for the bacterial community in estuarine environments. Therefore, according to Pearson's correlation analysis revealed that the sediments temperature was significantly correlated with *nifH* gene abundance. There was no significant correlation between abundance of *nifH* gene and other environmental factors in all sites. These results indicated that only temperature was the main environmental factor affecting on abundance of *nifH* gene in this study.

Nitrogen fixation rates were positively correlated with the abundance of *nifH* (nitrogen-fixing functional gene) present in the mangrove sediments (R^2=0.86, $p<0.005$).

In reports of Holguin *et al*. (2001) and Toledo *et al.* (1995), nitrogen fixation is a major bacterial activity in mangrove ecosystem, these bacteria might supply 40–60 % of the nitrogen requirements of the ecosystem. In my study, considering the contextual parameters that modulate the microbial assemblages in mangroves, physicochemical parameters and vegetation type stand out as the strongest agents. Evidence for low rates of nitrogen fixation found in the deepest layer (15-20 cm). All nitrogen fixation rates measured in my study were stabilized within 1 h to 6 h incubation and after 6 h incubation was gradually increased until 24 h. The level off activity with incubation times between 0 to 6 h may be related to bottle effects or to the imposed atmospheric changes inherent in the procedure. Evidence for the abundance of nitrogen fixation microbe has been confined chiefly to the sediments, with highest rates found within the top 0-5 cm depth sediment. Expressed in terms of nitrogen fixation rates in this zone ranged from 2.03 - 7.08 nmole/g (dry weight)/h. However, these phenomenon is probably not important as a nitrogen source in the mangrove sediments because of low fixation rates found at deep layers.

I have sufficient evidence to conclude that ARA in the sediments was directly related to nitrogen-fixing organisms. And the results imply that the interaction between the nitrogen fixation functional gene and the nitrogen fixation rate are mutually beneficial, that may support the use of nutrient as inoculants for reforestation and rehabilitation of partially or completely destroyed mangrove areas. All the previous research, *nifH* gene-based microbe is considered to be widespread and has been recently addressed in many

habitats, such as mangrove sediments (Dias *et al.* 2012), rhizosphere soil (Mårtensson *et al.* 2009), soybean field soil (Xiao *et al.* 2010) and terrestrial soil microenvironments (Izquierdo and Nüsslein 2006).

2.4.3. The relative correlation between the abundance of *nifH* and *alkB* genes in mangrove sediments

The correlation between the abundance of *nifH* and *alkB* genes in different sites represented a useful method for investigation factors involved in bioremediation sediments contaminated with oil. In my study, the copy number of *nifH* and *alkB* genes in sediments differed among the three sites. Jurelevicius *et al.* (2010) decreed that in bulk soil, rhizosphere, and root samples had a complex population of alkane-utilizing bacteria and a variable nitrogen-fixing population was observed via PCR - DGGE analysis of *alkB* and *nifH* genes. In this study, the qPCR data revealed statistically similar *nifH* and *alkB* genes abundances occurred across the samples of two sites (Site 1 and 2) in Shwe Thaung Yan mangroves forest (Figure 2.7 and 2.8). This suggests that the carrying capacities for such microbial guilds were similar across the sediments sampled in the two sites. However, a trend toward an increased abundance of *alkB* gene in the area with higher oil impact (Sites 2 and 3; maybe the oil-contaminated mangrove) was found. The oil contamination may have raised carbon input and limited O_2 availability at Sites 2 and 3, stimulating these populations compared to Site 1. In addition, the vegetation at Sites 2 and 3 was less dense and diverse, leading to the less intense cycling of organic matter, thus inducing the growth of nitrogen fixation bacteria to obtain inorganic forms of

nitrogen.

2.4.4. Microbial diversity in Shwe Thaung Yan mangrove forest, the Republic of the Union of Myanmar

According to a lot of previous researches, the nucleic acid-based techniques is a useful tool for diversity studies in natural habitats (Amann *et al.* 1995), and next generation sequencing (NGS) technique is a conventional tool to analyses of the composition and diversity of microbial communities in several habitats (Huber *et al.* 2007). In present study, Proteobacteria was the most abundant and largest phylum in all sediments, but its classes showed the different tendencies. Within the phyla, Deltaproteobacteria, Gammaproteobacteria, and Alphaproteobacteria enriched in four layers of Site 1, Deltaproteobacteria and Gammaproteobacteria in four layers of Site 2, Deltaproteobacteria, Gammaproteobacteria, Alphaproteobacteria and Betaproteobacteria in four layers of Site 3, and Deltaproteobacteria in all sites. The wide distribution of Alphaproteobacteria and Betaproteobacteria in estuarine environments had well documented, and pH and nutrients have been explained to be related to their abundances (Newton *et al.* 2011). In comparison, the bacterial groups enriched in mangrove sediment were mainly limited to Gammaproteobacteria and Deltaproteobacteria (Figure 2.11), and almost all of them were involved in the sulfur reduction (Campbell *et al.* 2006).

Site 1 and 2 showed a unique pattern of bacterial diversity that compare to Site 3. This pattern was concordant with recent reports about bacterial communities in a Brazilian mangrove (dos Santos *et al.* 2011) and Korean intertidal sediments (Kim *et al*.

2008). According to my analysis, the unique microbial communities of Site 1 and 2 correlated within the depth profile. A large number of photoautotrophic microbes, e.g., (Chloroflexi) significantly enriched in mangrove environments (Figure 2.10). Many chemolithoautotrophic bacteria form Epsilonproteobacteria contained in all sites. It has been reported that Epsilonproteobacteria was populated in high abundance at oxic-anoxic interfaces such as marine sediment surface meet with oxygenated seawater (Campbell *et al.* 2006), and my study suggests that the intertidal sediment also another typical interface. Additionally, a recent study reported that the novel groups of Gammaproteobacteria accounted for 40 to 70 % of CO_2 fixation using sulfur as the electron donor in the coastal intertidal sediment (Lenk *et al.* 2011). It also showed the high abundance of OTUs from unclassified Gammaproteobacteria within the mangrove sediments, and the tag sequence of these OTUs showed a relatively high similarity (97 %). Gammaproteobacteria orders Thiotrichales (2.4%), Alteromonadales (0.6%) and Oceanospirillales (0.1%) were hydrocarbon degradation bacteria that found in all sites. The most striking characteristic within the Gammaproteobacteria group was that found Microbulbifer and Congregibacter genera that degrade hydrocarbons. Their findings provided the target organisms for monitoring oil spills in mangrove environments. Santos *et al.* (2011) findings have supported this result, and it indicated that certain microbial groups can serve as bioindicators of oil pollution and that achievable identify which pollutants are present. All of these results suggested that the four layers of Site 3 were highly productive and had various types of primary producers that contributed to the high nutrient concentration

in the intertidal environment. It is rational to deduce that a high primary production supports diverse consumers, and many saprophytic microbes preferring eutrophic conditions, such as Bacteroidetes, Firmicutes, and Actinomycetales were enriched in all layers of three sites accordingly.

My results revealed that Site 2 and 1 had a significantly higher species diversity compared to site 3 (< 0.05). Overall, I observed the greater diversity along the tidal cheek environment (Site 2 and 1), that compared to the special marine park environment (Site 3). The reason for the enrichment of species could be due to a sustainable nutrient cycle (organic material sourced from the sea) in along the tidal cheek of mangrove environment. King (1984) and Saintilan *et al*. (2009), which suggests that the diversity increases as elevation increases. Because the tidal cheek is often submerged in salty or brackish water, which limits the species to those that can survive in an anoxic environment. Additionally, the mangrove sediment tends to be dominated and influenced by autotrophs that limit nutrient resources available to non-autotrophic species (Zedler and West 2008).

In concluded, microbial communities from three different sites showed obvious differences, which could not be fully explained by the limited factors determined in the present study. However, the present study provided a detailed comparison of three sites in twelve sediments using the same high-throughput sequencing method. The four layers in Site 2 had the highest diversity, with Gammaproteobacteria, Chloroflexi and Crenarchaeota (Archaea), and Deltaproteobacteria as indicators. The four layers in Site 1 had medium diversity, with many primary producers (such as Chloroflexi, Bacillariophyta,

Gammaproteobacteria, and Epsilonproteobacteria) and eutrophic microbes (such as Bacteroidetes, Firmicutes, and Actinomycetales). Although, Site 3 had the lowest diversity and it enriched with Gammaproteobacteria, Alphaproteobacteria and some orders of Betaproteobacteria. There were mainly involved with nitrogen-fixing bacteria, sulfate-oxidizing bacteria and sulfate-reducing bacteria under anaerobic conditions. In anaerobic sediments, sulfate-reducing bacteria play an important role in nutrient cycling and organic matter remineralization, and they can be especially important in oil-polluted locations where certain sulfate-reducing bacteria are capable of anaerobic hydrocarbon degradation. Alphaproteobacteria affiliated sequences (Figure 2.11) were abundant in four sediment layers of Site 3 (8.09 %, 6.01 %, 5.98 % and 5.81 %), respectively. Almost half of the Alphaproteobacterial sequences were affiliated with the photosynthetic purple non-sulfur order Rhodospirillales, while other sequences were affiliated with the mostly nitrogen-fixing order Rhizobiales. Numerous other sequences were dispersed through unclassified groups consisting of mainly marine related sequences.

2.5. Conclusions

The expanding genomic database has provided information on genome context as well as sequence information that can use in the next generation of environmental microbial studies. The use of *nifH* gene can provide additional supporting or contrast information for evaluating nitrogen fixation in the mangrove forest. The highest nitrogen fixation rate and *nifH* genes copy numbers found in sediments depth profile of Site 3.

Generally, the high copy numbers occurred in shallow layers in all sites. Also, *nifH* genes abundance was decreased with the increasing depth, and its structure was significantly correlated with the sediment characteristics and nitrogen fixation rate. In qPCR data revealed that statistically similar abundance of *nifH* and *alkB* genes occurred across the samples at two sites (Site 1 and 2) in Shwe Thaung Yan mangroves forest. The microbial communities showed considerable diversity in shallow layers and diversity decreased with the increasing depth. Bacterial phylum Proteobacteria, such as Deltaproteobacteria, Gammaproteobacteria, Alphaproteobacteria and Betaproteobacteria were more prevalent in all site. The bacterial composition shifted according to the depth and the affiliation of some groups suggests wide-scale participation in key nutrient cycles. This result again links the high occurrence of Deltaproteobacteria with the niches available for microbial colonization not only nitrogen fixation but also hydrocarbon degradation in mangrove sediments, making these groups candidates for more thorough assessment in future studies of mangrove microbiology. In recent decades, more interest has been given to the preservation of mangrove areas, due to its important functions performed in the environment. Further studies on the microbial ecology in mangroves are required for a better understanding of the functional role of the microorganisms in these environments. So, this study information will provide a better description of mangrove ecosystem and the development of preservation strategies.

Table 2.1. Measurement condition of gas chromatography.

GC-14B	
Detector	FID detector (hydrogen flame ionization detector)
Column	Sincarbon ST (50/80 mesh), packed, length 3 m × inner diameter 3 mm stainless-steel
Sample injection volume	1 mL
Column temperature.	60°C
Injector temperature	210°C
Detector temperature	210°C
Oven	160°C
Carrier gas	He (60 mL/min), H_2 (50 mL/min), O_2 (50 mL/min)

Table 2.2. Primer sets information for PCR amplification.

Target	Primer name	Sequence (5'-3')	Tm (°C)	Ref.
16Sr DNA	27F	AGAGTTTGATCMTGGCTCAG	52	Lane (1991)
	1492R	CGGTTACCTTGTTACGACTT	46	
nifH	PolF	TGCGAYCCSAARGCBGACTC	60.4	Poly *et al.* (2001)
	PolR	ATSGCCATCATYTCRCCGGA	58.4	
alkB	alkB- F	AAYACIGCICAYGARCTIGGICAYAA	59	Bell *et al.* (2011)
	alkB- R	GCRTGRTGRTCIGARTGICGY TG	61	

Table 2.3. Primer sets information for real-time PCR (qPCR).

Target	Primer name	Sequence (5'-3')	Tm (°C)	Ref.
16Sr DNA	1369F	CGGTGAATACGTTCYCGG	59	Suzuki *et al.* (2000)
	1492R	CGWTACCTTGTTACGACTT	56.5	
nifH	PolF	TGCGAYCCSAARGCBGACTC	60.4	Poly *et al.* (2001)
	PolR	ATSGCCATCATYTCRCCGGA	58.4	
alkB	alkB- F	AAYACIGCICAYGARCTIGGICAYAA	59	Bell *et al.* (2011)
	alkB- R	GCRTGRTGRTCIGARTGICGY TG	61	

Table 2.4. Physico-chemical environmental parameters of sampling three sites at Shwe Thaung Yan mangrove forest, the Republic of the Union of Myanmar.

Site	Dominant mangrove Species	Depth (cm)	Temp (℃)	pH	EC (µS/cm)	Sediment Texture	Salinity	
							Sediment	Seawater
1	*Bugueria gymnorrhiza*	5	28.5	5.9	1.750	Clay loam	35.0	32.0
		10	28.4					
		15	28.1					
2	*Ceriops tagal*	5	28.5	6.0	1.725	Mud-clay	34.5	31.0
		10	28.5					
		15	28.3					
3	*Rhizophora mucronata*	5	29.2	6.3	1.675	Mud- Clay- loam	33.5	32.0
		10	28.5					
		15	27.9					

Table 2.5. Rates of ethylene production and nitrogen fixation in Site 1 sediments incubated with 0.1 atm acetylene under (a) aerobic condition, (b) anaerobic condition.

(a) Aerobic condition

Depth (cm)	C_2H_4 (nmole/g(dry weight)/h)	N_2 – fixation rate (nmole/g(dry weight)/h)	Molar ratio	
0-5	2.03±0.31	0.68	3:1	(n=4)
5-10	2.38±0.48	0.80	3:1	(n=4)
10-15	0.52±0.13	0.17	3:1	(n=4)
15-20	0.73±0.13	0.24	3:1	(n=4)

(b) Anaerobic condition

Depth (cm)	C_2H_4 (nmole/g(dry weight)/h)	N_2 – fixation rate (nmole/g(dry weight)/h)	Molar ratio	
0-5	0.66±0.09	0.22	3:1	(n=4)
5-10	0.72±0.11	0.24	3:1	(n=4)
10-15	0.35±0.05	0.12	3:1	(n=4)
15-20	0.33±0.05	0.11	3:1	(n=4)

Table 2.6. Rates of ethylene production and nitrogen fixation in Site 2 sediments incubated with 0.1 atm acetylene under (a) aerobic condition, (b) anaerobic condition.

(a) Aerobic condition

Depth (cm)	C_2H_4 (nmole/g(dry weight)/h)	N_2 – fixation rate (nmole/g(dry weight)/h)	Molar ratio	
0-5	3.53±0.77	1.18	3:1	(n=4)
5-10	0.80±0.14	0.27	3:1	(n=4)
10-15	0.85±0.14	0.28	3:1	(n=4)
15-20	0.43±0.08	0.14	3:1	(n=4)

(b) Anaerobic condition

Depth (cm)	C_2H_4 (nmole/g(dry weight)/h)	N_2 – fixation rate (nmole/g(dry weight)/h)	Molar ratio	
0-5	0.53±0.77	0.18	3:1	(n=4)
5-10	0.64±0.10	0.21	3:1	(n=4)
10-15	0.40±0.06	0.13	3:1	(n=4)
15-20	0.15±0.01	0.05	3:1	(n=4)

Table 2.7. Rates of ethylene production and nitrogen fixation in Site 3 sediments incubated with 0.1 atm acetylene under (a) aerobic condition, (b) anaerobic condition.

(a) Aerobic condition

Depth (cm)	C_2H_4 (nmole/g(dry weight)/h)	N_2 – fixation rate (nmole/g(dry weight)/h)	Molar ratio	
0-5	7.08±1.38	2.36	3:1	(n=4)
5-10	3.31±0.69	1.10	3:1	(n=4)
10-15	1.84±0.33	0.61	3:1	(n=4)
15-20	2.32±0.45	0.77	3:1	(n=4)

(b) Anaerobic condition

Depth (cm)	C_2H_4 (nmole/g(dry weight)/h)	N_2 – fixation rate (nmole/g(dry weight)/h)	Molar ratio	
0-5	2.65±0.44	0.88	3:1	(n=4)
5-10	1.45±0.25	0.48	3:1	(n=4)
10-15	1.59±0.27	0.53	3:1	(n=4)
15-20	0.58±0.09	0.19	3:1	(n=4)

Table 2.8. Averaged alpha diversity indicator index Chao1, OTU numbers, PD whole tree, and Shannon index at three site sediments in Shwe Thaung Yan mangrove forest. In alpha diversity analysis, there were generated based on a species level of 97% similarity.

Site	Depth (cm)	Observe species	Chao 1	OTUs	PD whole tree	Shannon
1	0 – 5	14529	39846	14529	785	10.8
	5 – 10	118112	33926	11812	656	10.3
	10 – 15	11504	35115	11504	636	10.0
	15 - 20	10818	34439	10818	588	9.8
2	0 – 5	14849	41583	14849	790	11.0
	5 – 10	11680	36464	11680	653	9.9
	10 – 15	11437	33515	11437	631	9.9
	15 - 20	13711	39651	13711	723	10.5
3	0 – 5	11731	35452	11731	672	10.3
	5 – 10	9811	30386	9811	579	9.9
	10 – 15	10003	30802	10003	605	9.6
	15 - 20	10071	30874	10071	610	9.9

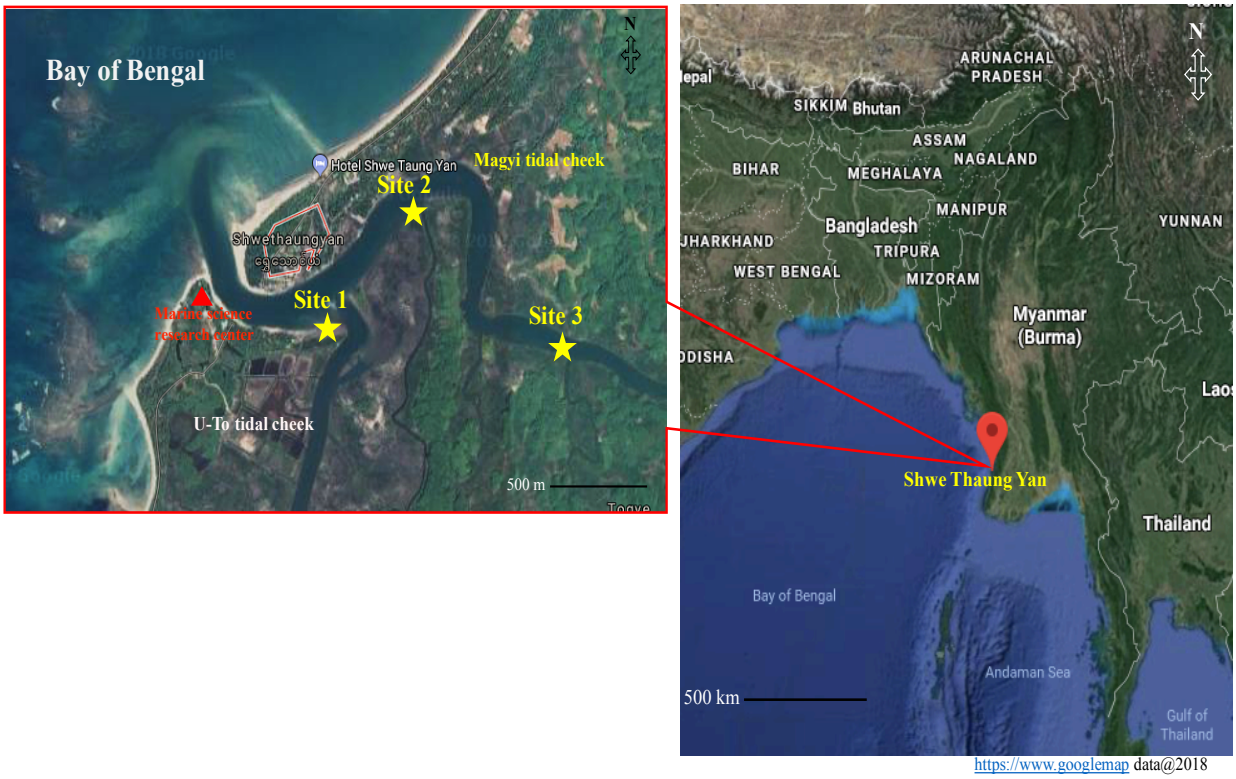


Figure 2.1. Study sites in Shwe Thaung Yan mangrove forest along the Magyi tidal cheek, the Republic of the Union of Myanmar.

Site 1. 17°04.222′N 94°28.913′E (at the mouth of tidal cheek: nearby village and harbour),

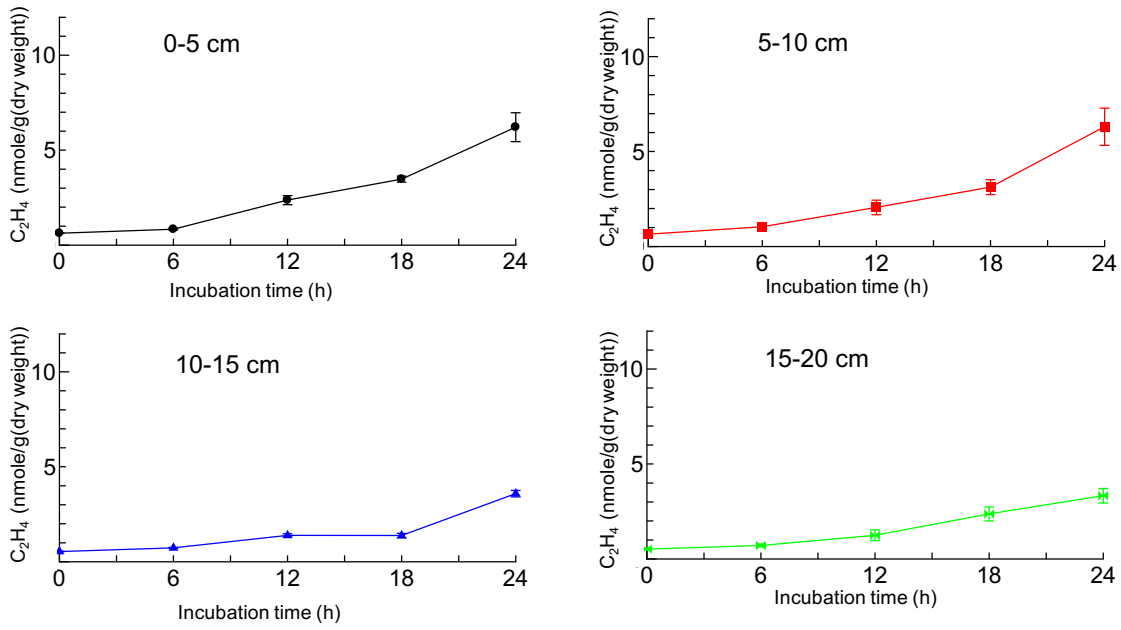
Site 2. 17°04.324′N 94°27.917′E (at the middle of tidal cheek: nearby village),

Site 3. 17°04.701′N 94°28.141′E (at the marine park: mangrove maintaining area).

Site 1	Site 2	Site 3
***Bugueria gymnorrhiza* spp.**	***Ceriops tagal* spp.**	***Rhizophora mucronata* spp.**
		
		

Figure 2.2. Study three sites information in Shwe Thaung Yan mangrove forest along the Magyi tidal cheek, Myanmar.

(A) Aerobic condition



(B) Anaerobic condition

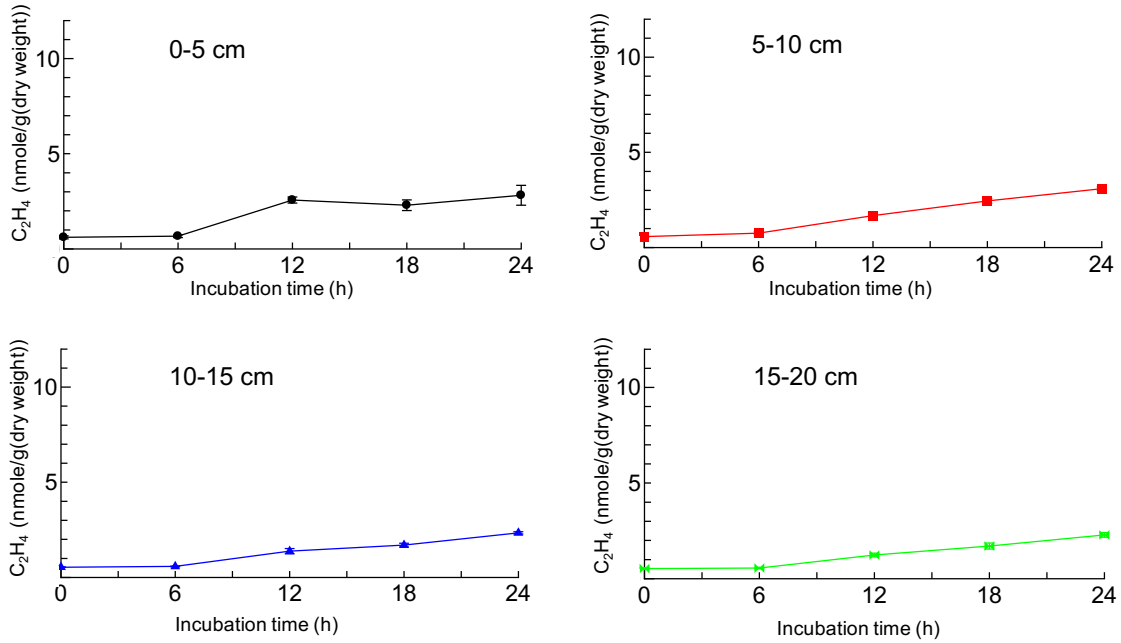
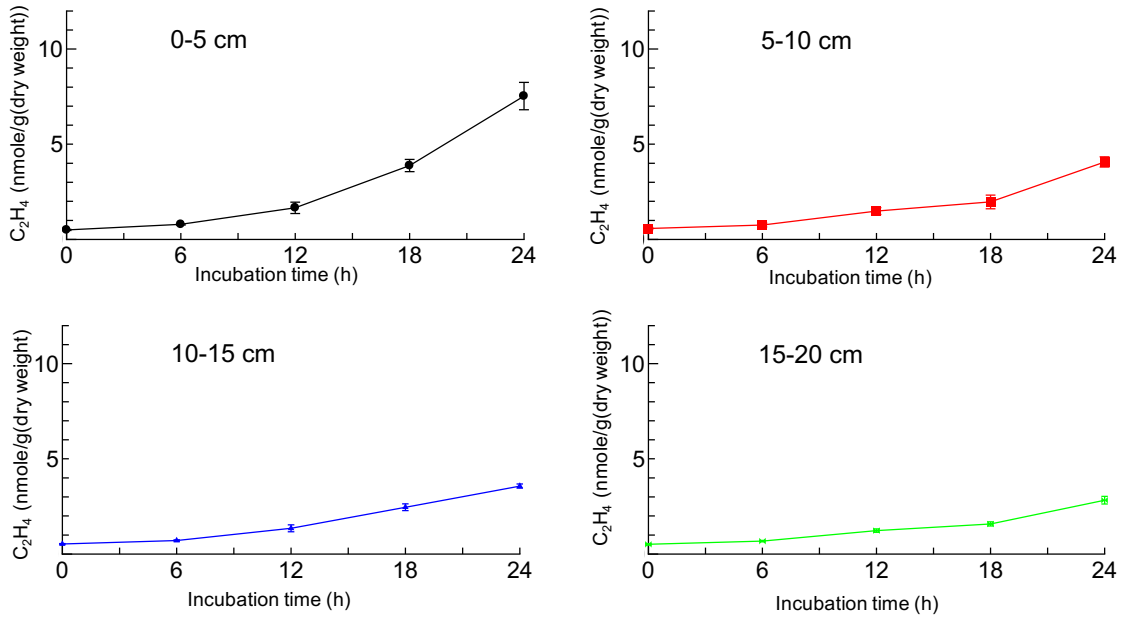


Figure 2.3. Acetylene reduction assay under aerobic condition (A), anaerobic condition (B) using sediment samples of Site 1.

(A) Aerobic condition



(B) Anaerobic condition

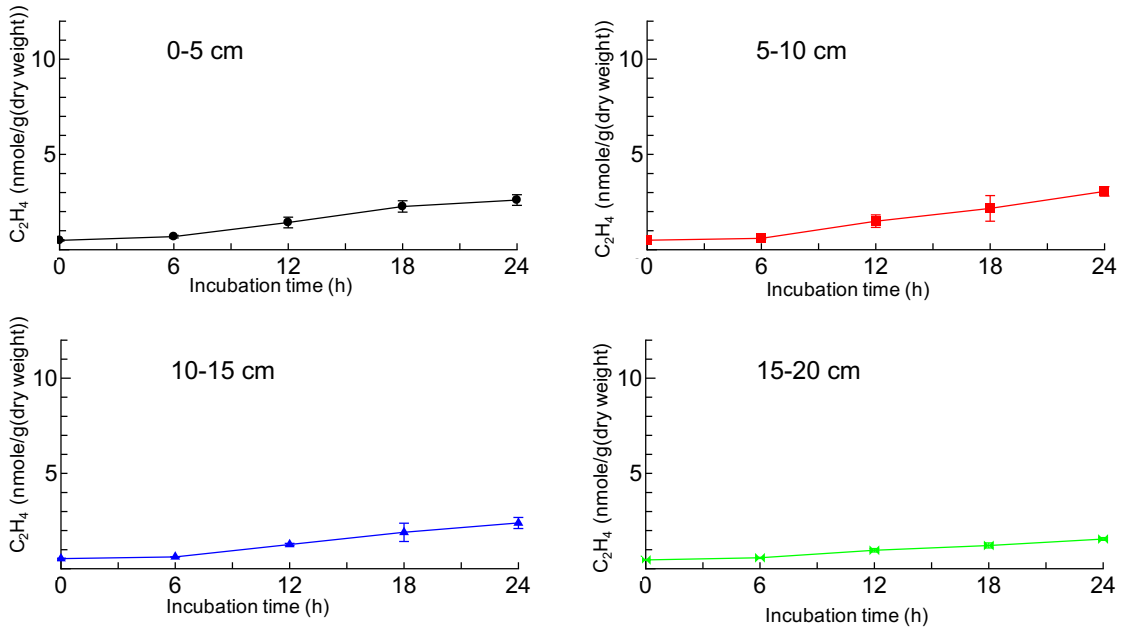
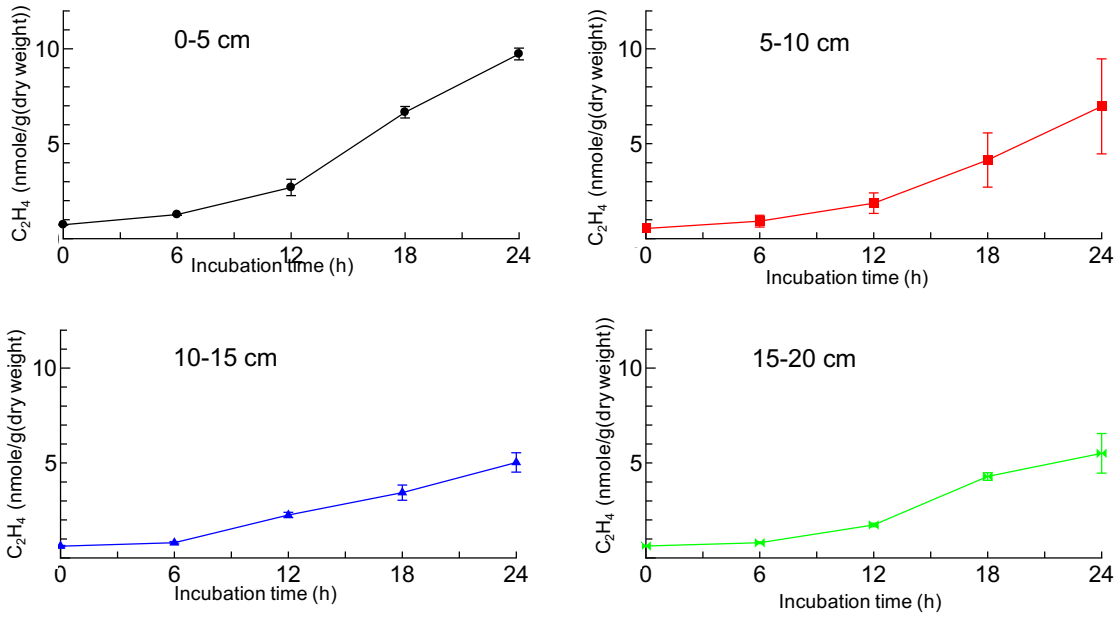


Figure 2.4. Acetylene reduction assay under aerobic condition (A), anaerobic condition (B) using sediment samples of Site 2.

(A) Aerobic condition



(B) Anaerobic condition

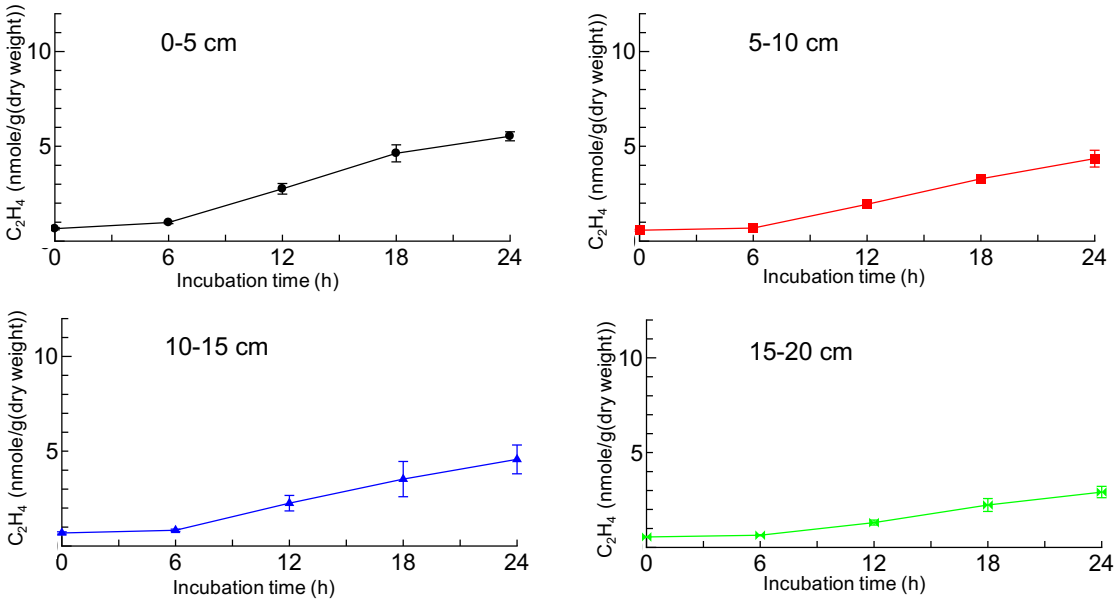


Figure 2.5. Acetylene reduction assay under aerobic condition (A), anaerobic condition (B) using sediment samples of Site 3.

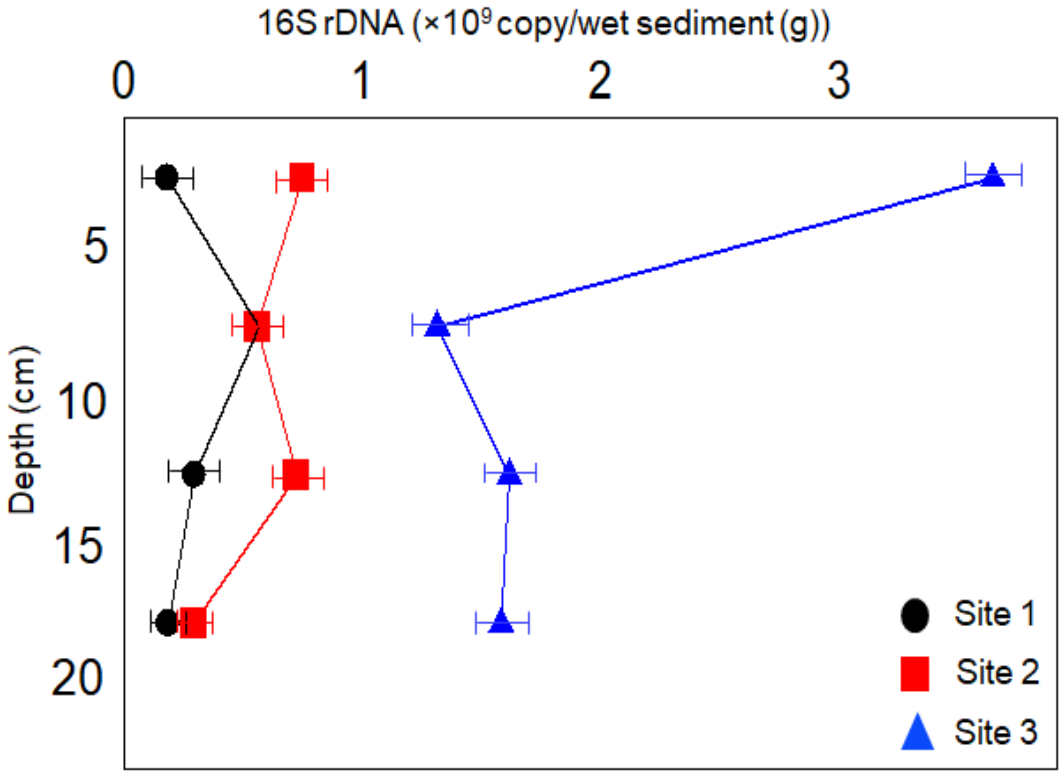


Figure 2.6. The depth profile of 16S rDNA gene abundance in Shwe Thaung Yan Mangrove forest, Myanmar (n=3).

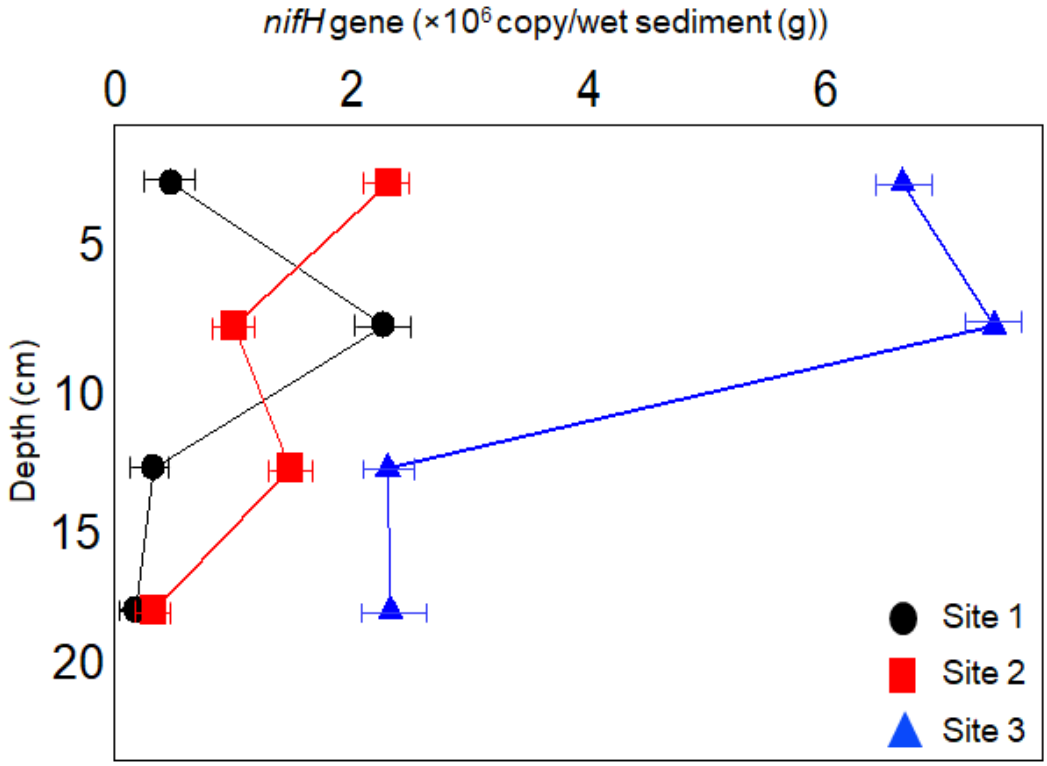


Figure 2.7. The depth profile of *nifH* gene abundance in Shwe Thaung Yan Mangrove forest, Myanmar (n=3).

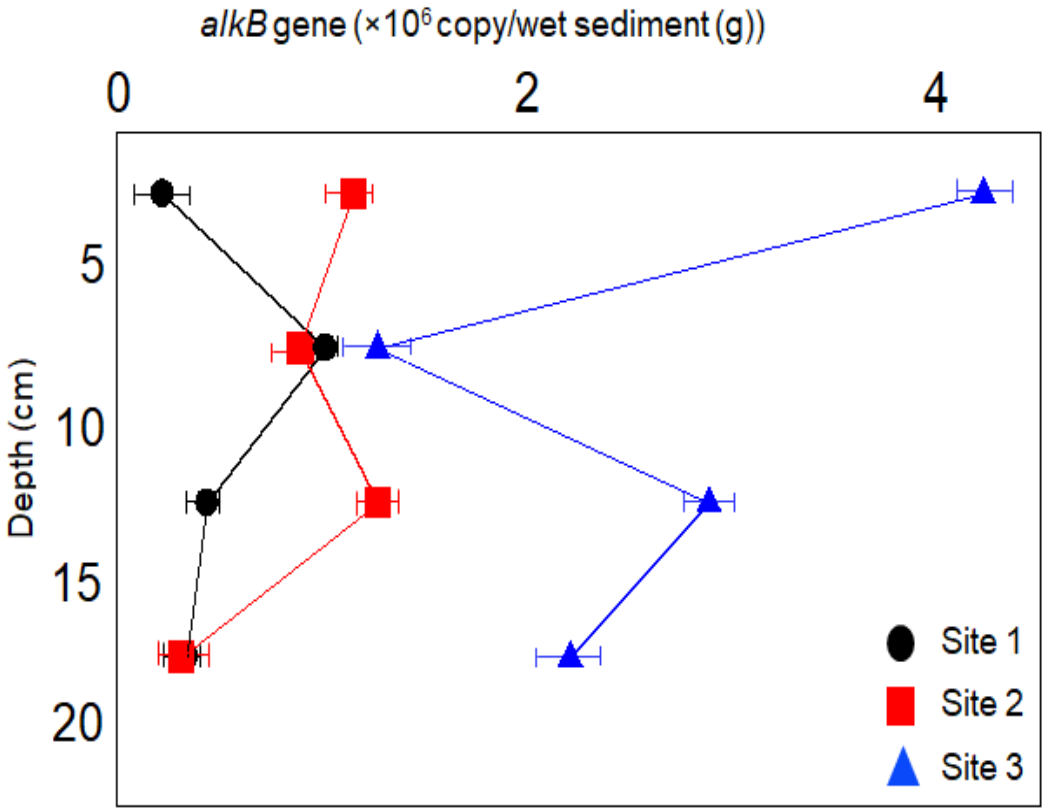


Figure 2.8. The depth profile of *alkB* gene abundance in Shwe Thaung Yan Mangrove forest, Myanmar (n=3).

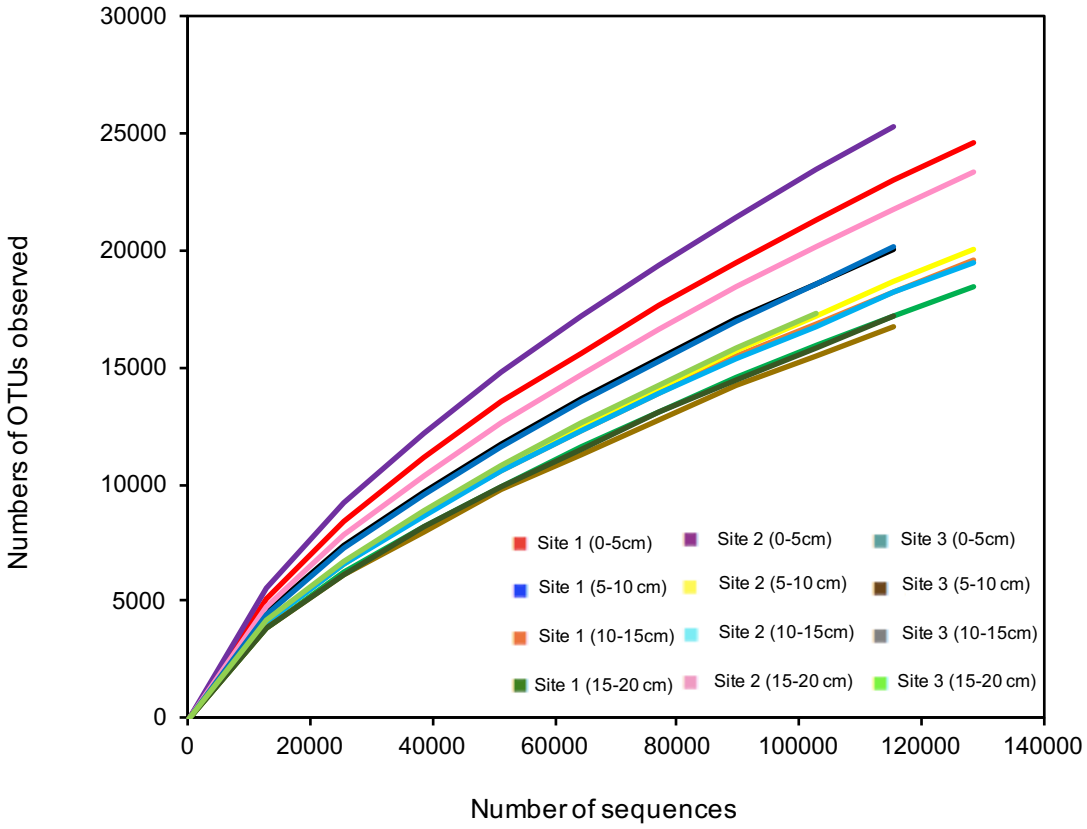


Figure 2.9. Rarefaction curves generated for 16S rRNA gene sequences from three sites in mangrove sediments. Sequences were grouped into OTUs based on distance sequences similarity of 97 %.

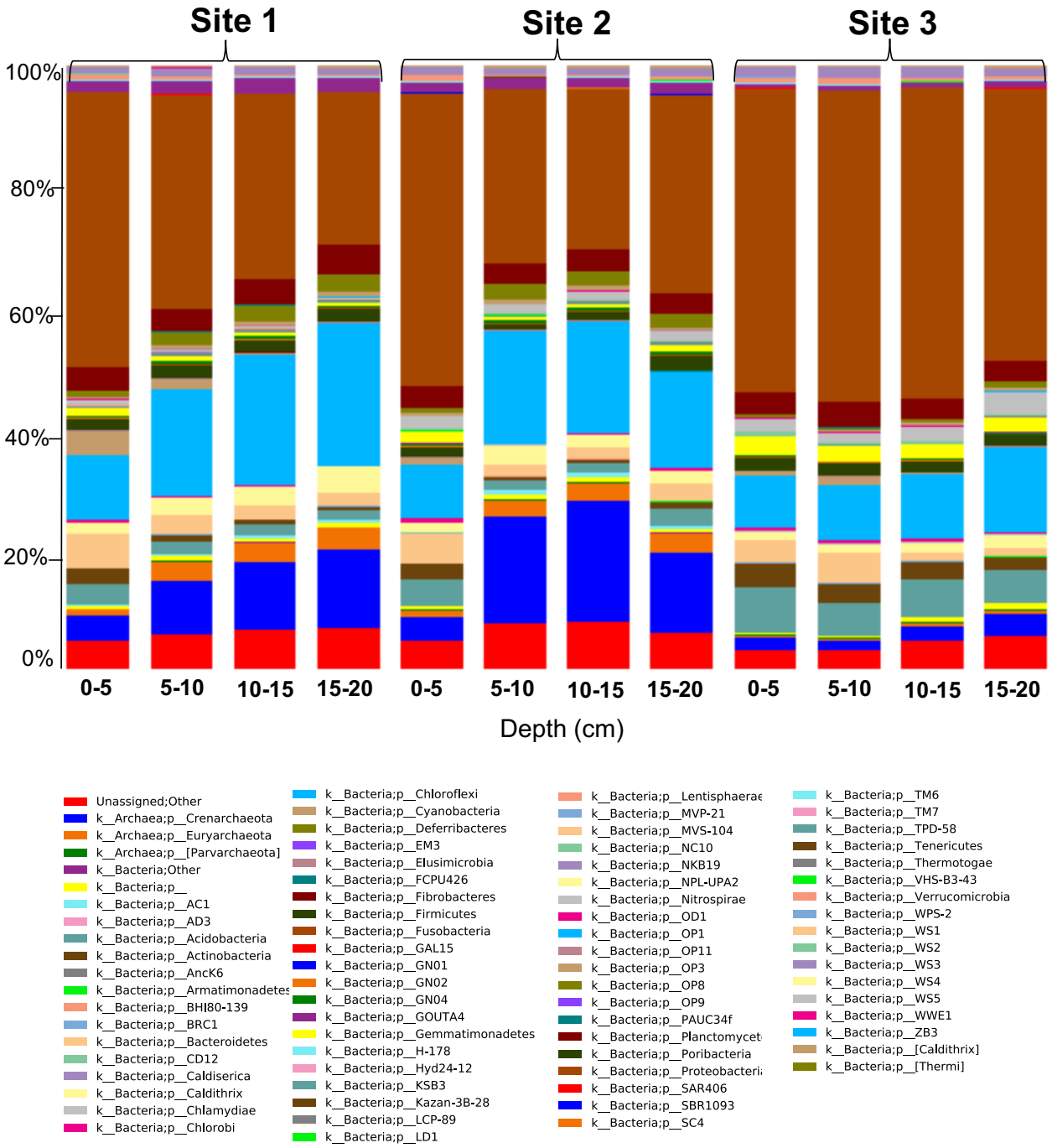


Figure 2.10. Taxonomic distribution of Phyla level at three site sediments in Shwe Thaung Yan mangrove forest.

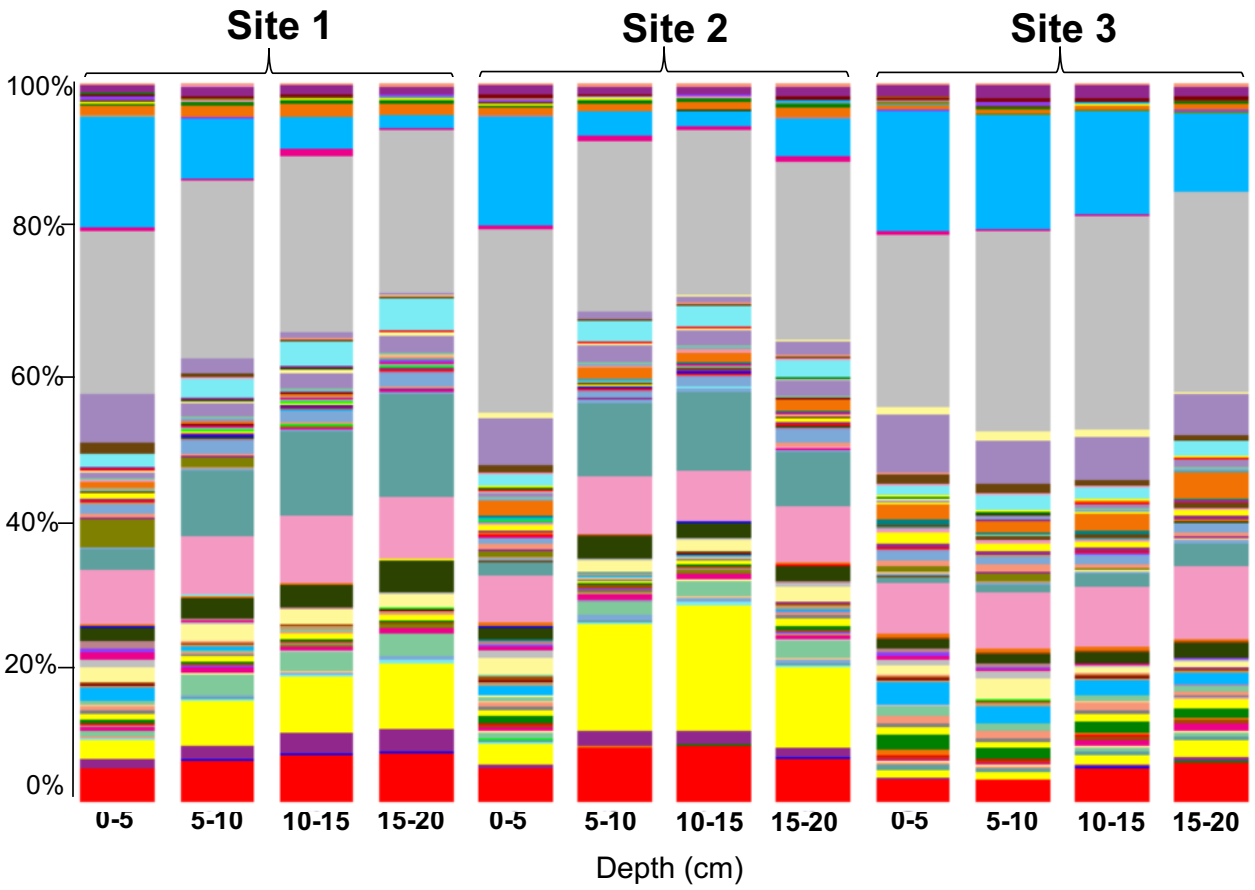


Figure 2.11. Taxonomic distribution of Class level at three site sediments in Shwe Thaung Yan mangrove forest.

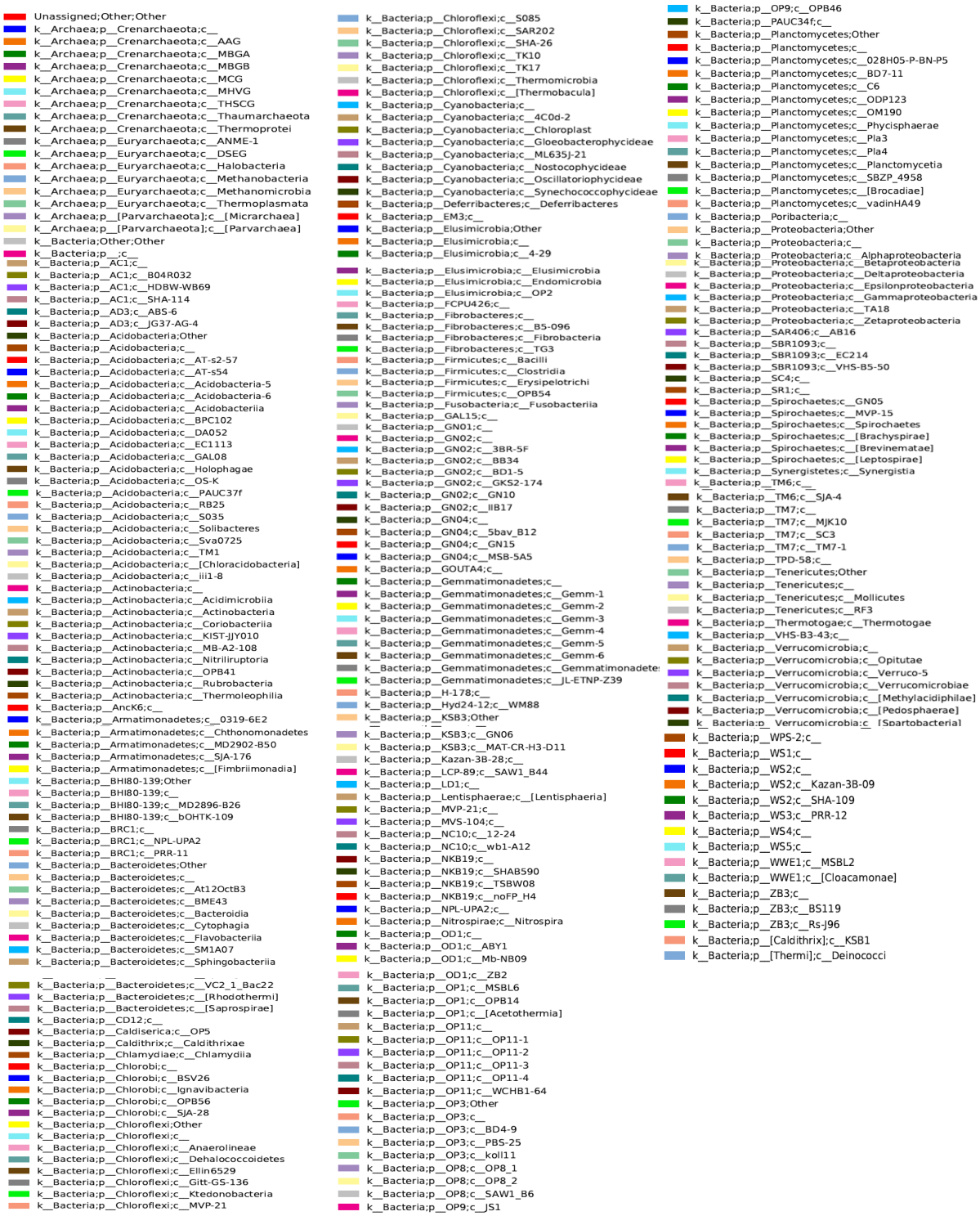


Figure 2.11. The legends for taxonomic distribution of Class level at three site sediments in Shwe Thaung Yan mangrove forest.

Principal component analysis (PCoA) Weighted-UniFrac

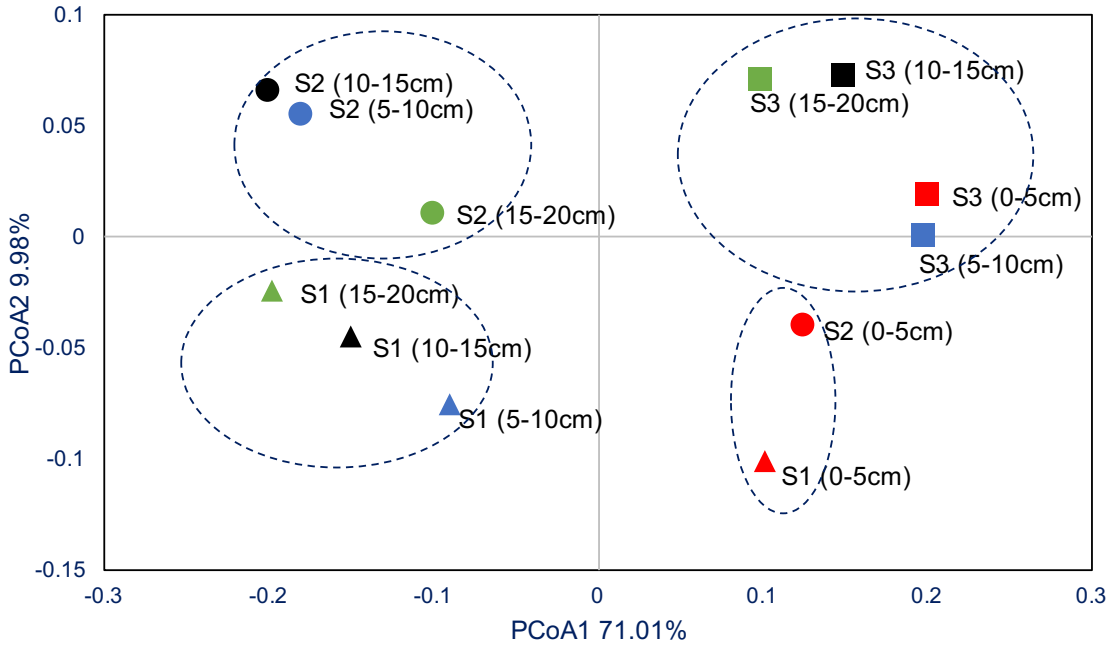


Figure 2.12. Principal component analysis (PCoA) of total 16S rRNA in Weighted-UniFrac showing species diversity over number of reads. The percentages of variance explained in each axis are indicated.

Chapter 3: Management in temperate coastal area by hydrocarbons bioremediation in oil-contaminated seawater

3.1. Introduction

With growing needs for humans' daily life, more and more crude oil has been explored and used exploited worldwide. However, releases of crude oil, due to spills from ship tankers, offshore platforms, and drilling wells, are now threatening the marine and coastal ecosystems. Crude oil spills that occurred in America, Korea, Spain, Singapore, and many other places have brought severe damage to the local ecosystems. Crude oil and other petroleum by-products are mainly composed of hydrocarbons of various molecular weights, such as alkanes, cycloalkanes, and aromatic hydrocarbons. These organic compounds are difficult to be metabolized into lighter forms or to be degraded and are toxic to most animals and humans. Nowadays, bioremediation, which uses microbial metabolism to break down petroleum hydrocarbons with additional nutrients supply, has been proven to be an effective method to remove petroleum pollution after crude oil spills.

Although bioremediation has already been introduced into some oil spill accident scenes, and increasing knowledge has been accumulated in bioremediation processes, information about biodegradation associated with carbon numbers of hydrocarbons as well as effects of temperature and other environmental conditions remains to be limited. Temperature is one of the important environmental factor affecting on the growth of

microorganisms, also influence the petroleum degradation not only through affecting the activities of petroleum-degrading microorganisms, but also changing the properties of petroleum itself (Nedwell 1999; Northcott and Jones 2000; Rowland *et al.* 2000). Activities of petroleum-degrading microorganisms could increase with the increase of temperature (Bossert and Bartha, 1984; Cooney, 1984), however, toxicity of petroleum components may also increase with temperature and the biodegradation of the fraction of recalcitrant petroleum components may still be poor (DeFoe and Ankley 2003; Leathy and Colwel 1990; Whyte et al. 1998). Although bioremediation efficiency has been tested on subpolar soil, estuary and seawater samples on low temperatures down to -1℃ (Akbari *et al.* 2015; Braskstad and Bonaunet 2006; Braskstad *et al.* 2015; Camenzuli and Freidman 2015; Colwell *et al.* 1978; Coulon *et al.* 2007; Venosa *et al.* 2007), further discuss on the influence of temperature are still needed, in aspects like seasonal bioremediation dynamics in temperate zones, and degradation efficiency associated with hydrocarbon structures, and so on.

In this study, I investigated seasonal changes of crude oil degradation efficiencies in simulated petroleum-polluted seawater collected from Tokyo Bay, and, for the first time, demonstrated degradation dynamics of individual hydrocarbons in different carbon number. At the meantime, I also connected the seasonal petroleum degradation to the shifts of potential functional genes of alkane-degrading bacteria. This study provides an integrated insight into the effect of temperature on the bioremediation of petroleum hydrocarbons in different molecular weights. These plans can serve as a secure platform

for remediation and restoration of oiled shoreline. A number of steps are required to build the scientific basis for the management of oil spills. This information becomes available from ongoing studies of coastal ecosystem responses to oiling. Knowledge of the spatial distribution of shoreline sensitivity, coastal resources and habitats vulnerable to an oil spill is crucial for effective coastal zone management.

3.2. Materials and methods

3.2.1. Sampling

Seawater sample was collected in two stations in Tokyo Bay (St. 1 Inner Tokyo Bay and St. 2 Central Tokyo Bay) seasonally (Figure 3.1). In February (Winter), May (Spring), August (Summer) and November (Autumn). One liter of surface seawater was collected for each station, and being stored in polyethylene bottles on ice before use. Seawater samples brought back to the laboratory were subjected to microcosm culture within 12 hours of sampling.

3.2.2. Water Quality Measurement

In this study, water temperature, pH and DO were measured at the site and measurement and analysis of nutrient salts (NO_3 - N. NOA, NH_4 - N, PO_4 - P) was carried out in the laboratory. The temperature was measured with an alcohol thermometer (AS ONE), DO was measured by a DO meter (Pro Do, YSI Nanotech), and pH was measured using a pH meter (pH/conductivity meter D-54, HORIBA). Each nutrient analysis method is as follows:

(1) Nitrate nitrogen (NO_3 - N)

Nitrate-nitrogen was measured by the brucine sulfanilic acid method (Saito *et al.* 1971). First, 1 mL of sodium chloride (30 w / v%) and sulfuric acid (30 w / v%) were added to 5 mL of sample water. Then, the amount of nitric acid ion was determined by measuring the absorbance of the yellow compound (4 + 1) was added, then mixed well, and cooled with running water. Thereafter, the water was kept in a constant temperature bath at 20°C, and 0.25 mL of the brucine sulfanilic acid solution was added to it. The mixture was allowed to stand in boiling water for 20 minutes. After sufficiently cooling with running water again, the temperature of the test water was kept in a constant temperature bath at 20°C. The absorbance at a wavelength of 415 nm was measured using a spectrophotometer (UV mini 1200, SHIMADZU).

(2) Nitrite nitrogen (NO_2 - N)

Nitrite nitrogen was measured by diazotization. The diazotization method is a method for measuring the red absorbance of a diazo compound generated by reacting nitrite nitrogen with an aromatic primary amine to produce an azo compound and adding aromatic amines for coupling. First, 0.1 mL of sulfanilamide solution was added to 5 mL of sample water and left for 5 minutes. Thereafter, ethylenediamine solution was added to 0.1 mL of sample water and allowed to stand at room temperature for 20 minutes. Then, the absorbance at a wavelength of 543 nm was measured using a spectrophotometer (UV mini 1200, SHIMADZU).

(3) Ammonia nitrogen (NH_4 - N)

The ammonia nitrogen was measured using the indophenol blue method. Indophenol blue method is a method of measuring and quantifying the absorbance of indophenol blue produced by reacting ammonia nitrogen with phenol under the coexistence of hypochlorite. First, 0.2 mL of a phenol solution in which 10 g of phenol was dissolved in 100 mL of 95% ethanol, 0.2 mL of a nitroprusside sodium solution and 5 mL of an oxidation reagent were added to 5 mL of sample water, and the mixture was sufficiently mixed by vortex and then left for 1 hour at room temperature. And then absorbance was measured at a wavelength of 630 nm using a spectrophotometer (UV mini 1200, SHIMADZU).

(4) Phosphorous phosphorus (PO_4 - P)

Phosphorus was measured by the molybdenum blue method. In the molybdenum blue method, phosphate ions were dissolved in ammonium molybdate and diantimony tartrate (11) acid. Reduction with p-toluic compound formed by reaction with potassium with L-ascorbic acid, measurement, and quantification of the absorbance of molybdenum blue produced. First, 2.5 mL/L sulfuric acid: ammonium molybdate solution: antimony potassium tartrate dissolved Solution: 0.5 mL of a mixed reagent prepared by mixing L-ascorbic acid solution at a volume ratio of 5: 2: 1: 2 was added and allowed to stand at room temperature for 5 minutes. Thereafter, the absorbance at a wavelength of 885 nm was measured using a spectrophotometer (UV mini 1200, SHIMADZU).

3.2.3. Preparation of microcosm and incubation experiment

The seawater sample was first filtrated with diameter 47 mm, pore size 3.0 μm (Nuclepore Membrane Filter, Whatman) to prevent decomposition of petroleum-degrading bacteria due to predation pressure of larger microorganisms. Then, 20 mL of filtered seawater was added to a 100 mL of glass vial bottle (Nichiden Rika).

The above seawater samples were then subjected to two different treatments: (1) NT; additional nutrients were supplied (20 mg/L NH_4NO_3 and 10 mg/L KH_2PO_4) (Coulon *et al.* 2007), and then loaded with an oil sheet (made by adding 20 μL vacuum distilled petroleum (prepared as petroleum of C_{19} to C_{36} alkanes, Cosmo Oil Co., Ltd.) to pre-sterilized glass fiber mesh (cut in circle with a diameter of 10 mm)). (2) CK; control blank without additional nutrients supplement, only loaded with an oil sheet. To exclude the possible loss of petroleum hydrocarbons due to reasons other than bioremediation, (3) WK; water blank, which is sterilized artificial seawater loaded with an oil sheet, was prepared in each incubation period. The artificial seawater (salinity: 33 %) was made by the ingredients of artificial seawater (Marine Art SF-1, Tomita Seiyaku Co.).

These microcosms were sealed with PTFE coated rubber stopper and an aluminum cap and incubated in an incubator for 28 days under dark condition (incubation temperature was controlled at 10℃ (winter), 23℃ (spring), 31℃ (summer), and 17℃ (autumn), respectively.

3.2.4. Determination of petroleum component

Determination of petroleum component was carried out on 0, 7, 14, 21 and 28th day of incubation and the procedures are described as followed. First, 20 mL of incubation sample flowed through a pre-conditioning SPE (solid phase extraction) tube (SupelcleanTM ENVI-18 SPE tube, SIGMA-ALDRICH). Then the microcosm vial bottle and oil sheet were rinsed with sterilized MiliQ water (for 1 time) and the mixed solution of hexane and dichloromethane (1:1) (for 3 times). The rinsed solvents also flowed through the SPE tube. All solvents that passed through SPE tube were collected in a test tube and dehydrated by gas injection (blow nitrogen gas onto the top of solvents for 1 h) in a draft under room temperature. Petroleum component that attached to the test tube was then dissolved in 1.5 mL of the mixed solution of hexane and dichloromethane (1:1), transferred to a 2 mL vial bottle (Mighty vial, Maruemu), and stored at -30℃ before analysis.

Concentrations of petroleum hydrocarbons in the extract were quantified by GC-MS (GC-MS 2010 plus, SHIMAZU). For standard curve, a mixture of n-alkanes ranging in different carbon number from C_9 to C_{40} dissolved in n-hexane (Wako Pure Chemicals) was diluted in 20 times, 100 times, 200 times and 1000 times. And the above-extracted samples were then diluted in 50 times before measured by GC-MS. Each hydrocarbon concentration was measured using by a purge & trap type GC-MS 2010 plus (SHIMADZU) by the University of Tsukuba Analysis Center. The GC-MS conditions were listed in Table 3.1.

3.2.5. Determination of total bacterial cell

Samples for cell count were also collected throughout the incubation period in all groups (on 0, 7, 14, 21, and 28^{th} days). DAPI staining method was used to measure the total number of bacteria. For these samples, Paraformaldehyde was added into each microcosm (final concentration vol/vol: 2.5%). Then 10 mL of each microcosm was filtered through 0.2 μm pore size filter (Whatman). These filters were then loaded with 2 μL/mL of adjusted DAPI solution (DAPI, PBS, Vectershid, PBS Glycerol) and dyed for 5 min at room temperature. The total bacterial cell was counted under the fluorescent microscope (BX-50, OLYMPUS). For dyeing, first drop the DAPI solution (DAPI, PBS, Vectorshild, PBS glycerol) adjusted to 2 μL/mL onto a sliding glass (Matsunami Glass), cover with a cover glass and filter. And soaked at room temperature of 25°C for 5 minutes. The total bacterial cell was counted under the fluorescent microscope (BX-50, OLYMPUS), and counting was 1000 times (100×/1.25 oil eyepiece). The cell density using the filter "WU" (λEX 330 - 385: 2EM 420) was measured with a microscope when cell count by DAPI staining. The number of cells was multiplied by the filtering area of the filtered filter and divided by the viewing area per visual field at the time of cell count and the filtration amount.

3.2.6. Analysis of real-time PCR (qPCR) for *alkB* gene

20 mL of incubation samples on day 0, 14 and 28 in each incubation group was through 0.2 μm pore size polycarbonate filter (Whatman), and the filters were stored at -80℃ before nucleic acid extraction. DNA samples were then extracted from these frozen

filters using ISOIL for Beads Beating DNA extraction kit (Nippon Gene), and dissolved in TE buffer. Amplification of the 16S rDNA and *alkB* gene regions of the DNA sample was attempted by PCR. In PCR reaction solution, an appropriate amount of ddH_2O, 0.2 μL of 10 × Ex Taq Buffer (Takara), 1.6 μL dNTP Mix solution (Takara), 0.5 μL each of primer solution (Forward and Reverse each 10 pmol /μL), 0.1 μL of TaKaRa Taq ™ (5 units / μL, Takara), and 1 μL of template genomic DNA was added (total volume of 20 μL) using by thermal PCR (Applied Biosystems). In addition, a mixture of 1.0 μL of PCR product and 1.0 μL of 6 ×dye was added to each cell and subjected to electrophoresis (85 v. 30 min). After the end of the electrophoresis, the band was confirmed by UV irradiation at 302 nm, and the presence or absence of amplification of target area was confirmed.

In order to quantify *alkB* gene, standard DNA used for calibration was prepared. The standard DNA was used on the 28th day of winter and spring culture experiments in the central bay. The primers targeting of *alkB* regions were used PCR amplification was carried out (Tables 3-1, 3-4 and 3-5), and the gel extraction from the PCR product was performed according to the manufacture protocol using the QIAEX® II Gel Extraction Kit (QIAGEN). Furthermore, DNA concentration of the PCR product was measured using pro analyzer (Amersham Pharmacia), and the copy number of the standard DNA sample of *alkB* gen was obtained by substituting into the calculation formula. These samples were also mixed with ddH_2O. 10^3-10^8 copies/μL and were used as standard DNA and subjected to Real-Time PCR.

To record the responsible petroleum-degradation bacteria during incubation, the copy

numbers of *alkB* gene (which encodes for alkanes monooxygenase enzyme, is the biomarker of n-alkanes-degrading bacteria) were measured by real-time PCR. Real-time PCR assays were performed on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) equipped with ABI 7500 software (ver. 1.4.1). Thermal cycle conditions were first optimized by adjusting annealing temperature and primer concentration to ensure the best assay specificity and efficiency. The copy numbers of *alkB* gene were determined in triplicate on non-diluted DNA samples, using primer set designed by Bell *et al.* (2011) in an optimized concentration of 0.2 μM (final concentration of both forward and reverse primer) and the Thunderbird Probe qPCR Mix (TOYOBO) according to the manufacturer's instruction. Known concentration of *alkB* gene amplification of environmental seawater samples was used as the standard DNA in real-time PCR analysis. Real-time PCR thermal cycles were conducted as follows: initial denaturation at 95℃ for 5 min; amplification for 35 cycles of 95℃ for 30 s, primer annealing at 57.5℃ for 35s, and extension at 72℃ for 30 s, with a plate read between each cycle; melting curve analysis at 60℃ to 94℃ with a plate read every 0.5℃ and held for 1 s between each read. Finally, the amplification efficiency was calculated from a calibration curve and its value was 100%.

3.3. Results

3.3.1. Environmental parameters

In situ environmental data during the seawater sample collection are shown in Table

3.2. Among all seasons, water temperature was highest in summer, and then in the order of spring, autumn, and winter; while DO was highest in winter, lowest in summer. In summer, a difference of 6°C was found between the inner Tokyo Bay and the central part of the bay in Tokyo Bay. It seems that the inner part of water temperature is lower than the central part of Tokyo Bay. Because it is influenced by Oyashio in the vicinity of the inner part. Although DO shows the highest value in winter, but the water temperature is low in winter. It may be the amount of saturated dissolved oxygen increases and DO is expected to rise. In summer, DO in the inner part of Tokyo Bay was as high as 2.7 - 3.7 mg / L compared with DO in central Tokyo Bay. From this, it is speculated that water pollution in the central part of Tokyo Bay is more advanced than Inner part of Tokyo Bay. Relatively high nutrients concentrations were observed at the two stations, which is considered as the result of large amount domestic wastewater inflow (Matsumura and Ishimaru 2004).

3.3.2. Dynamics of total bacterial cell density

Except for the WK group (artificial seawater control group), all the other groups showed observable cell growth during incubation (Figure 3.2), while the groups with nutrients addition demonstrated much more considerable and continuous growth compared to without nutrients addition. All groups with nutrients addition in 4 seasons reached approximately 2.0×10^8 cells/mL after 28 days of incubation, showing no obvious seasonal difference.

3.3.3. Degradation of total petroleum hydrocarbons (TPH)

In winter, TPH degradation was 37 ± 8.94 % and 40 ± 3.89 % after 7 days of incubation for central and inner Tokyo Bay respectively; and gradually reached 53 ± 2.47 % for central Tokyo Bay, and 46 ± 18.42 % for inner Tokyo Bay after 28 days incubation. In contrast, the TPH degradation (here may also consider as TPH loss in control groups) was close to 24% (SD: 1.54 to 19.97) in all three control groups (two groups without nutrients addition, one for artificial seawater) after 28-day incubation (Figure 3.3. upleft).

Increased by 13℃ on temperature, TPH degradation was more efficient in spring than in winter. After 28 days of incubation, TPH degradation was 86 ± 4.54 % and 87 ± 1.32 % for central and inner Tokyo Bay respectively, in contrast to 20 ± 8.36 % and 24 ± 9.18 % in respective groups without nutrients addition (Figure 3.3. upright).

Summer season showed the highest TPH degradation in all seasons. TPH degradation suddenly reached nearly 70% after 7 days in both groups with nutrients addition, when the rates were only 8% to 17% in the control groups. After 28 days, the TPH degradation increased to 80% (central) and 87% (inner) with nutrients addition, and 3% to 12% without nutrients addition (Figure 3.3. downleft).

Dynamics of TPH degradation in Autumn was similar to that in spring, despite 6℃ of difference in water temperature. After 28 days, TPH degradation was 66 to 67% for groups with nutrients addition, and 21 to 31% for groups without nutrient addition (Figure 3.3. downright).

3.3.4. Degradation of n-alkanes (C_{20} to C_{36})

In general, bioremediation efficiency decreased with the increase of carbon number in all seasons. The changes in the percentage of remaining alkanes (separated by its carbon number) during bioremediation in each season are shown in Figure 3.4. In winter, degradation could be observed between groups with nutrients (NT treatment) addition and groups without nutrient addition (CK and WK treatment) when carbon number is less than 25. However, the degradation rates in winter were much slower when compared to other seasons. No significant difference can be observed when carbon number is more than 25 neither with nor without nutrient addition.

In spring and summer, a significant difference can be found between nutrients groups and no nutrients groups when carbon number is up to 36. Nevertheless, bioremediation in summer was more active than in spring, as most alkanes were effectively degraded within 7 days in summer, and this took place for 14 to 21 days in spring.

In autumn, alkanes with more than 30 carbons were not significantly degraded within 28 days of incubation. Bioremediation rates for C_{20} to C_{29} were also not as fast as rates in summer, but close to rates in spring.

3.3.5. Dynamics of *alkB* gene in Tokyo Bay

alkB gene was detected in all samples during incubation in 4 seasons. At the beginning of incubation, 5.1×10^2 to 1.1×10^4 copies per Liter of *alkB* gene were detected and increased to 2.6×10^6 to 2.3×10^8 copies per Liter in the 28th day of incubation (Figure 3.3). The most rapid reproductively of *alkB* gene in all seasons was found in the first 14

days, while no or less significant growth in their copy number can be found from 14th day to 28th day. In general, copy number in winter was nearly 1 order of magnitude lower than in other three seasons.

3.4 Discussion

3.4.1. Seasonally changes in petroleum decomposition at Tokyo Bay

The residual ratio of total petroleum hydrocarbon (TPH) was calculated by measuring the residual amount of petroleum using GC/MS. The high petroleum decomposition in without nutrient addition system was not confirmed as compared with the sterilized artificial seawater system. Even in the incubation experiment of summer and spring, significant decomposition of petroleum was confirmed in the nutrient addition system. It is clear above that petroleum decomposition in Tokyo bay does not proceed under low water temperature in the winter season and nutrient addition is necessary for petroleum decomposition in high-temperature spring, summer, and autumn. In the past research, it was reported that active petroleum decomposition was carried out at the mouth of the Thames River in the UK at a water temperature of 7°C (Coulon *et al.* 2007). Although it was not confirmed, this is thought to be due to the difference in features of the sea area. Because of the water temperature at the mouth of the Thames River in England below 10℃ a least about 6 months. However, Tokyo Bay seawater temperature is 2 - 3 months for them to reach 10℃. As described above, since the low-temperature environment does not last long in Tokyo Bay. It is estimated that petroleum-degrading

bacteria could not adapt to low-temperature environment due to the short period to acclimatize to low-temperature environment. The results showed that the petroleum degradation was promoted by adding the nutrient addition and petroleum-degrading bacteria existed in Inner Tokyo bay, where there are no petroleum related facilities around.

This result is based on the previous study that local petroleum-degrading bacteria having petroleum resolution ability exist widely all over the world (Foght and Westlake 2008; Head *et al*. 2006, Leahy and Colwell, 1990; Yakimov *et al.* 2007). The decomposition rate of n-alkane for each carbon number is shown in Figure 3.4. The nutrient addition system in winter, significant decomposition of n-alkane was confirmed up to C_{24} as compared with the control system. However, n-alkane decomposition was not confirmed even in nutrient addition system in C_{25} and later. Decomposition of n-alkane was confirmed in nutrient addition system up to n-alkane of C_{33} in spring, summer, and autumn. However, in each season, n-alkane degradation rate was decreased as the carbon number increased. This tendency is consistent with the past study that the decomposition rate decreases with increasing number of carbons (Mc Kew *et al.* 2007). From the above, decomposition is carried out in n-alkane having a small number of carbons even in winter, but decomposition does not proceed with n-alkane having a large number of carbon atoms. On the other hand, decomposition of n-alkane having a large number of carbons was also promoted by adding nutrient in spring, summer, and autumn when the temperature was relatively high.

All my results were summarized, TPH degradation by petroleum-degrading bacteria progressed in nutrient addition systems in spring, summer and autumn, but TPH degradation was not confirmed in winter. In the inner part of Tokyo Bay with few petroleum-related facilities, but significant decomposition of petroleum was confirmed in nutrient addition system. However, the decomposition tendency differs between n-alkanes in petroleum, and it became clear that n-alkane having a smaller number of carbons tends to decompose more.

3.4.2. Quantitative analysis of oil spill remediation bacteria (*alkB*) in Tokyo Bay

The abundance of *alkB* gene was detected from each seasonal culture sample, and on 28th days of *alkB* gene was estimated 2.6 x 10^6 - 2.3 x 10^8 copies / L. It was indicating that when the petroleum flows into the Tokyo Bay, and *alkB* gene retaining petroleum-degrading bacteria will increase. In each season, *alkB* gene copy number reached a plateau on the 14^{th} day and did not increase or decrease until the 28^{th} day, which remained flat. Comparison of the copy number of the *alkB* gene among the sampling points in the inner part of the Bay and the central part of Tokyo Bay was shown the same value at both points. In addition, in winter where degradation did not progress in the TPH part but increasing *alkB* gene was confirmed after the start of the culture.

Based on this fact, I recognized that the petroleum-degrading bacteria carrying *alkB* exist even in the winter when active petroleum breakdown did not take place. Assuming that the degrading bacteria use petroleum as a carbon source for their own growth, and there is a correlation between hydrocarbon degradation genes and hydrocarbon

decomposition. Also in previous research, Coulon *et al.* (2007) and Salminen *et al.* (2008) has been reported that there is a correlation between petroleum-degrading bacteria and petroleum decomposition amount. These research findings that n-alkane degrading *alkB* gene increased copy number until the 14^{th} day after the start of culture, and it showed a flat value until the 28^{th} day after reaching the plateau on the 14^{th} day in each season. In winter, the number of *alkB* copies was less than in spring and summer autumn. It was suggesting the possibility that the decomposition of petroleum-derived from the field microorganism did not proceed.

3.5. Conclusion

As a result of analyzing the amount of petroleum decomposition during microcosm experiment using Tokyo Bay seawater, the following clarified. The highest degradation of total petroleum hydrocarbon (TPH) was found in NT (nutrient) treatment and in summer. But similar degradation to be found in autumn and spring, and the lowest degradation in winter. Significant petroleum decomposition was confirmed in nutrient addition system. The determination of n-alkanes degradation was shown the bioremediation efficiency, and that decreased with the increase of carbon number in four seasons. *alkB* genes were detected in all incubation samples for four seasons. But their copy number in winter was nearly one order of magnitude less than in other three seasons. This observation indicated that the effect of bioremediation efficiency was depended on temperature and nutrient availability.

Table 3. 1. Measurement condition of GC/MS.

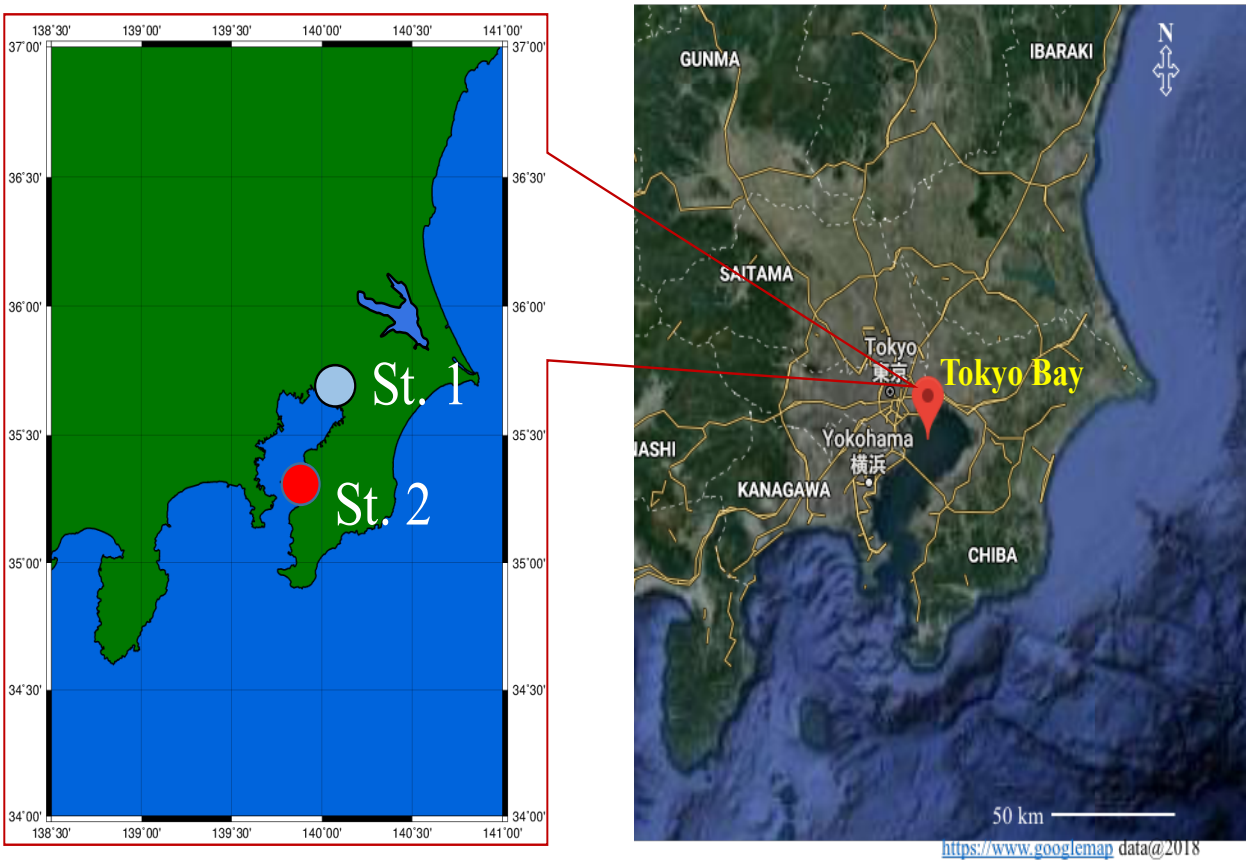
GC	
Column	Rtx - 5 MS (manufactured by RESTEK) Length 30 m diameter 0.25 mm × thickness 0.5 μm
Sample introduction method	Splitless method (high pressure injection mode 250 K min)
Sample injection volume	1 μL
Sample vaporization chamber temp.	330°C
Oven	85°C (2 min - 20°C / min to 330°C (25 min)
Carrier gas	He (5.19 cm/s)

MS	
Interface temperature	230°C
Ion source temperature	200°C
Ionization method	SIM method
Event time	0.3 s

Table 3.2. Environmental parameters *in situ.*

	Station	Water Temp. (˚C)	DO (mg/L)	pH	NH_4-N (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)	PO_4-P (mg/L)
Winter	St.1	10.8	12.1	8.03	0.050	0.93	0.042	0.059
	St.2	8.9	11.5	7.82	0.092	0.86	0.019	0.077
Spring	St.1	22.0	8.1	8.56	0.042	0.51	0.018	0.023
	St.2	21.0	9.0	7.84	0.043	0.42	0.003	0.033
Summer	St.1	28.6	7.8	8.56	0.217	0.32	0.073	0.081
	St.2	31.2	6.8	7.97	0.058	0.18	0.010	0.026
Autumn	St.1	17.5	8.9	7.85	0.085	0.94	0.038	0.040
	St.2	17.9	8.2	7.77	0.146	0.72	0.031	0.057

Winter: 2nd Feb, Spring: 4th May, Summer: 6th Aug, Autumn: 17th Nov; all in the year of 2015.



http://www.googlemap data@2018

Figure 3.1. Map of sampling stations at Tokyo Bay.

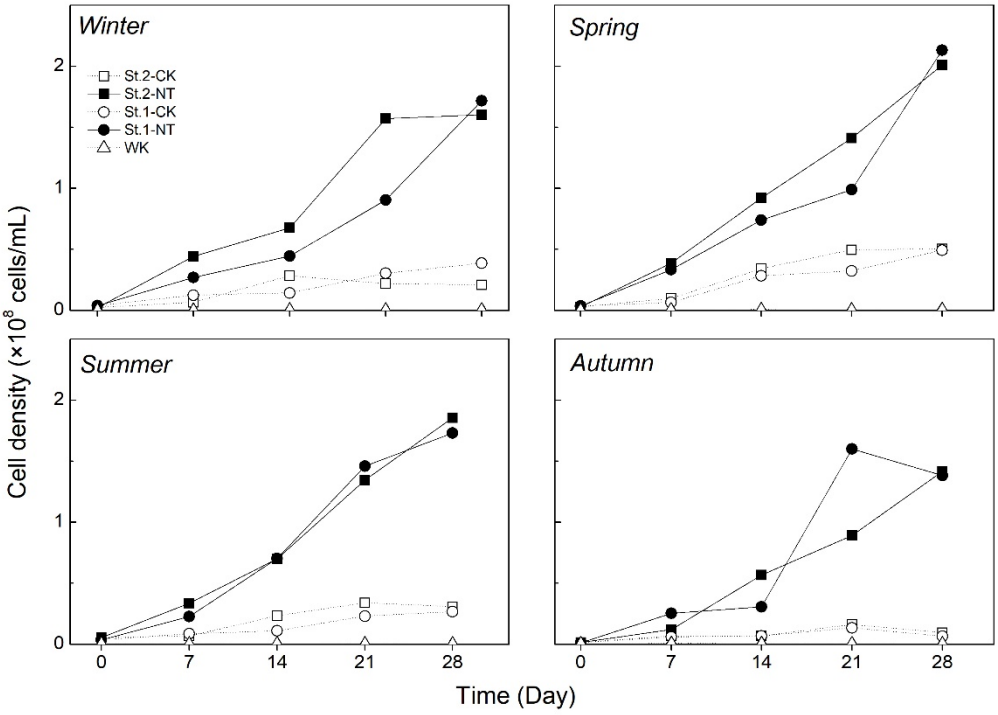


Figure 3.2. Changes of total bacterial cell densities during 28 days incubation.

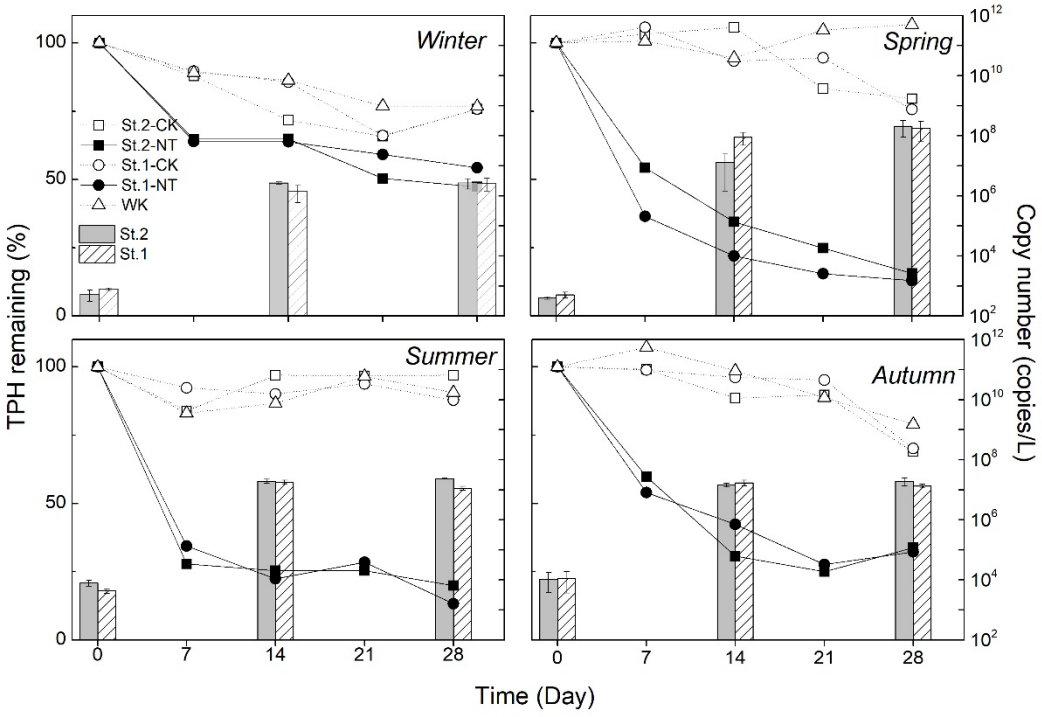


Figure 3.3. Degradation of TPH (show as lines and symbols, left Y-axis), and abundance of *alkB* copy number (show as bar, right Y-axis) during 28 days incubation.

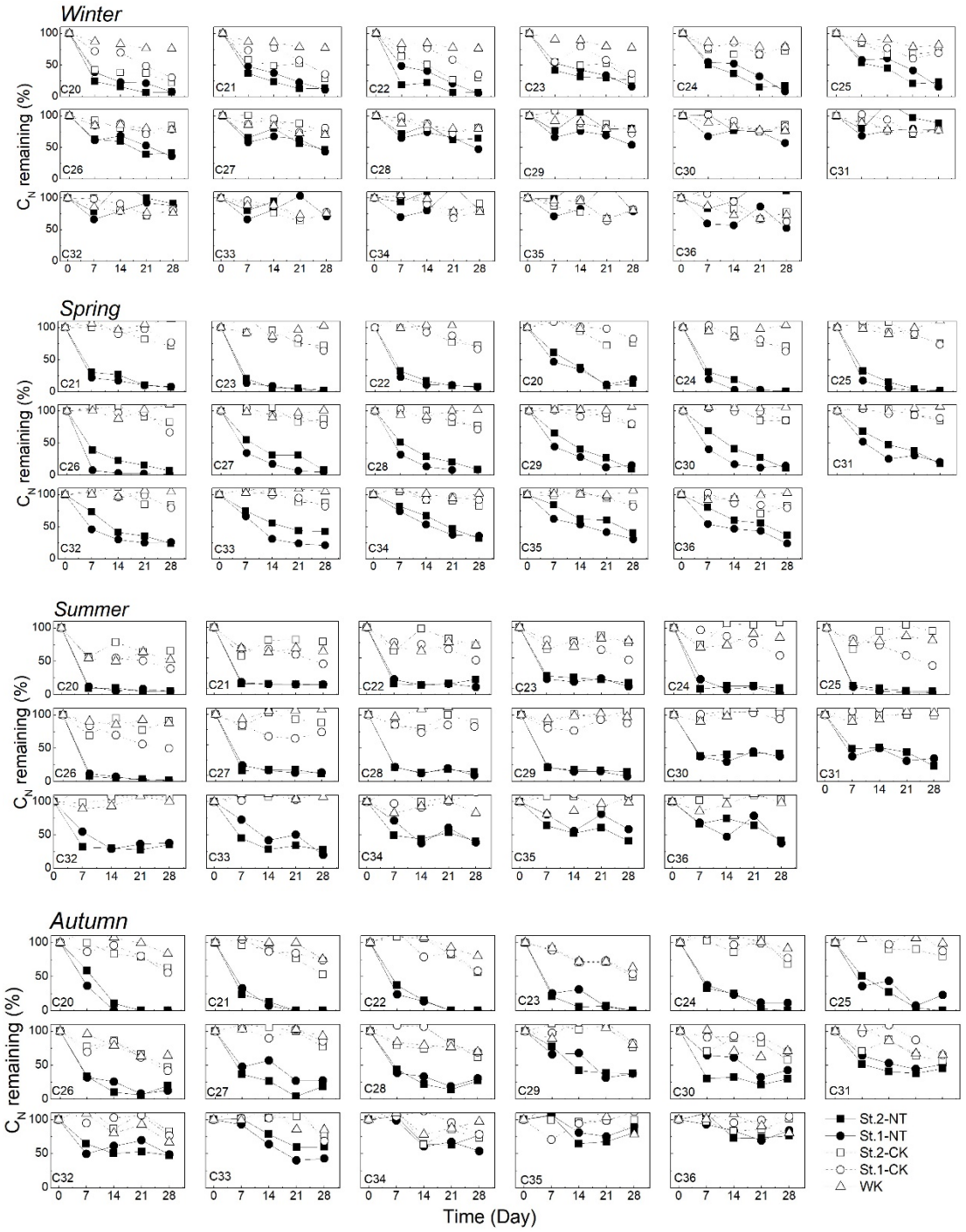


Figure 3.4. Degradation of n-alkane during 28 days incubation.

Chapter 4: General discussion and conclusion

4.1. General discussion

Coastal zone management depends on the information available on varied aspects of coastal habitats, coastal processes, natural hazards and their impacts, water quality, and living resources. The effective management practices depend on such knowledge and suitable response by concerned government agencies. The concept of these research supports the various functions of the coastal zone, has been resolving these conflicts, and has been making the coastal zone management which focuses on maintaining the health and stability of the coastal regions. In the current research was discussed by the two successions, 1; about the targeting on-site study of mangrove forest conservation and 2; about the targeting on laboratory study of oil spills bioremediation. For the site study was identified tropical mangrove forest in the Republic of the Union of Myanmar and the laboratory test was identified in temperate seawater of Tokyo Bay in Japan (Figure 4.1).

Here, it will be focusing on the coastal region of Shwe Taung Yan mangrove forest which connected with the Bay of Bengal. The sampling based on spectrally distinct and ecologically meaningful mangrove types, allowing for the determination of nitrogen fixation rate and microbial diversity in mangrove forest sediments. Biological nitrogen fixation is a key ecosystem process, and its activity is influenced by the resident functional microbe community and environmental conditions (Fasanella *et al.* 2012; Santos *et al.* 2011). I addressed questions about the abundance and diversity of nitrogen-fixing bacteria communities across sediment samples and the possible effect of oil pollution. I used the

nifH gene as a proxy for the nitrogen-fixing communities involved and *alkB* gene as a proxy for the hydroxylase degradation bacteria in mangrove sediment.

A few previous studies have addressed the microbial diversity in mangroves, most of them reporting taxonomical groups inhabiting this ecosystem and assessing shifts in the community structures in the face of environmental pressures (Dias *et al.* 2010, Liang JB *et al.* 2007, Marcial Gomes *et al.* 2008, Zhou *et al*. 2008). In my study, the qPCR data revealed statistically similar *nifH* gene community abundances across the samples of three mangrove sites. Considering the contextual parameters that modulate the microbial assemblages in mangroves, physicochemical parameters and vegetation type stand out as the strongest agents. The parameters of sediment temperature, as well as pH values, were revealed to impose distinct conditions on the assemblage of *nifH* host communities. It can be suggested that these variables modulate specifically the activity of nitrogenase, possibly determining the essentiality of *nifH* presence in such communities.

The present study implied its effectiveness for measurement of nitrogen fixation rate in Shwe Thaung Yan mangrove forest. The higher nitrogen fixation rate was occurred in mangrove park (Site 3) than other two sites. In this study, Site 1 was more frequently suffers waste pollution from the village, that would be likely to increase exogenous nitrogen inputs (Zedler *et al.* 2008) and inhibit nitrogen fixation rates (Shi *et al.* 2006). As a single block with high nitrogen fixation rates drove the difference between aerobic and anaerobic sediments, rather than consistent disparities between these sample types, abiotic environmental controls seem to have dominated biotic influences of the mangrove

on nitrogen-fixing bacteria. And oxygen is known to inhibit nitrogenase activities (Capone and Mantoya 2001) and may also affect microbial community structure, which was also the strongest environmental factor correlating with the nitrogen-fixing community structure. Soil structure may be another factor for increasing *nifH* gene copy number. All of my study sites were the clay type sediment. Gupta and Roper (2010) were described that clay fractions in soils are important in imparting specific physical properties, forming micro and macro-aggregates, and providing microaerophilic or anaerobic conditions that are favorable to nitrogen fixation. However, this knowledge is consistent with my findings, which showed higher abundances of *nifH* gene in the mud-clay loam soils compared with others. In another research, bacterial communities in tropical soil (Chotte *et al.* 2002), it has been shown 70 % of the free-living nitrogen-fixing bacteria are located in the clay fraction. Therefore, Roper and Smith (1991), and Nelson and Mele (2006) were found that alternatively the higher *nifH* gene abundance in the clay soils could be explained by the higher pH of these soils, which has been shown to favor the potential for biological nitrogen fixation (BNF). Interestingly, the effect of sediment layer habitats on the nitrogenase activities and the abundance of *nifH* gene varied across the sediment layers, being higher in the surface layer, when it explained almost half of the deepest layer in all samples. These observations are also underlined by Li *et al.* (2011), who defined mangroves as environments with particular nutrient cycling and site-specific anaerobic and aerobic conditions, where distinct microbially mediated transformations can occur. And mangroves offer very particular conditions to their inhabitants (mixed

influences from marine and terrestrial environments) and hence can be seen as reservoirs for such genetic and ecological novelty.

Previous mangrove sediments molecular studies, understanding consequences of disturbances such as biological studies for microbial diversity and function constitutes an important challenge in ecology such as soil biodiversity (Fitter *et al.* 2005), soil gene to ecosystem (Torsvik and Øvreås *et al.* 2002). Moreover, Dias *et al.* (2010), Liang *et al.* (2007), Marcial Gomes *et al.* (2008), and Zhou *et al.* (2008) have expressed the microbial diversity in mangroves, and most of them reporting taxonomical groups inhabiting and assessing shifts in the community structures in the face of environmental pressures. However, the composition and dynamics of the mangrove microbiome are still scarcely explored and understood in terms of its functional groups.

In this study, NGS data revealed statistically microbial diversity across the samples of three mangrove sites. Although, the microbial structure in Site 2 and 1 had a similar pattern, but Site 3 was different. The highest microbial ß-diversity index was found in Site 3 followed by Site 1 and Site 2. These results have shown the high microbial diversity in these environments indicating that they might play an important role in nutrient cycling. In my results, the four layers at Site 3 had less archaeal sequences compared to Site 1 and 2 samples. Totally 3 archaea phyla were dominated in three sites. Then, bacteria phylum Proteobacteria in Site 3 was more abundant than in other two sites. Additionally, the highest abundance of Cyanobacteria and Crenarchaeota were found in the surface layer (0-5 cm) and the deepest layer (15-20 cm) of Site 1. Moreover, I observed the greater

species richness within along of tidal cheek environment (Site 2 and 1) compared to the special marine park environment (Site 3). The reason for the enrichment of species could be due to a sustainable nutrient cycle (organic material sourced from the sea). Additionally, I observed that Site 2 is more species richer than Site 1. It has been reported that Epsilonproteobacteria was populated in high abundance at oxic-anoxic interfaces such as marine sediment surface meet with oxygenated seawater (Campbell *et al.* 2006), and my study suggests that many chemolithoautotrophic bacteria form Epsilonproteobacteria contained in all sites. Moreover, a recent study reported that the novel groups of Gammaproteobacteria accounted for 40 to 70 % of CO_2 fixation using sulfur as the electron donor in the coastal intertidal sediment (Lenk *et al.* 2011). It is rational to deduce that a high primary production supports diverse consumers, and many saprophytic microbes preferring eutrophic conditions, such as Bacteroidetes, Firmicutes, and Actinomycetales were enriched in all layers of three sites accordingly.

Yan (2006) reported that the importance of bacteria and fungi in mangrove biogeochemical cycles well established, but the knowledge of archaea in mangrove habitats are remaining extremely limited. In my results, phylogenetic analysis revealed archaea in mangrove sediments including Crenarchaeota (8.5 %), Euryarchaeota (1.9 %) and Parvarchaeota (0.2 %), and archaeal diversity in mangrove sediment is relatively high. This suggested mangrove forests and tidal flats, all located within the continent and marine contact zones, which are periodically flooded and exposed to seawater. The archaeal community in mangrove soil appears to be a mixture of organisms found in a

variety of environments with the majority being of marine origin. Moreover, the presence of numerous archaea in mangrove sediment may be of ecological significance. In order to gain new insight into the archaeal communities of mangrove soil and to build foundational information for future research.

Previous field studies and laboratory researches have revealed that bioremediation was cost-effective method to accelerate petroleum hydrocarbons degradation in oil-polluted seawater and soils, such as in the Sea of Japan (Maki *et al.* 2003), subpolar areas (Akbari *et al.* 2015; Camenzuli and Freidman 2017), Norwegian coastal seawater samples (Brasksta and Bonaunet 2006; Brasksta *et al.* 2015), Chinese soil sample (Wu *et al.* 2016), Kuwait oil lake sediments (Al-Mutairi *et al.* 2008), Brazil dystrophic red argisoil (Cerqueira *et al.* 2014) and so on. It is also found that the petroleum hydrocarbon degrading bacteria ubiquitously distributed in the marine environment (Atlas *et al.* 1995) and the population and the community of such microbes shift rapidly when the environment is contaminated with petroleum (Atlas *et al.* 1995; Liu *et al.* 2017; MacNaughton *et al.* 1999; Scoma *et al.* 2017; Shahi *et al.* 2016). Although the population of responsible microorganisms appears to grow rapidly when sudden crude oil inflow even without extra nutrient supplement, bioremediation process can be accelerated with proper nutrients addition (Rahman *et al.* 2003; Tellez *et al.* 2002). In my study, TPH were deduced more rapidly by NT treatment than by CK or WK treatment, indicating that groups with nutrients addition can lead to a faster petroleum hydrocarbon degradation. As a limiting factor in the natural marine environment, nutrients concentration largely

regulates the growth of most microorganisms. My results showed that total bacterial cell numbers in NT treatment groups (with nutrients addition) were significantly larger than in CK or WK treatment groups (without nutrients addition), indicating that nutrients addition accelerated the growth of microorganisms in the microcosms. On the other hand, the considerable increase in *alkB* gene was also observed during incubation, showing a microbial response to sudden environmental change (when added or contaminated with petroleum compounds). Numerous studies have reported this kind of microbial response to oil pollution (Hazen *et al.* 2010; Hu *et al.* 2017; Liu *et al.* 2017; MacNaughton *et al.* 1999; Scoma *et al.* 2017; Shahi *et al.* 2016), and this response can be observed in both microbial abundance and its community. Although microbial community has not been discussed in this study, remarkable increase of total bacterial cell as well as *alkB* gene were detected in groups with nutrients addition, suggested that, nutrients addition could promote the growth of microorganisms in microcosm, and when accompanied with petroleum contamination, this significant increase in cell number is mainly because of the growth of petroleum-degrading bacteria.

Although pH (Bossert and Bartha 1984; Foght and Westlake 1987), salinity (Zobell *et al.* 1973), DO (Bossert and Bartha 1984; Head and Swannell 1999), nutrients concentration in the indigenous environment (Al-Awadhi *et al.* 2012; Baelum *et al.* 2012; Hazen *et al*. 2010; Head *et al.* 2006; Wang *et al.* 2010a), and temperature (Brakstad and Bonaunet 2006, Venosa and Holder 2007) have been reported to affect bioremediation, this study focuses on the effect of temperature which generally covers the temperature

range in all seasons in temperate areas, and demonstrates of petroleum hydrocarbons individually in its carbon number. Bioremediation efficiency was highest in summer, followed by spring, autumn, and winter. As similar trends were also detected in the total bacterial cell number and *alkB* gene abundance, this seasonal difference showed in bioremediation efficiency can be explained by microbial activities under different temperature, most possibly, the growth of these responsible microorganisms thrive in warm temperature (like summer and spring) but be constrained in cold temperature (like winter). My results showed that bioremediation in winter was not as effective as in other seasons, especially for degradation of hydrocarbon with more than 25 carbon atoms. However, significant bioremediation can still be observed when water temperature was as low as 0 to 5℃ (Brakstad and Bonaunet 2006; Venosa *et al.* 2007), which is even lower than in my winter experiment (10℃). In the study conducted by Venosa *et al.* (2007), enriched degrading bacterial culture was used, which indicate a higher responsible microbial density compared with my study; and in Brakstad and Bonaunet (2006) use seawater samples collected from a high latitude marine area (Norwegian sea) for microcosm preparation, where the naturally distributed microbial community tends to have higher cold tolerance or cold adaptation than my study (which is collected from temperate zone). Thus, the differences showed in bioremediation efficiency in low temperatures are more likely to be the consequences of responsible microbial density and biological divergence of microorganisms in the indigenous environments. Bioremediation of n-alkanes have been confirmed up to C_{44}-alkanes (Atlas *et al.* 1981), my results showed

that degradation was becoming difficult when the carbon number increased. In particular, degradation of >C_{33}-alknanes was more difficult than its lighter companions in summer and spring; and this 'threshold' was >C_{30} in autumn, and >C_{27} in winter. These findings generally follow the finding by testing high-latitude originate enrichment (Venosa *et al.* 2007) and subpolar soil (Akabari *et al.* 2015), both of which reported more readily degradation of lighter n-alkanes than heavier n-alkanes.

Mangroves often situated in areas of high anthropogenic influence, being exposed to pollutants, such as those released by oil spills. In fact, oil has a major ecological impact on the marine and terrestrial systems. Crude oil pollution reduced productive outputs of the delta and coastal mangroves. Thus, efficient strategies to monitor oil in the environment must be developed, especially in mangroves ecosystems. Microorganisms are fundamental for the maintenance of productivity, conservation, and recovery of mangroves. They are directly involved in the transformation of nutrients, photosynthesis, nitrogen fixation, methanogenesis, phosphate solubility, sulfate reduction and production of other substances. The bacterial community structure and diversity in mangrove sediment was also evaluated using a 16S rRNA multiplex pyrosequencing approach. Such an approach is very useful to extend the knowledge about the microbial communities that abound in mangrove ecosystems and to evaluate not only nitrogen fixation rate but also the effects of oil spills. There need to understand bacterial community dynamics from mangrove sediments and to look for common patterns that may associate with oil biodegradation in such environments. The hypothesis of petroleum biodegradation

compared between the sample sites with different oil pollution history using by analyses approach based on the microbial resource management framework. When properly understood and managed of microorganisms including nitrifying bacteria (*nifH* gen) and hydrocarbon degradation bacteria (*alkB* gene) provide a wide range of ecosystem services, such as nutrient flow, bioremediation, and for supporting the recovery of impacted environments (Figure 4.1).

This research has provided the important information on the abundance of 16S rRNA, *nifH* and *alkB* genes in sediment depth profile and investigation of sediment microbial communities in Shwe Thaung Yan mangrove forest of the Republic of the Union of Myanmar. Using quantitative analysis technology, it can get a general view on basic characteristics of nitrogen fixation functional gene and affect factors determining on nitrogenase enzyme activities. They successfully link scientific discovery with practical applications by understanding the need to manipulate mangrove sediment function. Moreover, nitrogen fixation microbes and hydrocarbon degradation microbes are such a mysterious species that culturing in the laboratory phase is very difficult in Myanmar. However, this species is so essential to the global biogeochemical cycle that abundance and potential role cannot ignore. With more and more evidence accumulating, I will have a further understanding of responsible microbial function in these two functional genes. Therefore, continuous supervision over the use and management of mangrove required for the welfare of coastal communities. According to my knowledge, this is the first study on the understanding of microbial analysis in Myanmar mangrove ecosystem.

This research demonstrated the current state of knowledge in microbial diversity of mangrove sediment and illustrates the important contributions these microorganisms make to the productivity of mangrove ecosystems. The fundamental issues of ecosystem ecology are related to understanding how ecosystems maintain functional stability and predicting how ecosystems respond to environmental changes. For sustainable coastal zone management, it is necessary to describe the relevant criteria pertaining to the area under study, as well as site-specific indicators which may be significant in evaluating them. As quantitative data required for many environmental indicators are often not available, especially when a large coastal zone must be evaluated, qualitative indicators can be used as an acceptable alternative. Moreover, scientific researches can provide scientific advice on matters related to the utilization and management of marine resources in given regions.

4.2. Conclusion and perspective

The study method was using the results from a recent assessment of tropical and temperate coastal areas. The microbiological functional measurement is used to manipulate and analyze the data. In this study, I made the site study based on tropical and incubation test in temperate areas using microbial functional approaches. Tropical mangroves are considered one of the most productive ecosystems of the world. However, in temperate unlike tropical areas, vegetation is rarely in coastal areas. And then microbial communities in water and bottom sediment play an important role in maintaining for

coastal ecosystem. I would have tested both, nutrient cycling (especially for nitrogen fixation rate) and the ability of microorganisms exist in the mangrove ecosystem. Then calculated the abundance of *alkB* gene in mangrove sediments and also the community. I checked the balance of all the species in mangrove sediments. The data showed that the ability to respond to the oil spill. So, I carried out the incubation test in Tokyo Bay seawater. Although there are different environments assumed the same species. During the incubation test, the species mainly impacted by temperature. So, if the temperature is correct, the gene as the species will be activated when the oil spill happened.

Biological nitrogen fixation is the key in an ecosystem process, and its activity influenced by the resident functional microbial community and environmental conditions in the mangrove ecosystem. Sediment microbial communities play a vital role in the process of nutrient recycling and the maintenance of mangrove health. The quantification of *alkB* gene copy numbers in mangrove sediment provides an insight into the microbial response to the mangrove ecosystem of hydrocarbons and acts as a useful complementary tool for the understanding of this ecosystem response to hydrocarbons. The most important factor to the effect of degradation rate is temperature. And the best temperature in incubation test is very close to Myanmar. Moreover, hydrocarbon degradation efficiency and microorganism's activities were rapid with high temperature. Additionally, *alkB* gene detected in the mangrove sediments and the responsible species have existed in mangrove microorganism. So, the mangrove ecosystem will respond to oil spill if it happened in these ecosystems. Because oil-degrading bacteria ubiquitously distributed

not only oil-polluted seawater but also in mangrove sediment. If some pollutants groups impact on mangrove environment, they recover themselves by the activities of microorganisms in the mangrove ecosystem.

Microorganisms are directly involved in biogeochemical cycles, as key drivers of the degradation of many carbon sources (petroleum hydrocarbons) including not only sediment but also seawater. Microorganisms are cost-effective agents for *in-situ* remediation of pollution in soils, sediments, and marine environments. So, bioremediation is a potential new tool for the cleaning of certain oil-contaminated shoreline types. The microbiological methodology approach used in this study, despite limitations, provide insights into the structural diversity of bacterial communities, which support the various functions of the coastal zone and a proposal for comprehensive coastal zone management.

According to chapter 2 and chapter 3 studies, the function of microbiological measurements is very good tools for coastal zone management. From tropical coastal zone management, can be investigated the quantitative evaluation of links between microbial functions and environmental factors in the mangrove ecosystem. And in temperate, can be determined the development of clean-up strategic in environmental assessment technology and its application to degradation of oil spill pollution in the coastal ecosystem (Figure 4.1). So, these two case studies have to combine with the present case studies for coastal ecosystem conservation assessment in tropical and temperate regions and that will be supported to aquatic coastal zone management. Because of studies on microbial

ecology in this complex environment are required for a better understanding of bacterial responses to environmental stresses and ecosystem functioning, and to aid the development of mitigation strategies. The present study focus is the microbiological quality of selected sites in the coastal regions where are studying the extent of pollution, seasonal variations including the effect of microbiological finding. It will be collected the manage and disseminate data, and other information on coastal resources, issues and processes. This study results will be helpful and used for more detailed future investigations of aquatic coastal zone management by microbiological function measurement approaches. So far, the present study provides the interesting data and novel information on my understanding of the aquatic coastal ecosystem. However, this study confirmed the specific microbiological function of site study in tropical and laboratory incubation test in temperate communities that represented for world's coastal zone management.

In the near future, a lot of microbiological function measurement approach will be replaced by more large-scale for environmental management will be established. Some threats to coastal areas can be approached most effectively on a global scale, their individual characteristics and relevance tend to vary from region to region and from sea to sea. So, we should more be learning about microbiological function measurements, it is apparent that ignoring these benefits is detrimental to coastal management and planning.

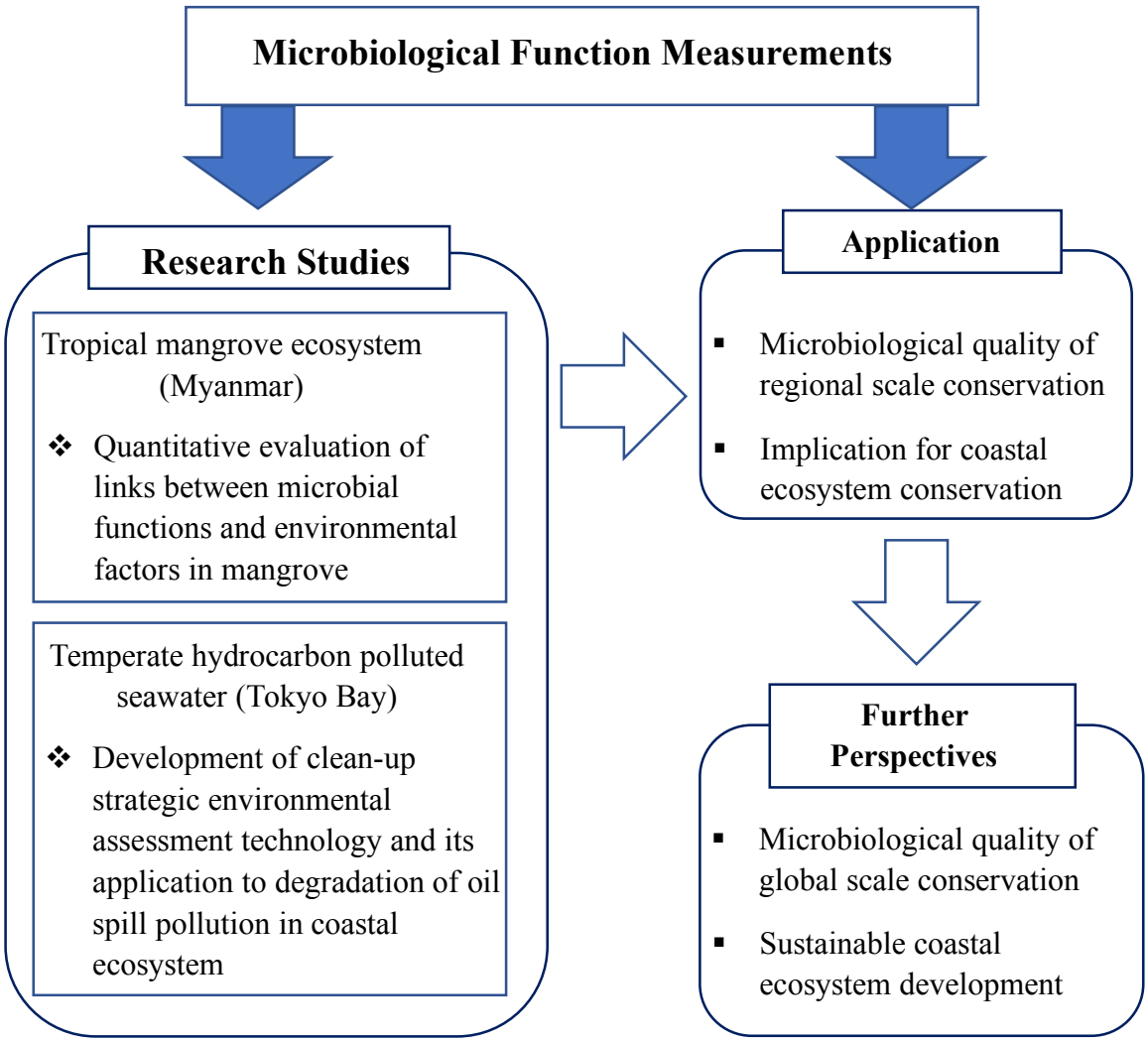


Figure 4.1. Application of the research studies from regional aquatic coastal zone management currently under the specific microbiological function measurement approaches.

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