ผลของแหล่งอาหารต่อชุมชนแบคทีเรียในทางเดินอาหารของเม่นทะเล *Diadema setosum* (Leske, 1778) โดยการศึกษาด้านเมทาจีโนมิกส์

นางสาวณัฐณิชา ตันรัตนพิทักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2559

EFFECTS OF FOOD SOURCES ON BACTERIA COMMUNITY IN GUT OF SEA URCHIN *Diadema setosum* (Leske, 1778) USING METAGENOMICS APPROACHES

Miss Natnicha Tanrattanapitak

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Marine Science
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Thesis Title	EFFECTS OF FOOD SOURCES ON BACTERIA COMMUNITY IN GUT OF SEA URCHIN *Diadema setosum* (Leske, 1778) USING METAGENOMICS APPROACHES
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ณัฐณิชา ตันรัตนพิทักษ์ : ผลของแหล่งอาหารต่อชุมชนแบคทีเรียในทางเดินอาหารของเม่นทะเล*Diadema setosum* (Leske, 1778) โดยการศึกษาด้านเมทาจีโนมิกส์ (EFFECTS OF FOOD SOURCES ON BACTERIA COMMUNITY IN GUT OF SEA URCHIN *Diadema setosum* (Leske, 1778) USING METAGENOMICS APPROACHES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.สุภณัฐ ไพโรหกุล, 62 หน้า.

การศึกษาความหลากหลายของชุมชนแบคทีเรียในลำไส้ของเม่นทะเลหนามดำ (*Diadema setosum*) โดยใช้แนวทาง metagenomics วิเคราะห์ลำดับนิวคลีโอไทด์ของยีน 16S rRNA ของตัวอย่างเม่นทะเลจากเกาะมุกด์ จังหวัดตรัง การศึกษาเก็บตัวอย่างเม่นทะเลจากแหล่งอาหารสามแหล่ง เพื่อเปรียบเทียบความหลากหลายของชุมชนแบคทีเรีย ได้แก่ บริเวณพื้นทราย บริเวณแหล่งหญ้าทะเล และบริเวณแนวปะการัง และทำการศึกษาแบคทีเรียในสองสภาวะ คือ เม่นทะเลที่เก็บมาจากธรรมชาติโดยตรง และเม่นทะเลในสภาวะอดอาหารเป็นเวลา 72 ชม. นอกจากนี้ยังมีการการเก็บตัวอย่างตะกอนดินโดยรอบบริเวณที่เก็บตัวอย่างเม่นทะเล และใบหญ้าทะเล เพื่อการวิเคราะห์แบคทีเรียด้วยเช่นกัน ผลการศึกษาพบว่าแบคทีเรียจากตัวอย่างตะกอนดินพบจำนวนชนิดและความหลากหลายมากกว่าตัวอย่างจากลำไส้และใบหญ้าทะเล ในลำไส้เม่นทะเลที่เก็บจากธรรมชาติจากทุกแหล่งอาหาร พบแบคทีเรียกลุ่มเด่น ได้แก่ ไฟลัม Bacteroidetes Proteobacteria และ Firmicutes และไม่พบความแตกต่างทางสถิติระหว่างชุมชนแบคทีเรียจากแหล่งอาหารที่แตกต่างกัน สำหรับในเม่นทะเลกลุ่มที่อดอาหาร สัดส่วนขององค์ประกอบแบคทีเรียจะมีการเปลี่ยนแปลงไปโดยแบคทีเรียในไฟลัม Proteobacteria มีแนวโน้มเพิ่มสูงขึ้น ในขณะที่ Bacteroidetes ลดต่ำลง ชุมชนแบคทีเรียในตัวอย่างลำไส้ของเม่นทะเลและตัวอย่างตะกอนดินมีความแตกต่างอย่างมีนัยสำคัญทางสถิติ (*p*-value = 0.001) จากผลการศึกษาแบคทีเรียที่พบในลำไส้ของเม่นทะเลค่อนข้างมีความจำเพาะและแตกต่างจากตะกอนดินและอาหารของโฮสต์ (host) อาจเนื่องมาจากสภาพแวดล้อมในลำไส้ที่มีลักษณะเฉพาะตัว และอาจจะเป็นไปได้ว่าแบคทีเรียในลำไส้นี้จะมีบทบาทเกี่ยวกับโภชนาการของโฮสต์ นอกจากนี้การศึกษาในครั้งนี้ พบแบคทีเรียกลุ่ม sulfate-reducing bacteria (SRB) ในลำไส้ของเม่นทะเล ซึ่งเป็นกลุ่มแบคทีเรียที่มีความเกี่ยวข้องกับวัฏจักรซัลเฟอร์ แบคทีเรียกลุ่มนี้อาจมีบทบาทเกี่ยวกับกระบวนการเมแทบอลิซึมของสารในกลุ่มซัลเฟตในลำไส้ของเม่นทะเล

ภาควิชา วิทยาศาสตร์ทางทะเล ลายมือชื่อนิสิต .....

สาขาวิชา วิทยาศาสตร์ทางทะเล ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: 16S RRNA / BACTERIAL COMMUNITY / DIADEMA SETOSUM / METAGENOMICS

NATNICHA TANRATTANAPITAK: EFFECTS OF FOOD SOURCES ON BACTERIA COMMUNITY IN GUT OF SEA URCHIN *Diadema setosum* (Leske, 1778) USING METAGENOMICS APPROACHES. ADVISOR: SUPANUT PAIROHAKUL, Ph.D., 62 pp.

16S rRNA metagenomics sequencing was applied for the study of bacterial communities in the gut of sea urchin, *Diadema setosum* from Mook Island, Trang province. Sea urchin samples were collected from three different food sources: around sand area, seagrass area and coral area for comparison between the bacterial community. In addition, bacterial community in the gut content of sea urchins were studied in two different conditions: the wild-caught and the starved sea urchin. The ambient sediment and seagrass leaves were also collected and analyzed. Both species richness and diversity indicies in the sediments were greater than the gut content and seagrass leaves samples. In the wild-caught sea urchins, Bacteroidetes, Proteobacteria and Firmicutes were the three most abundant phyla in all food sources. Surprisingly, there were no significant differences among bacterial community between the different food sources. In the starved condition, the proportion of bacterial composition was slightly changed. Proteobacteria tended to be increasing while Bacteroidetes was reduced. Bacterial communities between the gut content and the sediment samples were statistically significant different (*p*-value = 0.001). According to the results, bacteria detected in the gut are specific and differ from sediment and host diets. This may be result of the specific conditions of the intestine. It is also possible that resident microflora in the intestine can play the key role on their host nutrition. Moreover, sulfate-reducing bacteria (SRB), which involve sulfur cycle, were also detected in the present study. This group of bacteria might play a crucial role in sulfate metabolism in their host intestine.

Department:	Marine Science	Student's Signature	.....
Field of Study:	Marine Science	Advisor's Signature	.....
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Natnicha Tanrattanapitak

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CHAPTER 1

INTRODUCTION

Statement of Problem and Significance of Research

Sea urchin is a marine benthic invertebrate that consumes various kinds of food e.g. plants, animals, detritus and sediment (Muthiga and McClanahan, 2013). The previous study by Randall et al. (1964) indicated that algae is one of the most important diets of sea urchin especially sea urchin that lives around coral reef area. However, algae have thick cell wall; therefore, it is hard for sea urchin to digest and absorb them. It was reported that some components of algae cell wall couldn't be digested by the digestive juices produced by animal and presumed that bacteria may assist in algal digestion (Lasker and Giese, 1954). Furthermore, other studies of intestinal bacteria reported that gut bacteria of sea urchin are able to digest agar and some component of algae cell wall (Lasker and Giese, 1954; Prim and Lawrence, 1975). Also, the intestinal bacteria may enhance the digestion and assimilation abilities of sea urchin (Meziti et al., 2007; Nelson et al., 2010; Zhang et al., 2014). Nevertheless, there were limited numbers of studies on the identification of the entire bacterial community in the intestine (Harris, 1993; Nelson et al., 2010). In sea urchin, the studies were limited to only some edible and commercial species, for instance *Echinus esculentus* (Unkles, 1977), *Lytechinus variegatus* (Hakim et al., 2015; Nelson et al., 2010) and *Strongylocentrotus intermedius* (Zhang et al., 2014). However, there is little published research in sea urchin genus *Diadema*. This species is widely distributed and has the ecological important roles in the tropical ecosystem (Lessios et al., 2001; Muthiga and McClanahan, 2013)

One of the methods that has been applied for bacterial study is a conventional culture-based method. However, this technique has some challenges e.g. efficiency and accuracy of bacterial identification. Trying to overcome bacterial study conventional methods, 'metagenomics' approach has been developed and widely used in bacterial study. This cultured independent approach is able to identify whole genomes of bacteria directly from environments. Thus, it enable biologist to access both cultured and uncultured bacteria identification (Kennedy et al., 2008; Streit and Schmitz, 2004). A well-known example is the human gut microbial study using metagenomic approach by Qin et al. (2010)

Therefore, the present study will focus on the bacterial community in the gut of the sea urchin *Diadema setosum* using metagenomics approach. Also, the comparison of the intestinal bacterial community between the sea urchins collected from three different food sources: around sand area, seagrass area and coral area. The study will be based on the hypothesis that if the intestinal bacteria can contribute to digestion and assimilation of the host. Therefore, any differences between bacterial community should be detected in the sea urchin collected from the different food sources. Furthermore, bacterial communities varied from different parts or organs (Unkles, 1977). Consequently, the present study analyzed and divided the microbial community into two separate parts of intestine: the small intestine and the large intestine. Moreover, gut contents analysis was also conducted for observation the food that the sea urchin samples consumed. The results may, therefore, enhance more understanding about the relatedness of the intestine bacteria and their sea urchin host and probably apply from this long-spined sea urchin's intestinal bacteria.

Objectives

1. To study the diversity of bacterial community in the gut of sea urchins *Diadema setosum*

2. To compare bacterial community in the gut of the sea urchins between the sea urchin collected from the different food sources

3. To compare bacterial community in the gut of the sea urchins in the wild-caught condition and the starved condition.

Applications

The outcomes may contribute to an understanding about relationship of intestine bacteria and their sea urchin host physiology. Also, the results may further explain their roles of sea urchin in the marine ecosystem especially in terms of biogeochemical cycles.

CHAPTER 2

LITERATURE REVIEW

Biology and Ecology of Sea urchin Diadema spp.

The sea urchin *Diadema* is an invertebrate with the spherical shape and spines covering throughout the body. Sea urchin *Diadema* is classified into family Diadematidae; order Diadematoida; class Echinoidea; phylum Echinodermata. They live on the sea floor. The orientation of its body can be identified into 2 parts: the oral side, which is close to the sea floor, and the aboral side where anus and genital pores can be found (Pechenik, 2015). The body has ossicles, which are flat plates arranged vertically to enclose their body. Sea urchin test can be divided into an ambulacral plate, where tube feet can be found, and an interambulacral plate. Sea urchin *Diadema* has hard but brittle spines. The inside of these spines are hollow structures and contains some toxin. Sea urchin utilizes the spines for either movement or defense itself (Barth and Broshears, 1982; Pechenik, 2015). Sea urchin has sexual reproduction with separated sexes (dioecious species). There are five reproductive organs on the aboral side of the interambulacral plates (Pechenik, 2015). Most sea urchin releases its gamete and produces external fertilization (Barth and Broshears, 1982). According to the studied by Muthiga (2003), *D. setosum* can reproduce all year round.

Diadema setosum has a black test but some individual may also have white test (Coppard and Campbell, 2006). This long-spined species contains several black narrow brittle spines along their body. These hollow spines are slightly poisonous. The spines are greatly long in proportion to its body and also when compared with other species. (Muthiga and McClanahan, 2013). *D. setosum* has perforated and often also crenulated primary tubercles. Five distinct white spots on the aboral side and

the bright orange ring around the periproctal cone or the part compare to the anus are one of the major characteristics of *D. setosum*, as can be seen in figure 2-1. In addition, blue spots lining up along the middle of interambulacral plates can be found in this species (Coppard and Campbell, 2006; Muthiga, 2003).

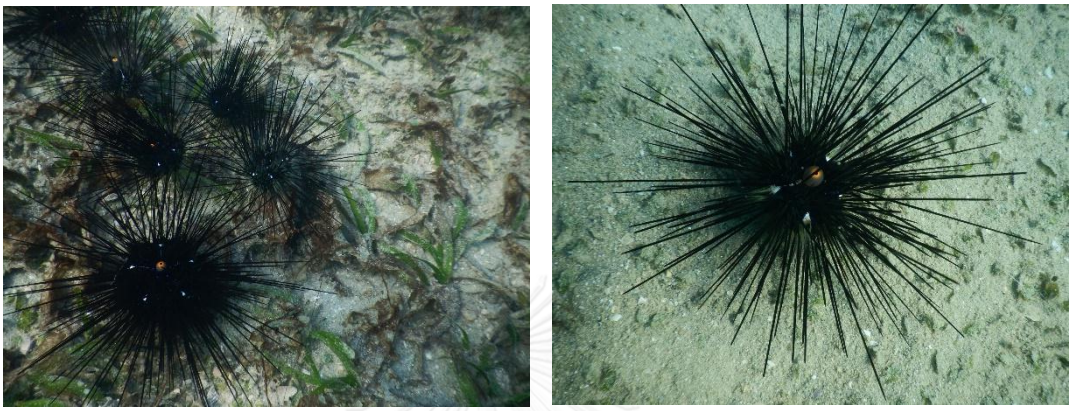

Figure 2-1 *Diadema setosum*

Sea urchin is marine benthic invertebrate. They can be found around coral reef areas, rocky shores, as well as in seagrass beds (Puspita et al., 2013). During daytime, they frequently hide in the crevice or under the rock cave and often found as the assemblage (Pearse and Arch, 1969). They normally leave their hiding places and feeding during nighttime by feeding in the vicinity of the daytime habitat (Lawrence and Hughes-Games, 1972; Magnus, 1967; Thornton, 1956). The study by Tuya et al. (2004) reported that night-time movement of *D. antillarum* was 3.7 meters on average.

Sea urchin *Diadema* lives in the intertidal zone. It is one of the most widespread and abundant genera. Also, they have ecological importances in the tropical ecosystem (Lessios et al., 2001; Muthiga and McClanahan, 2013). The distribution of *Diadema* is affected by several factors e.g. wave exposure, depth, sediment type and size, and composition of the sea floor (Muthiga and McClanahan, 2013). The distribution of *D. setosum* is also related to the size of the sediment. The sea urchin density varies according to an increasing size of the sediment (Dumas et al., 2007). Also, desiccation can

limit the distribution of sea urchin to the upper intertidal zone (Pearse, 1998). Each *Diadema* species has specific zonation. For example, *D. setosum* is widespread throughout the Indo-Pacific region e.g. East Africa, the Red Sea, the Gulf of Bengal, the South China Sea, Japan and the Philippines (Lessios et al., 2001; Pawson, 1972). In Thailand,it was reported that the distribution of *D. setosum* can be found both of the Gulf of Thailand and the Andaman Sea (Putchakarn and Sonchaeng, 2004).

Feeding Ecology of Sea urchin

Sea urchin is a benthic animal. They have a specialized organ called Aristotle's lantern which is a complex system of ossicles and muscles. The teeth of Aristotle's lantern can extend to feed various food at the sea floor or scape food from the rock and hard substrate. (Lawrence et al., 2013; Pechenik, 2015). The quantity and frequency of food intake depends on the physical and chemical characteristics of the food, physical conditions of sea urchins and the surrounding conditions (Lawrence et al., 2013). In addition, *Diadema*'s feeding behavior varies depends on the species and environment (Muthiga and McClanahan, 2013). Lawrence and Hughes-Games (1972) found that *D. setosum* in the Red Sea is a nocturnal animal, which they consume food at nighttime. According to the study by Shunula and Ndibalema (1986), it was also found that these sea urchins are active for nighttime feed.

Sea urchin can consume various kinds of foods, including plants, seaweeds, detritus and sediment. The composition of the intestinal food can reflect the type of food available in the habitat that the sea urchins were collected. (Muthiga and McClanahan, 2013; Randall et al., 1964). The previous study in sea urchins *D. antillarum* collected from seagrass bed by Randall et al. (1964), most of the intestinal food composition consists of *Thalassia* seagrass and some fine sand particles. In the case of sea urchins collected from the rocky area, sand particles were mainly found in gut contents. While sea urchin collected from the coral reef area, the intestine digesta are composed of algae and

detritus. According the study of *D. setosum* samples collected from coral reefs in Kenya, it was found that gut contents consisted of coral sediment, algae, seagrass and invertebrates (49%, 28%, 20% and 2%, respectively (McClanahan, 1988).

Digestive Biology of Sea urchin

Sea urchin digestive tract starts from the mouth. In buccal cavity, almost sea urchins, except the cidaroids, can produce mucous film to cover the food in the form of food pellet (Holland, 2013). These food pellets are then pass through the intestinal tract, which consists of pharynx, esophagus and long convoluted intestine. Finally, the faeces are egested at an anus on the aboral side (Barth and Broshears, 1982). The diagram of sea urchin can be seen in figure 2-1. The ingestion and digestion times vary from hours to days depending on an amount of food and feeding frequency of the urchin (Holland, 2013; Lawrence et al., 2013). In *D. setosum*, which has a diel rhythm, the transit time varies within a day (Lawrence and Hughes-Games, 1972). In term of time to empty gut or clearance time depends on the species of sea urchins, food type and feeding rate, which may take up to several weeks (Holland, 2013).

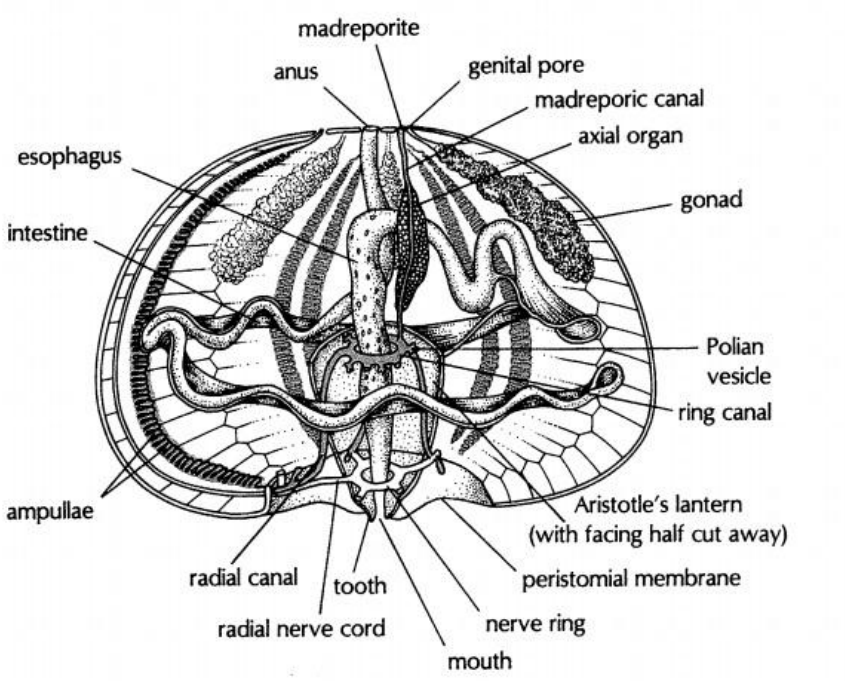

Figure 2-2 The anatomy and internal structures of sea urchin *Arbacia punctualata* (Adapted from Pachenik, 2015)

The experiments on sea urchin, *Strongylocentrotus purpuratus*, by Lasker and Giese (1954), found that if the sea urchins were on starvation, the digestion and egestion will occur slowly and take longer time than normal. Moreover, the egesta are rather completely digested and contains a lot of bacteria. On the other hand, if the sea urchin continue feeding, they can excrete a lot of feces and excretion in relatively shorter time. According to the revision by Lawrence, 2013 it was found that carbohydrases, glycogenase, agarase, chymotrypsin and trypsin can be found in the digestive tract of sea urchin. However, cellulase activity of this enzyme is rather low. Moreover, the previous study in sea urchin *S. purpuratus*, it was shown that amylase and proteinase activities were observed in intestine. Nevertheless, there were no enzymes that can digest whole algae. It was expected that bacteria may help sea urchins to digest algae (Lasker and Giese, 1954).

The previous study by Lawrence and Hughes-Games (1972), it was reported that *D. setosum* only has food in the stomach during the nighttime. In contrast to the stomach, food always store in the intestine even if they are not feeding on that time. Thus, the intestine is likely to be the primary site for food digestion and absorption (Lawrence and Hughes-Games, 1972; Michel and John, 1982). Consistent with Pechenik (2015) study, it was reported that echinoid has no true stomach, instead of, esophagus and then connected to the long convoluted intestine. This intestine is the place where both digestion and absorption can occur. So, the present study will be focused on bacterial community in the intestinal part of sea urchin *D. setosum*.

Bacteria Association with Digestive tract

The symbiotic relationship between gut bacteria and sea urchin digestive biology have been discussed by several studies. For example, it was found that sea urchins are able to digest agar and some components of plant cell walls. In addition, it was expected that an adequate amount bacteria may aid in terms of digestion in sea urchin (Lasker and Giese, 1954). Moreover, it is possible that slow digestion by bacteria

can occur in intestine (Lasker and Giese, 1954) because food in the digestive tract remains in the intestine for a long time (Lawrence and Hughes-Games, 1972). This concept corresponds with other report, Holland (2013) suggested that sea urchins which feeding algal as main food assist in digestion by bacteria is important. As many polysaccharides in algal cell wall composition can tolerate to digestive enzymes that produced by animals, it is expected that bacteria may break down large molecules into subunit for host utilization. According to the previous study of intestinal bacteria by Meziti et al. (2007), it indicated that bacterial diversities differed in two different parts of digestive tract. The results are rather interesting as the anterior part of the gut, which composed of pharynx and esophagus, bacterial diversities are similar to bacteria found in surrounding environment. While the posterior part of the gut, which composed of stomach and intestine, contains different bacterial composition compared with sediment and seawater samples. Bacteria in the posterior part may specific to some region of sea urchin intestine, probably due to the specific environments of the intestine (Unkles, 1977). So it is possible that these bacteria may act as sea urchin symbionts and could enhance the ability to digest complex structures or macromolecules (Meziti et al., 2007).

The sea urchin intestinal bacteria have been studied in some commercial species. For instance, Unkles (1977) studied intestinal bacteria of sea urchin *Echinus esculentus* using culture-based method. *Vibrio* and *Pseudomonad* were detected as the dominant species. Similar to study in the captive species *Lytechinus variegatus*, *Vibrio*, *Pseudomonad* and Gamma Proteobacteria were found as the most abundant from digestive tract (Nelson et al., 2010). However, in the case of gut bacteria in *S. Intermedius*, *Psychrononas* and *Shewanella* were the predominant taxa (Zhang et al., 2014). Furthermore, bacterial diversity are varied corresponding to the specific organs (Unkles, 1977). In consistent with the study by Zhang et al. (2014), the different diversities between bacteria collected from small intestine and large intestine of sea urchin *S. intermedius* were reported.

Several factors that impact the existence of the intestinal bacteria in invertebrates have been reported (Harris, 1993). Host diets are considered as one of the factors that might influent on intestinal bacterial composition and their activities (Harris, 1993). According with Zhang et al. (2014), biochemical composition of food may be one factor that determines the composition of intestinal bacteria. Moreover, intestinal bacteria of echinoids displayed different digestive abilities when host diets are differed (Prim and Lawrence, 1975). However, the effect of different diets on the intestinal bacterial community are still lacking. Understanding the relationship between host diet and bacterial diversities are, therefore, important to study of host's digestive physiology (Harris, 1993)

Sampling site: Mook Island

Mook Island is one of the large islands in Trang province Mook Island is part of Chao Mai national park. The island is located near the shore, formation of scleractinian can generally be found in many bays except in the east side is sandy area and seagrass bed. The coral reef is approximate 100 meters wide and limit at 4 meters depth. In term of seagrass, *Enhalus acoroides* and *Cymodocea serrulata* are dominate. Moreover, various marine animals can be found in this area (สถาบันวิจัยและพัฒนาทรัพยากรทางทะเล ชายฝั่งทะเล และป่าชายเลน, 2558). Three sampling sites: coral area, sandy area and seagrass area are in the north and the east side of the island as can be seen in figure 2-3.

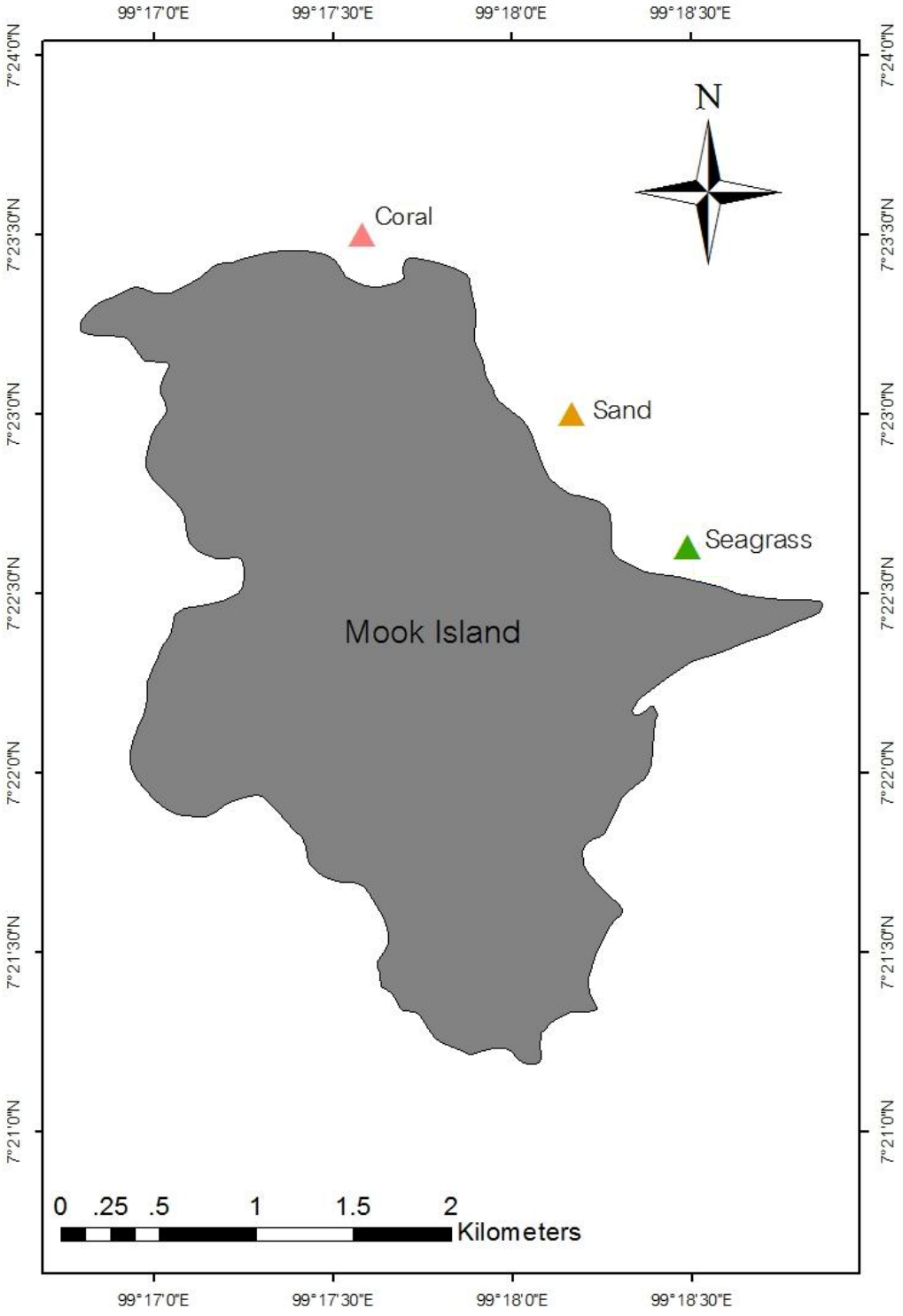

Figure 2-3 sampling site: Mook Island

16S rRNA Metagenomics Sequencing

There are several ways to study bacterial community. Culture-based method is one of the popular traditional methods (Munn, 2011). However, there are some limitations because the traditional method can only identify the culturable bacteria species (Munn, 2011; Streit and Schmitz, 2004). According to the fact that different bacteria requires the specific culture media and conditions for their growth so it is difficult to find some selective media that specific to bacterial intestine community which they are mainly anaerobe (Vaughan et al., 2000). Therefore, molecular techniques have been developed and allowed to access more species either cultured or uncultured bacteria (Munn, 2011; Thomas et al., 2012).

16S rRNA is a small subunit of ribosome found in Bacteria and Archaea while in Eukaryote presents as 18S rRNA.(Munn, 2011; Tringe and Rubin, 2005). In bacteria, the 16S RNA gene composes of the conserved and hypervariable regions. The hypervariable regions are located on nine positions (V1-V9), Fig. 2-2. These regions have different sequences. So they are very useful for bacterial identification (Petrosino et al., 2009). The rRNA sequencing is one of the most widely used methods for several reasons. First of all, every organism contains these gene because rRNA has an important function on protein synthesis. Secondly, rRNAs are highly conserved. Lastly, polymerase chain reaction (PCR) can amplify even if there are only some small amounts of DNA because growing bacteria frequently carry numerous copies of rRNA (Munn, 2011).

Figure 2-4 Hypervariable regions of 16S rRNA (V1-V9)

In the present study, metagenomic approach will be adopted in order to study bacterial community. This approach has been widely used because of the ability to classify the entire community of bacteria from the environment (Thomas et al., 2012). Also, it has been applied for study the genomes of the bacterial community which reside in host animals (Petrosino et al., 2009). The next generation sequencing (NGS) performs better sequencing than the traditional sequencing. This technique can performs high throughput analysis and more results can derived per sequencing run (Lluch et al., 2015; Munn, 2011). Roche 454 pyrosequencing has also been applied as a standard platform for 16S metagenomics sequencing. However, Lluch et al. (2015) indicated that MiSeq by Illumina have several advantages compared with Roche 454 pyrosequencing including higher output per run, cheaper price per read and simplified library preparation procedure. Therefore, the present study aims to study Intestinal bacteria of sea urchin *D. diadema* by 16S rRNA metagenomics sequencing using Illumina MiSeq platform.

CHAPTER 3

METHODOLOGY

Survey and Samples Collection

A. Sea urchin sampling

Adult sea urchins (35-45 mm) were collected from three different habitats including sand area, seagrass bed and coral area, around Mook Island, Trang province, Thailand. Sixteen sea urchins per site were collected and separated into two groups: one for gut contents analysis and another for bacterial community analysis using molecular approach.

Ten sea urchins per sites were collected for gut content analysis. Each sea urchin was dissected followed the method by Whalen (2008) using sterile scissors cut through the circumference to open the test. After that, all gut contents were removed and kept in sample collection bottles and preserved with 4% formalin fixative. The second group of the collected urchins were collected for bacterial community analysis using metagenomic. Three sea urchins per site were dissected *in situ* with the same method as described by Whalen (2008). However, some minor changes were applied to preserve the metagenome DNA i.e. the samples were rinsed with steriled sea water and then were sprayed with 70% ethanol to reduce any external contaminations. The intestines were removed and divided into two parts: small intestine and large intestine referring from Whalen (2008). Each part was kept in separate zip lock plastic bags and stored at -20 °C until laboratory analysis. Three remaining sea urchins were held in the starving condition for 4 days in order to eliminate the effects of food referring from the protocol by Zhang et al. (2014). Four days later sea urchins were processed as same as the *in situ* group.

B. Ambience sampling

The ambient sediment and food samples (seagrass leaves) were also collected. Sediment were sampled for 3 replications per food source from the location around the collected sea urchin samples. While, seagrass leaves samples were collected for 3 replications only from the seagrass area. Then, the samples were kept in zip lock plastic bag and stored at -20 °C until bacterial community analysis process.

Laboratory Analysis

A. Gut content analysis

Gut content of each sea urchin was placed on gridded petri dish and was examined through stereo microscope with the point-count method as describe by Jones (1968). All contents were divided for comparison between different food sources. Gut contents were divided into four categories: sediment, coral, seagrass and invertebrates (McClanahan, 1988). In addition, the abundances of each food type were calculated using the formula derived from Jones (1968) as follow:

$$\text{Abundance of food A compare with another} = \frac{\text{Intersection of all food A}}{\text{Intersection of all food}}$$

$$\text{Relative abundance of food A} = \text{Abundance} \times 100$$

B. Bacterial community analysis

DNA extraction from gut contents and environment samples

Genomic DNAs were extracted from the gut contents of the eighteen sea urchins, nine sediments and three seagrass leaf samples using Qiagen's QIAamp Fast DNA Stool Mini Kit (Valencia, CA, US; catalog no. 51604) following the manufacturer's protocol. For seagrass leaves, the sample was cut into the small size and then put into 2 ml microtubes with contain 1ml InhibitEX buffer. Microtubes

were vortex to remove the epiphytes that may cover the seagrass leaf. After the epiphyte removal, the seagrass leaf samples were centrifuged at 10,000 rpm for 3 minutes. The supernatant was discarded while the pellet was kept for performing the extraction with the same protocol with gut contents and sediment samples.

16S metagenomic sequencing library preparation

The Illumina primer pair, 16S Amplicon PCR Forward Primer: 5'TCGTCGGC AGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S Amplicon PCR Reverse Primer: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGGACTACHVGGGTATCTAATCC) with overhang adapters attached, forward overhang: 5' TCGTCGGCAGCGT CAGATGTGTATAAGAGACAG [locus specific sequence] and reverse overhang: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAG-[locus-specific sequence] were applied for amplification of the V3 and V4 regions of the 16 rRNA gene (Klindworth et al., 2013). The PCR preparation of 25 µl of total reaction volume was set up as follow: 2X KAPA HiFi HotStart ReadyMix, 200 nM of each primer and 12 ng of genomic DNA. PCR was performed in a thermal cycler using the following program: initial denaturation step at 94°C for 3 min; 25 cycles of 98°C for 20 sec, 55°C for 30 sec, and 72°C for 30 sec and final extension step at 72°C for 5 min. Then 1 µl of the PCR product was run on a Bioanalyzer DNA 1000 chip to verified the size. The 16S V3 and V4 amplicon products were purified away from free primers and primer dimer species using AMPure XP beads and attached dual indices using the Nextera XT Index Kit, then using AMPure XP beads to cleaned up the final library again before quantification. Purified 16S amplicons were normalized to a concentration of 4 nM and pooling 5 µl from each library. 250 bp paired-end read sequencing of a mixture library was performed on an Illumina MiSeq at the Omics Sciences and Bioinformatics Center, Chulalongkorn University, Bangkok, Thailand.

Bioinformatics analysis

Bioinformatics were conducted using QIIME v1.9.1 pipeline to analyze data (Caporaso et al., 2010). The pair-end sequencing reads from Illumina were de-multiplexed and stitched together, followed by cut off low quality reads using FASTX toolkit as well as removed chimeric reads using VSEARCH. Sequences were clustered into operational taxonomic units (OTUs) at 97%sequence identity and assigned taxonomic position using UCLUST algorithm as executed in USEARCH (Edgar, 2010) against the Greengenes v13.8 database (DeSantis et al., 2006). The alignment of the sequences was performed with PyNAST (Caporaso et al., 2010)

Statistical analyses

Alpha diversity of each sample was calculated using the observed OTUs, Good's coverage and the Shannon-Wiener and Simpson diversity indices. All metrics were computed by Qiime v1.9.1(Caporaso et al., 2010). For beta diversity PCA plot were generated with STAMP v2.1.3 (Parks et al., 2014) to compare bacterial communities between food source and sea urchin condition. Analysis of similarities (ANOSIM) was applied for statistical test whether there is any significant differences between beta diversity (Clarke, 1993). ANOSIM was calculated using *compare_categories.py* after weighted Unifrac distance matrix produced, all were executed by Qiime.

CHAPTER 4

RESULTS

Gut contents Analysis

Ten sea urchins f each food source were examined for gut content analysis to determine the proportion of each diet. The gut contents were separated into four categories: (i) seagrass, (ii) coral, (iii) sediment and (iv) invertebrates as mention earlier in the Chapter 3. In the samples from the coral area, corals were the highest abundant category accounted for 46.87%. Seagrass, sediment and invertebrate were following category, contributing 24.74%, 17.19% and 11.20% of the total gut contents, respectively. In the case of samples from the seagrass area, more than half of all gut contents were identified as seagrass category which accounted for 62.04 %. Subsequent categories were invertebrates and sediment (21.39% and 14.92% respectively). Coral category was found in slightly less proportion in the gut contents from seagrass area, only 1.65% (see figure 4-1). In the case of the sand area, seagrass was the most abundant proportion which accounted for 52.50%. Sediment was the second most observed category which accounted for 24.38. However, sediment proportion in the gut content from sand area was displayed as the highest amount compared with the others. Corals and invertebrates were the subsequent category in gut contents from the sand area which accounted for 15.31% and 7.81%, respectively.

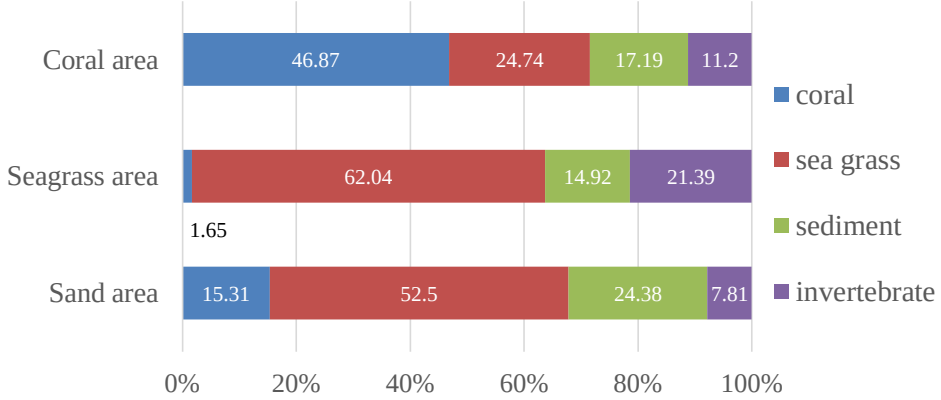

Figure 4-1 The proportion of the four categories (coral, seagrass, sediment and invertebrate) in gut contents of the sea urchins collected from three different food sources: sand area, sea grass area and coral area

Bacterial richness and diversity

After filtered out the low-quality reads and removed chimeric reads, the total 2,071,793 reads were obtained from 29 samples (the wild-caught sea urchin; n=9, the starved sea urchin; n=8, sediment; n=9, the seagrass leaves; n=3) by Miseq sequencing of the V3-V4 regions of 16S rRNA gene. Percentage of usable reads that ready for analysis from each sample were displayed in table 4-1. The samples from sea urchin's gut content, both wild-caught and starved condition, as well as from seagrass leaf shown rather higher coverage percentage comparing with the sediment samples.

The outcome reads were grouped into the total 107,434 OTUs using threshold cutoff at 97% nucleotide sequence similarity by using UCLUST (Edgar, 2010). Sediment samples have shown the highest in numbers of observed OTUs which obtained from 6,892 to 8,491 OTUs, compared with the groups of seagrass leaf and sea urchin's gut that obtain only 1,821 to 3,551 and 444 to 3,958 respectively (Table 4-1). Among different sea urchin condition, the wild-caught sea urchin have higher abundant of bacteria than the starved sea urchin (1,274 to 3,958 and 444 to1464, respectively). According to the richness indices, high number of Shannon index represent more diversity and evenness in community. While, Simpson index which is closer to 1.0 means that the diversity of community is high. As shown in table 4-1, Shannon and Simpson diversity indices of sediment samples were also indicated higher diversity in the sediment samples than the seagrass leaf and the sea urchin's gut content groups.

Table 4-1 Sample statistics and the diversity indices values (SA-W, natural sea urchins from sand area; SG-W, natural sea urchins from seagrass area; CR-W, natural sea urchins from coral area; SA-S, starved sea urchins from sand area; SG-S, starved sea urchins from seagrass area; CR-S, starved sea urchins from coral area; SA-SED, sediment from sand area; SG-SED, sediment from seagrass area; CR-SED, sediment from coral area; LEAF, seagrass leave)

Samples	Observed	Coverage (%)	Shannon	Simpson
SA-W1	2522	96.16	8.24	0.986
SA-W2	3958	92.58	8.15	0.974
SA-W3	1274	98.39	6.33	0.924
SG-W1	3434	94.15	8.30	0.981
SG-W2	1303	98.49	6.64	0.968
SG-W3	2945	94.35	7.83	0.986
CR-W1	3489	93.51	8.27	0.988
CR-W2	3951	93.16	8.80	0.991
CR-W3	1472	98.23	7.29	0.976
SA-S1	953	99.39	6.87	0.958
SA-S2	685	99.08	4.93	0.903
SG-S1	1251	98.21	6.09	0.946
SG-S2	1028	99.03	6.30	0.957
SG-S3	1464	99.27	9.40	0.997
CR-S1	444	99.48	4.00	0.839
CR-S2	896	98.74	5.02	0.918
CR-S3	834	99.32	6.59	0.971
SA-SED1	8491	86.37	11.48	0.998
SA-SED2	7769	87.46	11.28	0.998
SA-SED3	8352	86.33	11.44	0.998
SG-SED1	7038	89.28	11.15	0.998
SG-SED2	6982	89.65	11.02	0.997
SG-SED3	6892	89.59	11.06	0.998
CR-SED1	7189	89.03	11.01	0.997
CR-SED2	7471	88.76	11.16	0.998
CR-SED3	7283	88.76	10.94	0.997
LEAF1	3551	95.36	9.20	0.992
LEAF2	1821	98.46	8.63	0.991
LEAF3	2692	97.39	8.60	0.977

Bacterial Community across the Different Food sources in the Natural Sea urchins

The OTUs were classified into the most resolvable taxa, from phylum to genus. All natural sea urchin's gut content samples across the three different food sources were shown the similarity of the predominant phylum. Bacteroidetes was the highest abundant to be observed in all food sources, accounted for 38.55%, 30.94% and 28.51% of the reads from the sand area, the seagrass area and the coral area, respectively. The second place was Proteobacteria which revealed as 19.06% in the sand area, 18.22% in the seagrass area and 18.63% in the coral area. Firmicutes was also detected by 11.37% in the sand area, 16.07% in the seagrass area and 17.3% in the coral area. The forth resolved phylum in the sand area and the coral area was Tenericutes, which accounted for 2.77% and 2.56%, respectively. Contrast with the other samples, the forth rank in the seagrass area was Spirochaetes (3.45%). Other phyla were detected in the small proportion. The relative abundance of the 20 phyla were shown in figure 4-2. Some of bacterial clade that could not be classified were designated as the unassigned taxa (based on 97% similarity with greengene database), approximately 18.11%, 22.68% and 24.99% in the sand area, the seagrass area and the coral area, respectively.

At more specific level, the taxonomic results have shown that gut contents of the natural sea urchins collected from the sand area were dominated by order Bacteroidales and Flavobacteriales (29.68% and 5.27% respectively); family Christensenellaceae (2.73%); and genus *Fusibacter* (4.49%), *Desulfotalea* (2.68%). The samples collected from the seagrass area have shown some similarities. The abundance was represented by order Bacteroidales and Flavobacteriales (21.50% and 6.35% respectively); family Pseudoalteromonadaceae (3.72%); and genus *Fusibacter* (7.30%) and *Spirochaeta* (3.07%). In the case of the gut contents of the natural sea urchins collected from the coral area, Bacteroidales (22.00%) and Flavobacteriales (3.60%) were

shown as the most prevalent order. Moreover, family Christensenellaceae (2.73%) was observed as well as at the genus level *Desulfotalea* (4.17%) and *Fusibacter* (3.45%) were detected as the dominant taxa. These results were similar to the samples collected from the sand area but different in terms of proportion, see in figure 4-3.

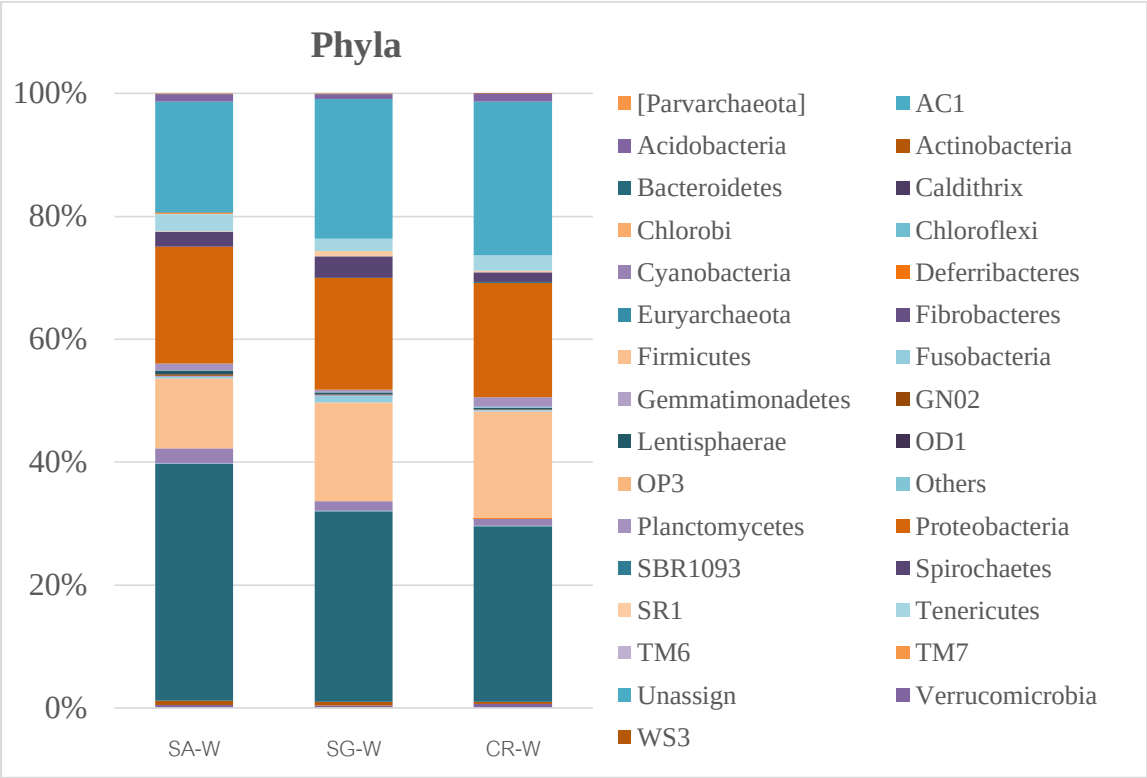

Figure 4-2 The top 20 most abundant phyla in the gut contents of natural sea urchins across different food sources. (SA-W, *natural sea urchins from sand area*; SG-W, *natural sea urchins from seagrass area*; CR-W, *natural sea urchins from coral area*)

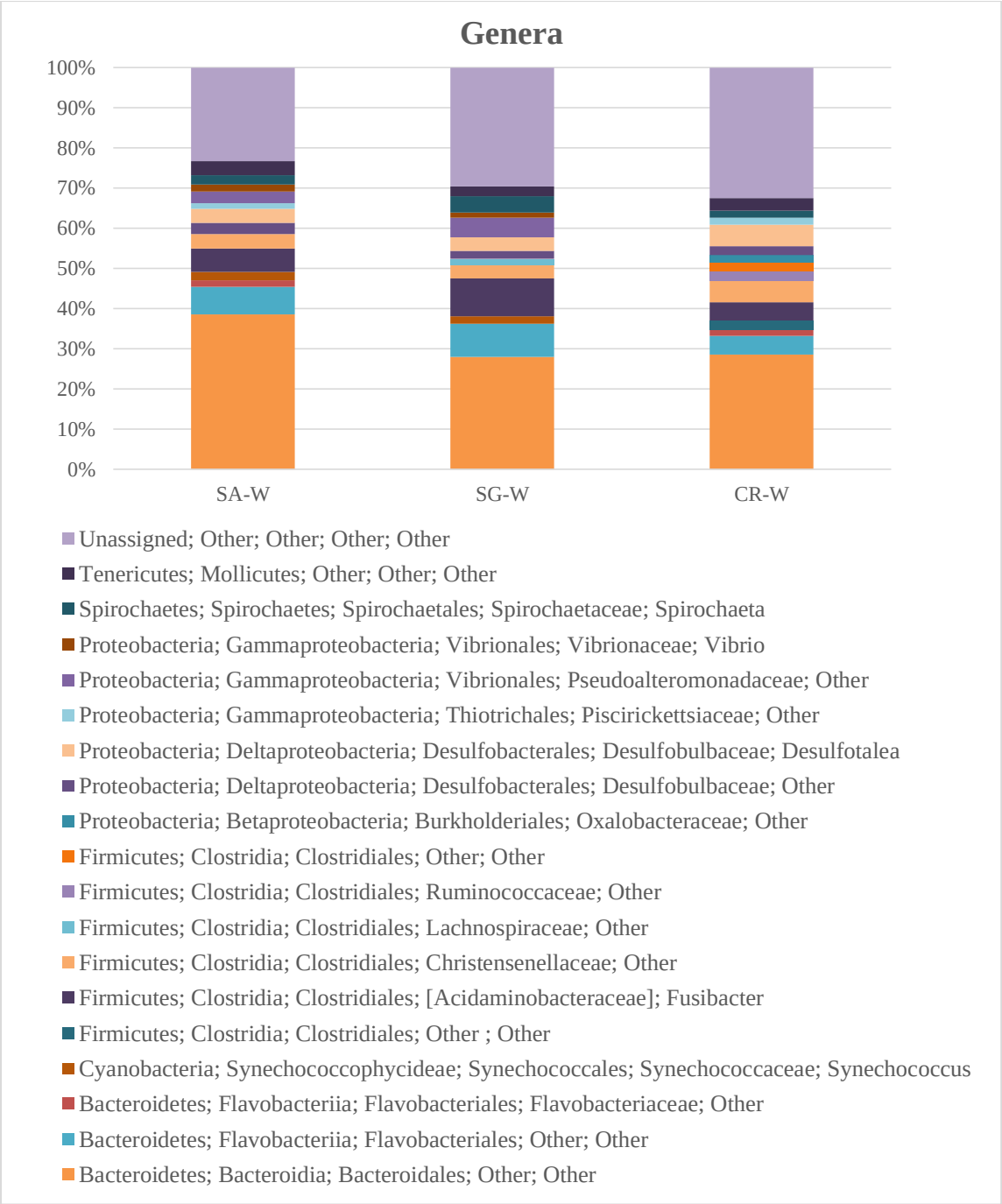

Figure 4-3 The most abundant genera (1% cut off) in the gut contents of natural sea urchins across the different food sources. (SA-W, *natural sea urchins from sand area*; SG-W, *natural sea urchins from seagrass area*; CR-W, *natural sea urchins from coral area*). The genera not detect were designated as 'Other' while the taxonomic level detected is shown.

Bacterial Community across the Different Food sources in the Starved Sea urchins

At the phylum level, the dominant bacterial taxa of gut content of the starved sea urchins were similar with the natural sea urchins. However, some degree of differences in proportion can be detected. Gut content of the natural sea urchins were dominated mainly by Bateroidetes. Whereas gut content of the starved sea urchins were revealed that Proteobacteria was the highest abundance, accounted for 39.13%, 41.53% and 55.40% in the sand area, the seagrass area and the coral area, respectively. Bacteroidetes was presented as the second rank, accounted for 30.95, 26.69% and 21.26% in the gut content collected from the sand area, the seagrass area and the coral area respectively. In the sand area, Fusobacteria was found as the third rank accounted for 10.19%. While in the seagrass and the coral area presented Firmicutes by 8.01% and 9.21%, respectively. The unassigned taxa were also presented but in the smaller amount than the gut content of the natural group, making up to 8.23%, 10.14% and 4.40% in the sand area, the seagrass area and the coral area, respectively. The composition of bacterial community of gut content of the starved sea urchins at the phylum level was displayed in figure 4-4.

At more specific level, the taxonomic results have been shown that the gut contents of the starved sea urchins collected from the sand area were dominated by order Bacteroidales and Fusobacteriales (21.29% and 8.85% respectively; family Pseudoalteromonadaceae (12.55%); and genus *Desulfotalea* (9.99%). In the samples collected from the seagrass area, the abundance was represented by order Bacteroidales (19.31%); family Vibrionaceae (12.74%) especially *Vibrio* which contribute to 1.58% and family Pseudoalteromonadaceae (5.09%). At the generic level, *Desulfotalea* was the most dominate taxa (4.92%). In the case of the gut contents of starved sea urchins collected from the coral area, Bacteroidales (13.25%) and Flavobacteriales (7.00%) have been shown as the most prevalent orders. Moreover, family Desulfovibrionaceae (5.98%) was observed as well as

at the genus level *Ferrimonas* (19.74%) and *Vibrio* (11.90%) were detected as the dominant taxa. These results were rather differed from the samples collected from the other area, see in figure 4-5.

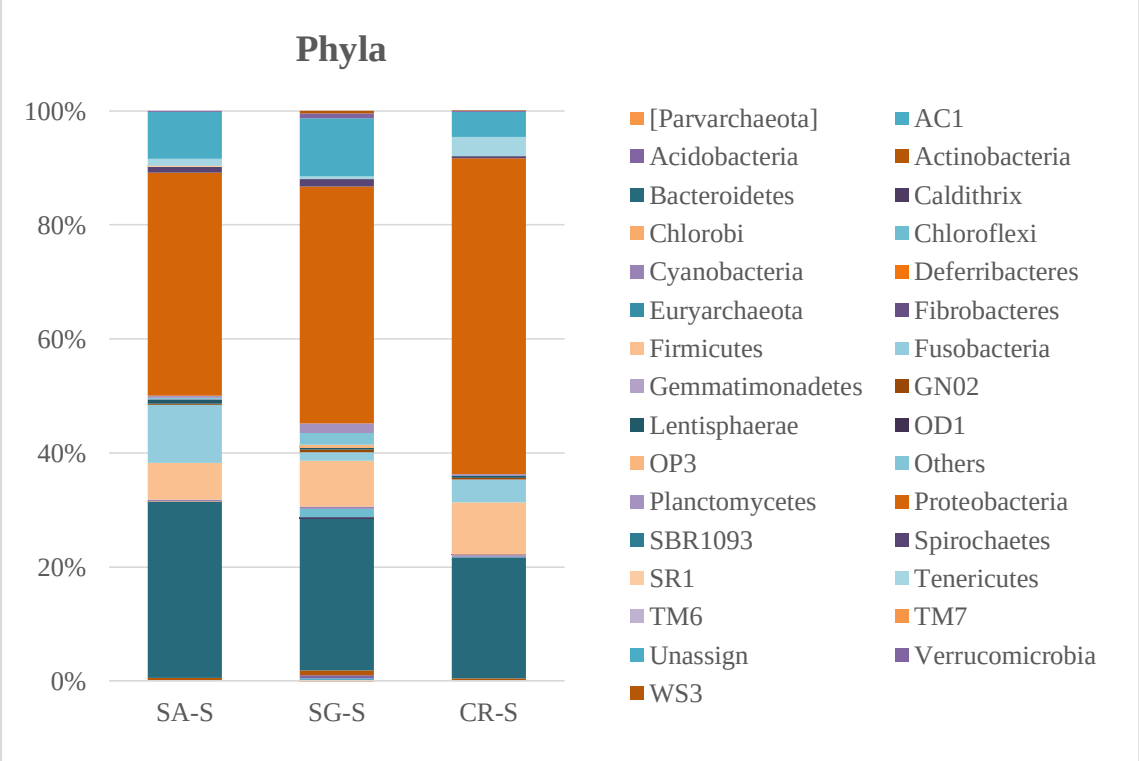

Figure 4-4 The top 20 most abundant phyla in the gut contents of the starved sea urchins across different food sources. (SA-S, starved sea urchins from sand area; SG-S, starved sea urchins from seagrass area; CR-S, starved sea urchins from coral area)

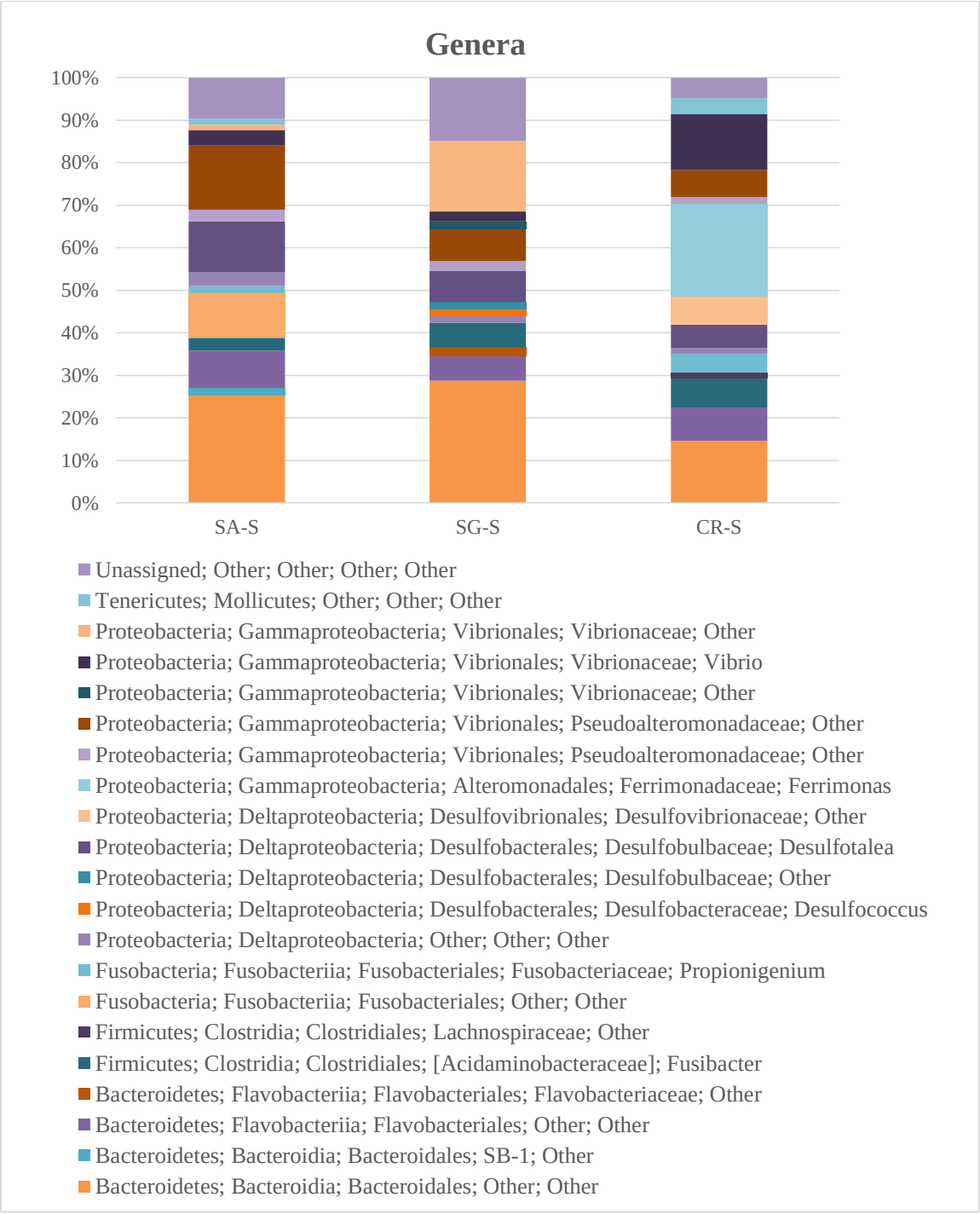

Figure 4-5 The most abundant genera (1% cut off) in the gut contents of starved sea urchins across different food sources. (SA-S, starved sea urchins from sand area; SG-S, starved sea urchins from seagrass area; CR-S, starved sea urchins from coral area). The genera not detect were designated as 'Other' while the taxonomic level detected is shown.

Bacterial Community in the Sediment and the Sea grass leave Samples

The sediment samples collected from all three food sources were applied as the circumstantial evidence for each food source. In addition, the seagrass leave samples were collected and analyzed from the seagrass area. The taxonomic data derived from the sediment samples collected from the sand and the seagrass area has shown the similarity between the dominant phylum as can be seen in figure 4-6. Proteobacteria was the predominant phylum, making up to 39.24% and 39.07% respectively. Bacteroidetes was detected in the second rank accounted for 12.09% and 12.75%, respectively. Next, Planctomycetes (8.03% and 8.08% respectively) and Cyanobacreia (6.26% and 7.18% respectively) were revealed. In the coral area, there were some dissimilarities, Proteobacteria was also detected as the highest abundance with 40.62% covered the total reads but the second rank was Cyanobacteria which accounted for 11.42%. The other predominant phyla were Bacteroidetes and Planctomycetes that presented as 10.33% and 9.55% respectively. Like the sediment samples, Proteobacteria (43.40%) was the dominant phylum in the seagrass leave samples. Bacteroidetes was revealed as the second place, accounted for 18.56%, followed by Cyanobacteria and Planctomycetes approximately 17.08% and 6.19% respectively. In addition, small amount of unclassified taxa were presented, accounted for 5.97%, 5.28%, 4.13% and 3.73% in the sediment samples from the sand area, the sea grass area and the coral area, as well as the seagrass leaves samples, respectively.

At more specific level, the sediment samples from the sand area were predominated by order Chromatiales (7.06%); family Thiotrichaceae (5.83%), Pirellulaceae (4.28%); and genus *Desulfococcus* (4.71%). The sediment samples from the seagrass area, predominance were order Stramenopiles and Chromatailes (6.02% and 5.61%, respectively); family Piscisrickettsiaceae (5.74%) and Pirellulaceae (5.35%). In addition, *Desulfococcus* was the dominant taxa detected at the generic level, accounted for 4.77%. In sediment from the coral area, the highest resolved order were

Chromatailes and Stramenopiles (7.48% and 5.67%, respectively). At the Family level, Pirellulaceae (4.42%) was also dominated as in the other sediment samples. In addition, *Desulfococcus* was detected as dominant taxa at the generic level which accounted for 4.65%. The samples of seagrass leaves were shown some differences, at the order level Streamenopiles and Streptophyta were predominated accounted for 6.37% and 6.12% respectively. At the family level, Rhodobacteraceae was presented as dominance (14.74%) which was detected in small amount in the sediment samples (sediment from sand area; 1.00%, sediment from sea grass; 1.41% and sediment from coral area; 0.62%). It was not detected in the gut content samples except in the gut content of starved sea urchin from the coral area presented 0.25%. Family Flavobacteriaceae was also found, covered 6.84% as can be seen figure 4-7

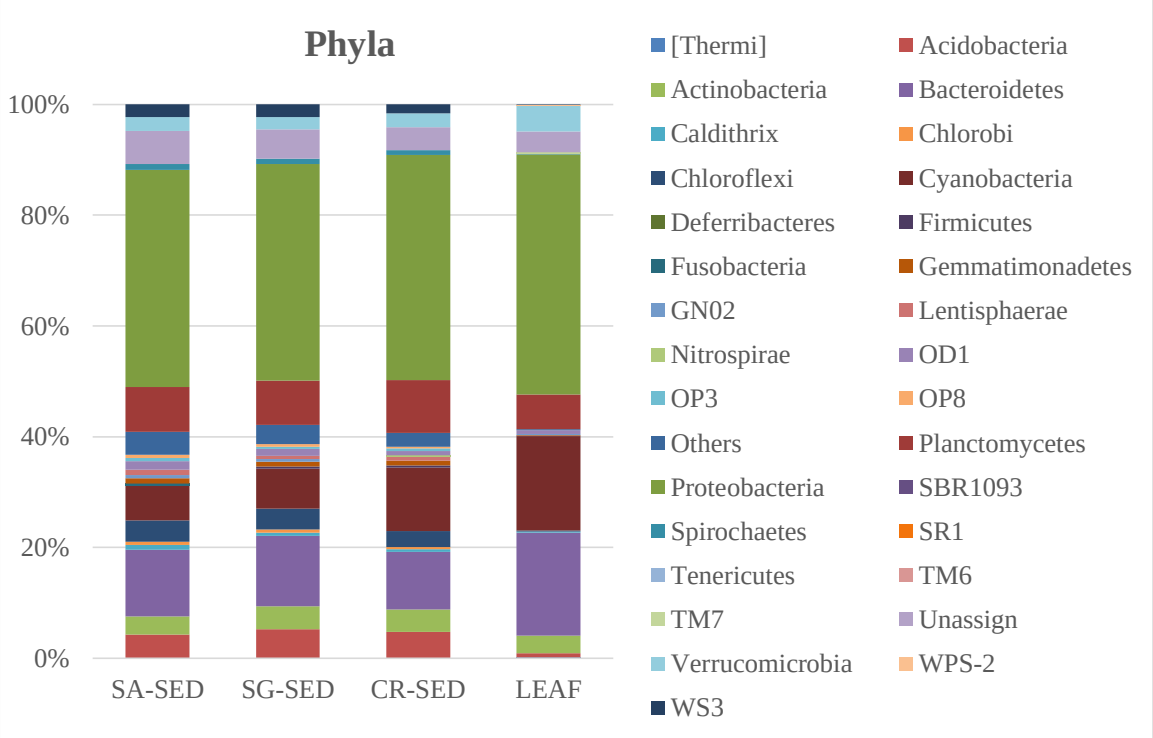

Figure 4-6 The top 20 most abundant phylum in the habitat ambient samples. (SA-SED, sediment from sand area; SG-SED, sediment from seagrass area; CR-SED, sediment from coral area; LEAF, seagrass leave)

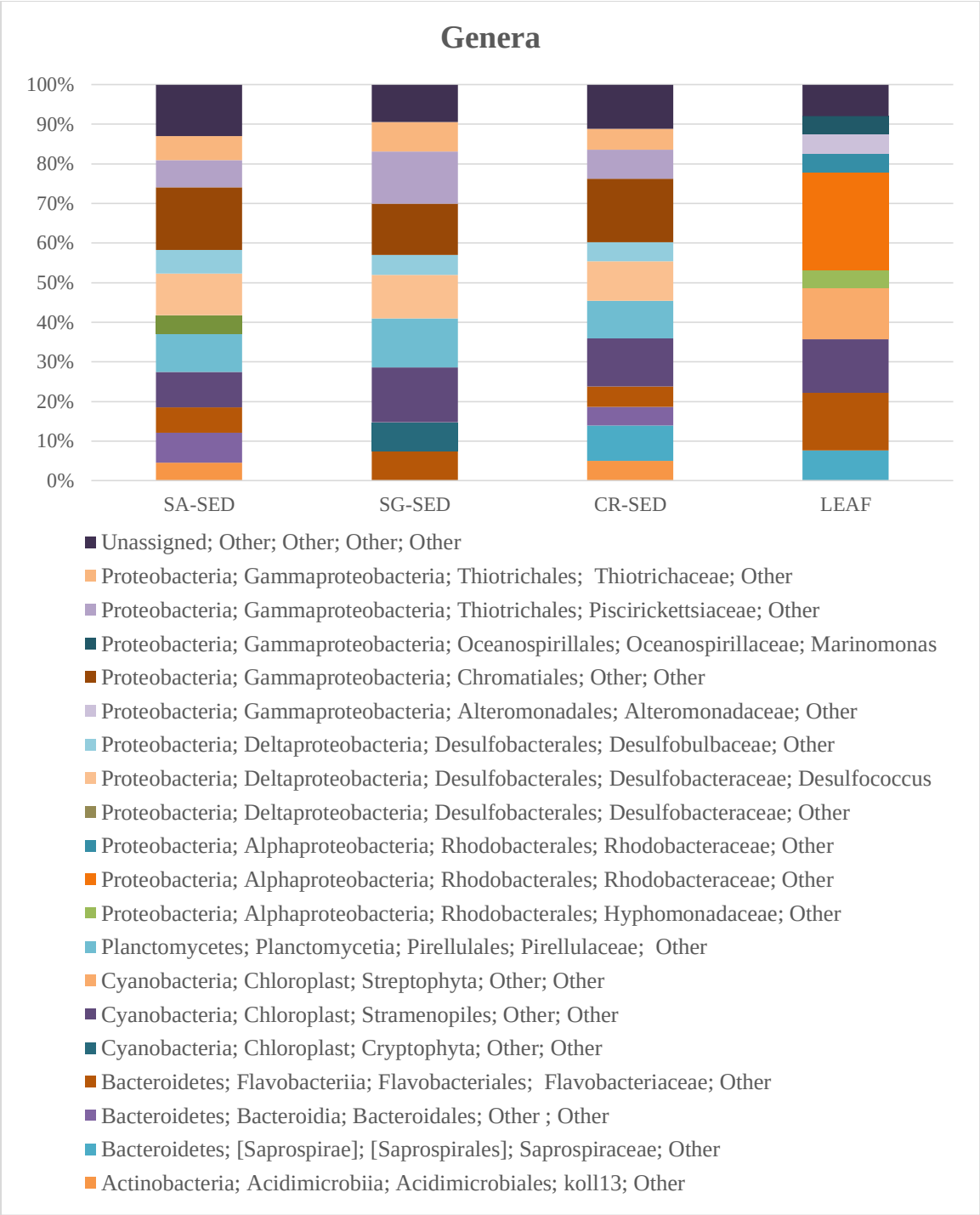

Figure 4-7 The most abundant genera (2% cut off) in the habitat ambient samples. (SA-SED, sediment from sand area; SG-SED, sediment from seagrass area; CR-SED, sediment from coral area; LEAF, seagrass leave) The genera not detect were designated as 'Other' while the taxonomic level detected is shown.

Comparison the Bacterial Community in Gut contents of Natural Sea urchins across the Different Food sources

Composition of bacterial community in gut contents of the natural sea urchins across the three food sources were presented some similarities from the dominant phyla down to the genus level. Bacteroidetes, Proteobacteria and Firmicutes were the three most abundant phyla in these samples groups. At the generic level, *Fusibacter*, *Defulfotelea*, *Spirochaeta*, *Synechococcus* and *Vibrio* were the most prevalent observed taxa. According to the beta diversity (diversity between groups) statistic computed by ANOSIM method (Clarke, 1993) using weight Unifrac metrics, the results indicated that bacterial community was not statistically significant difference between the gut contents of the natural sea urchins from the three different food sources ($p > 0.05$). Also, the samples were scattered and cannot be clustered into a distinct group in PCA plot, see figure 4-8. The bacterial composition, which have been shared between the natural sea urchins gut contents from the different food sources were: Proteobacteria *Desulfococcus*, *Defulfotalea*, *Desulfovibrio*, *Ferrimonas* and *Vibrio*); Bateroidetes (*Robiginitalea*); Cyanobateria (*Synechococcus*); Firmicutes-(*Alkaliphilus*, *Anaerofustis*, *Clostridium*, *Fusibacter* and *Tindallia_Anoxynatronum*); Fusobacteria (*Propionigenium*); Spirochaetes (*Spirochaeta*) and Verrucomicrobia (*Verrucomicrobium*)

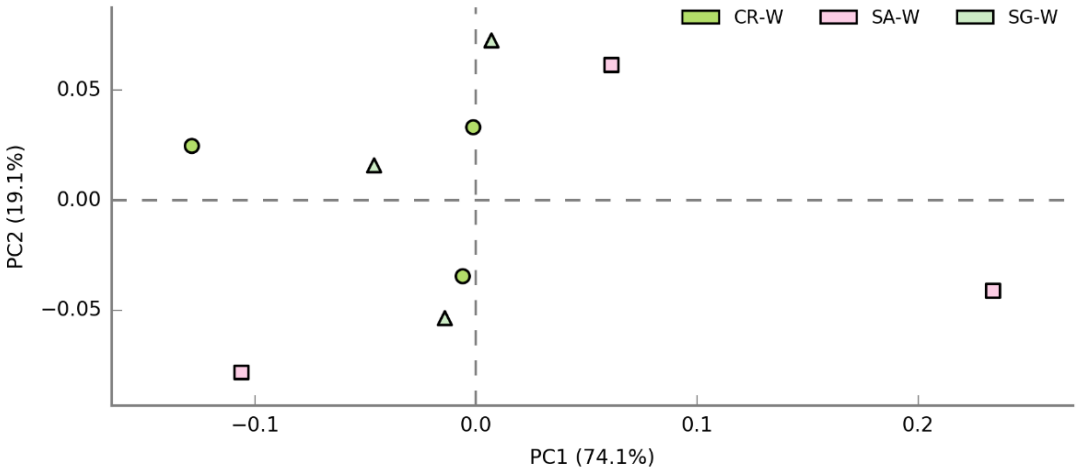

Figure 4-8 The PCA results generated at the phylum level, display relatedness of bacterial community in natural sea urchins' gut contents across the food sources (SA-W, *natural sea urchins from sand area*; SG-W, *natural sea urchins from seagrass area*; CR-W, *natural sea urchins from coral area*)

Comparison the Bacterial Community in Gut contents of Sea urchins between two Conditions

Bacterial community of the natural sea urchins gut contents was dominated by phylum Bacteroidetes followed by Proteobacteria and Firmicutes, respectively. Conversely, the proportion of Proteobacteria in the starved sea urchin was greater than in natural sea urchins and Proteobacteria becoming the most abundant phylum in the samples. Subsequently, Firmicutes and Fusobacteria were in the third rank. The comparison between the two conditions was displayed in figure 4-9. The increasing of Proteobacteria was statistical significant differences between the sea grass and coral area, with *p*-value 0.009 and 0.026 respectively. The bacterial compositions, which have been shared between the gut contents from the natural and the starved conditions, were: Proteobacteria-(*Desulfococcus*, *Defulfotalea*, *Desulfovibrio*, *Ferrimonas* and *Vibrio*); Bateroidetes (*Robiginitalea*); the Cyanobateria (*Synechococcus*); Firmicutes- (*Fusibacter* and *Tindallia_Anoxynatronum*); Fusobacteria (*Propionigenium*) and Spirochaetes (*Spirochaeta*)

Beta diversity of the natural and the starved samples was performed using PCA (Figure 4-10). According to the PCA results, the samples were separated into two groups. The group of the natural sea urchins' gut contents are cluster on the left side of PCA plot, as well as the group of the starved sea urchins gut contents are on the right side except one sample from sand area. The two groups were clustered mainly by the first principal component (PC1), which accounted for 78.2%. PCA was total explained 91.7% of variations between the bacterial communities of two conditions.

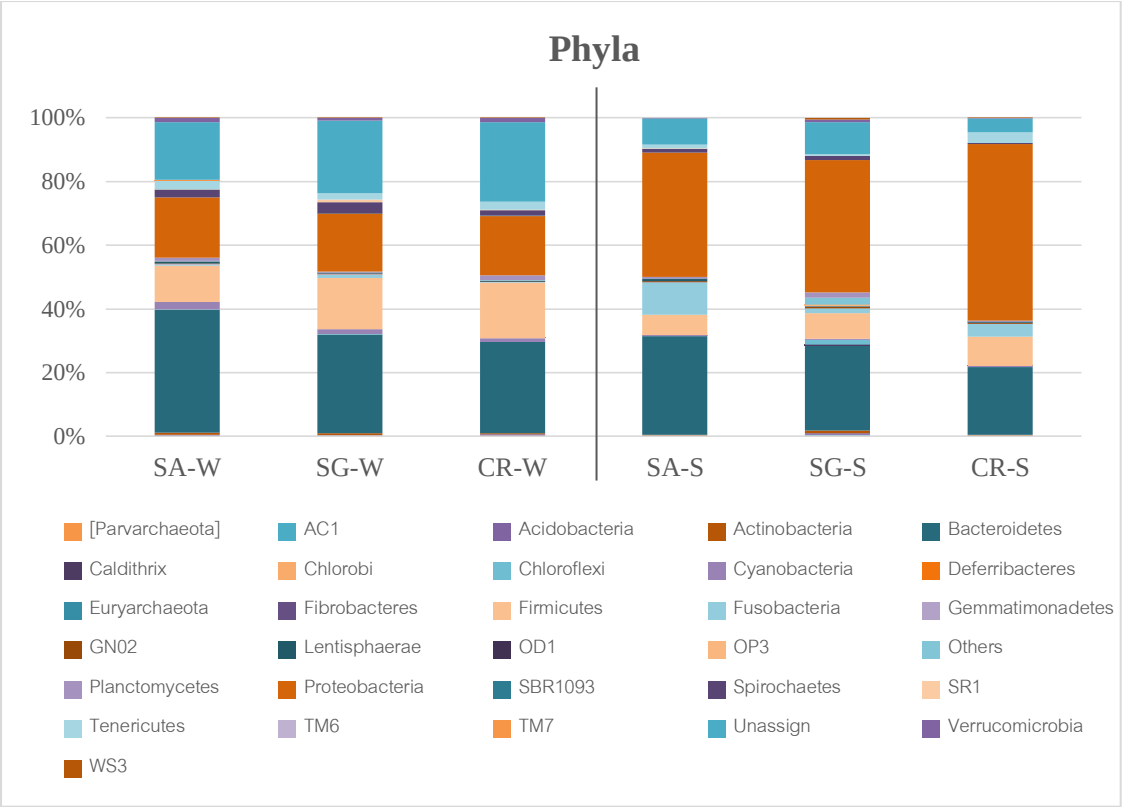

Figure 4-9 The most abundant genera (1% cut off) in the gut contents of the natural and the starved sea urchins across different food sources. (SA-W, natural sea urchins from sand area; SG-W, natural sea urchins from seagrass area; CR-W, natural sea urchins from coral area; SA-S, starved sea urchins from sand area; SG-S, starved sea urchins from seagrass area; CR-S, starved sea urchins from coral area)

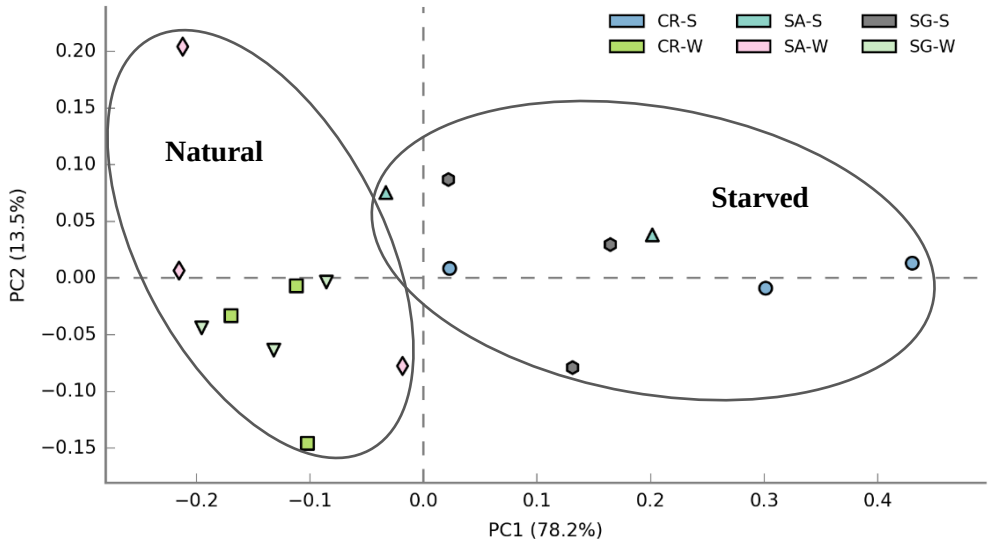

Figure 4-10 PCA analysis of the bacterial communities from the two conditions sea urchin across different food source. (SA-W, natural sea urchins from sand area; SG-W, natural sea urchins from seagrass area; CR-W, natural sea urchins from coral area; SA-S, starved sea urchins from sand area; SG-S, starved sea urchins from seagrass area; CR-S, starved sea urchins from coral area)

Comparison the Bacterial Community between Gut contents of the Sea urchins and the Surroundings

The ambient sediments and the seagrass leaves were collected as the circumstantial evidences of the food source at the sampling time. Bacterial community in the ambience was shown the dissimilarities characteristic compared with the gut content samples, both from the natural and the starved sea urchins. Proteobacteria, Bateroidetes, Firmicutes and Fusobacteria were resolved as the dominant taxa in the gut content samples. While in the ambient sediment and seagrass leaves samples, Firmicutes and Fusobacteria were the least presented and replaced by the phylum Cyanobacteria and Planctomycetes as shown in Figure 4-11. The difference between the gut content and the ambient samples was statistical significant differences at the *p*-value = 0.001. PCA plot was analyzed for comparison the beta diversity. All of the ambient samples were distinctly clustered on the bottom left area separated from the gut content samples as shown in figure 4-12. Overall, the PC1 and PC2 axes were explained 84.5% of the variations between the samples.

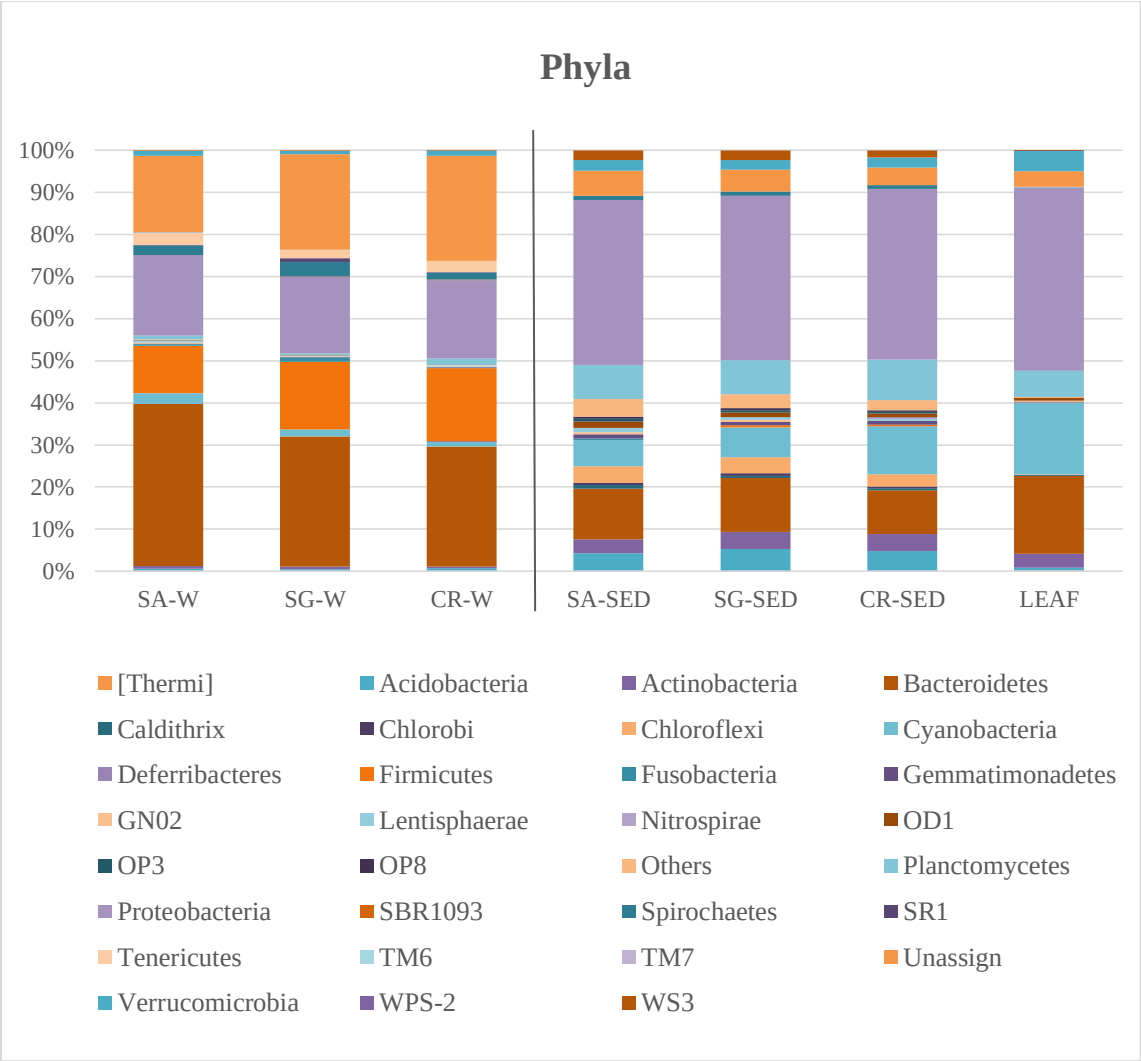

Figure 4-11 The 20 most abundant phyla in the gut contents and the ambience samples. (SA-W, natural sea urchins from sand area; SG-W, natural sea urchins from seagrass area; CR-W, natural sea urchins from coral area; SA-SED, sediment from sand area; SG-SED, sediment from seagrass area; CR-SED, sediment from coral area; LEAF, seagrass leave)

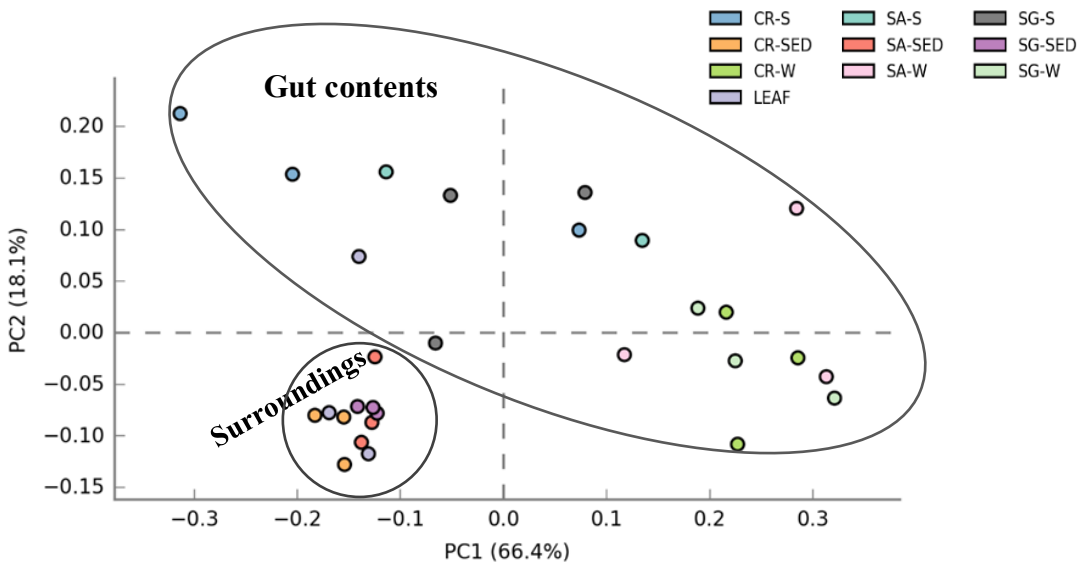

Figure 4-12 PCA analysis of the gut contents and the ambience samples. (SA-W, *natural sea urchins from sand area*; SG-W, *natural sea urchins from seagrass area*; CR-W, *natural sea urchins from coral area*; SA-S, *starved sea urchins from sand area*; SG-S, *starved sea urchins from seagrass area*; CR-S, *starved sea urchins from coral area*; SA-SED, *sediment from sand area*; SG-SED, *sediment from seagrass area*; CR-SED, *sediment from coral area*; LEAF, *seagrass leave*)

CHAPTER 5

DISCUSSION

The present study analyzed bacterial community in the gut content of sea urchin and their ambient sediment as well as seagrass leaves using 16S rRNA metagenomics sequencing. According to the results in the Chapter 4, this methodology was better to find out bacterial diversity in the samples than the conventional method using bacterial cultures. According to the previous study by Unkles (1977), bacterial flora of the sea urchin *Echinus esculentus* was examined by culture-based method. The results shown that three different parts of digestive system: intestine, peristomial membrane, and coelomic fluid contain a total of 85 strains of bacteria while only 26 bacteria strains were found from sand and seawater samples. However, the present study results are considerably differed in term of the quantity of the strains of bacteria. This may be as a result from some limitations of culture-based methods as the majority of microorganisms in the environment are not readily culturable especially the bacteria in the specific intestine environment (Vaughan et al., 2000). It is may be resulted from the limited availability of selective media for the most strict anaerobes (Streit and Schmitz, 2004; Vaughan et al., 2000). In order to overcome the restriction, metagenomics approach has developed as the culture-independent approaches and has been applied to the discovery of the vast novel species as well as the relatedness within the niches (Handelsman, 2004; Streit and Schmitz, 2004; Thomas et al., 2012). During the past 10 years, metagenomics has been widely utilized for bacteria studies i.e. the study of gut microbiome (Turnbaugh et al., 2009); marine symbioses (Fiore et al., 2010); bacterial communities in marine environments (Kennedy et al., 2008) and bacterial communities sediment (Somboonna et al., 2012).

Diversity of Bacteria Community in the Sea urchins' Gut content collected from the Different Food sources

Bacterial communities in the gut content of sea urchin collected from the three different food sources consisting of the sand area, the seagrass area and the coral area were analyzed. Surprisingly, there were no statistical significant differences among bacterial community between the different food sources. There were similarities among the predominant taxa; however, some differences in term of proportion may be found. The predominant phyla in the gut content were Bacteroidetes, Proteobacteria, Firmicutes, Tenericutes and Spirochetes, as shown in figure 5-1. At the genus level, *Fusibacter*, *Desulfotalea*, *Spirochaeta*, *Synechococcus* and *Vibrio* were revealed as prevalence, see in figure 5-2. Then, the ANOSIM shown that these results were not statistically significant. The studies of bacterial community in digestive tract have been limited in the sea urchin genus *Diadema*. Nevertheless, the predominant taxa observed from the present study are in consistent with the reports from several studies in other sea urchin species. For instance, Hakim et al. (2016) studied bacterial communities in different internal part of structures of the sea urchin *Lytechinus variegatus*: pharynx tissue, gut tissue, gut digesta and egest fecal pellet. The results of bacterial community in the gut digesta have shown that *Vibrio*, *Photobacterium*, *Propionigenium*, *Ferrimonas*, *Persicobacter* and *Tenacibaculum* were the dominant taxa. The reported taxa were also observed in the present study. Genera *vibrio*, *Photobacterium*, *Propionigenium* and *Ferrimonas* were found within the top 20. Although, *Persicobacter* and *Tenacibaculum* were detected, only small proportion was determined. Furthermore, bacteria in sea urchin *Paracentrotus lividus* digestive tract was reported from the study by Meziti et al. (2007). The major genera were *Desulfotalea*, *Desulforhopalus* and *Desulfovibrio*. Some study from other species of echinoderm, 16S rRNA gene pysosequencing was applied in the study of bacterial community from the gut content of sea cucumber *Apostichopus japonicus* by Gao et al. (2014).

Bacterial community composition was mainly composed of bacteria belonging to Proteobacteria. At the genus level, *Desulfosarcina*, *Rhodopirellala* and *prolixibacter* were the most abundant genera.

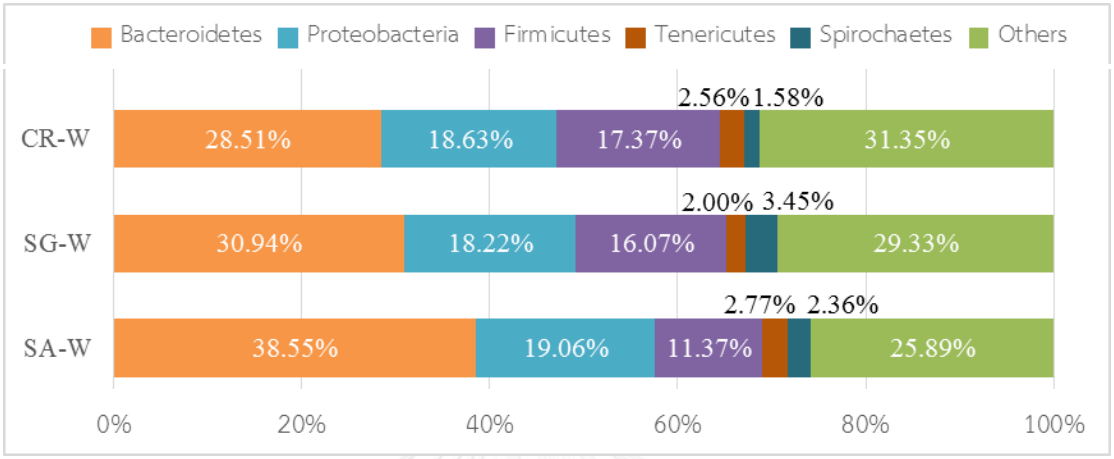

Figure 5-1 the top 5 phyla in the gut contents of the natural sea urchins across different food sources. (SA-W, *natural sea urchins from sand area*; SG-W, *natural sea urchins from seagrass area*; CR-W, *natural sea urchins from coral area*)

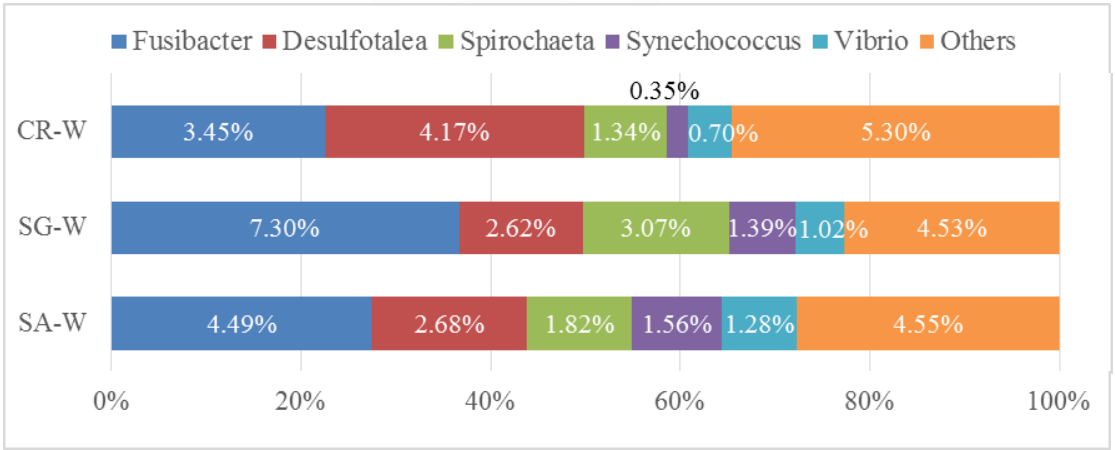

Figure 5-2 the top 5 genera in the gut contents of the natural sea urchins across different food sources (the unassigned taxa are excluded). (SA-W, *natural sea urchins from sand area*; SG-W, *natural sea urchins from seagrass area*; CR-W, *natural sea urchins from coral area*)

On contrary to expectations, any significant differences between the bacterial communities from three food sources cannot be found. These are in consistent with the assumption that the diet of the host is one of the contributing factors that could affect species composition and activity of gut bacteria (Harris, 1993). Similarly, Zhang et al. (2014) suggested that the biochemical composition of the food is also one of the factors that determine the bacterial composition. Also, Prim and Lawrence (1975) reported that the abilities of bacteria from the gut of echinoid correspond to their host diet. The large intestine is the posterior digestive tract which contains not only the complex structure but also the specific chemical condition. Consequently, these are limited to the distributions of bacteria to proliferate and colonize (Harris, 1993). A useful example is the study bacteria diversity from two different parts of digestive tract by Meziti et al. (2007). They found that bacteria from the anterior digestive tracts: pharynx and esophagus, were related to the bacteria samples from the surrounding environment. Whereas, bacteria from the posterior part: stomach and intestine, were presented by many of anaerobic species and sulfate-reducing bacteria groups. According to these reasons, the bacterial taxa, which were found in the present study, may be the groups that colonize as microflora in the sea urchin *Diadema setosum*. Moreover, several previous studies demonstrated that aquatic invertebrates can sustain permanent and consistent flora (Harris, 1993). For examples, giant prawn, *Macrobrachium rosenbergii* (Colorni, 1985); irregular sea urchin, *Echinocardium cordatum* (Thorsen, 1999) and sea urchins (Guerinot and Patriquin, 1981), *Echinus esculentus* (Unkles, 1977).

Therefore, the contribution of the present study has confirmed the intestinal bacterial diversity of marine invertebrate. Moreover, bacterial community among the difference food sources were not statistical significant difference. Also, there are some similarities with other intestinal bacterial community reported from several species of sea urchins. In conclusion, these results may support the possibility of bacterial symbiosis in the intestine of sea urchins.

Diversity of Bacteria Community in the Gut content between the Sea urchin in the Different Conditions

The physiology of the host can influence the shape of gut bacterial community (Harris, 1993). In the present study, the proportion of bacterial composition was slightly changed in the starved condition. Phylum Proteobacteria tended to be increasing while the Bacteroidetes was inverted in the starved group. Therefore, Proteobacteria becomes the most abundance replaced the Bacteriodetes as shown in figure 5-3. This finding is in consistent with the previous study in shrimp *Rimicaris exoculata,* in which the composition of the bacteria of long-starved (72 h) samples had changed from the Deferribacteres to the Gammaproteobacteria (Durand et al., 2009). Moreover, the study in human gut reported that the blooming of Proteobacteria can reflect microbial imbalance. Proteobacteria could be sensitively responded to the environment changes e.g. diets. Therefore, an increasing in numbers of Proteobacteria could lead to the unstable of bacterial community structure. (Shin et al., 2015). Although, the differences between the overall communities were not statistical significant different. This might be due to the fact that the starved sample group have higher variation of the data set compared with the natural group as can be seen in figure 4-10.

The community shift seems to be in consistent with other researches e.g. Atlas et al. (1982). The study found that gut bacterial populations in amphipod, *Boeckosimus affinis* can be shifted. The bacterial community have been changed continuously during the starvation period. Freese and Schink (2011) was found that the symbiotic bacteria in the digestive tract of *Daphnia magna* were reduced in the starvation group. Starvation could be one of the pressures that lead to the gut micro-environment changes (Harris, 1993). Hence, it could conceivably be hypothesized that each bacterial species has dissimilarities responses and toleration abilities. The tolerated group could endure and colonize better than the intolerated group that may die or depopulate during starvation. These could result in

the community changes in terms of proportion. As mentioned earlier, the study by Shin et al. (2015) have shown that Proteobacteria which responds sensitively to the diet could be outgrow than the other gut community. Other hypothesis is that bacteria may be translocated for avoiding the stress and finding more suitable condition. According to the previous studies there are literatures that either support or oppose this hypothesis. For instance, the study in Sprague-Dawley rats reported that over-starvation causes injury of intestinal mucosal and could promote bacterial translocation under high-altitude hypoxic environment (Zhou et al., 2011). However, the study in human by MacFie (2005) indicated that starvation by oneself do not induce bacterial translocation. Therefore, further investigations to resolve these explanations are required.

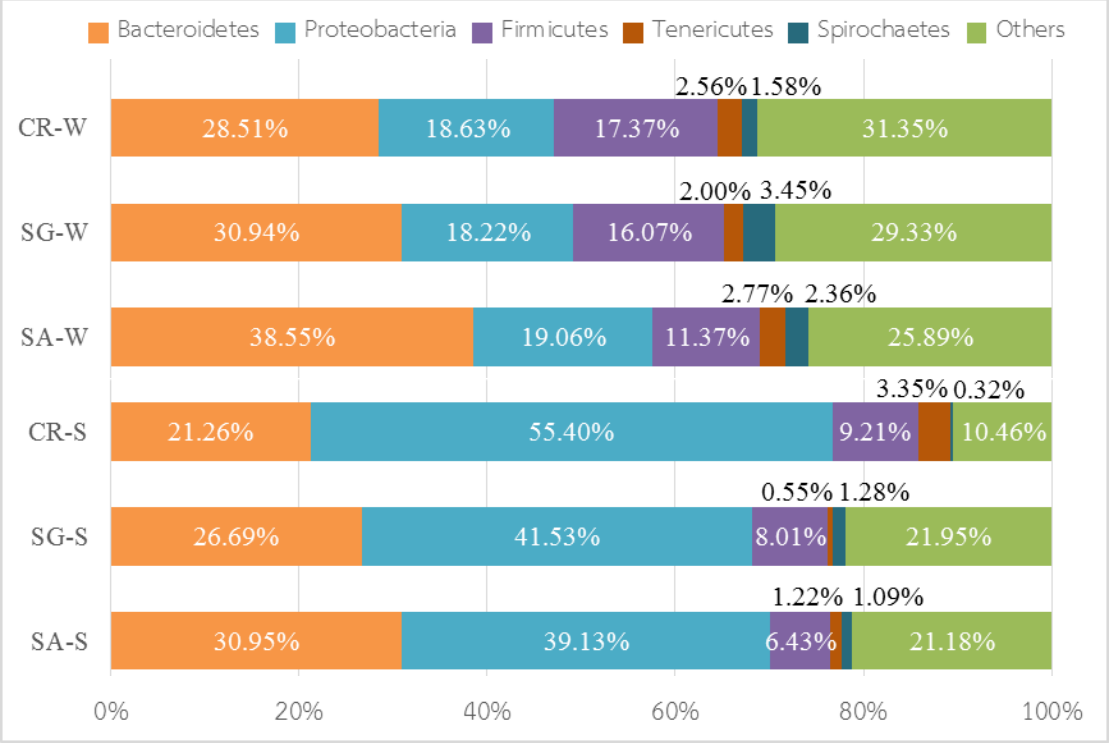

Figure 5-3 the top 5 phyla in the gut contents of the natural and the starved sea urchins across different food sources. (SA-W, *natural sea urchins from sand area*; SG-W, *natural sea urchins from seagrass area*; CR-W, *natural sea urchins from coral area*; SA-S, *starved sea urchins from sand area*; SG-S, *starved sea urchins from seagrass area*; CR-S, *starved sea urchins from coral area*)

Diversity of Bacterial Communities between Gut content and Sediment samples

Bacterial communities between the gut content and the sediment samples were statistical significant different (*p*-value = 0.001. From the gut content samples, Proteobacteria, Bateroidetes, Firmicutes and Fusobacteria were resolved as the most abundant taxa, respectively. At generic level, *Fusibacter*, *Desulfotalea*, *Spirochaeta*, *Synechococcus* and *Vibrio* were detected. In the case of the sediment samples, the most abundant phyla were Proteobacteria, Bateroidetes, Planctomycetes and Cyanobacteria as shown in figure 5-4. At the generic level, *Desulfococcus*, *Synechococcus*, *LCP-26* and *Spirochaeta*, see in figure 5-5. The According to the PCA results in figure 4-12, the two sample groups were distinctly separated with PC1 and PC2 explained total 84.5% of the variations. These results are in agreement with those obtained by Gao et al. (2014). Gut microbial community in sea cucumber, *A. japonicas*, and their surrounding surface sediment, were examined. They found that bacterial communities between the foregut content and the sediment were different. They suggested that selective feeding of *A. japonicas* may lead to the different bacterial communities between the two samples groups. Moreover, the data obtained in the recent bacterial communities studied in the same sea urchin species, *D. setosum* and their ambient sediment from Sichang Island was shown the similarity (Tanrattanapitak N. and Pairohakul S., unpublished data). The results also shown the different bacterial communities between the gut content and the ambient sediment.

These results may be explained by the fact that the intestine itself is the micro-ecosystem which defined by both critical physical and chemical environmental factors (Harris, 1993). These specific conditions can limit bacterial colonization and may cause differences between bacterial community in the gut content samples and the sediment. The study by Meziti et al. (2007) also reported that bacteria in the intestine of sea urchin, *P. lividus* was prevalent by anaerobic species and seemed to be the putative symbiotic bacteria that related to the degradation of the organic compounds. In addition, many studies in

aquatic invertebrate revealed that bacterial populations isolated from gut were differed with the habitat or diet (Harris, 1993; Harris et al., 1991; Unkles, 1977). These studies can support the idea that the resident microflora in the intestine of the animals and the microbes can play the role on their host nutrition (Holland, 2013; Lasker and Giese, 1954; Meziti et al., 2007).

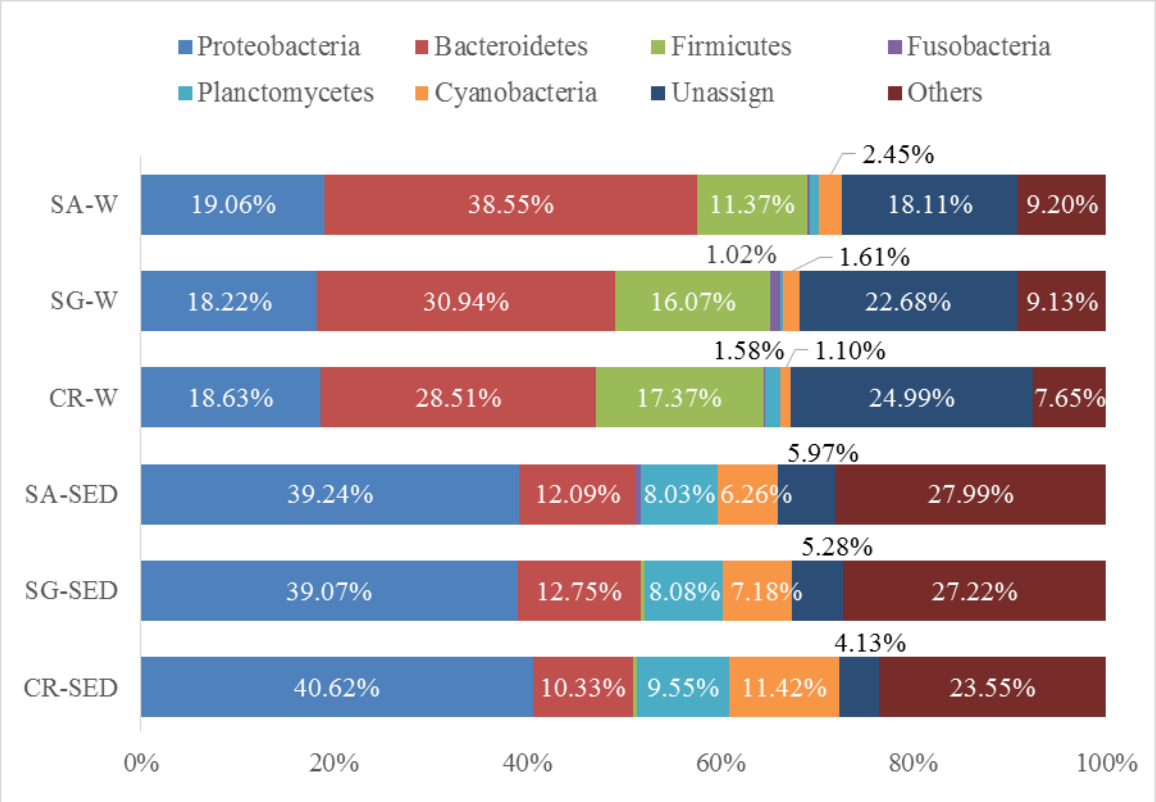

Figure 5-4 the top 5 phyla in the gut contents and the surroundings samples. (SA-W, natural sea urchins from sand area; SG-W, natural sea urchins from seagrass area; CR-W, natural sea urchins from coral area; SA-SED, sediment from sand area; SG-SED, sediment from seagrass area; CR-SED, sediment from coral area)

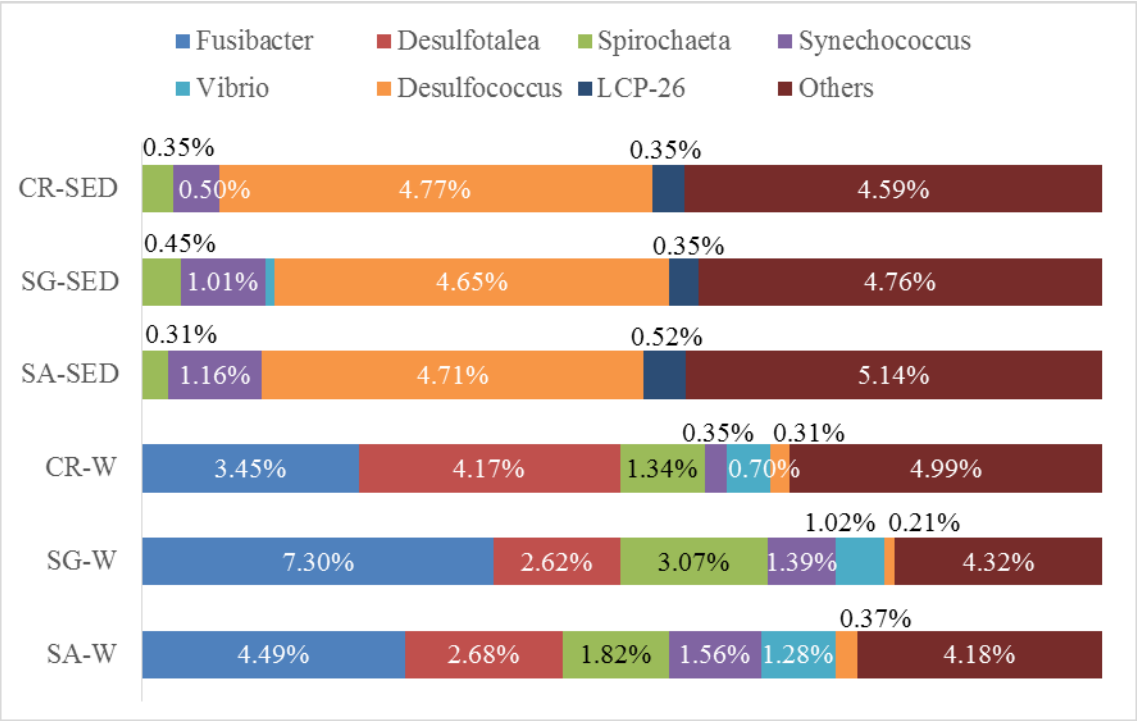

Figure 5-5 the top 5 phyla in the gut contents and the surroundings samples. (SA-W, natural sea urchins from sand area; SG-W, natural sea urchins from seagrass area; CR-W, natural sea urchins from coral area; SA-SED, sediment from sand area; SG-SED, sediment from seagrass area; CR-SED, sediment from coral area)

The Dominant Genus and their Applications

According to the results, *Fusibacter* and *Desulfotalea* were mainly detected in the present study (3.45% to 4.49% and 2.62% to 4.17% respectively). The dominant genera in the present study were differed from the previous research by Hakim et al., (2015); Zhang et al., (2014). One remarkable finding was that *Fusibacter* which hardly found in other species of sea urchin so far but present as the most abundant in the present study. According with the recent study by N. Tanrattanapitak and S. Pairohakul (unpublished data), bacterial community of gut contents of *D. setosum* collected from Sichang Island also present *Fusibacter* as the dominant taxa. *Fusibacter* is a strict anaerobic and halotolerant bacteria that first discovered in the oil producing well (Ravot et al., 1999). It has been detected in the intestine of shrimp *Penaeus monodon*

both from the natural and the captive-raised shrimps (Chaiyapechara et al., 2012; Rungrassamee et al., 2014). This genus can reduce sulfur elements e.g. thiosulfate, sulfur or sulfite to sulfide regarding to the capability of each species (Ben Hania et al., 2012; Fadhlaoui et al., 2015; Ravot et al., 2015; Ravot et al., 1999). The other bacteria involved with sulfur element were also detected, *Desulfotalea*, *Desulfovibrio*, *Desulfococcus*. These can be affiliated with sulfate-reducing bacteria (SRB) group. These bacteria can use sulfate as a terminal electron acceptor for the degradation of organic compounds (Muyzer and Stams, 2008). The SRB has been detected in sea urchin *P. Lividus*. Meziti et al. (2007)reported that the genera *Desulfotalea*, *Desulforhopalus* and *Desulfovibrio* deteced can be found in *P. lividus* intestine. Also the study in irregular sea urchin *Echinocardium cordatum* (Thorsen et al., 2003) is similar to Meziti et al. (2007) study. However, the further studies of the role of these bacteria group to their host biogeochemical physiology are required.

Limitations and Suggestions

As mentioned earlier, there have some limitations in the present study, the anterior digestive part such as pharynx and esophagus cannot be included in the present study as well as the small intestine samples. Due to the fact that gut contents in small intestine are barely exist especially in the starved condition; therefore, the amplification cannot meet the requirements for 3 replications. However, further repetitions sampling are not practicable because all ambient conditions may have changed. This complement is the supporting data to understand the diversity of bacterial community in the intestine as well as the association with surrounding environment.

In the present study, the effect of food sources from the natural samples was conducted in order to represent the natural bacterial community of the sea urchin in the nature. Nevertheless, this may not actually prove the effect of the food sources effectively because each food source in nature is not distinctly separated.

They may contains more than one components; therefore, in the case of diet effect study, controlled designed laboratory experiment may be an alternative way to study about the effect of diets. However, laboratory experiment is a trade-off by unnatural bacterial communities due to laboratory conditions may also affect bacterial diversity. Therefore, natural and laboratory-reared animals may have different bacterial communities (Nelson et al., 2010).

In conclusion, the present study is provide the intestinal bacterial diversity of sea urchin *Diadema setosum*. The community diversity of this tropical crucial sea urchin was revealed. Although, statistical significant difference between bacterial communities of sea urchin collected from three different food sources were not be found. These finding will serve as a basic knowledge for further intestinal bacterial studies. Nevertheless, further studies are still required in order to enhance understanding in terms of association between intestinal bacteria, diets and their host physiology.

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APPENDIX

VITA

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