เมตาจีโนมิกส์เพื่อการศึกษาความหลากหลายของประชากรจุลินทรีย์ในอ่าวไทยตอนกลาง

นางสาวดลพร ศรีพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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METAGENOMICS FOR STUDY DIVERSITY OF MICROBIAL POPULATIONS IN CENTRAL GULF OF THAILAND

Miss Donlaporn Sripan

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Microbiology and Microbial Technology
Department of Microbiology
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ดลพร ศรีพันธ์ : เมตาจีโนมิกส์เพื่อการศึกษาความหลากหลายของประชากรจุลินทรีย์ในอ่าวไทยตอนกลาง (METAGENOMICS FOR STUDY DIVERSITY OF MICROBIAL POPULATIONS IN CENTRAL GULF OF THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นราพร สมบูรณ์นะ, 70 หน้า.

เมตาจีโนมิกส์เป็นวิธีการศึกษาจุลินทรีย์โดยสกัดดีเอ็นเอจากสิ่งแวดล้อมโดยตรง และเป็นเทคนิคที่สำคัญที่ใช้ในการวิเคราะห์ความหลากหลายของจุลินทรีย์ในสิ่งแวดล้อมทำให้ทราบถึงความสัมพันธ์ที่แท้จริงของจุลินทรีย์ต่างๆภายในสิ่งแวดล้อม โครงการวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาความหลากหลายของจุลินทรีย์และช่วยให้เข้าใจเกี่ยวกับระบบนิเวศของจุลินทรีย์บริเวณอ่าวไทยตอนกลาง อันประกอบไปด้วยโปรคาริโอต ยูคาริโอตโดยใช้วิธีเมตาจีโนมิกส์ร่วมกับการซีเควนซิ่งยีน 16S และ 18S ไรโบโซมอลอาร์เอ็นเอ โดยงานวิจัยนี้ได้ศึกษาน้ำทะเลที่พิกัดต่างๆที่ระดับผิวน้ำ (ความลึก <5 เมตรจากผิวน้ำ) และระดับก้นทะเล (ประมาณ 1 เมตรเหนือพื้นทะเล) ทั่วบริเวณอ่าวไทยตอนกลางในช่วงเดือนมีนาคมถึงเมษายน (ฤดูแล้ง) พ.ศ. 2556 พบว่าบริเวณอ่าวไทยมีรูปแบบความหลากหลายของจุลินทรีย์เหมือนกันทั่วอ่าวไทยเนื่องจากโปรแกรม Mothur ไม่สามารถแบ่งกลุ่มประชากรจุลินทรีย์ได้ อย่างไรก็ดีพบปัจจัยที่ส่งผลต่อความหลากหลายของโปรคาริโอตได้แก่ อุณหภูมิ ($p \leq 0.012$) ส่วนปัจจัยที่ส่งผลต่อความหลากหลายของยูคาริโอตได้แก่ แสง ($p = 0.029$) และความเค็ม ($p=0.004$) OTUs ที่สำคัญต่อความหลากหลายของโปรคาริโอตคือ *Pseudoalteromonadaceae* และ *Oceanospirillacea* OTUs ที่สำคัญต่อความหลากหลายของยูคาริโอตคือ *Bilateria*, *Euglenida*, and *Cnidaria* และยังพบอีกว่ากระแสการไหลเวียนของน้ำและสิ่งมีชีวิตที่อาศัยอยู่ในน้ำอาจมีส่วนสัมพันธ์กับความหลากหลายของจุลินทรีย์ โดยพบการจัดกลุ่มของจุลินทรีย์ในรูปแบบ PCoA ที่สอดคล้องกัน อย่างไรก็ดีงานวิจัยนี้ศึกษาความหลากหลายของจุลินทรีย์ในฤดูแล้งเท่านั้นจึงควรมีการศึกษาความหลากหลายของจุลินทรีย์ในฤดูอื่นๆ เพื่อเปรียบเทียบด้วย

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา จุลชีววิทยาและเทคโนโลยีจุลินทรีย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559

5671961723 : MAJOR MICROBIOLOGY AND MICROBIAL TECHNOLOGY
KEYWORDS: METAGENOMICS/ DIVERSITY/ GULF OF THAILAND

DONLAPORN SRIPAN: METAGENOMICS FOR STUDY DIVERSITY OF MICROBIAL POPULATIONS IN CENTRAL GULF OF THAILAND. ADVISOR: ASST. PROF. NARAPORN SOMBOONNA, Ph.D., 70 pp.

Metagenomics is a useful technique to study microbial diversity directly in a culture-independent manner. Thus, metagenomics finds nearly 100% of all microorganisms in the environment. This approach becomes a major tool to analyze the microbial diversity (microbiota) worldwide. This research studied the diversity of microbial populations, including prokaryotes and eukaryotes, in the central Gulf of Thailand, using metagenomics combined with 16S and 18S rRNA gene sequencing. The research area covers various geographic coordinate throughout the central Gulf of Thailand, at the sea surface (<5 m from the sea surface) and a seafloor (1 m above the seafloor), during March to April (dry season) of 2013. The communities, both prokaryotes and eukaryotes, were determined homogeneous, by the function of the program Mothur (get.communitytype). Yet, some abiotic factors were found to affect the prokaryote population: temperature (p=0.012) and for eukaryote population fluorescence Seapoint (p=0.029) and salinity (p=0.004). Significant representative OTUs in prokaryotes were *Pseudoalteromonadaceae* and *Oceanospirillacea*; and eukaryotes were *Bilateria*, *Euglenida*, and *Cnidaria*. Moreover, the direction of water circulation and the type of organisms might affect the microbial diversity, observed possible correlation in PCoA. Overall, this research successfully studied the microbial diversity of the dry season; meanwhile does not represent other seasons. Therefore, it is advisable to also study the diversity of microbes in other seasons as well.

Department: Microbiology
Field of Study: Microbiology and Microbial Technology
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Student's Signature .....
Advisor's Signature .....

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CHAPTER I

INTRODUCTION

1.1 Background

Most of the world is covered by large bodies of water. It covers about 3 in 4 or 71 percent of the world surface. And a total volume of water is more 4,000,000,000 cubic km^2.

Thailand is surrounded with two sea borders: Gulf of Thailand and Andaman Sea. These account for a total of more than 2,400 km of coastal length. The Gulf of Thailand is divided into: the upper, from Hua Hin, Prachuap Khiri Khan province in Samae San island; the central, from Samae San island on the east coast of Surat Thani province; and the lower, from Surat Thani downward. The geography of the Gulf of Thailand, especially, the central part, is like basin where nutrients and minerals from sediments from the inflowing rivers through several major rivers come in and accumulate. Nutrient-rich in this area also come from the runoff mangrove forests and coral reefs surrounding the Gulf of Thailand. Subsequently, the area is fruitful of diverse aquatic resources and lives, shelters for breeding and young animals, and of refugee animals; and one of the most popular tourism sites in Thailand. In addition, the Gulf of Thailand serves one central marine transport route, and contains an oil rig site.

Hence, the study of the biodiversity of marine organisms and microorganisms in this area is important to better understand its current marine condition and the information is in part essential for marine environmental management plan. Limited studies have investigated their association in marine water ecosystems around the Gulf of Thailand. Previously, Suvapepun (1)studied the changes of ecosystems but has not studied the diversity of microbes in the Gulf of Thailand. However, the marine biodiversity are likely changed over time as a result of the human activities, causing increased temperature, sunlight and carbon dioxide, and even leaking of

toxic substances. In spite all of this, no such research has analyzed the microbial diversity and the relationship to environmental conditions by a culture-independent approach. Metagenomics is the study of the metagenome that is directly extracted from a sample in nature. The metagenome thereby contain all microorganism species represented in the nature sample, both cultured and uncultured microorganisms (2, 3). This represents the complete marine prokaryotic profiles and allow the true correlation analysis with biotic and abiotic, such as environmental factors. Similar to the research of Ghai (4) that used the metagenomic sequencing to create a database of bacterial diversity in the Amazon River. And from the research of Chan *et* al. (5) water samples were collected at a depth of two levels (0 m. and 17.2 m.) and used 16S rRNA gene sequencing to identify the communities of bacteria and their potential metabolisms. This central Gulf of Thailand same approach has been used to study microbial diversity in the coastal Si Chang island and elsewhere can provide a database of bacteria, archaea, and small eukaryotes, and help understand the microbial ecosystem (2). This is in part to establish a Thailand marine microbiota database.

1.2 Objectives

1. To study the diversity of microbial populations prokaryotes and small eukaryotes) in the central gulf of Thailand

2. To better understand the ecosystem of prokaryotes and eukaryotes in the central Gulf of Thailand

1.3 Hypothesis

If samples were collected in the central Gulf of Thailand at different depths to study the diversity of microorganisms, there would be variations in the diversity of microorganisms and the factors that affect the diversity of microorganism

CHAPTER II
LITERATURE REVIEW

2.1 Gulf of Thailand

The Gulf of Thailand (6) is a semi-enclosed tropical sea located in the South China Sea (Pacific Ocean) and enclosed by the countries Malaysia, Thailand, Cambodia and Vietnam. The GoT covers an area of 320,000 km² (7, 8). These account for a total of more than 2,400 km coastal length. The Gulf of Thailand is divided into: the upper, from Hua Hin, Prachuap Khiri Khan province in Samae San island; the central, from Samae San island on the east coast of Surat Thani province; and the lower, from Surat Thani downward (9) (Figure 2.1). The GoT is an important resource to the national economy of Thailand. Especially the central part, the basin where nutrients and minerals from sediments are accumulated, and the inflowing rivers that bring marine animals for the fishing industry, and so do the tourism, and other piers (7). The GoT serves one central marine transport route, and contains an oil rig site. The water circulation of GoT is weak compared with that of the South China Sea, so GoT the flow of water is countered by the South China Sea, and thus causes the shift in the GoT water circulation by seasons (7, 8) (Figure 2.2).

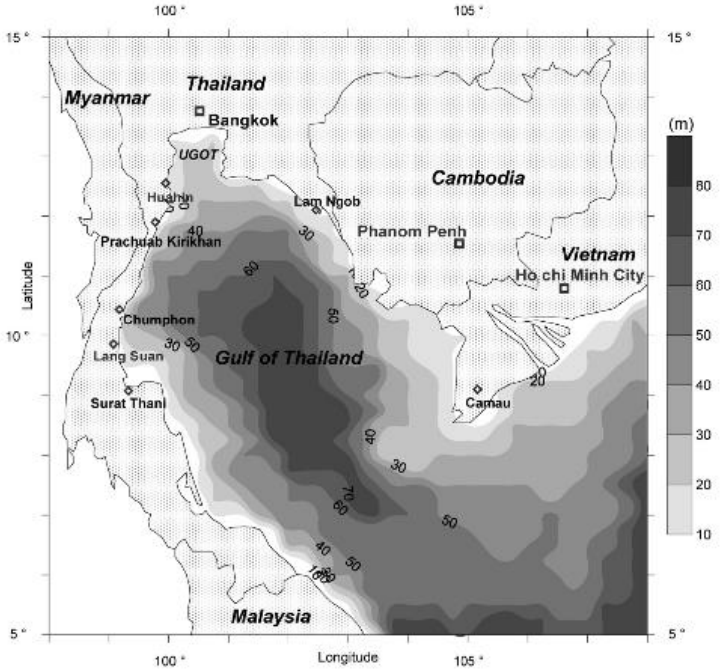

Figure 2. 1 Map of Gulf of Thailand

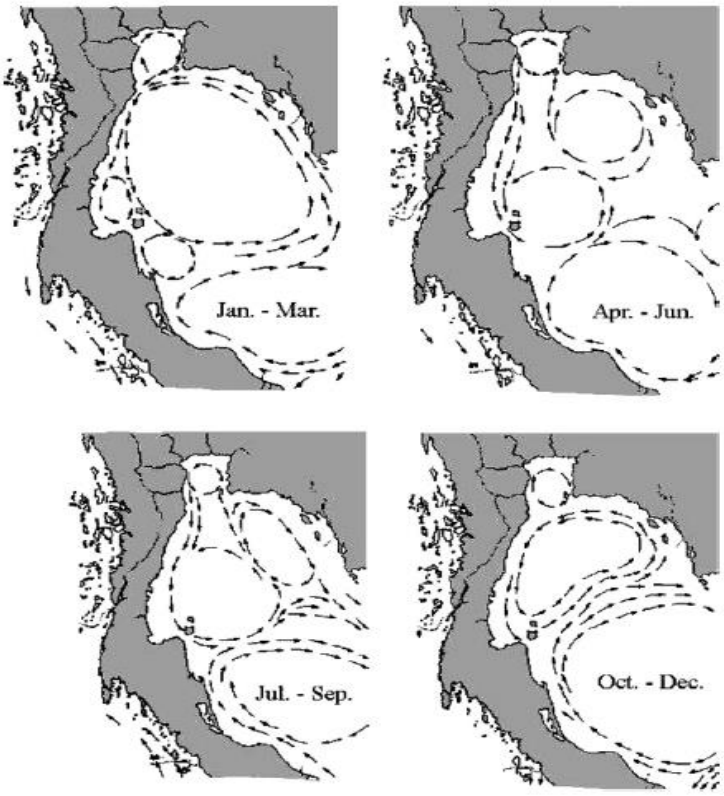

Figure 2. 2 Seasonnal circulation in gulf of Thailand

2.2 Metagenomics

Metagenomics is a study of all the DNA (metagenome) extracted from natural specimens, which includes genomes of all the microbes contained in the sample. The microbes in the natural specimen are mostly uncultured (3). Metagnomics is therefore a popular and most reliable method of study today because it provides a comprehensive study of culture-unbias microorganisms. For metagenomic processes to study diversity starting from the extraction of DNA from the sample directly from the environment. Then, DNA analysis can be carried out using three main principles (10): (1) target gene, such as 16S or 18S ribosomal RNA (16S rRNA, 18S rRNA) genes in prokaryotic and eukaryotic microorganisms (11). (2) A more traditional method by cutting into pieces and cloned into a vector, of the largest DNA fragment, this method is suitable for use in analyzing the functional properties of the interested genes (12). (3) Shotgun metagenomic sequencing, to provide a coverage of both the protein genome for function and the 16S rRNA and 18S rRNA genes to indicate species. However, because of the diversity in nature is high while, the amount of the DNA that can be obtained by sequencers is limited, the 3rd method may result in low diversity compared to method one.

2.3 Biodiversity

A variety of plant and animal life in the world or in any habitat is generally preferred. Biodiversity is a term that is used to describe the number of different species that live within a particular ecosystem. Biodiversity is important not only for the variety of beautiful and interesting species, it offers us, but it is also very important and vital to the stability of an ecosystem and our entire planet (13) . Biodiversity plays a direct role in climate regulation and the environment. A variety of microorganisms infer the nature of the area (14). 70% of the surface of the earth is

covered by oceans, which are different consist of mangroves, coral reefs and seagrass. Climate change will lead to the transformation of things in the oceans, including: salinity in the sea, floods, higher storm surges, increase in sea level, coastal erosion, and ocean surface temperatures, coral bleach mangroves and millions of immigrants from climate change (15).

Examples of research on the marine microbial diversity such as those of Benny et al. (2016) (5)studied community structure and environmental parameters in Shallow Water Hydrothermal Vents off Kueishan Island, Taiwan found that acidic chemistry effect the distribution of microorganisms, algae and coral. Microbial was found at shallow sea can found in the deep sea as well, but at deep sea has microbial more than shallow sea due to the area of deep sea the toxins will be diluted.

The Global Ocean Sampling (GOS) is originated with the aim to complete the global marine microbial database, for the benefit of the major world's food chain resources. However, the database is still inconclusive due to the large entire world's marine. GOS developed bioinformatics method to find the relationship of microorganisms and environment, and reveal the genomes of cultured and uncultured microbial (16).

2.4 Sequencing

Sequencing is the process of determining the precise order of nucleotides within a DNA molecule. The first sequencing technology, we know is Sanger Sequencing, which Dideoxy-nucleotide chain termination refers to the introduction of nucleotides to label fluorescent colors in different colors. Then, the enzyme synthesizes the DNA from the prototype DNA. However, there is the limitation that one sequence of readings can be obtained per read (17). Later developments have

been made to find more sequences of reads as a source of next generation sequencing (18). There are currently three tools available for this technique: 454 / Roche, Illumina / Solexa, and SOLiD (19) (20, 21). These tools are different at DNA template immobilization, DNA synthesis, error rate, length of reads, and cost. The third generation sequencing technique, which is capable of providing longer reads and 99.99% reliability, has recently been introduced in research of Gullapalli et al. (2012) (22) (Table 2.1). For example, Pacific Biosciences (PacBio) is developed from the depletion of 454 / Roche, Illumina / Solexa, and SOLiD (19) but in Thailand is not commonly used (23).

Table 2. 1 compares the features of next generation sequencing (Gullapalli et al., 2012).

NGS technology	454/Roche	Illumina/Solexa	SOLiD/ABI
Chemistry	Pyrosequencing	Polymerase-based	Ligation-based
Read length	400 bp	2x150 bp	2x60 bp(avg)
Run time	10 hrs.	26 hrs	6 days
Data	400 Mb/run	3000 Mb/run	4000 Mb/run
Raw accuracy	99.5 %	99.5 %	99.94 %

2.5 Illumina sequencing technology

Illumine sequencing uses principles based on a sequence based labeling with fluorescent dye and increased amounts of DNA strands were connected with bridges on solid state. DNA library preparation on Illumina starts by repairing ends and phosphorylating the 5' end. DNA is fragmented either enzymatically or by sonication to create smaller strands. Add dA-tail to the repaired products at the 3 'end and link the adapters. Separation of DNA strands and DNA amplification occurs by Bridge PCR technique (Figure 2.3). After the DNA strand separation procedure, a single stranded

DNA was placed on a random flow cell channel. The slide was coated with an adapter and a matching adapter. (Complementary adapters), which function as primers, were used to amplify DNA. Nucleotides and enzymes were added to start the bridge amplification. Single strands of DNA attaches to the primer as a curved bridge (Double-stranded bridges) and separate double strands to a single strand DNA for use as DNA template again. After the amplification with PCR technique will get a cluster of DNA (Figure 2.4). Finding base Illumina uses sequencing by synthesis by separating the DNA strands in each group into single stranded DNA. Give the primer to find the base pair with the specific DNA, which four nucleotide polymorphisms were added to the luminescent with different colors.

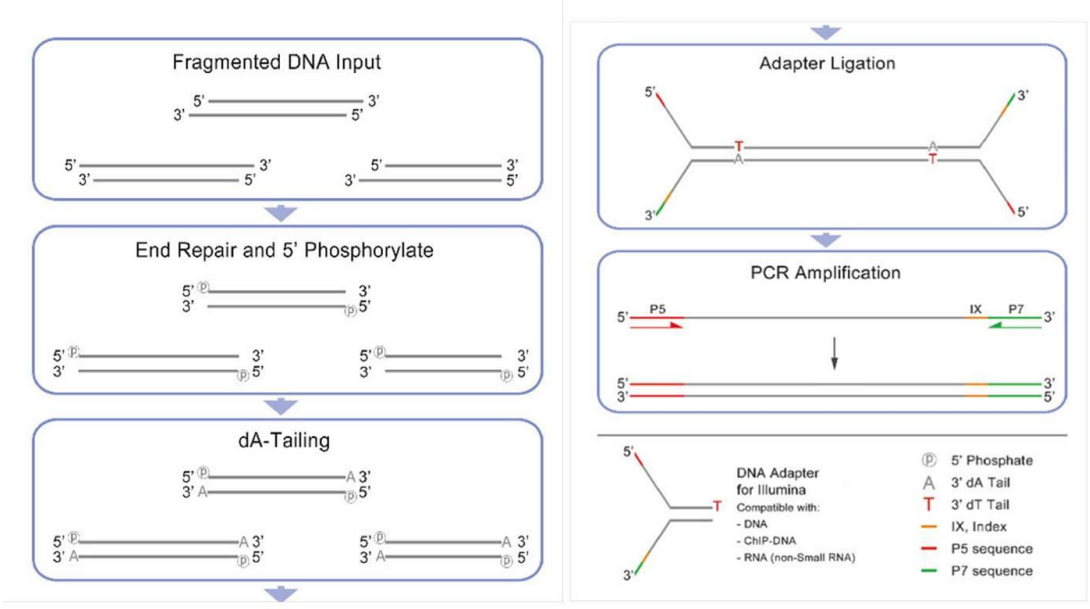

Figure 2. 3 DNA library preparation on Illumina

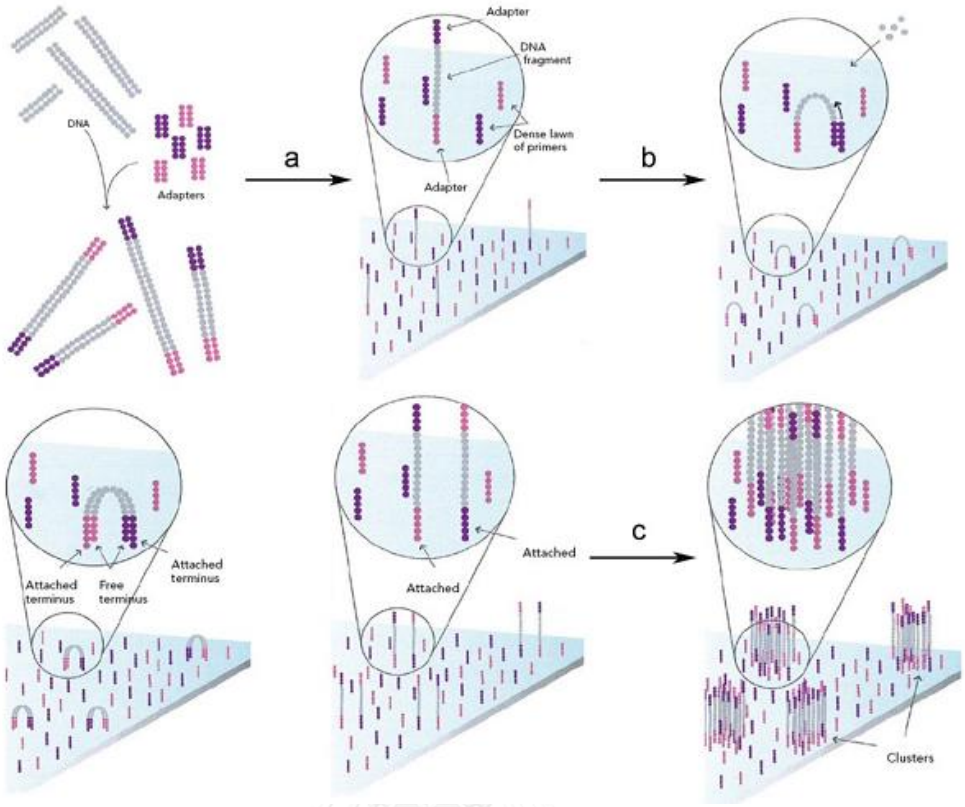

Figure 2. 4 Bridge amplification of Illumina

2.6 Bioinformatics

Bioinformatics is a new field of life sciences. This study is based on the integration of knowledge from Molecular biology, genetics, biochemistry, microbiology, applied mathematics, statistics, informatics, and computer science to assist in data storing, processing and a systematic biology search. Bioinformatics has three main components. First, a database is created to store and execute a large set of data. Second, algorithms and statistics are developed to examine the relationship between members in the large datasets. Using these tools to analyze and interpret a variety of data types, bioinformatics can be used to answer biological problems or to compute models from the data to predict biological possibilities.

Mothur is a free software package for microbiota bioinformatics and analyses. Mothur is the ability to process raw sequences that come from 454 pyrosequencing, Illumina HiSeq and MiSeq, Sanger, PacBio, and IonTorrent, given the beginning preprocessing steps of these raw sequences are different. This software can identify, classify by operational taxonomic (OTU) up to species, and perform alpha and beta diversity calculations (24).

STAMP is a statistical analysis of taxonomic and functional profiles that a graphical software package for analyzing taxonomic and metabolic profiles. STAMP identifies features based on genes that had been assigned to metabolisms by KEGG and COG databases (11, 25).

CHAPTER III

METERIALS AND METHODS

3.1 Instruments

Autoclave: Kokusan, Shizuoka, Japan

Freezer 4°C MITSUBISHI, Tokyo, Japan

Deep freezer -20°C: SANYO, Osaka, Japan

Deep freezer -80°C: SANYO, Osaka, Japan

Agarose Gel Electrophoresis System: GE-100, Hangzhou Bioer Technology CO., LTD. Hangzhou, China

Gel Documentation: Gel DOC 2000™, Bio-Rad Laboratories, California, USA

Laminar flow: BossTech, Hampshire, UK

Microcentrifuge: Hettich, Massachusetts, USA; and WiseSpin CF-10, DAIHAN Scientific, Seoul, Korea

Rotary vacuum evaporation: EYELA, Japan

3.2 Chemicals

0.5xTBE buffer

1.0xTBE buffer

0.85% NaCl

1 kb plus DNA ladder: Invitrogen, USA

70% ethanol

Agarosepowder: AMRESCO®, Ohio, USA

Double distilled water

Ethidium Bromide: AMRESCO®, Ohio, USA

GeneRuler™100 bp Plus DNA Ladder: Invitrogen, New York, USA

Glycerol

Isopropanol: MERCK, Darmstadt, Germany

3.3 Supplies

0.22 micron membrane filters: Whatman international, UK

Blade

Cheesecloth

Forceps

Glassware

Laboratory bottles

Microcentrifuge tubes: Bioline, Massachusetts, USA

Micropipette: Labnet international, Inc., New Jersey, USA

Petri Dish: Thermo Fisher Scientific., Inc., New York, USA

Scissors

Stainless spoons and spatula

3.4 KITS

Metagenomic DNA Isolation Kit for Water, EPICENTRE, Wisconsin, USA

EmeraldAmp® GT PCR Master Mix TAKARA BIO INC., Shiga, Japan

PureLink® Quick Gel Extraction Kit: Invitrogen New York, USA

3.5 Sample collection

Water samples were collected during March and April of 2013 in sterile containers by SEAFDEC survey vessels. Each sample included 20 liters (5 liters 4 independent repeats) at a depth of 5 meters from the surface and the seabed totaling 12 positions following picture (Positions 1, 4, 10, 13, 18, 21, 26, 28, 33, 42, 45, 36). The physical properties of color and scent were determined using the naked

eye. Temperature, salinity, fluorescence Seapoint and dissolved oxygen were measured at the sampling site.

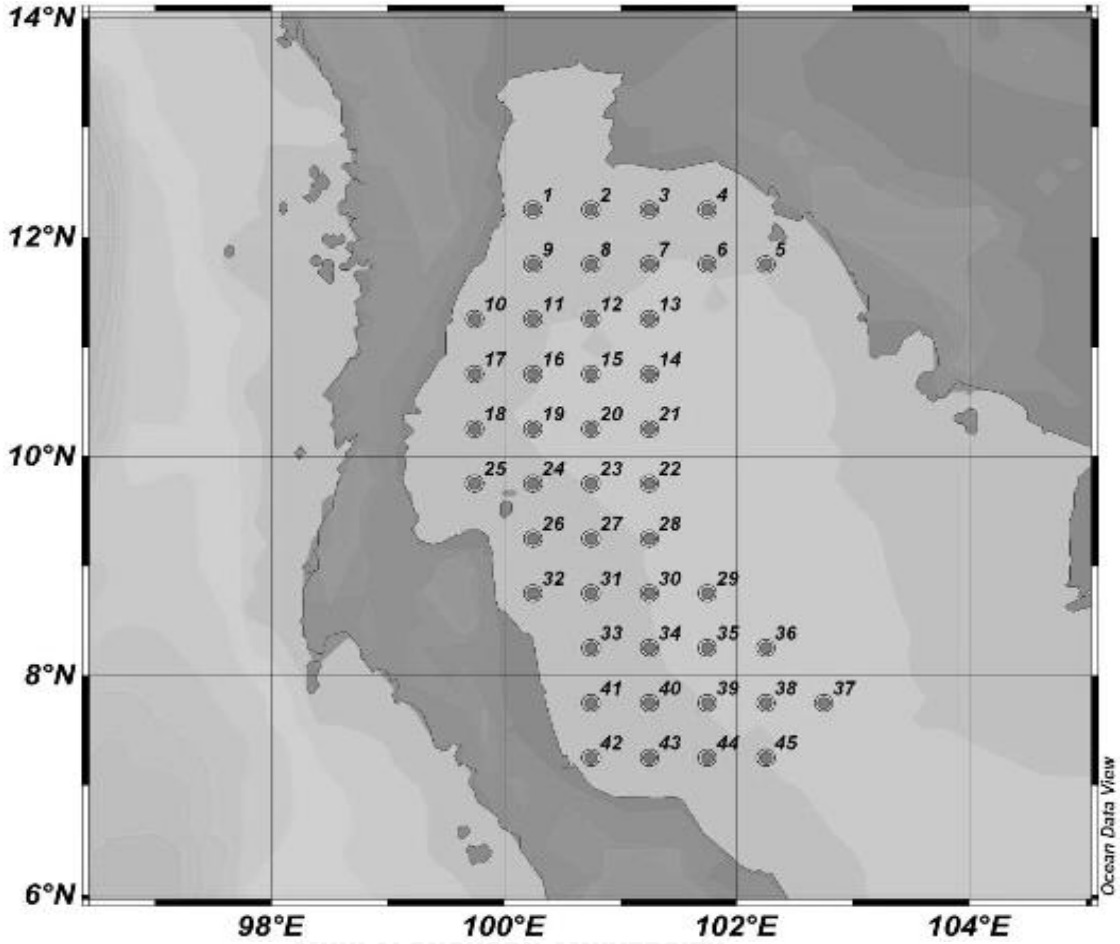

Figure 3. 1 The sampling location of the Gulf of Thailand

Table 3. 1 Sampling position (GPS coordinates)

St. no.	Position		St. no.	Position	
	Latitude	Longitude		Latitude	Longitude
1	12 20.55 N	100 14.53 E	**45**	07 18.44 N	102 15.21 E
10	11 14.04 N	095 47.59 E	**36**	08 25.54 N	101 04.53 E
18	10 15.25 N	099 44.58 E	**28**	09 15.47 N	101 05.32 E
26	09 14.14 N	100 14.31 E	**21**	10 15.53 N	101 05.32 E
33	08 14.40 N	100 46.11 E	**13**	11 14.32 N	101 06.40 E
42	07 20.31 N	100 46.18 E	**04**	12 15.32 N	101 45.39 E

3.6 Metagenomic extraction

Of each independen sampling, 2 metagenomic extrations were performed. Pour water sample through a 4-layer sterile cheesecloth to remove microorganisms larger than 30 µM. Then pour water sample through 0.22 micron filter membrane (Whatman international, UK) to obtain a microorganism larger than 0.22 µM. Then total microorganism was extracted following by Metagenomics DNA Isolation Kit for water (Epicentre, Madison, USA). First, wash microorganisms on the filter membrane with wash buffer and transfer cell suspension to the microcentrifuge tube. Next, centrifuge to precipitate, discard supernatant and resuspend the cell pellet by TE buffer, RNase A, Ready-Lyse Lysosyme. Incubate at 37°C for 30 minutes and then add Meta-lysis solution (2X), Protenase K. After, incubate at 65°C for 15 minutes, then cool down on ice 3-5 minutes and add MPC Protein precipitation Reagent. Centrifuge at 4°C and transfer supernatant to microcentrifuge tube. Later, add isopropanol, inverting, centrifuge at 4°C and discard any residual liquid. Clean the pellet by 70% ethanol and centrifuge at 4°C. Remove ethanol by pipet and air dry. Finally, DNA were dissolved in elution buffer and stored at -20 °C.

Figure 3. 2 Filtering water sample left) filtering through cheesecloth right) filtering through the filter membrane

3.7 Quality and concentration of extracted DNA

The quality and concentration of the extracted DNA were determined by agarose gel electrophoresis using 0.55% agarose gel. Run at 100 V 20 minutes and 50 V 15 minutes, and use 1 kb plus DNA ladder (Invitrogen, Massachusetts, USA) was a DNA standard. Visualized under UV transilluminator and Gel documentation, and cut the band by sterile blade. The metagonome of each sample was further purified using GenepHlow ™ Gel Extraction Kit (Geneaid, Taipei, Taiwan).

3.8 Construction and examination of prokaryotic 16S rRNA and eukaryotic 18S rRNA gene libraries

Construction of DNA fragments prokaryotic library by Enrichment the gene 16S rRNA using the universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 786R (5'-CTACCAGGGTATCTAATC-3'). And eukaryotes library constructs by gene 18S rRNA using the universal primers Euk1A (5'-CTGGTTGATCCTGCCAG-3') and Euk516R (5'-

ACCAGACTTGCCCTCC-3') (26, 27). The design features a barcode, attached to one sample per one barcode. PCR with the initial conditions of 94 °C for 4 minutes, followed by a process of synthesis of DNA 30-35 cycles at 94 °C for 0.45 minutes and 50 °C for 0.55 minutes and 72 °C for 1.30 minutes (28). Determine the quality and concentration of metagenomic DNA by agarose gel electrophoresis using 1.75% agarose gel and use 100 bp DNA ladder (Biolabs, London, UK) was a DNA standard. Visualized under UV transilluminator and Gel documentation.

3.9 Library purification and sequencing

Cut intersecting DNA band from 1.75% agarose gel to microcentrifuge tube with a sterile blade and cleaning products follow steps in GenepHlow ™ Gel Extraction Kit (Geneaid, Taipei, Taiwan). Firstly, incubate gel slice and DF buffer at 60 °C. Transfer sample to a DF column by pipette, centrifuge, discard the flow-through and replace DF column to Collection tube. Add W1 Buffer into the DF Column and centrifuge. Next, discard the flow-through and place the DF Column back in the Collection Tube. Add Wash Buffer into DF column and centrifuge. After, discard the flow-through, replace DF column to Collection tube and centrifuge to dry column. Transfer DF column to a new microcentrifuge tube and let stand. After adding elution buffer to ensure DF column is completely absorbed. Finally, centrifuge to elute the purified DNA. Determine concentration by Qubit® 2.0 Fluorometer and Qubit®dsDNA HS Assay kit (Invitrogen) (6). Pool library of DNA fragments with HiYield ™ Gel/PCR DNA Fragments Extraction Kit (Life Biomedical Limited, Cambridge, UK), total concentration of 2.5 micrograms in 50 microliter. Attach adapter by using TruSeq®DNA Library Prep Kit (Illumina) and sequenced with Miseq illumina (500 laps) at the research center, faculty of Medicine Chulalongkorn University.

3.10 Bioinformatic analysis

Bioinformatics based on Mothur program (24). First, remove low-quality reads and categorize reads based on the appended barcodes. Then, combine 2 sets of reads (R1 and R2) and align sequences by BLASTN with Greengenes and SILVA databases for 16S rRNA and SILVA databases for 18S rRNA (29). After that, remove undesirable and compare population from different sample sources to find relationships or demographic groups. Analysis community type to find out how many community types of microbes. Clustering of microbial by PCoA based on OTUs and create a tree by using the Mothur program base on thetayc distance. Abiotic factor analysis to find factors that effect to microbial communities. Then, correlation coefficient analysis was done to find out OTUs that significantly influence the microbial diversity based on P-value ≤ 0.05. Analysis potential functions using picust based on KEGG database (30). Using STAMP to reconstruct heatmap for finding out the statistic significant and abundance of metabolisms at genus and species levels. STAMP also clustered by PCA communities based on the genes assigned to metabolisms (25).

CHAPTER IV

RESULT

4.1 Water characteristics of the central Gulf of Thailand

From Table 4.1 shows that temperature due to 21-31 ℃, salinity due to 31-33 PSU, fluorescence Seapoint due to 9.77E-02 -1.37E+00 and oxygen due to 2.7-5.8, are very similar. Whereas, depth due to 5-60 m. and distance of oil leak are quite different. This may indicate that these factors may affect the microbial.

Table 4. 1 characteristic water of central Gulf of Thailand

sample	Depth [m]	Temperature [°C]	Salinity [PSU]	Fluorescence seapoint	Oxygen [mg/l]	Density [Kg/m^3]	distance oil leak
st1	5	29.8621	31.4237	1.81E-01	5.79131	8.074	133.2
st10	10	29.5324	31.4521	1.13E-01	5.77602	8.094	632.8
st18	5	29.639	31.4961	1.08E-01	5.7503	8.154	317.4
St26	5	29.7039	31.5603	7.13E-02	5.69658	8.244	393.5
St33	23	28.7284	31.8381	3.00E-01	5.57995	8.015	487.1
St42	5	29.3468	31.9867	1.09E-01	5.66256	8.055	586.4
St45_s	5	29.6617	32.6071	9.76E-02	5.66609	8.07	591.8
St45_b	43	27.0739	33.1796	1.37E+00	4.86402	8.007	591.8
St36_s	5	30.1007	31.645	3.47E-02	5.60632	8.169	462.6
St36_b	46	28.8905	31.9786	2.53E-01	4.93788	8.102	462.6
St28_s	5	30.0083	31.8048	7.67E-02	5.66003	8.157	370.3
St28_b	60	28.7969	32.0688	9.88E-01	4.54585	8.099	370.3
St21_s	5	30.598	31.5896	7.23E-02	5.64754	8.246	259.9
St21_b	60	28.7831	31.8036	1.17E+00	5.16839	8.194	259.9
St13_s	5	30.6502	31.6982	9.77E-02	5.59313	8.231	152.5
St13_b	55	28.7744	32.3963	1.22E+00	2.7014	8.02	152.5
st4_s	5	31.0676	31.5505	1.16E-01	5.31869	8.235	48.5
st4_b	24	30.8652	31.5386	9.03E-01	5.12831	8.21	48.5

* Fluorescence Seapoint is the amount of fluorescent compounds in the water, such as chlorophyll and fluorescence. This relates to sunlight exposure and activities.

* distance oil leak, the sample was collected before the oil was leaked, so it was used as a reference for research on oil spills in the Gulf of Thailand.

4.2 Sequencing and bioinformatics of prokaryotes

After removal of low-quality and undesirables read, and categorize reads based on the appended barcodes, suggested that total of reads were 2168091 and the average reads length were 421.53 bp. The number of sequence reads and average reads the length of each sample were in Table 4.2. From community analysis to find out how many community types existed in the data using Mothur found indicated that prokaryotic communities in the central Gulf of Thailand have 1 community type in Table 4.3.

Table 4. 2 Number of 16S rRNA sequence reads

sample	Number of sequence reads	Average read length (bp)	sample	Number of sequence reads	Average read length (bp)
st1	58964	424.50	st28_s	219674	424.08
st10	65436	424.44	st33	36119	417.83
st13_b	71521	415.98	st36_b	6693	419.22
st13_s	50701	422.34	st36_s	302168	421.87
st18	39988	422.43	st42	155527	418.09
st21_b	4853	422.32	st45_b	123649	423.53
st21_s	218827	425.84	st45_s	111741	420.97
st26	73828	419.75	st4_b	319720	425.94
st28_b	285297	415.60	st4_s	23385	422.71

* Subsampling at 4853 reads for following analyses

Table 4. 3 Prokaryote community type in central Gulf of Thailand

sample	communitytype	sample	communitytype
st1	Partition_1	st28_s	Partition_1
st10	Partition_1	st33	Partition_1
st13_b	Partition_1	st36_b	Partition_1
st13_s	Partition_1	st36_s	Partition_1
st18	Partition_1	st42	Partition_1
st21_b	Partition_1	st45_b	Partition_1
st21_s	Partition_1	st45_s	Partition_1
st26	Partition_1	st4_b	Partition_1
st28_b	Partition_1	st4_s	Partition_1

The sample coverage, the number of observations OTUs, Chao, the Shannon and the Inverse Simpson diversity estimate using Mothur shows coverage and prokaryote diversity at phylum in Table 4.4. The coverage, quality score considers if a quality value greater than 97% indicates that a good value of sampling the communities found that all samples have a quality score around 99% indicate sampling was coverage communities of prokaryotes. At the phylum level of prokaryote composition profiles is shown in Figure 4.1. Suggest that the communities of prokaryotes were rather similarity corresponding to Table 4.3 that get same community type, and founded that *Proteobacteria*, *Firmicutes* and *Actinobacteria* were abundant, respectively.

Table 4. 4 Sample coverage and prokaryotic diversity at phylum

sample	coverage	OTUs	chao	invsimpson	shannon
st1	99.98	39	48.17	2.021989	1.102983
st10	99.98	44	57.2	2.271042	1.19463
st13_b	99.97	84	107	4.051236	1.844023
st13_s	99.99	44	46.33	3.491796	1.586341
st18	99.97	48	57.43	2.950393	1.481598
st21_b	99.77	35	42.86	3.423171	1.692379
st21_s	99.99	68	87	1.41483	0.738572
st26	99.98	61	74.13	4.773401	1.868047
st28_b	99.99	99	102.46	4.379338	1.898687
st28_s	99.99	64	86.67	2.368606	1.174892
st33	99.97	43	52	4.765529	1.77795
st36_b	99.86	36	45	4.641457	1.910135
st36_s	99.99	67	127	2.469572	1.379327
st42	99.99	88	109	5.803795	1.988354
st45_b	99.99	58	80.67	2.360105	1.276889
st45_s	99.98	74	91	2.813966	1.464456
st4_b	99.99	81	91.91	1.378632	0.70511
st4_s	99.95	47	53.6	3.44139	1.572659

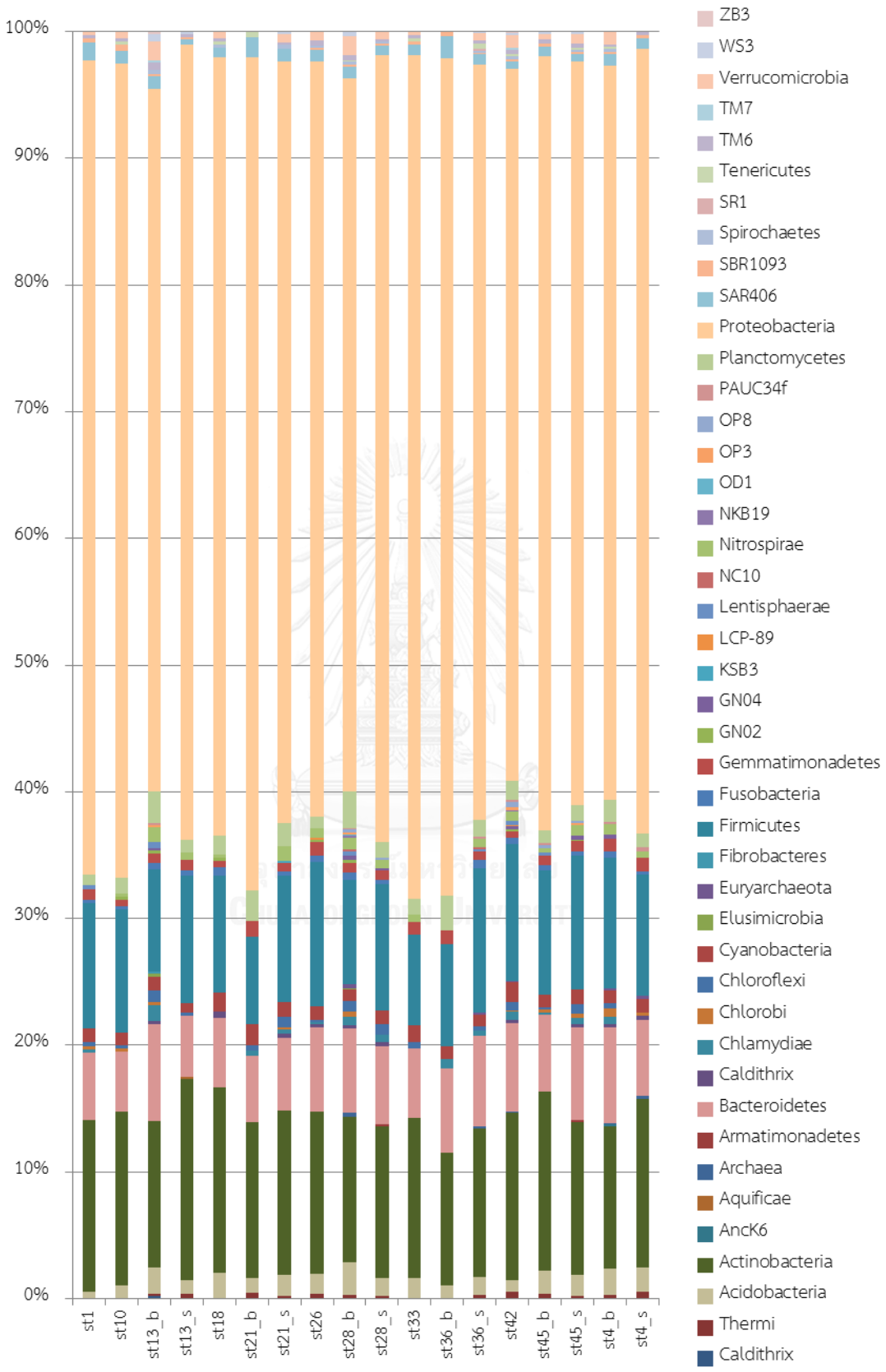

Figure 4. 1 OTUs microbial diversity of prokaryotic communities at phylum-level

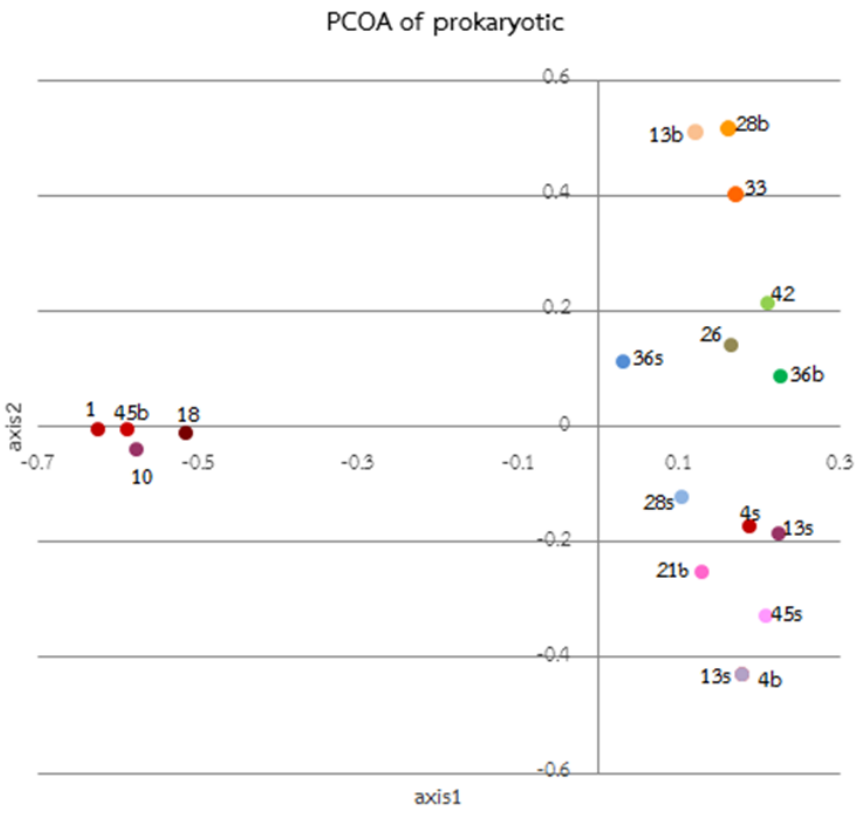

Figure 4. 2 Principal Coordinate Analysis (PCoA) of prokaryote

The Principal Coordinate Analysis (PCoA) of prokaryote showed a potential 2 clusters, although from get.communityType (function in motor) get 1 homogenous community. That grouping shown in Figure 4.2 was correlated to a phylogenetic tree analysis by tree.shared (Figure 4.3). In details, the samples may be grouped according to the Table 4.5. The 2 groups of communities were consistent with the bar chart species distribution pattern, were similar within the group. And compare cluster from the PCoA with characteristic founded that the same type have similar temperature and salinity. So the analysis of abioticseapoins factor compared with Principal Coordinate Analysis (PCoA) was done, and showed that factors affecting microbial grouping was temperature, based on significant *P*-value ≤ 0.05 (Table 4.6). *P*-value of density was 0.056 which is close to 0.05, so it may also be important.

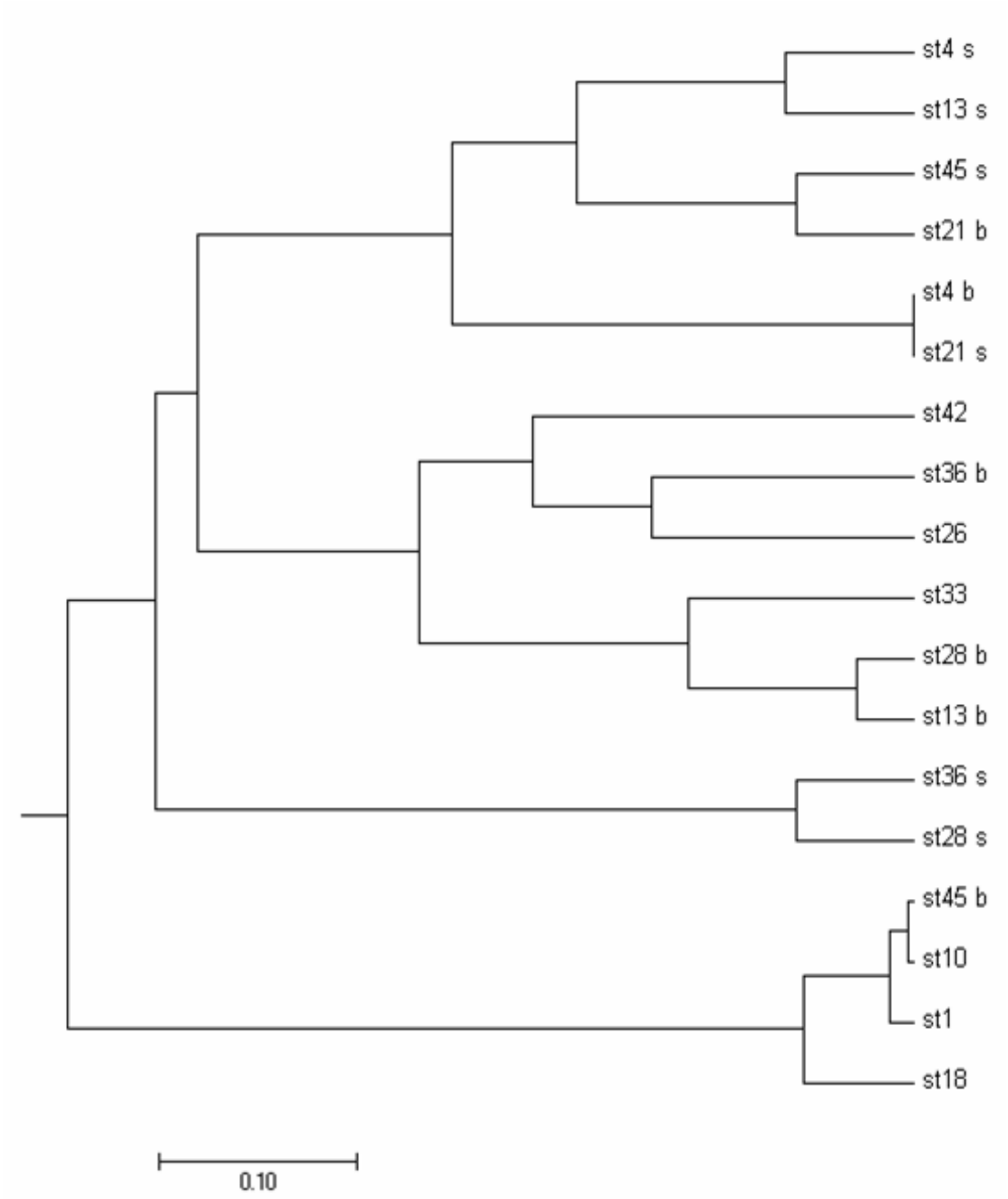

Figure 4. 3 Dendrogram of prokaryotes communities constructed by Mothur, based on thetayc

Table 4. 5 Cluster group of prokaryote base on phylogenetic tree

type	group	sites	source
1	st13_s	site13	surfacesource13
1	st4_s	site4	surfacesource4
2	st21_b	site21	bottomsource21
2	st45_s	site45	surfacesource45
3	st21_s	site21	surfacesource21
3	st4_b	site4	bottomsource4
4	st26	site26	surfacesource26
4	st36_b	site36	bottomsource36
4	st42	site42	surfacesource42
5	st13_b	site13	bottomsource13
5	st28_b	site28	bottomsource28
5	st33	site33	bottomsource33
6	st28_s	site28	surfacesource28
6	st36_s	site36	surfacesource36
7	st1	site1	surfacesource1
7	st10	site10	bottomsource10
7	st18	site18	surfacesource18
7	st45_b	site45	bottomsource45

Table 4. 6 Abiotic factor analysis of prokaryote

Factors	p-value	length
Depth	0.290036	0.266907
Temperature	0.012195	0.629858
Salinity	0.177389	0.419849
Fluorescence Seapoint	0.191455	0.359681
Oxygen	0.329859	0.29869
Density	0.056462	0.459321
distanceOilLeak	0.230381	0.303909

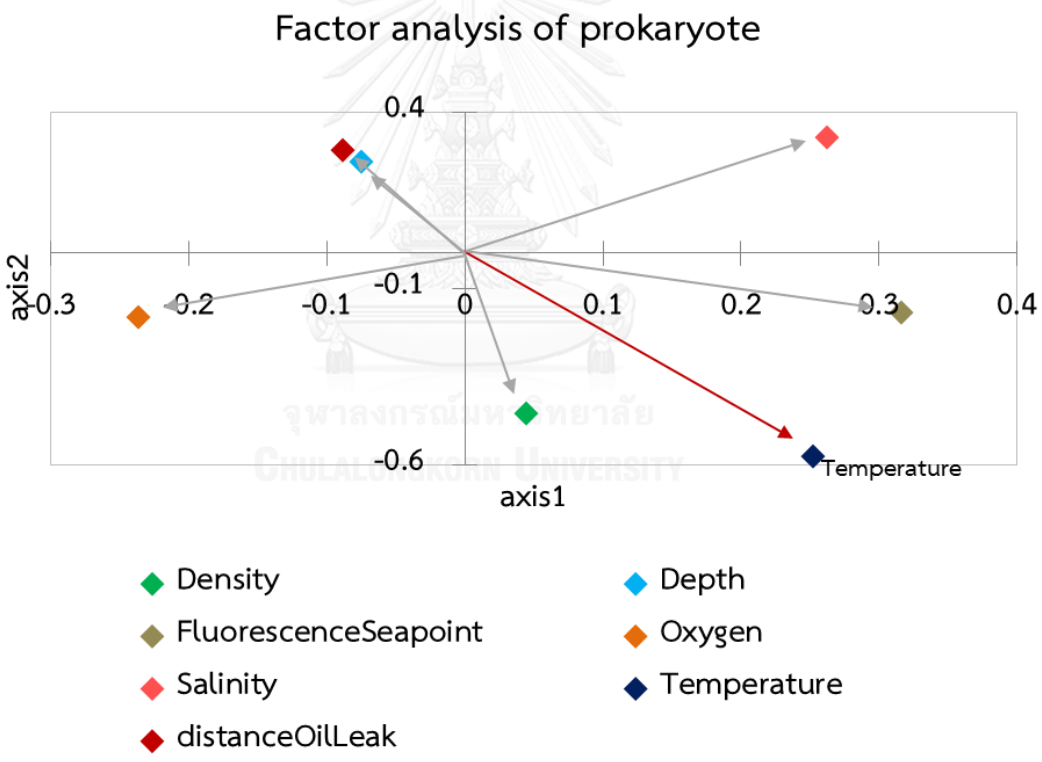

Figure 4. 4 Factor analysis of prokaryote base on PCoA

Table 4. 7 Correlation coefficient OTUs of prokaryote

OTU	p-value	p-value	length
Otu0001	0.004663	0.000073	1.019404
Otu0075	0.000335	0.006252	0.972118
Otu0062	0.007711	0.000264	0.970727
Otu0013	0.00541	0.000589	0.961636
Otu0019	0.015569	0.000335	0.936431
Otu0044	0.00028	0.018554	0.934081
Otu0058	0.000807	0.015111	0.91146
Otu0024	0.001661	0.009747	0.905895
Otu0012	0.028006	0.000939	0.879147
Otu0043	0.004841	0.011818	0.857541

Table 4. 8 Correlation coefficient taxonomy OTUs of prokaryote

OTU	Taxonomy
Otu0001	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales; f__Pseudoalteromonadaceae;g__Pseudoalteromonas;unclassified;
Otu0012	k__Bacteria ;p__Proteobacteria ;c__Gammaproteobacteria ;o__Oceanospirillales ;f__Halomonadaceae;g__Cobetia;unclassified;
Otu0013	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales; f__Pseudoalteromonadaceae; g__Pseudoalteromonas; s__luteoviolacea;
Otu0019	k__Bacteria ;p__Proteobacteria ;c__Gammaproteobacteria ;o__Vibrionales ; Unclassified; unclassified; unclassified;
Otu0024	k__Bacteria ;p__Proteobacteria ;c__Gammaproteobacteria ;o__Oceanospirillales; f__Halomonadaceae ; unclassified ; unclassified ;
Otu0043	k__Bacteria ;p__Proteobacteria ;c__Gammaproteobacteria;o__Pseudomonadales; f__Pseudomonadaceae ;g__Pseudomonas ; unclassified ;
Otu0044	k__Bacteria ;p__Proteobacteria ;c__Gammaproteobacteria ;o__Vibrionales ; f__Vibrionaceae; g__Vibrio; unclassified;
Otu0058	k__Bacteria ;p__Proteobacteria ;c__Gammaproteobacteria ;o__Vibrionales ; f__Pseudoalteromonadaceae; unclassified; unclassified;
Otu0062	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales; f__Pseudoalteromonadaceae;g__Pseudoalteromonas;s__piscicida;
Otu0075	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales; f__Oceanospirillaceae; unclassified; unclassified;

Then, correlation coefficient analysis was done to find out OTUs that significantly influence the microbial diversity based on *P*-value ≤ 0.05. The most significant OTUs of prokaryotes shown in Table 4.7, of which many cannot be identified be the species but at the genus level. They were of *Oceanospirillacea, Pseudoalteromonadaceae, Halomonadaceae* and *Vibrionaceae* families. These significant OTUs were affected the community pattern.

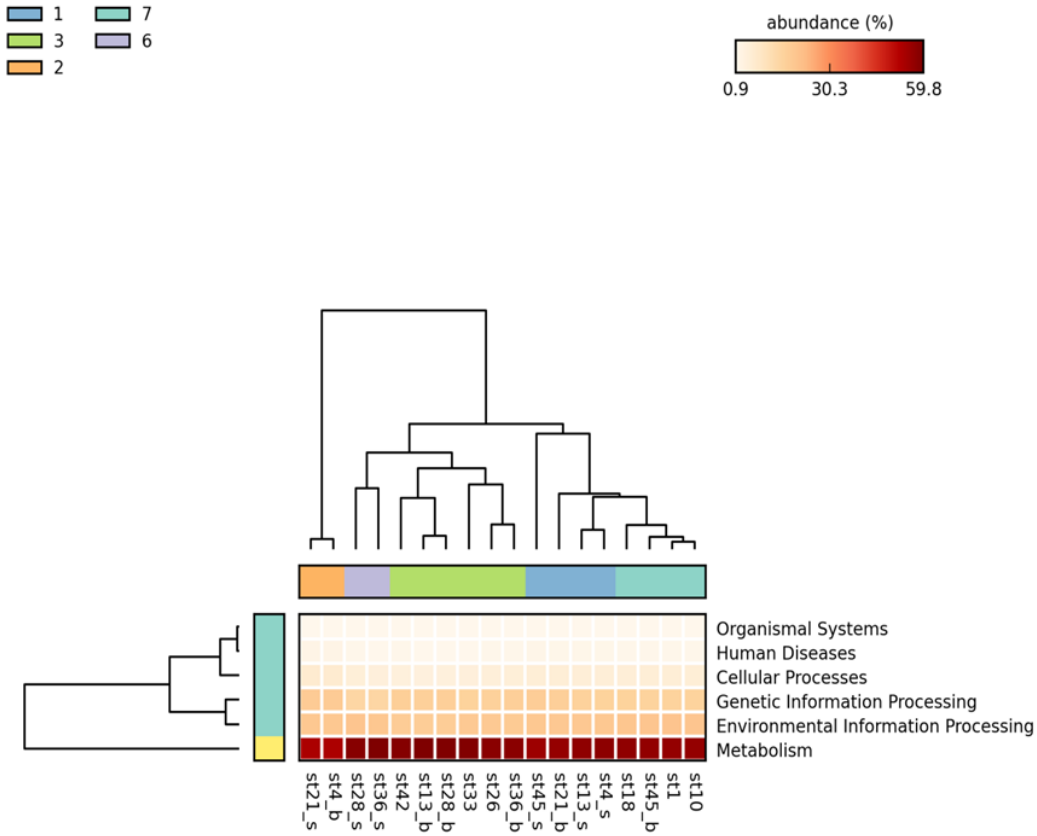

Figure 4. 5 Potentials functional metabolic pathway using Statistical Analysis of metagenomic Profiles (STAMP)

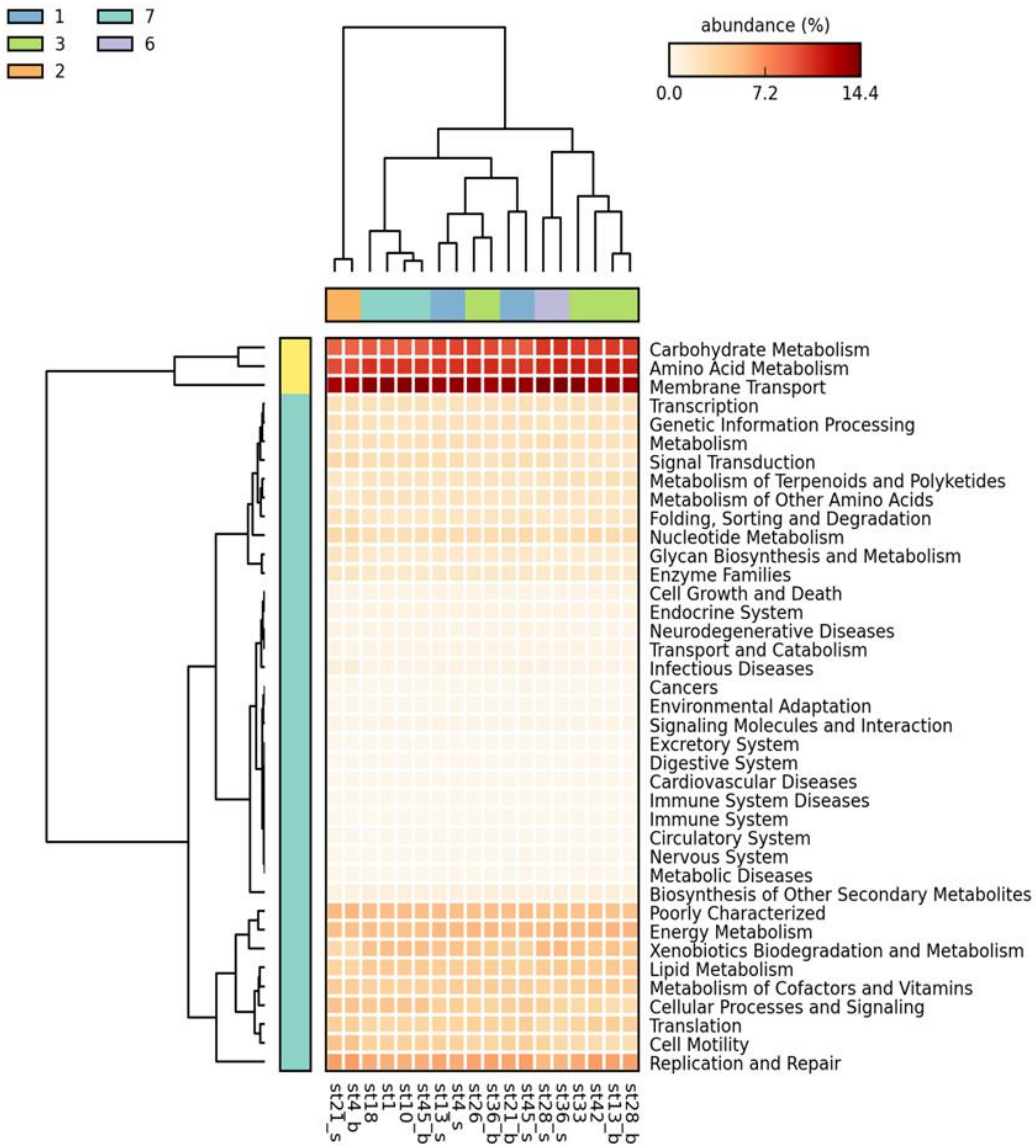

Figure 4. 6 Potentials functional metabolism using Statistical Analysis of metagenomic Profiles (STAMP)

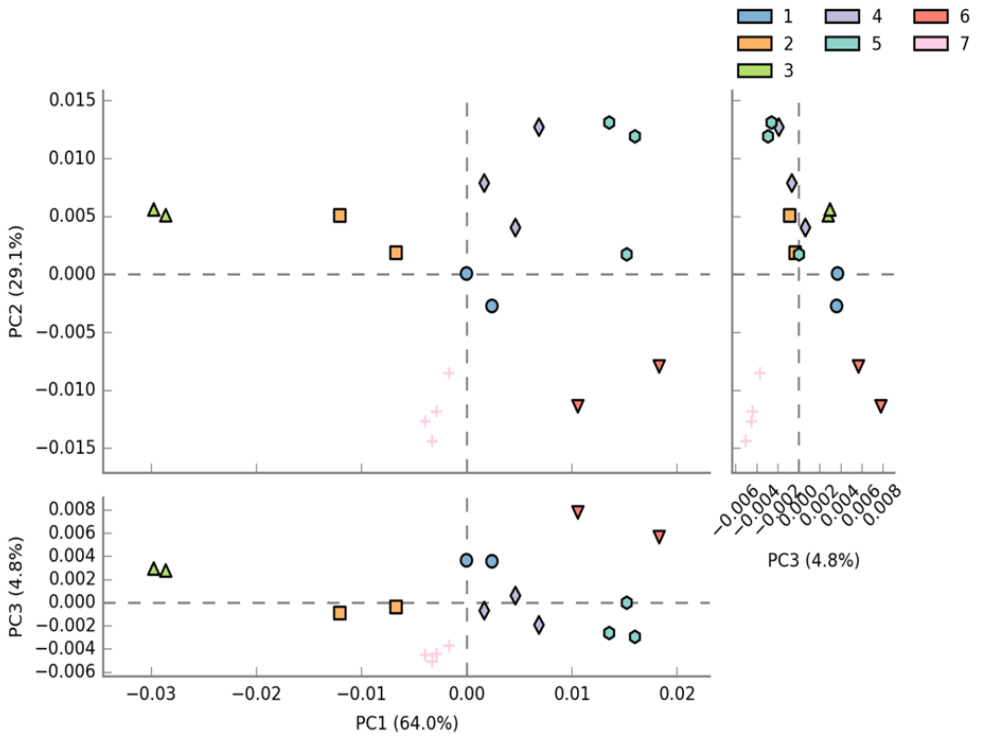

Figure 4. 7 Principal Component Analysis (PCA) based on potential metabolism profiles by STAMP. A, PC1&PC2; B, PC1&PC3; C, PC2&PC3.

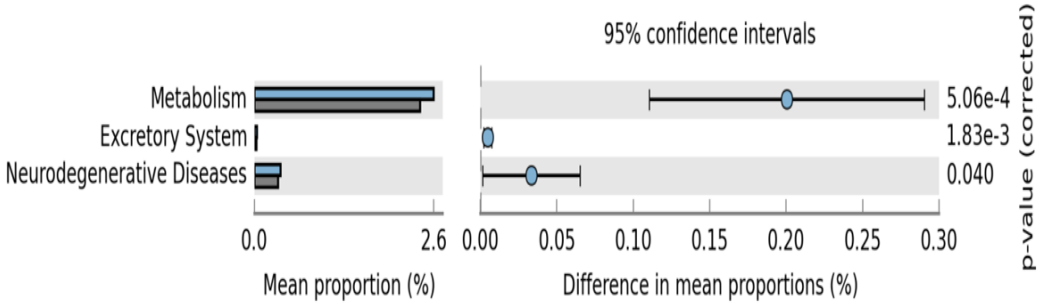

Figure 4. 8 Significant potentials metabolisms of group 1 against prokaryote life

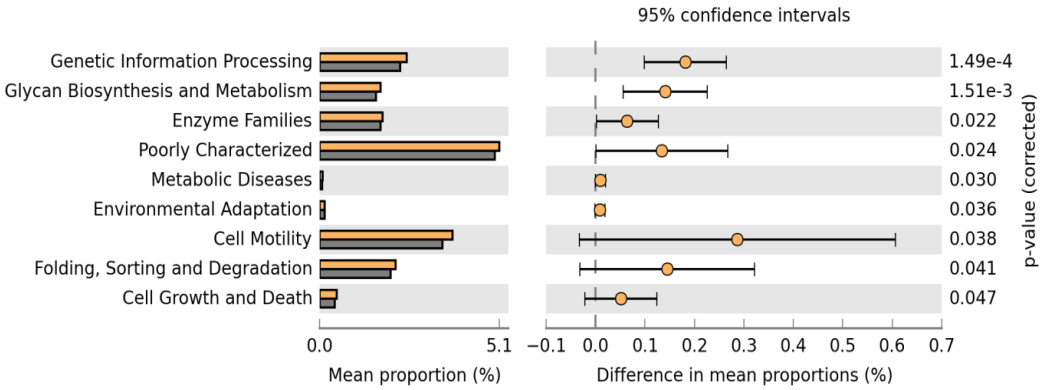

Figure 4. 9 Significant potentials metabolisms of group 2 against prokaryote life (the rest 6 groups)

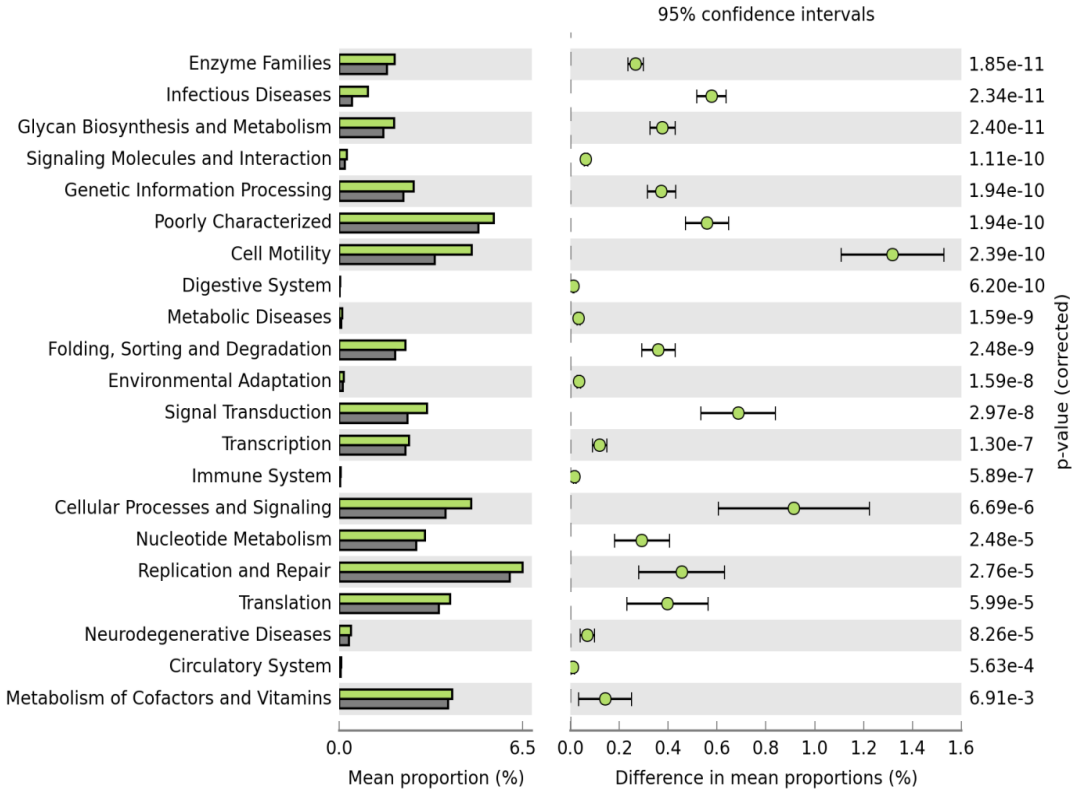

Figure 4. 10 Significant potentials metabolisms of group 3 against prokaryote life (the rest 6 groups)

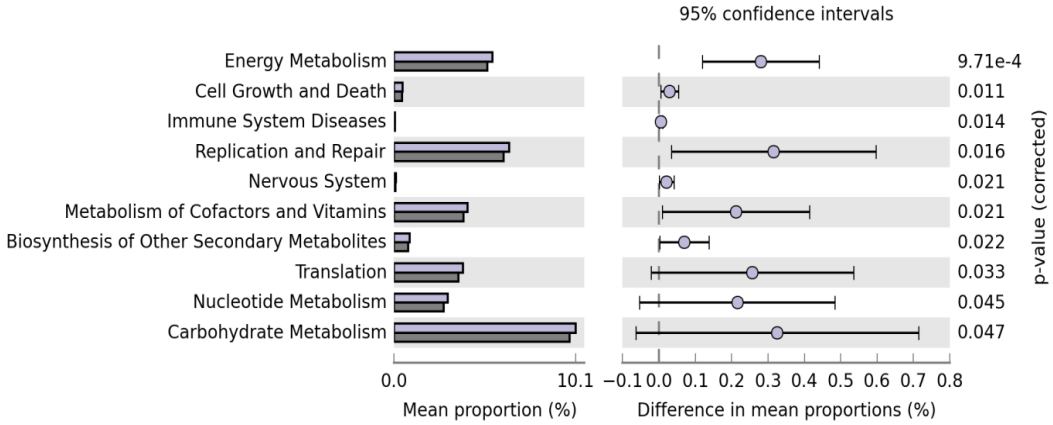

Figure 4. 11 Significant potentials metabolisms of group 4 against prokaryote life (the rest 6 groups)

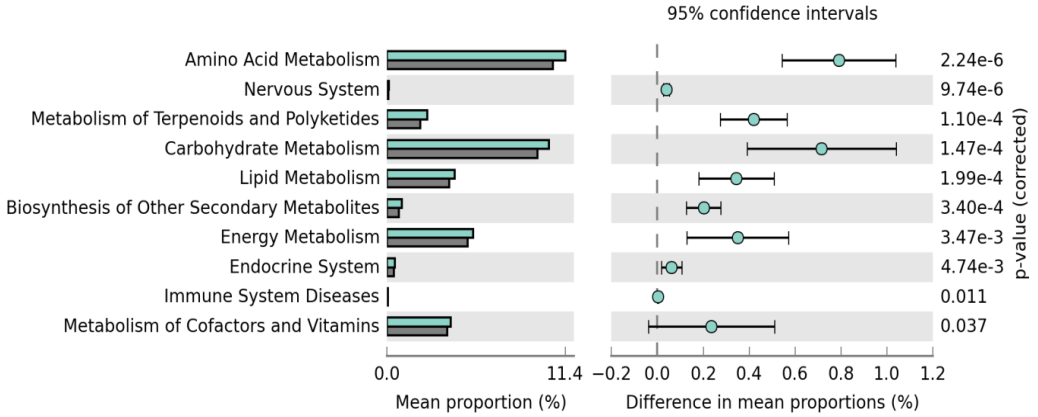

Figure 4. 12 Significant potentials metabolisms of group 5 against prokaryote life (the rest 6 groups)

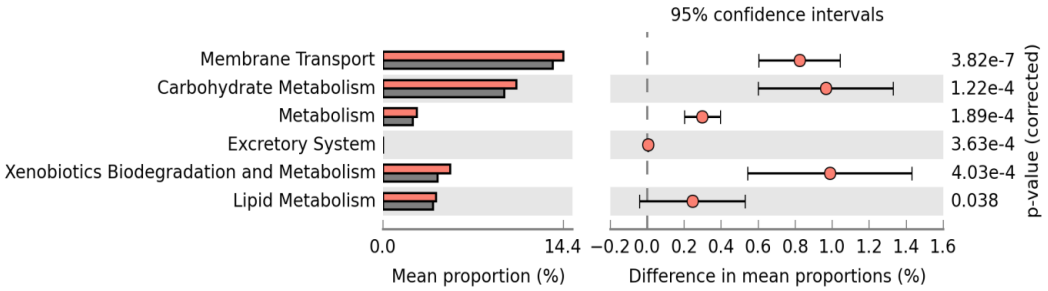

Figure 4. 13 Significant potentials metabolisms of group 6 against prokaryote life (the rest 6 groups)

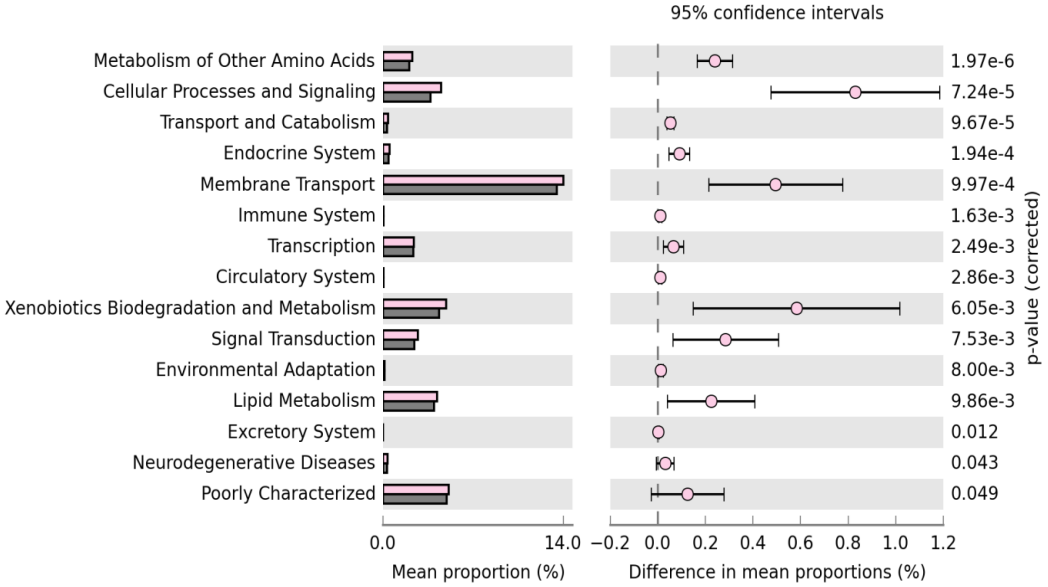

Figure 4. 14 Significant potentials metabolisms of group 7 against prokaryote life (the rest 6 groups)

After a cluster group, we analyzed functional profiles using STAMP (Parks *et* al., 2014), a heatmap indicating the proportion of sequences assigned to each function. Dendrograms on the sides of heatmap were used to cluster both the shared features (left) and samples (top). The red color of heatmap shows the relative abundance of metabolic found that the most relative abundance are metabolized.

Across communities, the abundance of metabolisms of organismal systems, human diseases and cellular processes, environmental information processing and genetic information processing were of similar. Suggest that these functional potentials metabolic are correlated (Figure 4.5). The metabolism was abundant to include amino acid metabolism, carbohydrate metabolism and membrane transport. And also found that metabolism related to cellular processes and environmental information processing. Organismal systems, metabolism related to human diseases as well and relatively abundance (Figure 4.6). Figure 4.6 shows that the previous grouping (Table 4.4) with a cluster group of metagenomics profiles were rather consistent, except group 4 and 5. Some example of group 4 be Inserted between samples of groups 5, therefore, Principal component analysis (PCA) exhibited the difference between each group. Overall, the metabolism was grouped a grouping of microbial from Mothur program were A. and B. in Figure 4.7. Principal component analysis (PCA) using Statistical Analysis of Metagenomic Profiles (STAMP) (Figure 4.7) at C. axis PC2 & PC3 show that group 4 close group 2 and 5. Group 4 which close group 5 was st42 that corresponds with a metagenomics profile in Figure 4.6. Grouping of the Mothur and STAMP programs may be different because the Mothur program are grouped using the OTUs of the microbes as an indicator, while the stamp uses the metabolic as an indicator. Therefore, the grouping is based mainly on grouping of Mothur. After we grouped the samples, we will find out significant metabolism of each group. Extended error bar indicates the difference in mean proportion between the groups. In Figure 4.8 - 4.14 show significant functional potentials each group. Significant potentials metabolism of group 1 has 3 metabolic were metabolism, organismal and human diseases. Significant potentials metabolisms of group 2 has 4 metabolic were cellular processes, metabolism, environmental information processing and human diseases. Significant potentials metabolic of group 3 and 7 have 5 metabolisms were metabolism, cellular processes, environmental information

processing and human diseases. Significant potentials metabolisms of group 4 and 5 has 4 metabolic were cellular processes, metabolism, Organismal systems processing and human diseases. Significant potentials metabolism of group 6 has 3 metabolic were metabolism, organismal and environmental information processing.

4.3 Sequencing and bioinformatics of eukaryotes

After removing low-quality reads and undesirables read, and categorize reads based on the appended barcodes suggest that a total of the roads were 394837 reads and average reads length were 196.209 bp. The number of sequence reads and average reads the length of each sample were in Table 4.9. From community analysis indicated that eukaryotes community in the central Gulf of Thailand has 1 community type in Table 4.10.

Table 4. 9 Number of 18S rRNA sequence reads

sample	Sequence reads	Average read length (bp)	sample	Sequence reads	Average read length (bp)
st1	135660	216.046	st28_s	8724	193.874
st10	29483	216.046	st33	8273	202.258
st13_b	14858	172.156	st36_b	426	184.215
st13_s	32371	215.356	st36_s	17569	211.04
st18	63328	216.007	st42	76	204.086
st21_b	2567	201.286	st45_b	7252	185.728
st21_s	29227	182.557	st45_s	6648	207.89
st26	540	184.436	st4_b	31488	182.972
st28_b	2195	168.779	st4_s	4152	187.035

Table 4. 10 Eukaryotes community type central Gulf of Thailand

sample	communitytype	sample	communitytype
st1	Partition_1	st28_s	Partition_1
st10	Partition_1	st33	Partition_1
st13_b	Partition_1	st36_b	Partition_1
st13_s	Partition_1	st36_s	Partition_1
st18	Partition_1	st42	Partition_1
st21_b	Partition_1	st45_b	Partition_1
st21_s	Partition_1	st45_s	Partition_1
st26	Partition_1	st4_b	Partition_1
st28_b	Partition_1	st4_s	Partition_1

Table 4. 11 Sample coverage and eukaryotic diversity at phylum

group	coverage	OTUs	chao	invsimpson	shannon
st1	100	11	11	3.295563	1.368076
st10	99.99	11	11	3.575883	1.451154
st13_b	99.99	9	9	2.678372	1.221744
st13_s	99.99	11	11	2.439282	1.208014
st18	99.99	11	12	3.452187	1.440637
st21_b	100	8	8	2.217941	1.104474
st21_s	100	9	9	2.619912	1.256002
st26	100	7	7	2.138669	1.10456
st28_b	99.95	9	9	2.32417	1.165619
st28_s	100	9	9	3.051329	1.315775
st33	100	9	9	3.719215	1.462043
st36_b	100	7	7	2.567794	1.260965
st36_s	99.99	9	9	2.022247	0.998989
st42	98.68	6	6	3.187919	1.354628
st45_b	100	8	8	3.218342	1.318299
st45_s	99.98	9	9	2.879436	1.260384
st4_b	100	10	10	3.858681	1.466631
st4_s	99.98	10	10	2.997736	1.282314

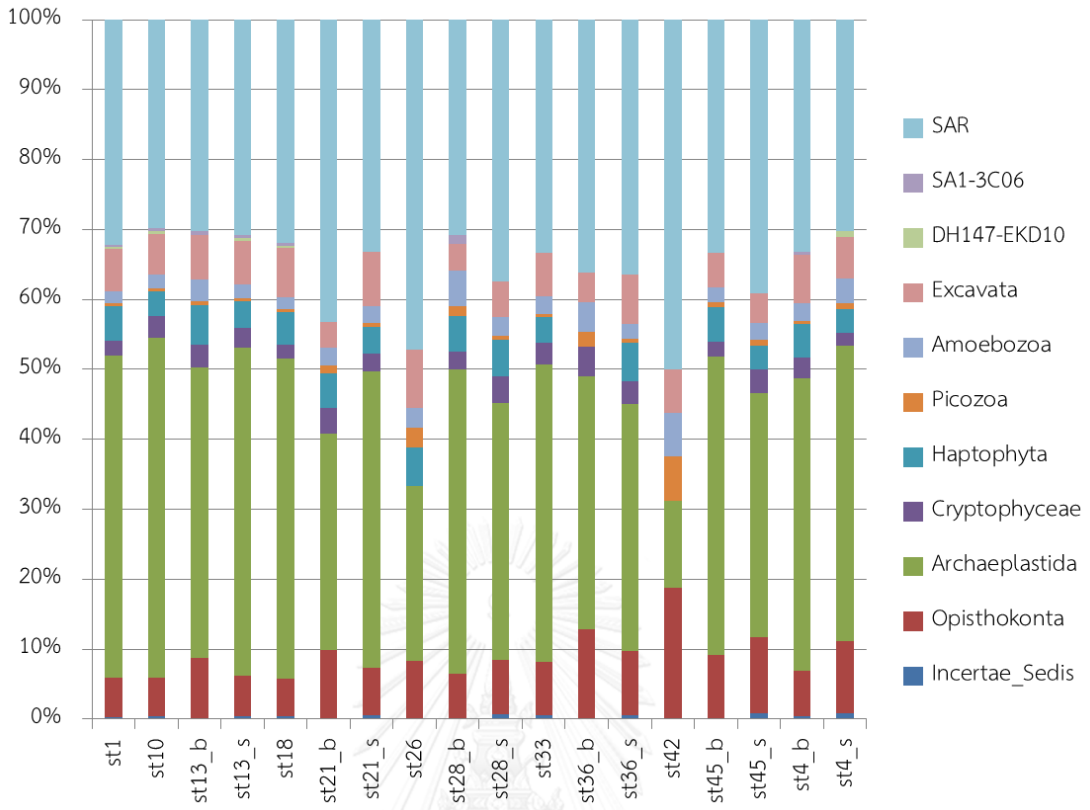

Figure 4. 15 OTUs diversity at supergroup level of eukaryote

The sample coverage, the number of observation OTUs, Chao, the non-parametric Shannon and the Inverse Simpson diversity estimated the coverage quality score of average ± S.D., and prokaryote diversity at supergroup were at Table 4.11. All samples with a coverage quality, value greater than 97% indicates that a good value of sampling the communities. At the supergroup level of eukaryote composition profiles shown in Figure 4.15 suggested that *Archaeplastida*, SAR (*Stramenopiles + Alveolata + Rhizaria*) (31) and Opisthokonta were abundant, respectively. Considering barchart found that pattern OTUs diversity relates to the community type in Table 4.10.

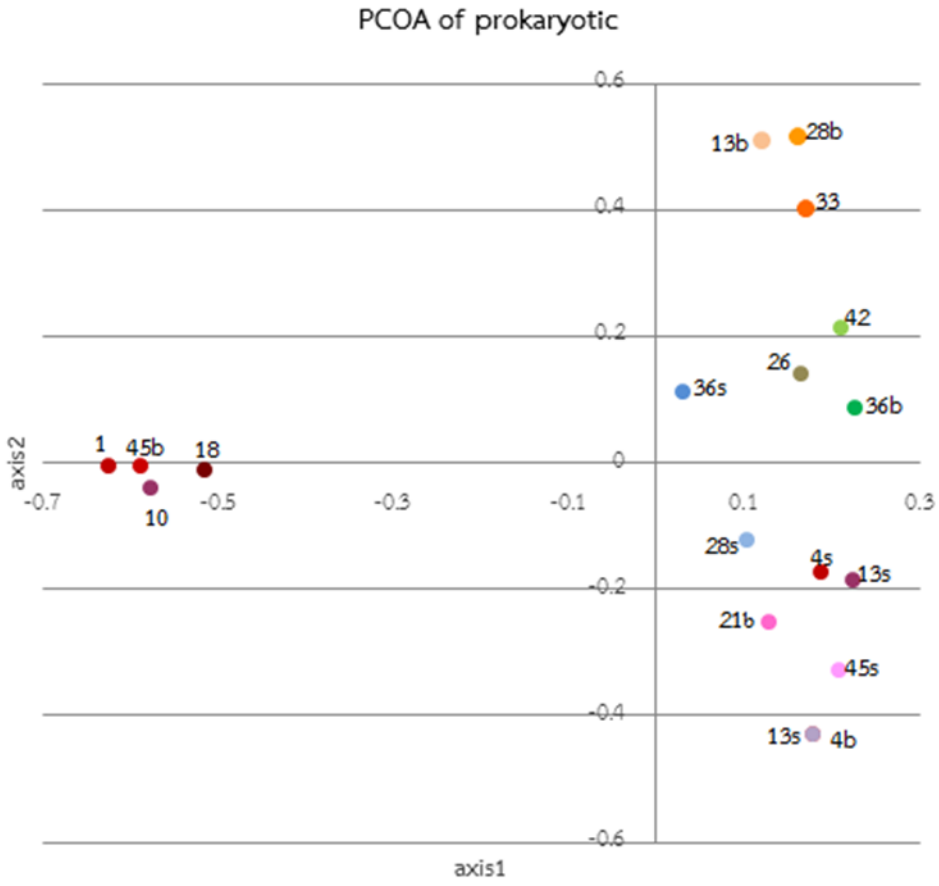

Figure 4. 16 PCoA of eukaryote communities

The Principal Coordinate Analysis (PCoA) of eukaryote (Figure 4.16) for clearly a distinguished clusters found that sample groups was scattered in the bottom of the PCoA chart except the sample st4_b, which separated from each group. That graph related to the grouping of the phylogenetic tree (Figure 4.17). In a phylogenetic tree found that st4_b far from st4_s probably due to this area has two rivers flow down and also the area that has changed the flow of the ocean from the counterclockwise to the clockwise. Therefore, the samples can be grouped according to the Table 4.8. And consider with characteristics founded that the same type has salinity was similar.

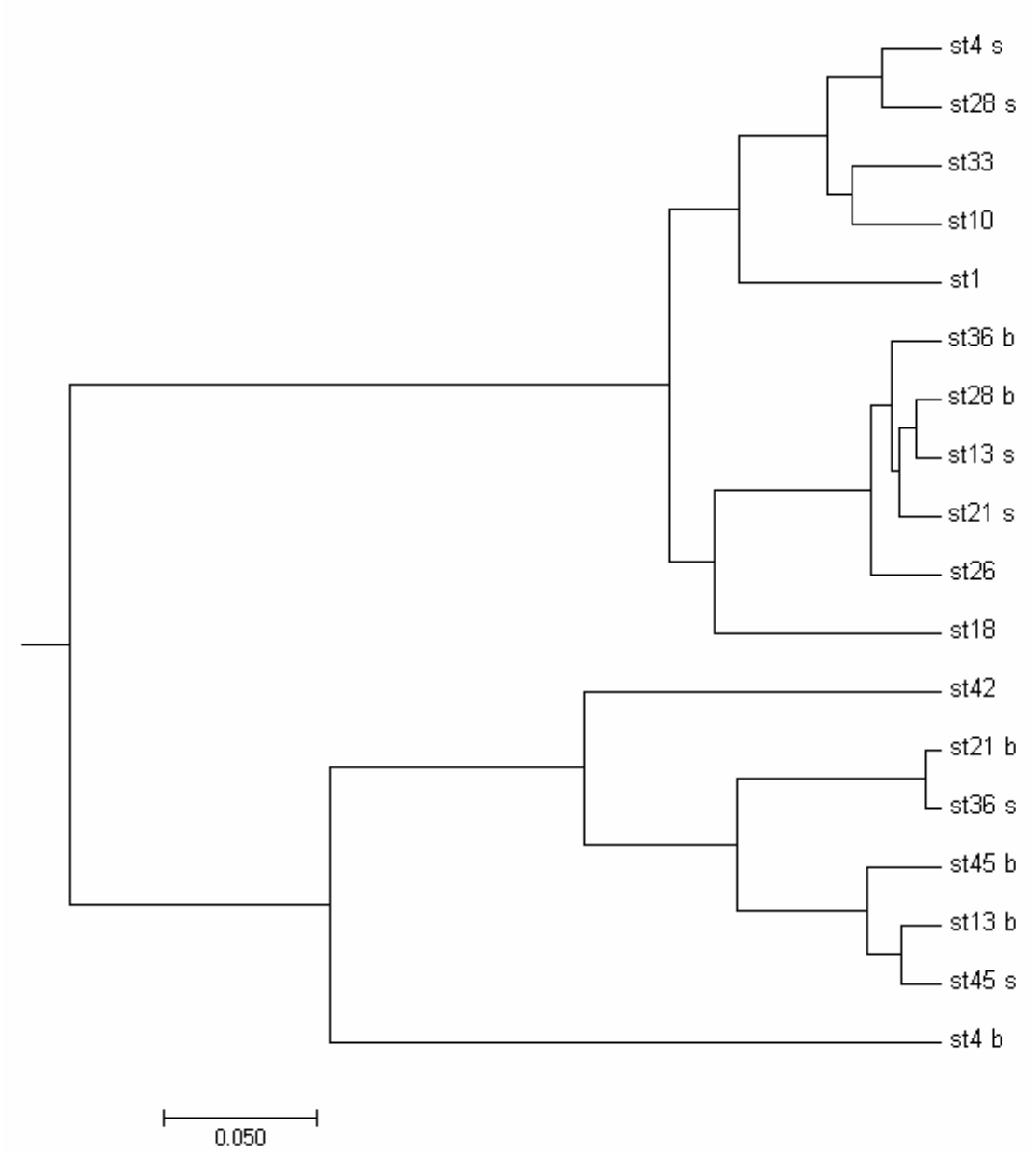

Figure 4. 17 Dendrogram of eukaryotes communities constructed by Mothur, based on thetayc

Table 4. 12 Cluster group of prokaryote base on phylogenetic tree

type	group	sites	source
1	st1	site1	surfacesource1
1	st10	site10	surfacesource10
1	st33	site33	bottomsource33
1	st28_s	site28	surfacesource28
1	st4_s	site4	surfacesource4
2	st36_b	site36	bottomsource36
2	st28_b	site28	bottomsource28
2	st13_s	site13	surfacesource13
2	st21_s	site21	surfacesource21
2	st26	site26	bottomsource26
2	st18	site18	bottomsource18
3	st45_s	site45	surfacesource45
3	st13_b	site13	bottomsource13
3	st45_b	site45	bottomsource45
3	st36_s	site36	surfacesource36
3	st21_b	site21	bottomsource21
3	st42	site42	surfacesource42
4	st4_b	site4	bottomsource4

Table 4. 13 Abiotic factor analysis of eukaryote

Feature	p-value	length
Depth	0.198117	0.358299
Temperature	0.351415	0.26023
Salinity	0.135304	0.381175
Fluorescence Seapoint	0.029507	0.600703
Oxygen	0.351415	0.303991
Density	0.079389	0.486551
distanceOilLeak	0.319979	0.334588

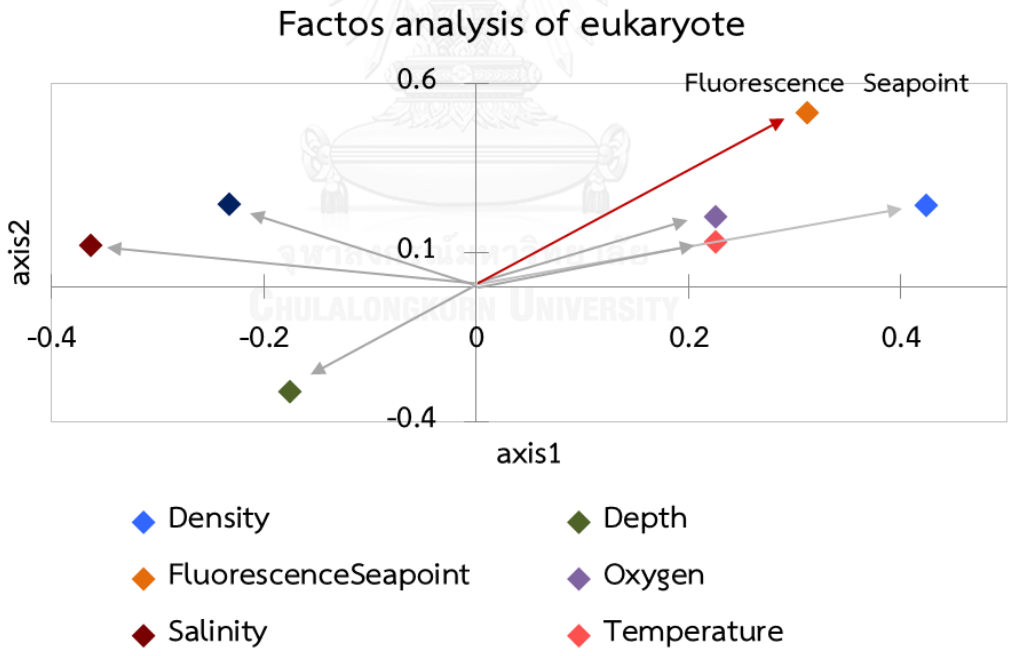

Figure 4. 18 Factors analysis base on PCoA Factors

From the analysis of abiotic factor compared with Principal Coordinate Analysis (PCoA) found that factors affecting microbial grouping ware fluorescence

seapoint, based on *P*-value ≤ 0.05 (Table 4.13, Figure 4.18). Fluorescent Seapoint effected to communities of eukaryotes probably due to the most abundance of eukaryotes that be founded were planted kingdom that have much fluorescent compounds and have photosynthesis (Figure 4.19).

Table 4. 14 Correlation coefficient OTUs of prokaryote

OTU	p-value	p-value	length
Otu002	0.031069	0.000001	1.020315
Otu001	0	0.089539	1.013146
Otu068	0	0.565679	0.966748
Otu200	0.000721	0.019847	0.903254
Otu235	0.002478	0.132991	0.760389
Otu112	0.000767	0.436176	0.743677
Otu065	0.001882	0.216424	0.743618
Otu017	0.00745	0.096171	0.729584
Otu020	0.026566	0.055216	0.694623
Otu157	0.00195	0.55583	0.693739

From the correlation coefficient analysis of eukaryotes to find out OTUs that significantly influence the microbial diversity suggest that the most significant OTUs of eukaryotes were OTUs2, OTUs1 and OTUs68, that were *Bilateria*, *Euglenida*, and *Cnidaria* genus, respectively (Table 4.14, Table 4.15). *Euglenida* genus was protista kingdom and *Bilateria* and *Cnidaria* genus were in fungi kingdom. From OTUs diversity at kingdom level of eukaryote found that protista and fungi kingdom have less than plant and a SAR kingdom (figure 4.19).

Table 4. 15 Correlation coefficient taxonomy OTUs of eukaryote

OTU	Kingdom	Taxonomy
Otu001	Protista	Eukaryota;Excavata;Discoba;Discicristata;Euglenozoa; Euglenida;
Otu002	Animalia	Eukaryota;Opisthokonta;Holozoa;Metazoa_(Animalia); Eumetazoa;Bilateria;
Otu017	SAR	Eukaryota; SAR;Stramenopiles;Ochrophyta;Chrysophyceae; Incertae_Sedis;
Otu020	Plantae	Eukaryota;Archaeplastida;Rhodophyceae;Florideophycidae; Rhodymeniophycidae;Rhodophyllis;
Otu065	SAR	Eukaryota; SAR;Alveolata;Dinoflagellata;Incertae_Sedis; Haplozoon;
Otu068	Animalia	Eukaryota;Opisthokonta;Holozoa;Metazoa_(Animalia); Eumetazoa;Cnidaria;
Otu112	SAR	Eukaryota; SAR;Alveolata;Dinoflagellata;Dinophyceae; SL163A10;
Otu157	SAR	Eukaryota;SAR;Alveolata;Ciliophora;Intramacronucleata; Spirotrichea;
Otu200	Protista	Eukaryota;Amoebozoa;Discosea;Longamoebia;Centramoebida ;Acanthamoeba;
Otu235	Plantae	Eukaryota;Archaeplastida;Rhodophyceae;Florideophycidae; Nemaliophycidae;Camontagnea;

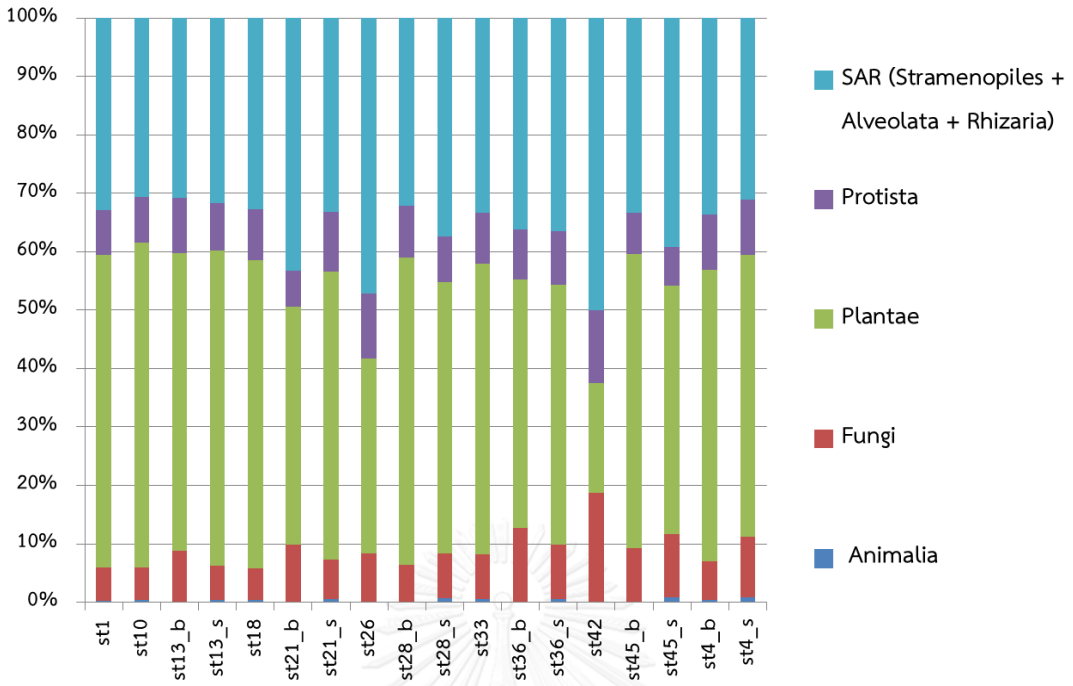

Figure 4. 19 OTUs diversity at kingdom level of eukaryote

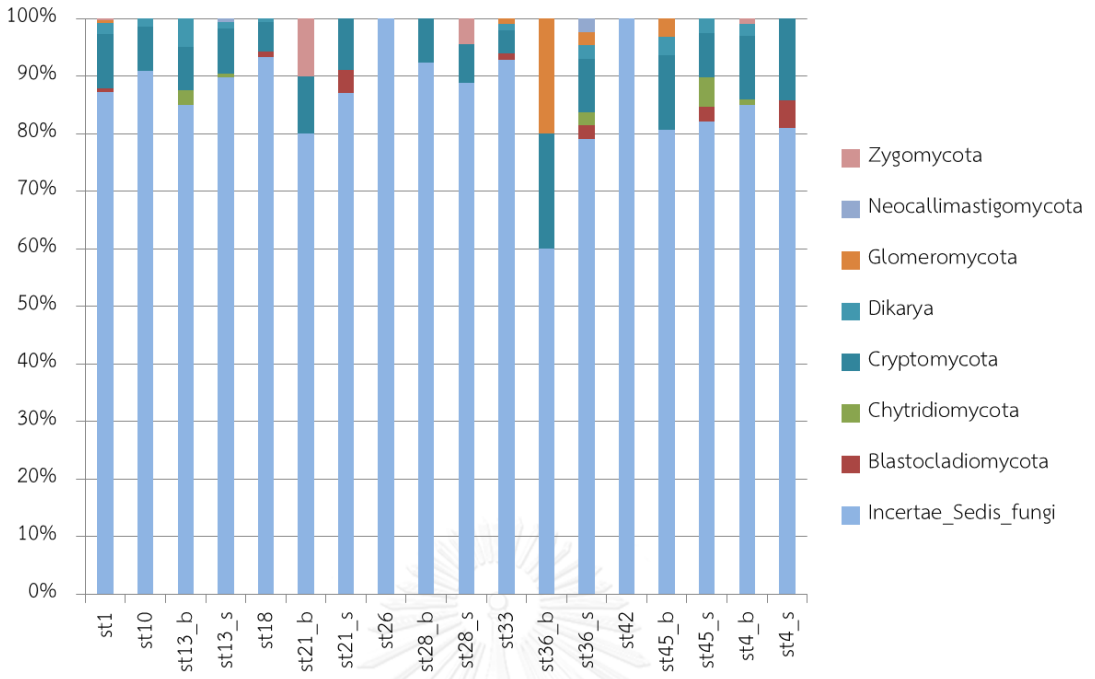

Figure 4. 20 OTUs diversity at phylum level of Fungi kingdom

Because of this research is interested in microbial, so we studied fungi kingdom. In Figure 4.19 show that fungi found just around 8% that less than plant and SAR (*Stramenopiles + Alveolata + Rhizaria*. So, we studied fungi at the genus level, of the fungi kingdom show in Figure 4.20 found that the most of the phylum were *Incertae Sedis* and *Crytomycota*, another genus found in similar amounts.

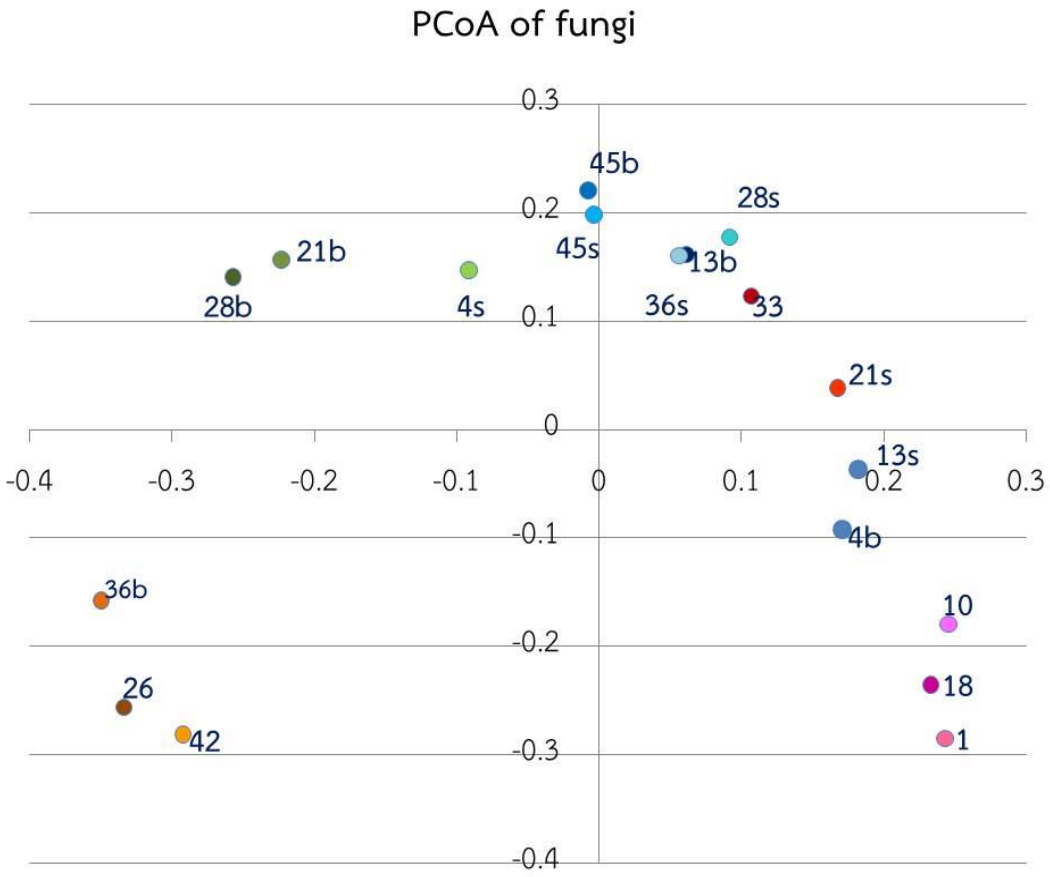

Figure 4. 21 Principal Coordinate Analysis of fungi

The Principal Coordinate Analysis (PCoA) of fungi show in Figure 4.21 found that the sample was rather scatter. And consider at phylogenetic tree suggests that corresponds to the tree, which all samples are related (Figure 4.21). Grouping of fungi different grouping of eukaryote due to the most communities of eukaryotes were plantae and SAR (*Stramenopiles + Alveolata + Rhizaria*) were around 70%, while fungi were around 8%. So the communities of eukaryotes related to plantae and SAR (*Stramenopiles + Alveolata + Rhizaria*).

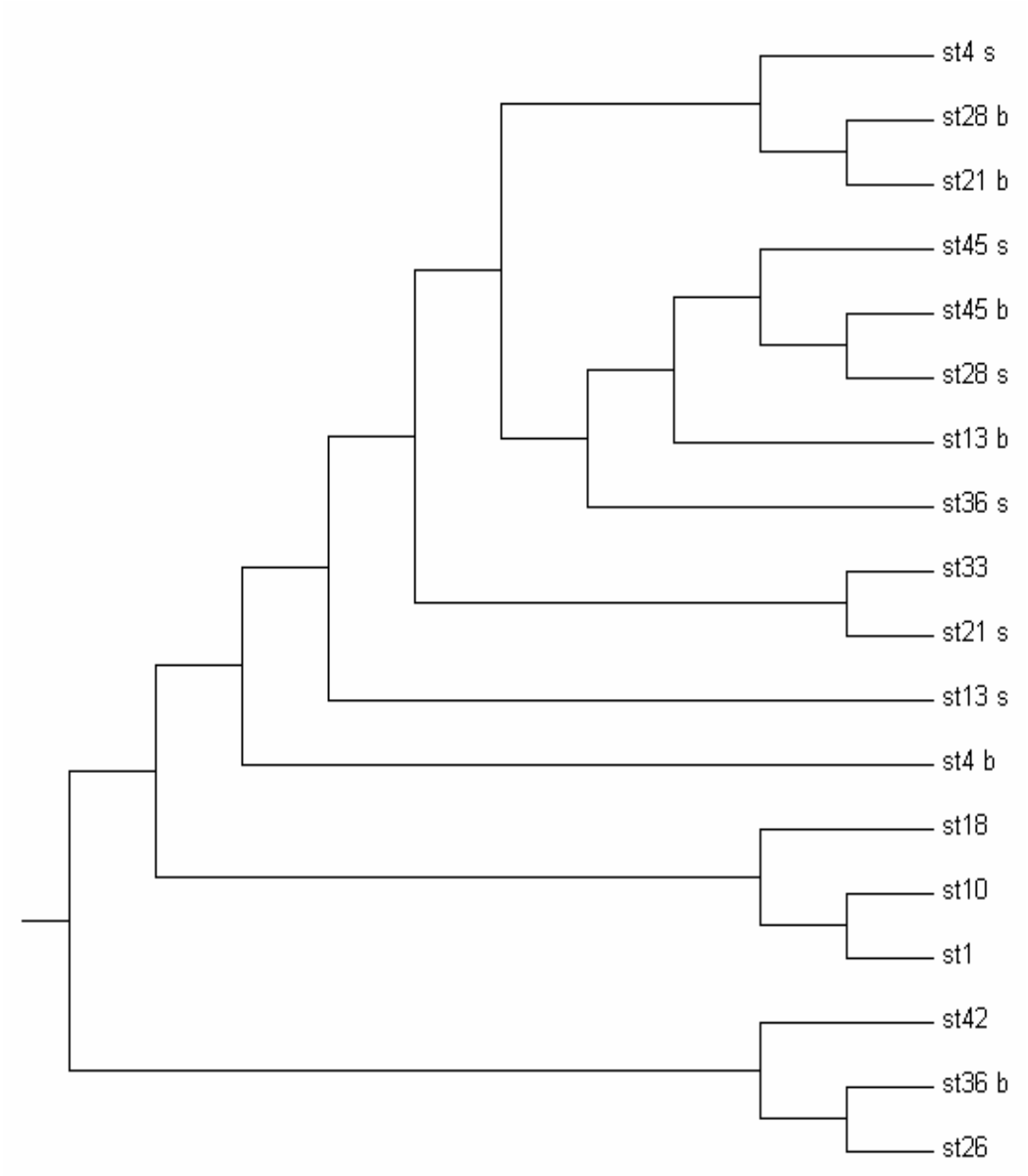

Figure 4. 22 Dendrogram of fungi constructed by Mothur, based on thetayc

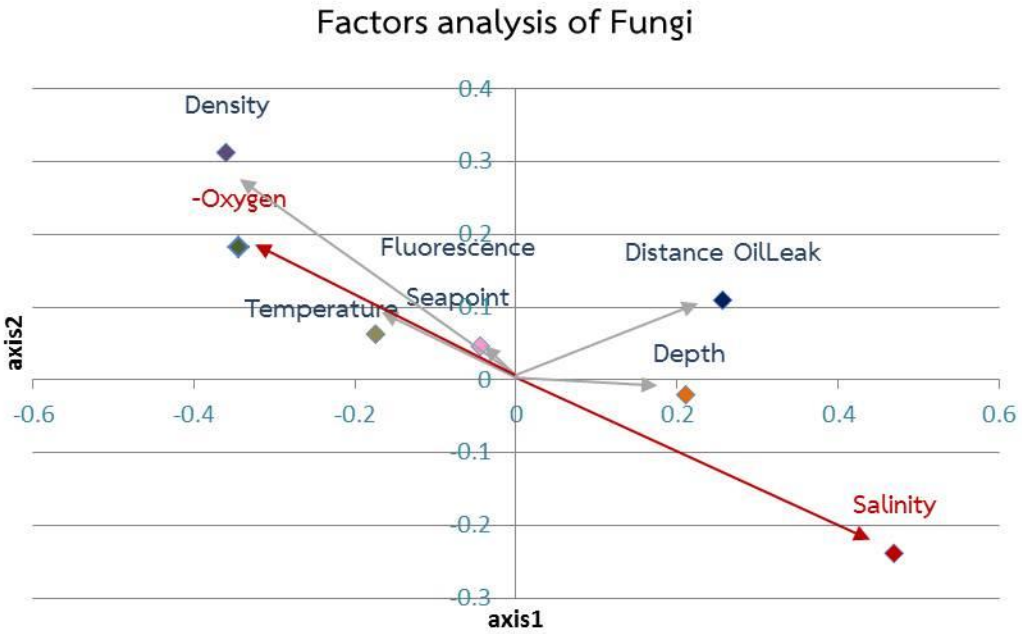

Figure 4. 23 Factors analysis of fungi based on PCoA

Table 4. 16 Factors analysis of fungi

Feature	p-value	length
Depth	0.263239	0.379205
Temperature	0.206324	0.357865
Salinity	0.004002	0.851509
Fluorescence Seapoint	0.180139	0.325092
Oxygen	0.020783	0.681379
Density	0.110572	0.417968
DistanceOilLeak	0.352124	0.266538

Factor analysis of fungi indicates that salinity and oxygen effects fungi community based on *P*-value ≤ 0.05 which is 0.004002 and 0.020783, respectively (Figure 4.22, Table 4.11). Significant abiotic factors of fungi, which is salinity, founded that related clustering of eukaryotes considers with characteristics of water. From the correlation coefficient analysis of eukaryotes founded that the most significant OTUs of eukaryotes were *Bilateria*, *Euglenida*, and *Cnidaria*, respectively (Table 4.14, Table 4.15).

CHAPTER V
DISCUSSION

This research studied microbial diversity in the central Gulf of Thailand by metagenomics, which is culture-independent method. From research of Khitmoh (2017) supported the use of culture-independent method, and that can detect species much more than by culture-dependent method (32). However, since no one has ever studied the diversity of microorganisms in the Gulf of Thailand, it is not possible to compare the results of research with the culture-dependent results.

From the measure water characteristics of the central Gulf of Thailand (6) (Table 4.1) it is suggested that temperature, salinity, fluorescence Seapoint and oxygen are very similar. Whereas, depth, and distance from an oil leak are quite different. From the research of Pramot and the researcher team in 2010 studies (8), seasonal variation of sea in GoT reported that the temperature, salinity, and density across the GoT has very little difference. The temperature difference is 3 degrees, the salinity difference is 2 PSU and the density difference is 3 Kg/m^3, suggests that these abiotic factors all stations of GoT is relatively similar. Microbial communities were relatively homogeneous due to the circular water of GoT which is influenced by the South China Sea (7). The flowing out of the GoT to South China Sea is weak compared with flowing of South China Sea, so the flowing water of GoT is being countered in Figure 5.1 (8). As a result, communities of microbes are alike.

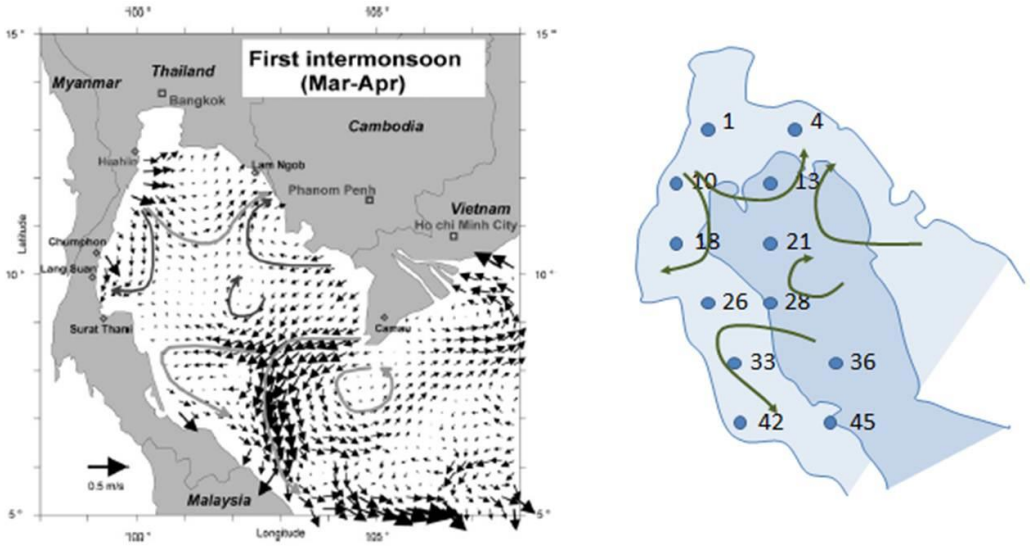

Figure 5. 1 Circulation water in the Gulf of Thailand left) circulation water in Mar-Apr right) circulation water with sampling collected

In terms of prokaryotes, abiotic factor analysis suggest that factors effect microbes of prokaryote where temperatures that range between 27°C – 31°C. Microbes that grow at optimal temperature in range 20°C to 40°C, are mesophilics. Mesophiles are found in warm blooded animals and in a terrestrial or aquatic environment (33). Temperature correlates to cell structure, cellular metabolism, and cell components, important members of this group are pathogenic bacteria, symbiotic bacteria that live in the human body without harming it and microbial spoilage (34). This is consistent with functional potentials metabolism using Statistical Analysis of Metagenomic Profiles (STAMP), found that the relative abundance of metabolism is a human disease and organismal systems (Figure 4.6). It was found that these microbes are commonly used in the process of beer and wine making, food preservation and waste water treatment.

At the phylum level of prokaryote composition profiles shown in Figure 4.1 found that *Proteobacteria*, *Firmicutes* and *Actinobacteria* were abundant,

respectively. These microbes correlate to human disease, which relate to functional potentials metabolism. *Proteobacteria* includes many bacteria that are part of the normal human microbiota as well as many pathogens (35, 36). *Firmicutes* are the intestinal microbiota of the human (37). *Actinobacteria* are commonly found in soil, but are also found in lakes, oceans and other freshwater (4). This microbe is commonly used in medical and pharmaceuticals such as the use of antibiotics against bacteria, and use in agriculture and industry (38).

Although from the get.communitytype (Mothur) get homogenous communities, we did the Principal Coordinate Analysis (PCoA) of prokaryote that clustering similar with phylogenetic tree (Figure 4.2, Figure 4.3). This suggests that type of communities included only 2 samples, has bar graph pattern and amount of each OTUs were similar. And compare cluster from the PCoA with characteristic water found that the same type have temperature and salinity were similar. The sample st1, st10, st18, st45_b clearly separate from other groups that from water characteristics are similar to other groups, so expect to be influenced by other factors from the environment. Samples st1, st10 and st18 closely with national parks, and st45_b, where area is the whole point of an ocean that flows together from the Gulf of Thailand and South China Sea. In Figure 4.2, st45_s and st45_b is same stations, but microbial population are very different. From comparing with other stations, where sampling of surface and bottom, found that abiotic factors are different in the same amount. It can be expected from this area is the whole point of an ocean that flows together from the Gulf of Thailand and South China Sea as mentioned previously. At st1, st10 and st18 different from st26, st33 and st42 expect geographical features, and circulation that st1, st10 and st18 flow to Samui island at Suratthani province, and st26, st33 and st42 flow to Malaysia.

The analysis of abiotic factors compared with Principal Coordinate Analysis (PCoA) was done, and showed that factors affecting microbial grouping was

temperature, based on significant *P*-value ≤ 0.05 (Table 4.6). *P*-value of density was 0.056 which is close to 0.05, so it may also be important. Microbes must live in optimum temperature to grow well due to temperature correlating to cell structure, cellular metabolism, and cell components as mentioned previously.

Then, correlation coefficient analysis was done to find out OTUs that significantly influence the microbial diversity based on *P*-value ≤ 0.05. The most significant OTUs of prokaryotes shown in Table 4.7, were *Pseudoalteromonadaceae* and *Oceanospirillacea* families. These significant OTUs were found to affect the pattern community. Many species of *Pseudoalteromonadaceae* family produce a variety of primary and secondary metabolites such as proteins and protein inhibitors, and unusual brominated compounds with antibacterial and antiviral properties (39, 40). And due to their versatile metabolic capacities, members of this family are highly adaptable to dissimilar ecosystem habitats and play important ecosystem roles in marine environments (13). *Oceanospirillacea* and *Pseudoalteromonadaceae* were marine microbes that have high molecular weights that dissolves organic matter and has important implications for the ecology of the sea (41) (39).

From functional potentials metabolic analysis found that the most relative abundance are metabolism. Organismal systems, metabolism is related to human diseases as well and relative abundance suggests that microbial organisms relate to human disease. These metabolism correlate to prokaryote composition profiles shown in Figure 4.6 found that mostly mimerabolism were carbohydrate metabolism, amino acid metabolism and membrane transport. Significant potentials metabolic of group 3 and 7 have 5 metabolisms were metabolism, cellular processes, environmental information processing, and human diseases, that most metabolism compared with other groups. Considering circulation found that group 3 received microbes from group 7.

In terms of eukaryotes, from the data analysis based on the Mothur (24), after aligning sequences by BLASTN with SILVA databases (29), the sample coverage, the number of observations OTUs, Chao, the non-parametric Shannon and the Inverse Simpson diversity estimate using Mothur shows coverage quality score and prokaryote diversity at phylum in Table 4.11. All samples with a quality value greater than 97% indicates that a good value of sampling the communities and stations had a low number of reads are also good coverage due to the diversity analysis, based on the number of OTU were found close to stations that had a lot of number of reads. Considering barchart found that pattern OTUs diversity relates to the community type in Table 4.10 just like prokaryotes.

At the phylum level of eukaryote composition profiles is shown in Figure 4.15 suggests that *Archaeplastida*, SAR (*Stramenopiles + Alveolata + Rhizaria*) and Opisthokonta were abundant, respectively (42). *Archaeplastida* were a group of the plantae kingdom that were unicellular algae (43) and the research of Denis et al., (2015) (44) found that *Archaeplastida* and *Opisthokonta* were multicellular organisms (45). *Opisthokonta* were a group of fungi kingdom.

The Principal Coordinate Analysis (PCoA) of eukaryote (Figure 4.16) distinguished clusters found that sample groups was scattered in the bottom of the PCoA chart except the sample st4_b, which separated from each group. That graph related to the grouping of the phylogenetic tree (Figure 4.17). In a phylogenetic tree found that st4_b far from st4_s probably due to this area having two rivers flow down and also the area that has changed the flow of the ocean from the counterclockwise to the clockwise in March to April. This area gets water that flows from many sources. One of them is from AO Udom and Laem Chabang, where there is a harbor. This area receives various substances from ships that ships leave bilge water and sewage, that are waste water (marine department of Thailand). And flow from rivers mentioned previously, and from Vietnam. From Figure 5.3 found that the

concentration of mercury (Hg-T) were 47.7 ng/l, that concentration of Hg-T is highest (46). The concentration of Hg-T at st4_b (bottom of station 4) different from st4_s (surface of station 4) and other stations. So st4_b different from other stations due to level of Hg-T. The Hg-T concentration parameters from Sompongchaiyakul (2013) (46) were showed on Table 5.1 for correlation analysis. Table 5.1 indicated Hg-T no significant correlating to the GoT prokaryotes, eukaryotes and fungi diversity.

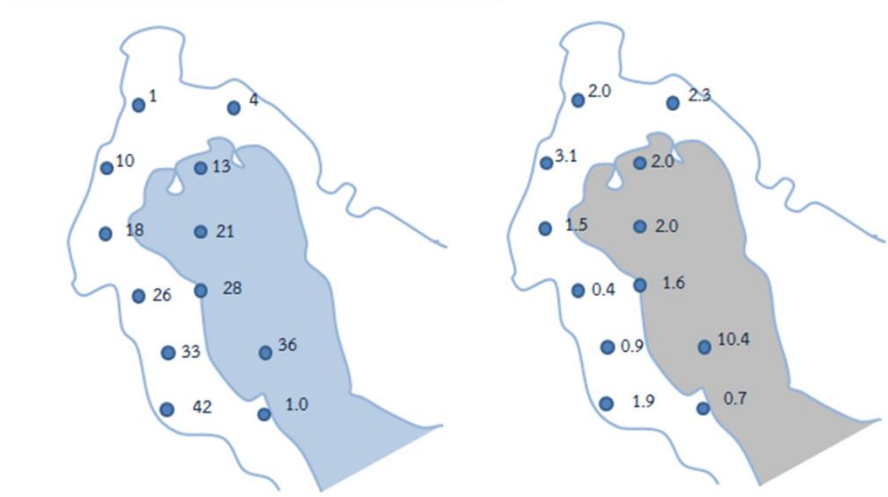

Figure 5. 2 Concentration of Hg-T (ng/l) in surface seawater left) stations right) total Hg-T

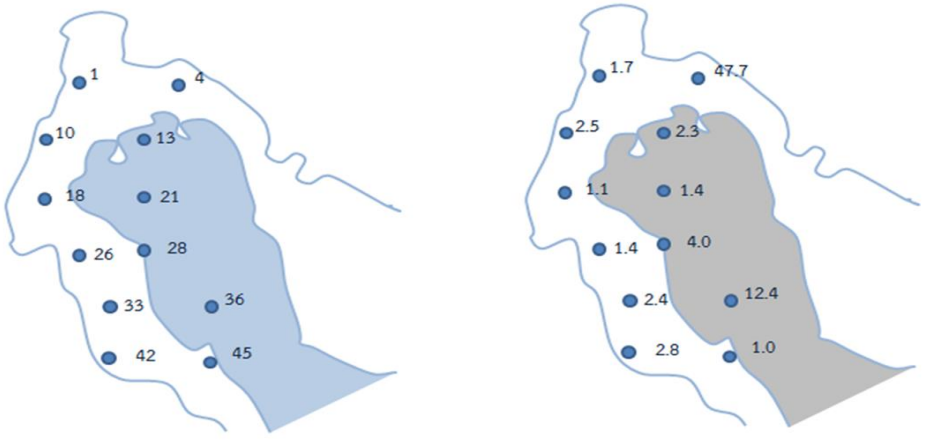

Figure 5. 3 Concentration of Hg-T (ng/l) in bottum seawater left) stations right) total Hg-T

Table 5. 1 Correlation analysis of Hg-T with prokaryote, eukaryote and fungi communities using Mothur

Group	p-value	p-value	length
Prokaryote	0.171319	0.28477	0.421171
Eukaryote	0.752489	0.831264	0.092309
Fungi	0.814683	0.364024	0.227376

The samples can be grouped according to the Table 4.8 and consider with characteristics found that the same type has salinity was similar. From the analysis of abiotic factors compared with Principal Coordinate Analysis (PCoA) found that factors affecting microbial grouping ware fluorescence Seapoint (based on *P*-value ≤ 0.05, Table 4.13, Figure 4.18). Fluorescent Seapoint affected communities of eukaryotes probably due to the most abundant of eukaryotes that were found to be the plant kingdom that have many fluorescent compounds and can do photosynthesis (Figure 4.19) (47).

Because of this research there is interest in microbes, so the fungi kingdom shown in Figure 4.19 shows that fungi found just around 8%**.** At the genus level, of fungi kingdom shown in Figure 4.20 found that the most of the genus were *Incertae Sedis* and *Cnidaria* genus. *Incertae Sedis* is fungal live in zoos and fossil leaves and related human gastrointestinal microbiota (40). *Cnidaria* genus is marine fungi that have metabolites related industrial and degrade several pollutants, and usually can screen from corals (48).

The Principal Coordinate Analysis (PCoA) of fungi show in Figure 4.21 found that the sample was rather scatter. And consider at phylogenetic tree suggests that corresponds to the tree, which all samples are related (Figure 4.21). Grouping of fungi different grouping of eukaryote due to the most communities of eukaryotes were planted and SAR (*Stramenopiles + Alveolata + Rhizaria*) were around 70%, while fungi were around 8%.

Abiotic factor analysis of fungi indicates that salinity and oxygen effects fungi community (Figure 4.22, Table 4.11). From the research of Jones E.B.G., (2000) (49) reported salinity is important in affect composition of species. Non-marine fungi were significantly less than marine fungi due to saline help maintaining (50). That salinity, if consider from groups eukaryotes samples according to the Table 4.8 with characteristics founded that the same type has salinity was similar. Suggest that salinity may be is significant abiotic factors of eukaryotes, too.

From the correlation coefficient analysis of eukaryotes suggest that the most significant OTUs of eukaryotes were OTUs2, OTUs1 and OTUs68, that were *Bilateria*, *Euglenida*, and *Cnidaria*, respectively (Table 4.14, Table 4.15). *Euglenida* was protista kingdom and *Bilateria* and *Cnidaria* genus were in animalia kingdom. *Cnidaria* is marine animal that have metabolites related industrial and degrade several pollutants, and usually can screen from corals (48). Significant abiotic factors of fungi, which is salinity, founded that related clustering of eukaryotes considers with characteristics of water(48). So salinity were significant abiotic factors that affected to communities of eukaryotes (Figure 4.20).

From demersal fishery resources (seafloor animal) survey in GoT reported more aquatic species in the central of GoT than the coastal due to the deep water level. Each station was thus found the spread of aquatic animals different both the member of individuals and the total weight (Table 5.2) (51). Communities of prokaryote, eukaryote and fungi were different (Figure 4.2, Figure 4.16, Figure 4.21), and maybe in part correlated to this. Subsequencetly, the demersal fishery resources survey data (Table 5.2) were analyzed for correlation analysis using Mothur. However, no significant correlation yielded (Table 5.3). Considering from Table 5.3 demersal fishery affected prokaryote more than eukaryote and fungi.

Table 5. 2 Demersal fishery resources (seafloor animal) survey catagoried by total members and weight in GoT (Promjinda, 2013)

Station	Total individual (fold-diff)	Total weightl (fold-diff)	Station	Total individual (fold-diff)	Total weightl (fold-diff)
st1	1	2	st28_s	2	3
st10	1	1	st33	2	2
st13_b	2	3	st36_b	1	2
st13_s	2	3	st36_s	1	2
st18	0	0	st42	3	3
st21_b	5	4	st45_b	3	3
st21_s	5	4	st45_s	3	3
st26	2	4	st4_b	5	4
st28_b	2	3	st4_s	5	4

* fold difference to the lowest (0) of the lowest

Table 5. 3 Correlation analysis of demersal fishery resources with microbial communities

Group	Total weight (fold-diff)			Total individual (fold-diff)		
	p-value	p-value	length	p-value	p-value	length
Prokaryote	0.112559	0.115586	0.548257	0.095288	0.044449	0.630895
Eukaryote	0.808449	0.158396	0.363611	0.182896	0.717017	0.343169
Fungi	0.116609	0.39739	0.441896	0.182896	0.717017	0.343169

CHAPTER VI

CONCLUSION AND RECOMMENDATION

This study obtained the metagenomics coupled 16S and 18S rRNA gene sequencing to unravel massive biodiversity of microbial prokaryotes and eukaryotes in the central Gulf of Thailand. The community type of the central Gulf of Thailand is relatively homogeneous (1 community type) due to the depth of GoT has 5-60 meters and the factors are similar.

In prokaryotes, abiotic factors effect to prokaryotes population is temperature. The most significant OTUs correlated abiotic factors are *Pseudoalteromonadaceae* and *Oceanospirillacea.* The significant functional potentials of prokaryotes is metabolism and the most relative abundance of metabolism is human diseases.

In eukaryotes, abiotic factors effect to eukaryotes population is Fluorescence Seaapoint and salinity. The most significant OTUs correlated abiotic factors are *Bilateria*, *Euglenida*, and *Cnidaria*. The abiotic factors affect to fungi population are salinity and oxygen.

Factors affecting the diversity of microbial diversity, in addition to the physical factors mentioned above also found that circulation of water and chemicals dissolved in water affected microbial diversity, too. Various organisms that live in the sea were likely affected by the similar factors.

As recommendation, when pooling samples together for the NGS, be careful of the pipette technique, as it migth result in the very different amount of reads. Further, this research studied the diversity of microbes in the Gulf of Thailand, in the summer; it is advisable to study the variety of microbes in other seasons in order to see the oceangraphic patterns diversity of microorganisms.

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APPENDICES

APPENDIX A

Stock reagent

10x TBE buffer

Tris	54.0	g
Boric	27.5	g
0.5 M EDTA	20	ml

Dissolved all compositions with distilled water to 1 L

0.5 EDTA pH 8.0

EDTA	18.6	g

Adjust pH to 8.0 and adjust volume to 100 ml with distilled water

0.8x TBE buffer for agarose gel

10x TBE buffer	80	ml

Adjust volume to 1 L with double distilled water

0.5x TBE buffer for eletrophoresis

10x TBE buffer	50	ml

Adjust volume to 1 L with double distilled water

0.55% agarose gel

Agarose powder	0.28	g
0.8x TBE	50	ml

1.75% agarose gel

Agarose powder	0.88	g
0.8x TBE	50	ml

VITA

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She graduated with a bachelor of Science and technology in Biotechenology from Thammasat university in 2012. She has further studied for the Master of Science in Microbiology Department, Chulalongkorn University since 2013. She presented hr research proceeding paper in title "18S rRNA sequences to reval microbial eukaryotes in the Central Gulf of Thailand" at the 27th Annual Meeting o the Thai Society for Biotechnology and International Conference, Bangkok, Thailand in november 2015.