CHAPTER 1 INTRODUCTION

1.1 Background and Rationale

Asia, including Thailand, China, Indonesia, Vietnam, and other countries, is a major source of shrimp production in the world. Thailand has been a major shrimp exporter for over 15 years [1] and is a current leader in shrimp production, providing more than 500,000 tons in 2012, which accounts for 15 % of the world production [2]. Thailand's shrimp production is valued at approximately US $3 billion in 2012, and the current major shrimp markets include the USA (38 %), Japan (23 %) and the European Union (15 %) [3]. For shrimp cultivation in Thailand, there has been a shift from *Penaeus monodon*, black tiger shrimp, to *Penaeus vannamei*, white shrimp, with a production ratio of 1:99 since 2008 [4]. Most farmers raise *P. vannamei* because of higher profits with a high production rate, low mortality rate, and low infection rate, especially under intensive culture conditions [5]. Good shrimp quality from hatcheries, including production of postlarvae (PL), supports the high production rate of the cultivation from PL to adult shrimps [4]. In the *P. vannamei* cultivation system, the larval diet has been commercialized for all stages of shrimp larviculture, from Zoea to PL stages. The diets are used in commercial hatcheries with varying levels of live food replacement. After the eggs hatch, Nauplii do not feed, but live on their yolk reserves. Zoea, Mysis, and early PL (PL1–PL2) feed on plankton and live zooplankton, whereas the later stages (PL1–2 to adult shrimps) are fed dry diets [6], [7]. Live diets present advantages such as high stability in water, which results in a lower nutrient release compared with pellet diets [8]. Therefore, there are fewer water quality problems during Nauplii, Zoea, Mysis, and early PL cultivation [6], [7]. Water exchange is an important factor in controlling water quality, including nitrogenous waste and disease [9], during the cultivation of PL2–PL12 and also in maintaining an environmentally friendly system. The water discharge from shrimp cultivation affects the environmental water quality, including light penetration, dissolved oxygen concentration, and the diversity of the benthic zone [10]. Management methods, such as a reduction in water or zero discharge rates, have been emphasized with two main practical approaches involving either bacteria with dissimilation process into gases or plants with assimilation process into biomass [10]. As biofilters, bacteria are highly effective, especially in aquaculture recirculation systems [11], [12]. However, conventional technology consists of many

complicated factors, such as oxygen concentration, particulate organic matter, and turbulence level [13]. Microalgae cultivation on wastewaters for biomass production, as well as the removal of organic and inorganic pollutants [14] has been widely studied [15]. A previous report used suspended cyanobacterium *Spirulina* for water quality control in shrimp culturing ponds [16]. However, harvesting the algal cells from the culture system was troublesome. Algal cells that are not removed produce nitrogen compounds that are released back into the water. Moreover, high microalgae concentrations can cause dissolved oxygen depletion during the night due to high respiration rates. Recent research efforts have increasingly focused on the use of non-suspended algae, either attached or immobilized, to avoid harvesting problems [17] and also increase growth and survival rates of aquaculture PL [18]. We previously constructed immobilized *Spirulina* films, or ‘‘*Spirulina* mats,’’ from *Spirulina* cells that adhere to fibrous polyester sheets and are non-toxic, non-reactive, cheap, stable in long-term cultures, and available in hatcheries [19], [20]. The mats showed efficiency of nutrient removal by more than 80 %. In addition, *Spirulina* has been used as supplemental feed due to its rich sources of protein, essential amino acids, vitamins, minerals, essential fatty acids (particularly gamma-linolenic acid), and antioxidant pigments, such as carotenoids and phycocyanin [21]. The microbial mat was preferentially assimilated and supported as a source of C and N for high-level growth and survival rate of shrimp [18]. In this study, we evaluated the use of *Spirulina* mat co-culture with *P. vannamei* PL for water quality control and aquaculture management. The water quality, growth performance of shrimp PL, and bacterial communities in the mat and water were investigated in a closed re-circulating system and in grow-out pond. Moreover, the microbial activity test and comparison of microbial community between in hatchery and grow-out pond were also studied.

1.2 Objective of Study

To study dynamic of microbial community in shrimp hatchery and grow-out pond and their relationship with the shrimp cultivation system

1.3 Scope of Study

1.3.1 To study dynamic of bacterial community in shrimp hatchery and grow-out pond, by Denaturing Gradient Gel Electrophoresis (DGGE).

1.3.2 To study the correlation between the bacterial community dynamic and the cultivation system, including quality of water and shrimp.

1.3.3 To compare the microbial community in the shrimp cultivation system between the hatchery and the grow-out pond.

1.4 Expected Output/Outcome

1.4.1 Information/knowledge of the correlation between microbial communities and water/shrimp quality in the shrimp hatchery and grow-out ponds for further application/cultivation improvement.

1.4.2 Development of microbial/*Spirulina* mats that could be applied for water quality control in aquaculture system for sustainable environmental friendly development, *i.e*. reducing of the water exchanging rate.

CHAPTER 2 THEORY AND LITERATURE REVIEWS

2.1 Shrimp

Shrimp and Prawn, the former sometimes is applied to smaller species whereas the latter is more often used for larger forms [22], are widespread and abundant. These species play important role in commercial food industry for human consumption. Significant shrimp farming was initiated in 1970s, particularly in China, before accelerated during the 1980s as demand for shrimp exceeded supply [23] and exceeded the capture of wild shrimp in year 2007 (Based on FishStat database) [24]. The commercial shrimp species support an industry worth 50 billion dollars a year [23]. The total commercial production of shrimp was nearly 7 million tonnes in 2010 (Table 2.1) [25].

2.1.1 Shrimp Cultivation in Thailand

Black tiger shrimp (*Penaeus monodon*) was very famous in Thailand, whereas white shrimp (*Penaeus vannamei*) at that time had low production yield and unstable supply [26]. Since 1980s, however, black tiger shrimp farming failure was occurred in Samut Prakan and Samut Songkram provinces, Thailand, because of the rapid and unplanned expansion in the farming area with little attention to proper site selection, pond construction, water supply and pond management [27]. Consequently, Thai shrimp culture has been currently suffering serious losses due to infectious diseases [28], including those caused by yellow-head baculovirus, baculovirus (white spot), and luminous bacterium (*Vibrio harveyi*) [29].

After many years of developing the white shrimp raising strategy, Thai farmers now can control the process of white shrimp cultivation. They have been succeeded in size-increasing white shrimp, which is larger than black tiger shrimp and free from diseases. Moreover, white shrimp hatcheries can bring high output tonnes/ha, which is 2-3 times more profit than that of black tiger hatcheries. Therefore, nowadays white shrimp gain more market share with 10-15% cheaper than black tiger shrimp [26], (Table 2.1).

Table 2.1 List of commercial shrimp species (modified form Khan *et al.* [25])

Common name	Scientific name	Description	Production rate* (Thousand tonnes)		
			Wild	Farmed	Total
White shrimp	*Penaeus vannamei*	The most widely farmed species of shrimp, developed by breeding programs as selecting for increased growth and disease resistance [5].	1	2721	2722
Giant tiger prawn	*Penaeus monodon*	The shrimp life cycle comprised of an off-shore planktonic larval phase [30], an estuarine post-larvae and juvenile phase, and an inshore adult and spawning phase [31].	210	782	992
Akiami paste shrimp	*Acetes japonicus*	The main uses are as fermented food or as a dry product [32].	574	nd	574
Southern rough shrimp	*Trachysalambria curvirostris*	The species occurs in the coastal waters of Japan, Korea, China, Taiwan, Thailand, and eastern Australia [33]. It is exploited mainly by bottom-trawl fisheries in coastal regions [34].	294	nd	294
Fleshy prawn	*Fenneropenaeus chinensis*	This shrimp is one of the most economically valuable and widely cultured species in China. During the last decade its development has been seriously affected on a regular basis by the outbreak of viral and bacterial diseases [28].	108	45	153
Banana prawn	*Fenneropenaeus merguiensis*	This shrimp is distinguished from the other alien penaeid prawns in the Mediterranean [35].	93	20	113
Northern prawn	*Pandalus borealis*	This shrimp has provided and important source of income for New England Fisherman [36].	361	nd	361
Total			3129	3788	6917

Remark: *according to year 2010; nd, no data

2.1.2 Shrimp Life Cycle

The shrimp life cycle (Figure 2.1) begin from egg that female penaeid shrimp lays as many as half to approximately three quarter of a million eggs per spawning. After hatching, the sequential early larval stages are nauplius, protozoea, mysis and early postlarvae [37], [38]. The first larval stage, nauplius, is unsegmented body which is pyriform in shape possesses three pairs of appendages. The larvae undergo six moltings within 50 hours into a protozoea. The body becomes elongated with a distinct cephalothorax. The early protozoea stage has a pair of protruded compound eyes, the next stage is characterized by the presence of a rostrum and the late protozoea stage has a pair of uropods. After 4–6 days, the protozoea finally metamorphoses into a mysis. At this stage, the larvae assume the form of a juvenile shrimp at which the pleopods are developed. At this stage, tiny protrusion known as pleobases are seen on the ventral side of the abdominal segments. The next stage is marked by the development of first segment of the pleopods development. The mysis remain drifting in the water column until they metamorphose into post larvae within 10–12 days. At post larval stages, the pleopods become fully developed and functional. The shrimps grow very fast in terms of size and are able to swim freely although early postlarvae are still planktonic in offshore waters. The shrimps spend their juvenile, adolescent and sub-adult stages in estuarine waters. Then, they gradually move toward deeper water as growing and eventually returning to offshore water when they attain sexual maturity.

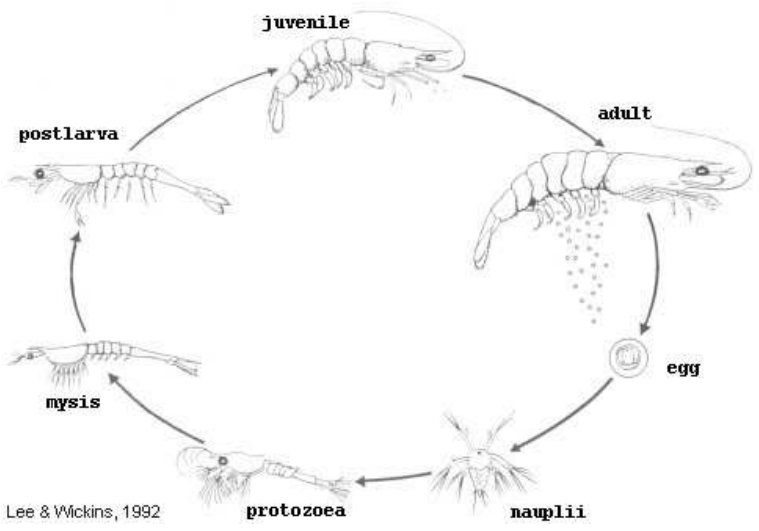

Figure 2.1 Shrimp life cycle [38]

2.2 Type of Shrimp Cultivation

2.2.1 Extensive Aquaculture

Extensive aquaculture is the most similar to natural production in the sea. Seawater (or estuarine water) containing larvae or post-larvae is captured in an impoundment. Nutrients and dissolved oxygen in the seawater are typically not supplemented with fertilizers and aeration, so that less than 10 post-larvae per cubic meter (PL m^{-3}) are sustainable. Constant or periodic replacement of water in these impoundments generally removes excess organic matter and ammonia and replenishes nutrients. Disease may be brought in the wild shrimp or replacement water. Conversion of mangrove and other swampland to shrimp ponds may cause low pH. Adjust to 8.2-8.7 as needed. Dissolved oxygen should be maintained at > 5 mg L^{-1} [39].

2.2.2 Semi-intensive Aquaculture

Semi-intensive aquaculture involves stocking the shrimp pond with captured or hatchery produced post-larvae (10-50 PL m^{-3}). Salinity may be controlled by addition of salt to fresh water (in inland production) with salinity of 20-39 parts per thousand (ppt) typically used. Fertilization with commercial fertilizers or heat-treated chicken manure is often used to encourage algal growth as a food supplement for the shrimp. In order that, commercial fertilizers are easier to use (to control nitrogen and phosphorus concentrations) and do not introduce coliforms and other undesirable bacteria. Dissolved oxygen should be maintained at > 5 mg L^{-1}. Salinity of 20 ppt is preferred for maximal growth of microbes. When nutrients are present in excess, ammonia concentrations may inhibit growth of shrimp, algae may over-grow and produce excessive dissolved oxygen, cyanobacteria may bloom causing off-flavor or odor [39].

2.2.3 Intensive Aquaculture

Intensive aquaculture uses high stocking rates of >50 PL m^{-2}. Water is usually completely contained to prevent disease introduction. Microbial growth is used as a food supplement instead of algae. Therefore, molasses or other cheap organic matter is added. Due to high shrimp density, nutrient concentration, pH, organic matter concentration and dissolved oxygen should be carefully maintained. Chemical oxygen demand (COD), ammonium, nitrite, nitrate, and phosphate are recommended tests to monitor water quality.

2.3 Factors affecting Shrimp Quality

Water quality is a key factor for shrimp quality control. The physical and biological management in shrimp ponds are basically water quality management to keep the ponds in optimal conditions for the growth of shrimp. This is very important for preventing the shrimp from experiencing stress that can make the shrimp susceptible to various diseases.

2.3.1 Water Quality

2.3.1.1 pH and nitrogen - The normal pH of seawater ranges from 7.5 to 8.5 [39]. The pH value is a key indicator of changes in the concentrations of ionized and un-ionized ammonia. The NH_3/NH_4^+ ratio in water is pH dependent. If pH value is high, unionized ammonia (NH_3) which is toxic to larvae increases. Ionized ammonia (NH_4^+) is not toxic because it is unable to pass through the gill membrane of the larvae. Safe ammonia concentrations in water should not exceed 1.5 ppm for NH_4^+ and 0.1 ppm for NH_3 [39].

2.3.1.2 Salinity - Biologically most penaied shrimps do not breed in brackish water. Mating, spawning and hatching of eggs take place in the open sea. Salinity in spawning grounds normally ranges from 30 to 36 ppt [40]. Seawater salinity in spawning tanks should be maintained at 30 - 32 ppt to ensure good hatching rates [41]. Moreover, low salinity affects larval growth during the first 15 days of rearing [41].

2.3.1.3 Dissolved oxygen - Dissolved oxygen is a critical factor in larval rearing. High mortalities can occur if aeration stops even for only one hour [39].

2.3.1.4 Temperature - Temperature directly affects the metabolic system of any species. In penaeid shrimps, eggs do not hatch at temperatures lower than 24 ^oC [41]. Larvae usually grow and moult faster at higher temperature. The optimum temperature is 26-31 ^oC. Below this level, larvae do not grow well and moulting may be delayed. The protozoea of *P.monodon*, for instance, molt to mysis stage within 4 days at temperatures ranging from 28 ^oC to 31 ^oC. However, moulting takes 6 days when temperature drops to 24 - 26 ^oC [41]. Slightly increase in water temperature above threshold may be lethal in the tropical species. Gradual variation in temperature

throughout the day is not critical. However, sudden changes, even as lower as 2 ^{o}C causing of high mortalities due to stress and temperature shock [41].

2.3.2 Microbial Community during Shrimp Cultivation

In shrimp cultivation system, nutrient (particularly nitrogen and phosphorus) concentration in the water is high, which results as a growth medium for heterotrophic or opportunistic bacteria. Many aquaculture diseases are implied by opportunistic pathogens. The number of marine pathogens has been observed in particles in the sea, and the microflora of marine seaweed, invertebrates, and plankton. Whereas *Aeromonas salmonicida* was found in water, sediments, seaweeds, and various invertebrates [42], *Vibrio anguillarum* was found in seawater that particles were present [43]**.** However, there are some beneficial bacteria that have positive effect on shrimp health, for example, *Lactobacillus plantarum*, lactic acid bacterium from the aquatic intestinal [43] which has been extensively used as probiotics [44]. They produce growth inhibiting factors towards various pathogenic *Vibrio* spp. [43]. The lactic acid bacteria have been fed to rotifers used as food for turbot larvae. This bacteria inhibited pathogens in rotifer cultures, and also resulted in a significant increase in the weight of turbot larvae [45]. The heterotrophic bacterial community is basically dominated by Alpha-proteobacteria and Gamma-proteobacteria, for example *Pseudomonas* and *Vibrio* which were the most abundant. Both genera are typical of the marine environment. *Pseudomonas* comprises non-fermentative, gram-negative bacteria of great metabolic diversity and includes biofilm-forming species. This bacteria is able to produce bioactive substances and antibiotics [46]. In addition to heterotrophic and nitrifying bacteria, the presence of some other groups mostly linked to the nitrogen cycle include denitrifying heterotrophs (*Pseudomonas* sp., *Aquaspirillum* sp., and various alpha-Proteobacteria), sulfide-dependent autotrophic denitrifiers (mainly belonging to Bacteroidetes, Proteobacteria, Furmicutes and Synergistetes). Moreover, bacteria related to the genera *Paracoccus*, *Aminobacter*, *Alcaligenes*, *Azoarcus* and *Desulfovibrio* that able to perform dissimilatory nitrate reduction to ammonia were also found, as well as the presence of the anammox bacteria, *Planctomycetes*, which are able to oxidize ammonium and nitrate into nitrogen gas, and bypass the denitrification reaction [47].

2.4 General Problems in Shrimp Cultivation

During shrimp cultivation, there are several parameters involving water and shrimp quality, *i.e.* ammonia and nitrite concentration and toxicity.

2.4.1 Ammonia and Nitrite

In shrimp feeding, nearly 10% of protein in the feed is converted into ammonia. There are a number of different pathways involving the conversion from proteins to ammonia (Figure 2.2). The eaten feed protein may be assimilated, pass through the gut and finally be excreted in the feces. Though the feed protein is converted into shrimp protein (e.g., muscle), but ammonia is produced as a by-product and excreted by through their gills. Additionally, there are heterotrophic bacteria that metabolize the uneaten feed proteins and fecal material and also excrete ammonia as a by-product of the protein metabolism [13]. Nitrifying bacteria utilize inorganic nitrogen as nitrogen sources for protein synthesis. *Nitrosomonas* and *Nitrobacter* are the two most important genera of nitrifying bacteria. *Nitrosomonas* uses ammonia as its source of nitrogen and excretes nitrite (NO_2^-). *Nitrobacter* uses nitrite as its source of nitrogen and excretes nitrate (NO_3^-). All three forms inorganic nitrogen (ammonia, nitrite, and nitrate) are present in the water at any given time [39].

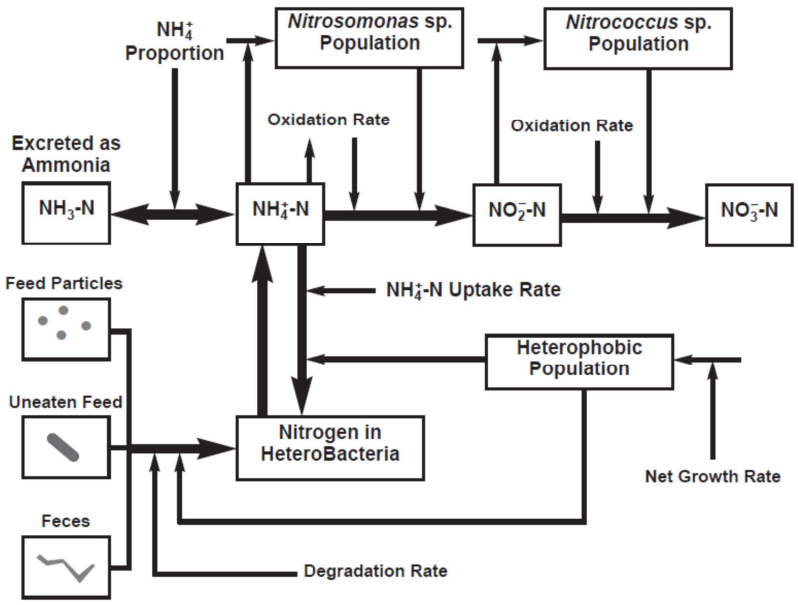

Figure 2.2 Bacterially mediated processes in a shrimp culture system [13]

2.4.2 Ammonia and Nitrite Toxicity

Ammonia exists in two different forms in the water: unionized ammonia (NH_3) and ionized ammonia (NH_4^+). Both of these forms of ammonia are generally present in the water simultaneously. The combined total concentration of unionized and ionized ammonia is named Total Ammonia Nitrogen (TAN) concentration. TAN is what is measured by most ammonia test kits. The fraction of TAN in the unionized form of ammonia is dependent upon the pH and temperature of the water. At a pH of 7.0 most of the TAN is in the ionized form. The fraction of TAN in the unionized form increases with both temperature and pH. At a pH of 9.0 about 50% of the TAN is in the unionized form. The unionized form of ammonia is highly toxic to shrimp, while ionized ammonia is relatively non-toxic. The unionized ammonia 48-hr LC50 for juvenile shrimp (the concentration that will kill 50% of the shrimp in 48 hours) was 1.26 mg NH_3-N L^{-1} [48]. Concentrations below 0.1 mg L^{-1} of unionized ammonia are safe, that these concentrations are non-lethal. However, chronic exposure to concentrations above 0.03 mg NH_3 L^{-1} will produce a number of non-lethal effects on the shrimp, including reduced growth rates, increased feed conversion, swollen gills, reduced tolerance to low dissolved oxygen conditions, and decreased resistance to disease.

Nitrite is also toxic to shrimp. The 96-hr LC50 of nitrite for shrimp is 3.6 mg NO_2^- L^{-1} [49]. The mechanism for nitrite toxicity in shrimp is not as well understood as it is in fish. In fish, high nitrite levels interfere with the uptake of oxygen by hemoglobin in the blood of fish and can cause the fish to die. Shrimp have a different blood pigment, hemocyanin, which transports oxygen to the tissues. It is likely that the mechanism for nitrite toxicity is similar to the mechanism in fish.

2.5 Mechanism for Nitrogen Controlling in Shrimp Cultivation System

There are 3 basic strategies to control ammonia: water exchange, plant uptake, and nitrification.

2.5.1 Water exchange

Water exchange is a conventional method, but effective for ammonia controlling. During water exchange is an effective way of rapidly reducing ammonia levels in case of emergency, it should not be the primary strategy for ammonia concentration controlling in a recirculating system. The very high exchange rates would be required to adequately control ammonia levels in a high density system. In addition, exchanging

large volumes of water would greatly increase the required size of the post-treatment system for treating effluent from the culture system. Jackson *et al*. [50] reported that exchanged water consist of dissolved organic nitrogen (DON), comprising 37–43% of total nitrogen (TN); and total ammonia nitrogen (TAN), comprising 12 – 21% of TN.

2.5.2 Nitrification

Most recirculating aquaculture systems depend on nitrification processes to control ammonia and nitrite levels. Nitrification is a two-step process in which ammonia is converted first to nitrite by *Nitrosomonas* sp. and then to nitrate by *Nitrobacter* sp. [39]. *Nitrosomonas* and *Nitrobacter* are normally present in all aquaculture systems, growing on surfaces such as tank walls and the inside walls of pipes [39]. The numbers of these bacteria can be greatly enhanced, however, by the addition of a biofilter to a system. A biofilter is simply a device that provides vast amounts of surface area on which nitrifying bacteria can grow, and a chemical environment suitable for nitrification to take place [51]. There are many different types of biofilter media and biofilters. There are several conditions that must be present for the nitrifying bacteria to be able to efficiently carry out the process of nitrification. Nitrification is an oxidative process, so there must be an adequate supply of oxygen. The minimum oxygen level at which nitrification can efficiently proceed is about 2 mg L^{-1} [39]. A stable pH in the range from 7.0 - 9.0 should be provided [39]. There are many different species of *Nitrosomonas* and *Nitrobacter*. Each species has its own optimum pH range, which is narrower than the range given above. A stable pH will allow the dominant species to function near its optimum pH. Bicarbonate ion is required by the nitrifying bacteria as a source of carbon. Nitrification consumes 7.14 g of bicarbonate ion for each gram of ammonia that is converted to nitrate [52]. However, it is difficult to give an exact minimum bicarbonate level that must be maintained, because bicarbonate chemistry in the water is very complex. To be on the safe side, bicarbonate alkalinity should be maintained above 50 mg L^{-1} as calcium carbonate. Light levels have also been found to have an important influence on the nitrifying bacteria. Olson R.J. [53] found that nitrifying bacteria are inhibited at light levels less than 1% of full sunlight intensity, and that complete darkness was superior to diurnal cycling of light regimes. Stable salinities are also important for efficient biofilter operation. Akai *et al.* [54] found that rapid changes in salinity significantly slowed the growth rate of nitrifying bacteria.

Nitrification occurs most efficiently when the bacteria are arrayed in a thin film on the surface of some type of media. Over time, older bacteria die and are replaced by newer bacteria. If the dead bacteria are allowed to accumulate on the surface, heterotrophic bacteria will attach to the surface and begin to decompose the dead nitrifying bacteria. These heterotrophic bacteria begin to compete with the nitrifying bacteria for space and oxygen. Solid particulates may also adhere to the surface of the biofilter media, smothering the nitrifying bacteria and providing additional organic material for the heterotrophic bacteria to grow on. In order to maintain a healthy, thin film of nitrifying bacteria on the biofilter media, some type of shearing force must be applied to the biofilter media to slough off the dead bacteria and to keep organic material from accumulating on the surfaces of the media. The shearing force may be the hydraulic force of water flowing over the media surface or abrasion caused by collisions between individual units of biofilter media.

2.5.3 Phytoplankton Uptake

Phytoplankton, includes microalgae are able to extract inorganic nitrogen directly from the water and use it as a nitrogen source for protein synthesis and growth. In aquaculture systems with good exposure to sunlight, the nutrients from the feed, including nitrogen and phosphorus, will enhance phytoplankton blooms, consisting of many different species of algae. These algae will extract inorganic nitrogen from the water, and maintain lower levels of ammonia and nitrite. Once algal bloom is established, the algal death rate is as about the same rate as new cells forming. The dead cells are decomposed by heterotrophic bacteria, which will break down the algal proteins and excrete ammonia. If the algal bloom crashes, there may be a rapid release of ammonia into the system as the algae undergo bacterial decomposition. During the day, algae extract carbon dioxide from the water to utilize for photosynthesis. As carbon dioxide concentrations in the water drop, pH rises. In systems with dense algae blooms, pH levels of 9.0 or greater are not uncommon [39]. At this pH level, a very high percentage of the TAN will be in the toxic, unionized form. Even relatively low concentrations of TAN may be lethal to the shrimp at high pH.

Application of using suspended *Spirulina platensis,* a cyanobacterium, for water quality control in shrimp culturing ponds was reported [55]. The algal cells showed

significantly decreased (P<0.05) inorganic nitrogen concentrations (NH_4^+, NO_2^- and NO_3^-) in the system, compared with control condition (without *S. platensis*) of which ammonium and nitrite concentrations ranged from 0.5 to 0.6 mg L^{-1}, while nitrate concentrations ranged from 16 to 18 mg L^{-1} by day 44. However, harvesting the algal cells from the culture system was troublesome. Algal cells that are not removed produce nitrogen compounds that are released back into the water. Moreover, high microalgae concentrations can cause dissolved oxygen depletion during the night due to high respiration rates. Recent research efforts have increasingly focused on the use of non-suspended algae, either attached or immobilized, to avoid harvesting problems [17] and also increase growth and survival rates of aquaculture PL [18].

2.6 Removal of Nutrients by Immobilized Microalgae

Microalgae are immobilized in various polymers for different biotechnological purposes, such as morphology studies, production of fine chemicals, energy production, and wastewater treatment [56], Immobilization is specifically important in wastewater treatment because it solves the inherent problem of biomass produced by suspended microalgae in the wastewater, as explained above [57]. Many examples show capacity of immobilized microalgae and cyanobacteria to remove nutrients from wastewater. *Chlorella vulgaris*, immobilized in carrageenan and alginate was used to treat primary domestic wastewater. Although algal cells in both kinds of polymer beads grew more slowly than suspended cells, the immobilized cells were more metabolically active. Over 95% of ammonium and 99% of phosphates were removed from wastewater in 3 days. This was higher efficiency than suspended cells that removed only 50% of nitrogen and phosphorus in the same time frame [58]. *Scenedesmus* spp. cells, immobilized in chitosan, showed high viability after the immobilization process. One of the immobilized strains of *Scenedesmus* sp. demonstrated a higher growth rate than its free-living counterpart. The immobilized cells accomplished a removal of 70% nitrate and 94% phosphate within 12 h of incubation compared to that of the free-living cells (20% nitrate and 30% phosphate removal) within 36 h of treatment. However, similar to the previous study, blank chitosan beads were responsible for removing up to 20% nitrate and 60% phosphate [59]. Immobilized cells of *Dunaliella salina* show a better uptake capacity of nitrate, ammonium, and phosphate than free-living cells [60]. The cyanobacterium *Phormidium laminosum*, immobilized on polymer foams, was

demonstrated to have potential value for removing nitrates in a continuous-flow system [61]. *Spirulina maxima* immobilized in polymers enhanced removal of ammonium from swine waste. More than 90% of ammonium was removed [62]. Immobilization of *Chorella vulgaris*, *C. kessleri*, and *Scenedesmus quadricauda* in several polymers was used to remove nutrients from raw sewage and pretreated cattle manure. Immobilization with alginate pellets of *C. vulgaris* and *C. kessleri* performed best with raw sewage under natural light, but not with pretreated cattle manure because the dark color of the latter substrate probably had a negative effect on the photosynthetic microorganisms [63]. Removing nutrients can be accomplished at high temperature in immobilized systems. Removal of nitrate and phosphate ions from secondarily treated sewage with a thermophilic cyanobacterium *Phormidium laminosum*, immobilized on hollow cellulose fibers in a tubular photobioreactor at 43 ^{o}C was achieved [64]. Similarly, removal of ammonium from wastewater at high temperatures was recently demonstrated for a heat and intense sunlight-tolerant strain of *C. sorokiniana*, after an acclimation period [65]. Small technical parameters are often importance for success in this technologically oriented field. The physical shape of the immobilization system's beads or screens should be considered. The capacity of *Scenedesmus bicellularis* to treat municipal wastewater was compared under different conditions: free cells with air bubbling; cells immobilized in alginate beads, and cells immobilized on alginate screens, all conditioned in synthetic culture medium depleted in nitrogen and phosphorus at relatively low temperatures of 18 ^{o}C [66].

2.7 Molecular Techniques for Microbial Community Analysis

Conventional microbiological techniques, based on isolation of pure cultures and morphological, metabolic, biochemical and genetic assays, have provided extensive information on the biodiversity of microbial communities in natural and engineering systems [67]. However, the drawbacks of the existing conventional methods, such as incomplete knowledge about their physiological needs and symbiotic relations, which are abundant in nature, make it impossible to obtain pure cultures of most microorganisms in natural environments. Moreover, most culture media tend to favor the growth of certain groups of microorganisms, whereas others that are important in the original sample do not proliferate. The techniques are based on the RNA of the small ribosomal subunit (16S rRNA for prokaryotes) or their corresponding genes was chosen because of its universality and abundance in all living beings and the fact that it

is a highly conserved molecule throughout evolution although bears some highly variable regions. These features allow comparison of organisms within the same domain, as well as differentiation of strains of the same species. Moreover, the gene sequence is sufficiently long to generate statistically relevant data and can be easily sequenced with current technology. For the first time it is possible to survey the biodiversity of a natural habitat in a relatively simple, but complete way.

2.7.1 Cloning of 16S rDNA

Cloning and sequencing of 16S rRNA gene is the most widely used in the field of microbial ecology. Extraction, amplification and cloning of the 16S rRNA genes are followed by sequencing, identification and affiliation of the isolated clone with the aid of phylogenetic software (Fig. 2.3) [68]. Amplicons generated from pure culture of bacteria could be sequenced directly, whereas the genomic DNA extracts from microbial communities include more steps of cloning, in order to isolate the different copies of 16S rDNA from the mixture. However, cloning is time consuming for analyzing larger sets of samples. Examples of using the cloning for phylogenetic affiliation of microbial communities: determination of green non-sulfur bacteria in filamentous bacteria in granular sludge [69]; sulfate reducing bacteria in a biofilm [70]; microbial composition and structure of a rotating biological contactor biofilm for the treatment of ammonium-contaminated wastewaters [71].

2.7.2 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is based on the different mobility on a gel of denatured DNA-fragments of the same size but with different nucleic acid sequences. The generating band patterns directly reflect the genetic biodiversity of the sample. The number of bands corresponds to the number of dominant species. Coupled with sequencing and phylogenetic analysis of the bands show a good overview of the composition of a microbial community (Figure 2.4) [68].

Muyzer *et al.* [72] reviewed on the potential, problems and applications of DGGE, including the first use in a study of complex microbial populations [72], characterization of a wide array of habitats, *i.e.* soil, bacterioplankton, hot springs, continental water, etc.

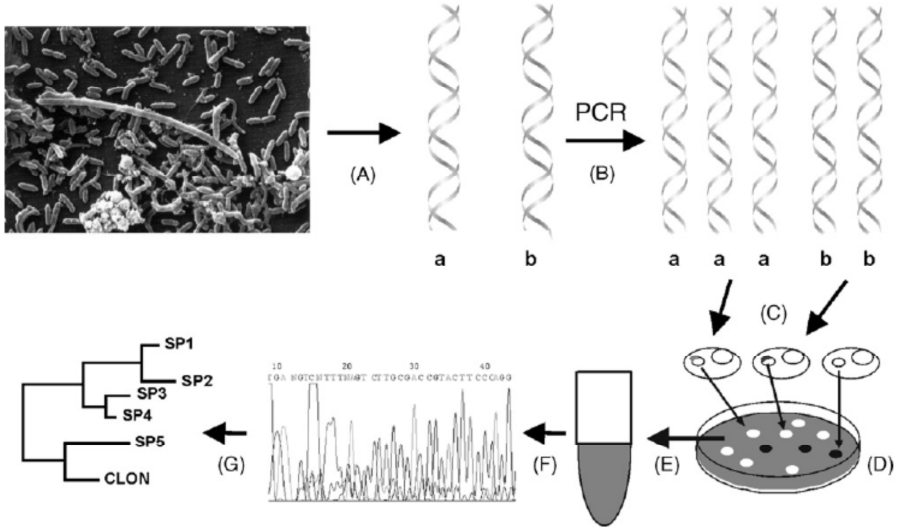

Figure 2.3 Outline of the cloning procedure for studying a microbial community

(A) direct nucleic acid extraction; (B) amplification of 16S rRNA by PCR; (C) cloning of the PCR products obtained from high copy number plasmid in the *E. coli* transformants; (D) selection of transformed clones; (E) extraction of the plasmid DNA; (F) sequencing of the cloned gene, creating a clone library; (G) determination of the phylogenetic affiliation of the organisms by dedicated computer programs. Modified from Sanz *et al.*[103].

DGGE has been used for the evaluation of microbial diversity from aquaculture for many objectives. As bacterial communities characterization in culturing system, there were high similarities in the community composition between different water samples and between larvae samples collected in scallop larvae (*Pecten maximus*) cultivation [73]. Liu *et al.* [74] reported that the most commonly reported genera of gut microflora in aquatic invertebrates are *Vibrio*, *Pseudomonas*, *Flavobacterium*, Micrococcus and *Aeromonas* sp. As disease investigation and protection, Reid *et al.* [75] reported that three pathogens, *Vibrio anguillarum*, *V. logei* and *V. splendidus* were found to be capable of causing mortalities in larval cod. In wastewater treatment system, Zhang *et al.* [76] used statistical methods, e.g. cluster analysis, to determine the similarity of bacterial and archaeal populations found in a UASB reactor for treating municipal wastewaters. In an ethylbenzene-degrading bacterial consortium, Nakagawa *et al.* [77] monitored changes in enrichment cultures under anaerobic, sulfate reducing conditions. By monitoring the predominant bacterial species over a period of 127 days, they identified a dominant bacterium that was present throughout the whole incubation period and most likely to be the microorganism responsible for ethylbenzene degradation.

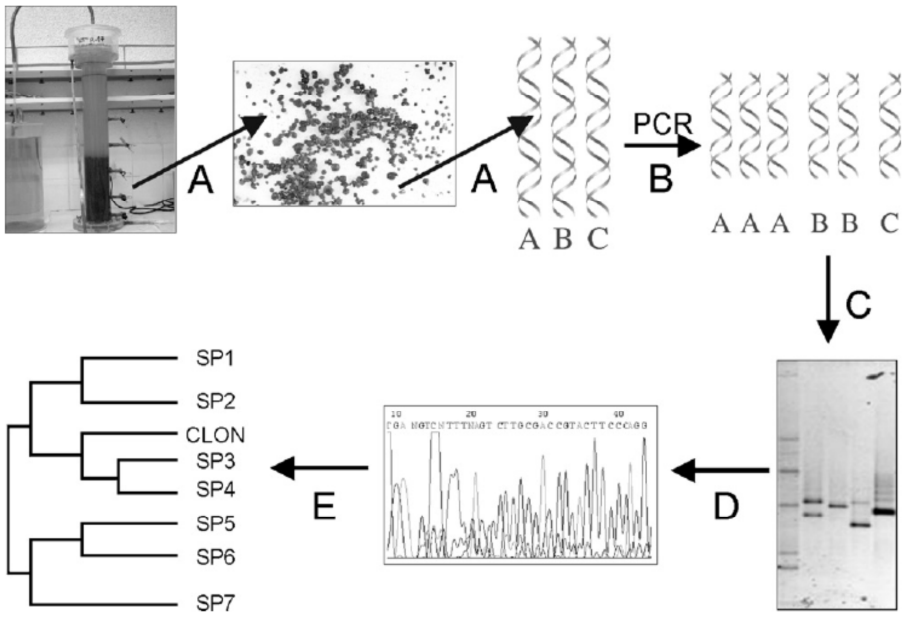

Figure 2.4 Schematic representation of DGGE.

DNA is extracted from sample, (A). The 16S rRNA gene is partially amplified by PCR usually with universal primers to give a mixture of DNA fragments, all of the same length, (B). The DNA mixture is then separated by denaturant gradient electrophoresis on an acrylamide gel with an increasing urea/formamide gradient. Every band on the gel corresponds to a different microorganism in the sample, (C). The bands can be cut from the gel and the DNA extracted and sequenced (D). Comparison of the sequences with a 16S rDNA database allows determining the phylogenetic affiliation of the microorganism (E). Modified from Sanz *et al.* [68].

CHAPTER 3 MATERIALS AND METHODS

3.1 Experimental Set up

In this thesis, experiments were investigated microbial community during shrimp cultivation in shrimp hatchery and grown-out pond:

3.1.1 Experimental set up in Shrimp Hatchery: The experiment was performed at Bangkok Aquaculture Farming Co., Ltd. (BAFCO) in Nakhon Si Thammarat, Thailand. Pacific white shrimp (*P. vannamei*) were cultured with or without the *Spirulina* mat, and ten replicates were performed for each condition as shown in Figure 3.1. Three sets of testing included

Set 1: shrimp culture (control; without *Spirulina* mat)

Set 2: shrimp culture with *Spirulina* mat

Set 3: shrimp culture with artificial mat

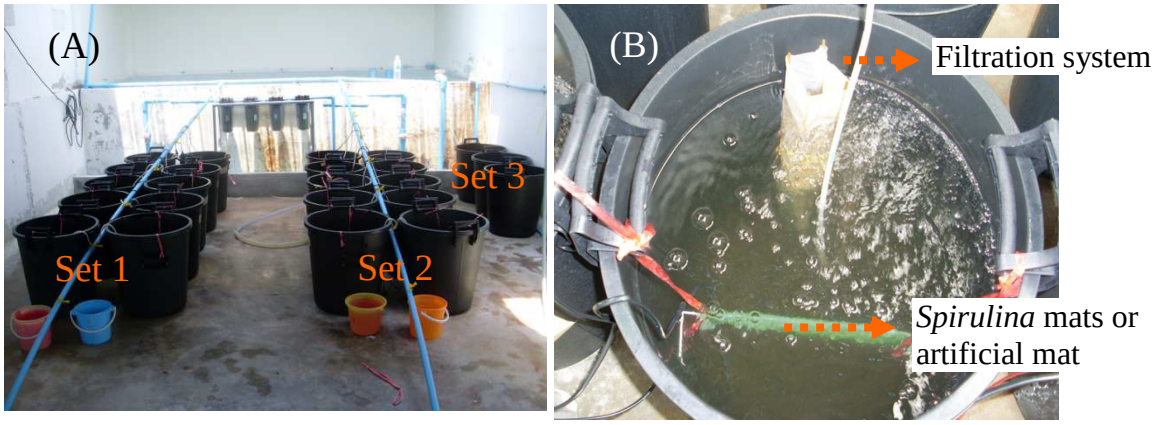

Figure 3.1 (A) Experimental set up of 35 L of plastic tank with Set 1: shrimp culture (control); Set 2: shrimp culture with *Spirulina* mat; Set 3: shrimp culture with artificial mat, respectively.
(B) Top view of the closed-recirculation system in 35 L plastic tank

One *Spirulina* mat was hung in the tank for each set of the experiment for the *Spirulina* mat condition. Feeding rates and cultivation conditions of the *P. vannamei* rearing system followed BAFCO protocols. Nauplii from a single spawn were first acclimated to seawater (35 ppt) for 60 min and then transferred to a tank at a density of 100 Nauplii L^{-1}. Protozoea were fed live diatoms (*Chaetoceros* sp.) at a density of 30,000 cells mL^{-1}, before feeding with freshly hatched Artemia nauplii (1 Nauplii mL^{-1}) in Mysis to early PL1 stage. In this study, we started the cultivation from PL2 for ten

days (named PL12), as shrimp farmers request this stage for grow-out ponds or earthen pond cultivation because of the highest organic loading rate, resulting in the highest wastewater production rate. The density of the shrimp larvae was 100 PL L^{-1} with 30 L of working volume in 35 L plastic tanks. The tanks were enclosed with a re-circulating system with natural sunlight, ambient temperature (less than 32°C), and salinity at 25 ppt. The commercial feed TNT#3 (CP feed, Thailand), less than 16 mesh (or 112 microns) in size and consisting of 45% protein and 9% fat, was used, as it is recommended for shrimp PL. Shrimp PL were fed TNT#3 in six meals day^{-1} (1 g per 1000 PL^{-1}) for the first three days, before reducing to four meals day^{-1} (8 g per 1000 PL^{-1}), according to BAFCO cultivation methods.

3.1.2 Experimental Set up in Shrimp Farming (Grow-out/Earthen pond)

Shrimp farming water samples were collected from a semi-intensive shrimp culture pond of white shrimps (*Penaeus vannamei*) located in Thung-Kru district, Bangkok, Thailand. A total of 4 water samples were studied. W_0 represented water samples taken before shrimp were released into the earthen pond. W_1, W_2 and W_3 represented water samples taken one, two and three months, respectively, after the shrimp were released. Sample collection, stabilization, and transportation to the laboratory as well as sample storage were done according to the Standard Methods of Strickland and Parsons [78]. Water samples were taken from the shrimp pond at 3 different depths: 10, 50 and 80 cm.

3.2 *Spirulina* mat Preparation

Spirulina mats were prepared by immersing a 5 cm (width) x 30 cm (length) x 0.3 cm (height), or 321 cm^2 (2wh + 2lw + 2lh = 3 cm_2 + 300 cm^2+ 18 cm^2), fibrous polyester mat (Figure 3.2) in one column containing suspended cells of *Spirulina platensis* strain BP [79] grown in Zarrouk's medium as described. The initial optical density of the cells at 560 nm (OD_{560}) was 0.35. The immobilized *Spirulina* film, or *Spirulina* mat, was exposed to a light intensity of 18 μmol photon m^{-2} s^{-1} for ten days without additional supplied cells as shown in Figure 3.3. The initial amount of the *Spirulina* mass of the mat was 56.2 g dry wt m^{-2}. After ten days, the immobilized *Spirulina* mass was 52.4 g dry wt m^{-2}. The mat was then applied for water quality control during the shrimp cultivation process.

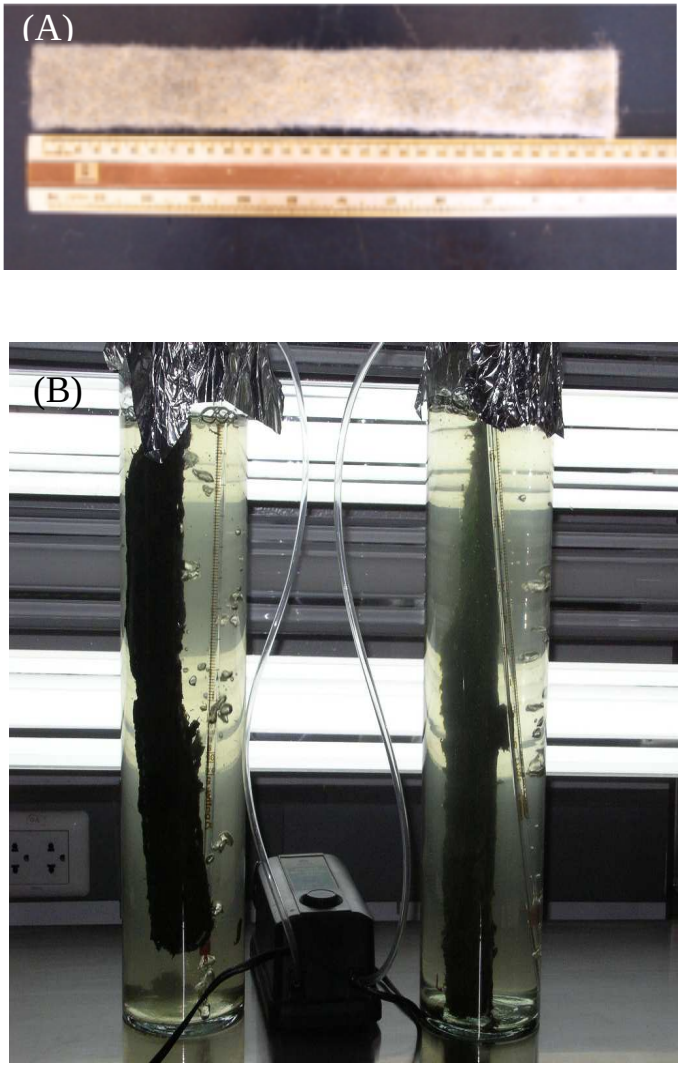

Figure 3.2 (A) Fibrous polyester mats; (B) *Spirulina* mat immobilization

3.3 The Closed-recirculation System

For closed-recirculated batch system reactor, during shrimp cultivation, filtration system (filter media with aquarium pump) was installed in all tanks in each set (Figure 3.3). The filtration system operated 2 hours per day to remove suspended solid.

(A)

(B)

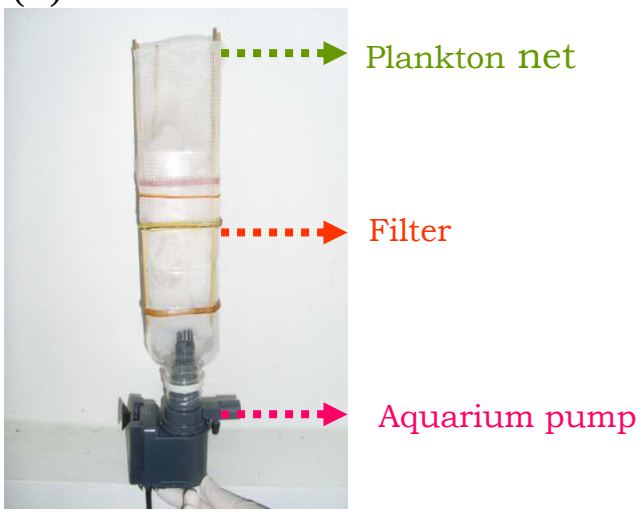

Figure 3.3 (A) Filter medium; (B) Filtration systems for suspended removal in closed-recirculation system during shrimp cultivation

3.4 Shrimp Quality Measurement

For shrimp quality measurement**,** to measure the survival shrimp from each tank compare with initial PL quantity. On day 0, density of PL2 in all sets was 100 PL L^{-1} (3,000 PL for 1 reactor). Specific growth rate (SGR), the SGR of the PL was calculated based on increasing larval length. On day 0, length of PL2 in all set was 4.32$\pm$0.37 mm. The survival rate in stress test, at the end of the experiment, survival shrimp at PL12 was calculated and reverse in salinity stress test (commonly practiced by commercial hatcheries to distinguish between healthy and weak PLs). For the stress test, shrimp PLs

were transferred from the culture tanks with ~ 25 ppt salinity to 5 ppt water and survival recorded after 2 hours.

3.5 Water Quality Measurement

3.5.1 Determination of Total Ammonia Nitrogen

Total ammonia nitrogen concentration (mg-N L^{-1}) was analyzed by Phenate method [132] with modification. Five milliliter of filtrated water sample was placed into 10 mL tube and then added with 200 µL of phenol solution (dissolve 20 g of crystalline phenol in 200 mL of 95% (v/v) ethyl alcohol). For the following step, the mixture was added with 200 µL of sodium nitroprusside (dissolve 1.0 g of sodium nitroprusside ($Na_2Fe(CN)_5NO \cdot 2H_2O$) in 200 L de-ionized water) and 500 µL of oxidizing solution before mixing. The oxidizing solution was prepared by mixing alkaline reagent (dissolve 100 g of sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) and 5 g of sodium hydroxide (NaOH) in 500 mL of de-ionized water) and sodium hypochlorite solution (commercial hypochlorite with the concentration approximately 1.5 N), at the mixing ratio of 4:1. Reaction tube was incubated at room temperature for one hour then ammonia concentration was measured by UV-visible spectrophotometer (Genesys 10 series, Thermo Spectronic, USA) at 640 nm against standard curve of 0.1-1.0 mg-N L^{-1} NH_4Cl.

3.5.2 Determination of Nitrite (NO_2-N)

Nitrite was measured on the basis of colorimetrical analysis by Griess-Ilosvay Diazotization method [132] with modification. Five milliliter of filtrated water sample was transferred to 10 mL tube and then added with 0.2 mL of sulfanilamide solution (dissolve 5 g of sulfanilamide and 50 mL of conc. HCl in 500 mL of dH_2O). After mixing for 1 minutes, 0.2 mL of N-(1-naphthyl)-ethylenediamine dihydrochloride (NNED) solution dissolve 0.5 g of NNED in 500 mL of dH_2O) was added, mixed and incubated for at least 30 minutes prior to color development. Nitrite concentration was measured at 543 nm using UV-visible spectrophotometer. For determination of nitrate (NO_3--N), nitrate concentration (mg-N L^{-1}) was analyzed by UV screening method. Water sample was filtered through GF/C filter and measured directly by UV visible spectrophotometer at 220 and 275 nm [80]. Calculation of nitrate concentration was as following:

$$\text{Nitrate (mg-N L}^{-1}) = \frac{(Abs_{220nm} - Abs_{275nm})XA}{B}$$

Where A = concentration of nitrate in standard curve (mg-N L^{-1})

B = absorbance of standard curve (220nm-275 nm)

Standard nitrate solution was prepared using 1-10 mg-N L^{-1} of sodium nitrate. It has to be noted that, this method must be strictly used with nitrate concentration between 1-10 mg-N L^{-1}. Water sample containing high nitrate concentration (over 10 mg-N L^{-1}) can be diluted with de-ionized water prior to analysis but water containing Low nitrate concentration (below 1 mg-N L^{-1}) was not applicable with this method. Moreover, high nitrite concentration can interfere with nitrate measurement hence concentration of nitrate must be subtracted with nitrite concentration.

3.5.3 Ammonia and Nitrite Oxidation Test

Ammonia and Nitrite Oxidation were measured in the laboratory using a microcosm approach. The 2.5 L glass bottles were fitted with aerators to ensure mixing and oxygen saturation and incubated 500 mL of sample in the dark for 24 hours with ammonium chloride (NH_4Cl) was added to 10 mg-N L^{-1} for ammonia oxidation test while added sodium nitrite ($NaNO_2$) was added to 5 mg-N L^{-1}. Level of pH was monitored regularly over the course of the experiment. Aliquots were removed from the microcosms every 4 hours and analyzed for ammonia and nitrate as describe previously [78]. The nitrification rate (mg-N L^{-1} d^{-1}) was calculated as the slope of a plot of ammonia concentration versus time over the incubation. Algal uptake of ammonia and nitrate was assumed not to occur in the dark.

3.6 Microbial Community Profiling by DGGE

In this study, microbial community was studied in three parts. Part I, microbial community in water column and on *Spirulina* mats in shrimp hatchery were investigated. Moreover, the microbial community in water column during shrimp cultivation in grow-out pond was studied as in Part II. In Part III, the comparison of microbial community in water column between hatchery and grow-out pond were investigated. The summarized of methodology was described briefly in Figure 3.4.

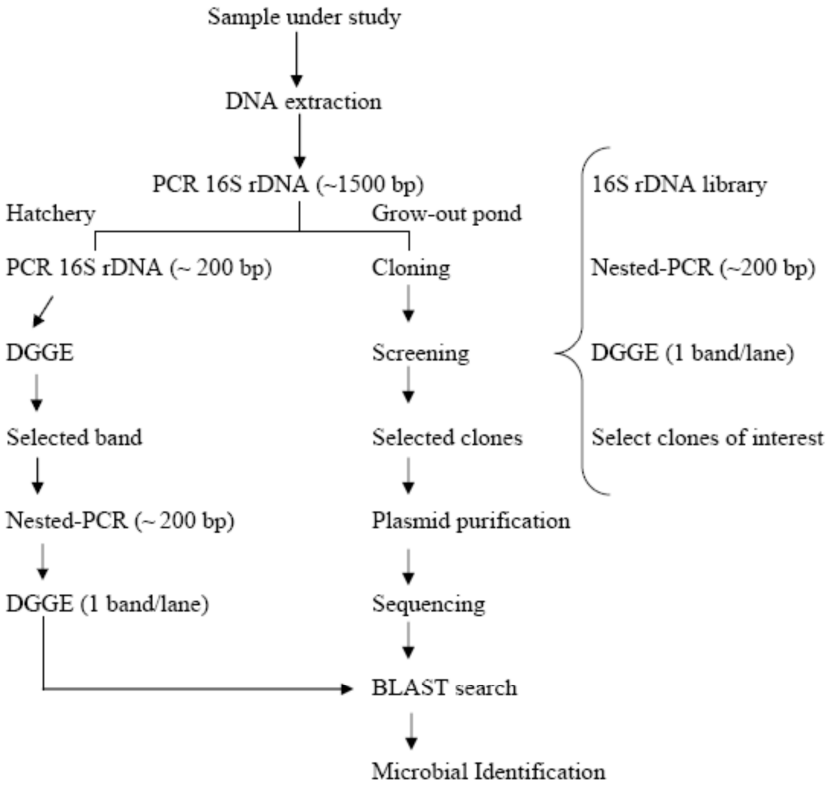

Figure 3.4 Outline procedures to analyze microbial community between samples from hatchery and grow-out pond (modified from Gonzalez *et al.* [81]).

3.6.1 DNA Extraction

3.6.1.1 Samples from Hatchery

Water columns were evaluated daily in a ten-day larval-rearing experiment. Samples of biofilm on the *Spirulina* mats were randomly sampled (size: 3 cm x 4 cm) on days 0, 2, 5, 8, and 10 of the shrimp cultivation process. Two liters of shrimp water were filtered successively with 0.45-µm and 0.2-µm filters. The filtered material and biofilm from the mats were re-suspended in 10 ml of 1X phosphate buffer solution (PBS) and frozen at -20°C. The biofilm was removed from the mat by a sonicator (Coleparmer Q55) before being frozen. DNA was extracted from the filtered material and the mats as previously described [82].

3.6.1.2 Samples from Grow-out

The shrimp water column 5 liters were filtrated with 0.45 and 0.2 µm, respectively. The filtrate was re-suspended in 10 mL of 1X Phosphate buffer solution (PBS) and frozen in –20 ^{o}C. The sample was extracted the DNA by the quarter of filtered material was resuspended in 300 µL of Lysozyme solution (10 mg Lysozyme L^{-1}, 0.15 M NaCl,

0.1 M Na_2EDTA, pH 8.0) and incubated at 37 ^oC for 1 h with mixing by inversion every 15 min. After cooling on ice, 300 μL SDS buffer (0.5 M Tris–HCl, 0.1 M NaCl, pH 8.0, 4% sodium dodecylsulfate) was added and the sample was incubated for 10 min and then placed at 55 ^oC for 10 min. This process was repeated three times. Genomic DNA was then extracted and purified from lysates by three sequential phenol–chloroform extractions [83] followed by precipitation with isopropanol. DNA pellets were washed with 70% ethanol and resuspended in sterile TE (50 mM Tris–HCl, 1 mM Na_2EDTA, pH 8.0) and stored in -20 ^oC.

3.6.2 DNA Amplification by PCR

3.6.2.1 Samples from Hatchery

The bacterial 16S rRNA gene was amplified by PCR with the primer EUB8F (primer sequences were shown in Table 3.1) and U1492R [84] for the first step, followed by amplification with the specific primer 338GC-F and 518R [85]. The 200 bp PCR fragments were analyzed by DGGE using a DGGE-2000 apparatus (CBS Scientific Company, USA).

3.6.2.2 Samples from Grow-out Pond

For cloning and sequencing of PCR-amplified products, the primer sequences for 16S rDNA gene for nested PCR amplification was shown in Table 3.1. PCR products of 16S rDNA genes were purified and cloned into *Escherichia coli* DH5α using the Promega pGEM-T Easy vector system (Promega, Madison, WI) to generate 16S rDNA gene library as described in the manufacturer's instructions. PCR was performed on cell lysates of white, ampicillin-resistant transformants using vector-specific primers T7 and Sp6 to confirm the size of the inserts. PCR with the correct size were screened by DGGE analysis as describe above to group 16S rDNA clones into operational taxonomic unit (OTU) before DNA sequencing. Approximately 200 transformants were grouped by DGGE analysis. Plasmids of representative transformants, with different DGGE patterns were purified and subjected to DNA sequencing analysis. DNA sequencing was performed by Genset, Singapore.

3.6.3 Denaturing Gradient Gel Electrophoresis (DGGE)

The PCR products were loaded on a 6.5% polyacrylamide gel in 0.5× TAE (Tris-acetate-EDTA) buffer. Optimal separation was achieved with a parallel and linear denaturing gradient ranging from 30–55% 7 M urea to 40% (V/V) formamide. Gels were run for five hours at 60°C and 200 V, stained with SYBR Gold (1:10,000 dilution; Invitrogen, Belgium) [86] for 20 min at room temperature, and photographed under a UV transilluminator. The image was visualized on a UV transilluminator and was captured using Biovision CN 1000/26 M (Vilber Lourmat, France). Most of the bands were excised from the gel using Gel Cutting Tips (Cleaver Scientific, England) and re-amplified with the primer 338F and the primer 518R. The PCR products were purified using a Gel/PCR DNA fragment extraction kit (Geneaid, Taiwan) according to the manufacturer's instructions. The band similarity was analyzed by Percentage of Nei and Li's similarity coefficients [87]**.**

3.6.4 Phylogenetic Analysis

The purified PCR products were sequenced using 1st BASE Laboratories (Malaysia). Sequences were first compared to known 16S rRNA sequences in the GenBankTM database using BLASTn to locate nearly exact matches in the GenBank database [88]. Phylogenetic analysis was performed by the computer program Ribosomal Database Project v. 9 (http://rdp.cme.msu.edu/) using default settings for the various algorithms. Phylogenetic relationships were inferred by distance matrix.

Table 3.1 Primer sequences for 16S rDNA gene for nested PCR amplification

Sampling Station	Primer	Sequences (5'------- 3')
Hatchery, Grow-out	EUB8F	GAG TTT GAT CCT GGC TCA G
Hatchery, Grow-out	U1492R	GGT TAC CTT GTT ACG ACT T
Hatchery, Grow-out	338F-GC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GG GAG GCA
Hatchery, Grow-out	518R	ATT ACC GCG GCT GCT GG
Hatchery, Grow-out	338F	CAC GGG GGG ACT CCT ACG GGA GGC A
Grow-out	T7	TAA TAC GAC TCA CTA TAG GG
Grow-out	Sp6	ATT TAG GTG ACA CTA TAG AAT AC

CHAPTER 4 RESULTS AND DISCUSSION

Experimental Framework

There are three major parts in this investigation; the first was focused on effect of *Spirulina* mats on water quality (*i.e.* salinity, total ammonia nitrogen, nitrite, and nitrate), water exchange rate and shrimp quality (*i.e.* survival rate, PL weight, PL length and survival rate in stress test) during shrimp cultivation in hatchery; the second was also focused on correlation between microbial community, water quality and shrimp quality, but in grow-out pond; the last was comparison of the microbial community between in hatchery and grow-out pond.

4.1 Dynamic of Microbial Community during Shrimp Cultivation

Dynamic of microbial community in the water column during shrimp cultivation in three experiments; shrimp without *Spirulina* mat (Set 1), shrimp co-culturing with *Spirulina* mat (Set 2), and shrimp co-culturing with artificial mat (Set 3) were daily investigated by denaturing gradient gel electrophoresis (DGGE).

4.1.1 Changes of Microbial Community with Different Cultivating Condition

DGGE fingerprints were obtained from water column and mats. The microbial community in water column of without *Spirulina* mat (Set 1), co-culturing with *Spirulina* mat (Set 2), and co-culturing with artificial mat (Set 3) was shown in Figure 4.1–4.6, respectively. All experiments were started from water column of PL2 cultivation. The microbial community in water (Table 4.1) was represented by Band C, H, M as microbial communities in water column of Set 1, 2 and 3, respectively, whereas band S and A was represented microbial communities on *Spirulina* mat and artificial mat, respectively. The dynamics of the microbial communities between Set 1 and Set 3 were similar, while Set 2 was different.

The microbial community in all sets showed similar microbial structure. This could be generated in 2 main groups: *Cytophaga-Flavobacterium-Bacterroids* and *Proteobacteria*. In *Proteobacteria,* the main bacteria in all sets were *Pseudomonas* sp., *Vibrio* sp., *Exiguobacterium* sp., *Aeromonas* sp., *Nitrosomonas* sp. and *Nitrobacter* sp.

Table 4.1 Summarized microbial community in water columns and on mats based on partial 16S rRNA gene sequences of EUB domain and organisms with the best-matching sequences determined by BLAST searches

Band	Affiliation	Similarity (%)	Taxon	Accession Number
H1,M14, A1	Uncultured *Firmicute*	98	Firmicute	HQ 219323
H2, S1, C1, M1, A2	*Nitrosomonas marina*	96	β-proteobacteria	HQ 219324
H3, S2, C2, M2	Uncultured Flavobacteriales *bacterium clone FX*	96	Bacteriodetes	HQ 219325
H4, S5, C5, M5, A4	*Pseudomonas* sp. *ITRI66*	98	γ-proteobacteria	HQ 219328
H5	*Marine bacterium SIMO-2515*	98	α-proteobacteria	HQ 219318
H6	Uncultured *Enterobacter* sp. *clone CA01014H02*	97	γ-proteobacteria	HQ 219319
H7, S7	*Spirulina platensis*	98	Cyanobacteria	HQ 219314
H8	Uncultured *Flavobacteriaceae bacterium clone A1905*	97	Bacteriodetes	HQ 219320
H9	*Sphingomonas* sp. *PC5.28*	96	γ-proteobacteria	HQ 219312
H10, S10, C7, A9, M7	*Fusobacterium ulcerans strain KCTC5932*	98	α-proteobacteria	HQ 219330
H11, S12, C8, M8	*Exiguobacterium arabatum*	95	γ-proteobacteria	HQ 219331
H12, S13, C9, M9	*Exiguobacterium arabatum*	95	γ-proteobacteria	HQ 219332
H13	*Marine bacterium 30ORI8*	98	Bacteriodetes	HQ 219321
H14	*Marine bacterium 30ORI8*	98	Bacteriodetes	HQ 219322
H15, S3, C3, M3	*Nitrobacter winogradskyi*	96	α-proteobacteria	HQ 219326
S4, C4, M4, A3	*Stenotrophomonas maltophilia*	98	γ-proteobacteria	HQ 219327
S6, A5	Uncultured *bacterium clone SWB23*	98	γ-proteobacteria	HQ 219313
S8	Uncultured *Bacteroidetes bacterium clone CC85*	98	γ-proteobacteria	HQ 219315
S9, A7	*Vibrio* sp. *MSSRF64*	97	γ-proteobacteria	HQ 219329
S11	*Klebsiella* sp. *NAP_L*	95	γ -proteobacteria	HQ 219317
C6	*Vibrio* sp. UST061013-028	95	γ -proteobacteria	JN 171846
C10, C11	Uncultured *bacterium FX6F*	97	γ -proteobacteria	JN 171847
C12, M10	*Vibrio* sp. P047C	95	γ-proteobacteria	JN 171848
C13, C14	*Aeromonas bivalvium* strain 665N	93	γ-proteobacteria	JN 171849
C15, M13	Uncultured Flavobacteriales *bacterium clone A830*	89	Bacteriodetes	-
C16	*Cytophaga* sp.	97	Bacteriodetes	JN 171850
C17, M11	*Uncultured bacterium* clone B-10	91	β-proteobacteria	-
C18, M12	*Uncultured bacterium* clone MTB3	80	α-proterobacteria	-
C19	*Epsilon proteobacterium*	96	ε-proteobacteria	JN 171851
M6	*Vibrio* sp. NH 89-11	99	γ-proteobacteria	-
M15	*Marine sediment bacterium ISA-6371*	99	Bacteroidetes	-
M16	*Salmonella enterica* sub sp. SL476	97	γ-proterobacteria	-
A6	*Unculture bacterium GZKB*	97	γ-proterobacteria	-
A8	*Unculture bacterium F1Clone81*	83	γ-proterobacteria	-

Remark: H: microbial community of shrimp larvae cultivated with *Spirulina* mats; C: microbial community of shrimp larvae without *Spirulina* mats; M: microbial community of shrimp larvae cultivated with artificial mats; S: microbial community on the *Spirulina* mats; A: microbial community on the artificial mat.

4.1.1.1 Microbial Community in Water Column without *Spirulina* mat

At the starting time (day 0), when microbial communities consisted of band C1 to C9, the dynamics of microbial communities in without *Spirulina* mat (Set 1) was shown in Figure 4.1 and Table 4.2. The similarity of microbial communities in water column by UPGMA analysis (Figure 4.2), bands C1, C2, C5, C6, C8, and C9 disappeared while bands C10, C11, C12, C13, C14, C15, C16, C17 and C18 were found. This might be an effect of water exchanging. Since on day 1, 3, 5, 7 and 9, ammonia concentration increased close up to 2 mg L^{-1}, therefore water exchanging was necessary, resulting DGGE fingerprints of water columns changed particularly on day 4 (CW4) and day 7 (CW7). The day 4^{th}, bands C1, C16, C17 and C18 were found and remained until day 6, but disappeared on the day 7^{th}. However, C19 was found in Set 1 only.

4.1.1.2 Microbial Community in Water Column of Co-culturing with *Spirulina* mat

As seen in Figure 4.3 and Table 4.3, when microbial communities consisted of band H2, H3, H10, H11, H12 and H15, in water column of co-culturing with *Spirulina* mat (Set 2) at day 0. The similarity of microbial communities in water column by UPGMA analysis (Figure 4.4), bands H1, H7, H8, H9, H13 and H14 were investigated in water column from day 1 to day 3. Since on day 3, 6 and 9, ammonia concentration increased close up to 2 mg L^{-1}, therefore water exchanging was necessary. However, DGGE fingerprints of water columns revealed minor changed particularly on day 9 (WH9) and day 10 (WH10).

4.1.1.3 Microbial Community in Water Column of Co-culturing with Artificial mat

In DGGE profile as shown in Figure 4.5 and Table 4.4, the dynamics of microbial communities at starting time in water column co-culturing with artificial mat (Set 3) consisted of band M1 to M9. The similarity of microbial communities in water column by UPGMA analysis (Figure 4.6), bands M1, M2, M3, M4, M5, M6, M8 and M9 disappeared while bands M10 and M13 were found on day 1. Since on day 1, 3, 5, 7 and 9, ammonia concentration increased close up to 2 mg L^{-1}, therefore water exchanging was necessary, resulting DGGE fingerprints of water columns changed particularly on day 1 (WA1), day4 (WA4) and day 7 (WA7). The day 4^{th}, bands M1, M2, M3, M11 and M12 were found and remained until day 6. On the day 7^{th}, bands M4, M14, M15 and M16 were additional found in system.

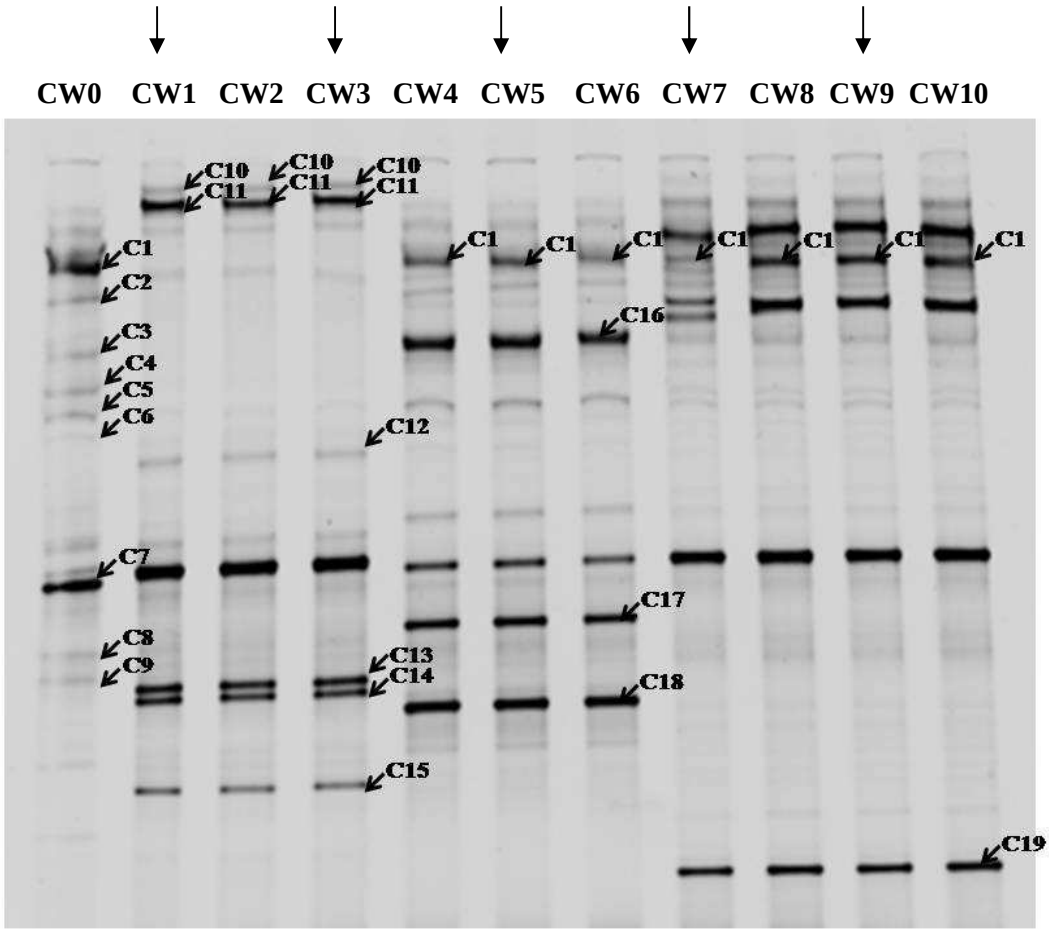

Figure 4.1 Microbial community in water column of without mat condition. CW0-CW10 was microbial community in water column from day 0 to day 10 that reared shrimp from PL2 to PL12 in without *Spirulina* mat condition. The arrow shows the water exchanging and the water samples were taken before water exchanging.

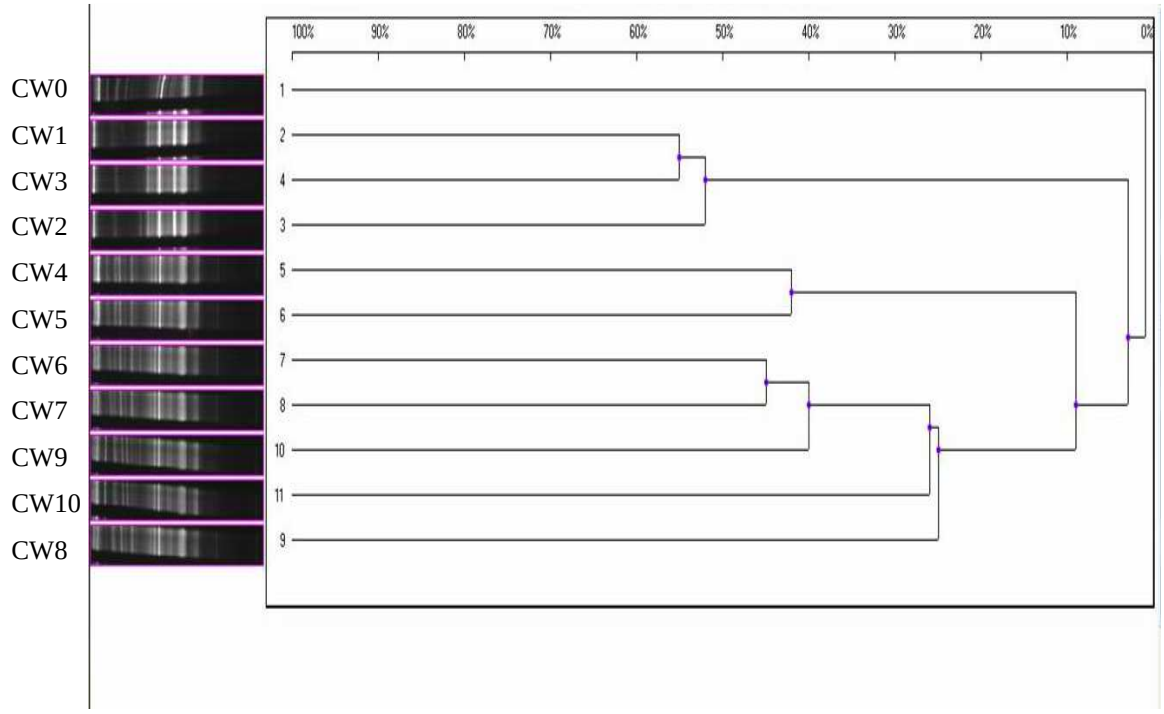

Figure 4.2 16S rDNA-based denaturing gradient gel electrophoresis (DGGE) profiles (Left) with cluster analysis or percentage of Nei and Li's similarity coefficients [87] (Right) of the bacterial community in water column of Set 1. Lane CW0 to CW10 was bacteria community in water column in Set 1.

Table 4.2 Microbial community in water column of without mat based on partial 16S rRNA gene sequences of EUB domain and organisms with the best-matching sequences determined by BLAST searches

Band	Affiliation	Similarity (%)	Taxon	Accession Number
C1	*Nitrosomonas marina*	96	β-proteobacteria	HQ 219324
C2	*Uncultured Flavobacteriales bacterium clone FX*	96	Bacteriodetes	HQ 219325
C5	*Pseudomonas* sp. *ITRI66*	98	γ-proteobacteria	HQ 219328
C7	*Fusobacterium ulcerans strain KCTC5932*	98	α-proteobacteria	HQ 219330
C8	*Exiguobacterium arabatum*	95	γ-proteobacteria	HQ 219331
C9	*Exiguobacterium arabatum*	95	γ-proteobacteria	HQ 219332
C3	*Nitrobacter winogradskyi*	96	α-proteobacteria	HQ 219326
C4	*Stenotrophomonas maltophilia*	98	γ-proteobacteria	HQ 219327
C6	*Vibrio* sp. UST061013-028	95	γ -proteobacteria	JN 171846
C10, C11	Uncultured *bacterium FX6F*	97	γ -proteobacteria	JN 171847
C12	*Vibrio* sp. P047C	95	γ-proteobacteria	JN 171848
C13, C14	*Aeromonas bivalvium* strain 665N	93	γ-proteobacteria	JN 171849
C15	*Uncultured Flavobacteriales bacterium A830*	*89*	Bacteriodetes	-
C16	*Cytophaga* sp.	97	Bacteriodetes	JN 171850
C17	*Uncultured bacterium* clone B-10	91	β-proteobacteria	-
C18	*Uncultured bacterium* clone MTB3 16S	80	α-proterobacteria	-
C19	*Epsilon proteobacterium*	96	ε-proteobacteria	JN 171851

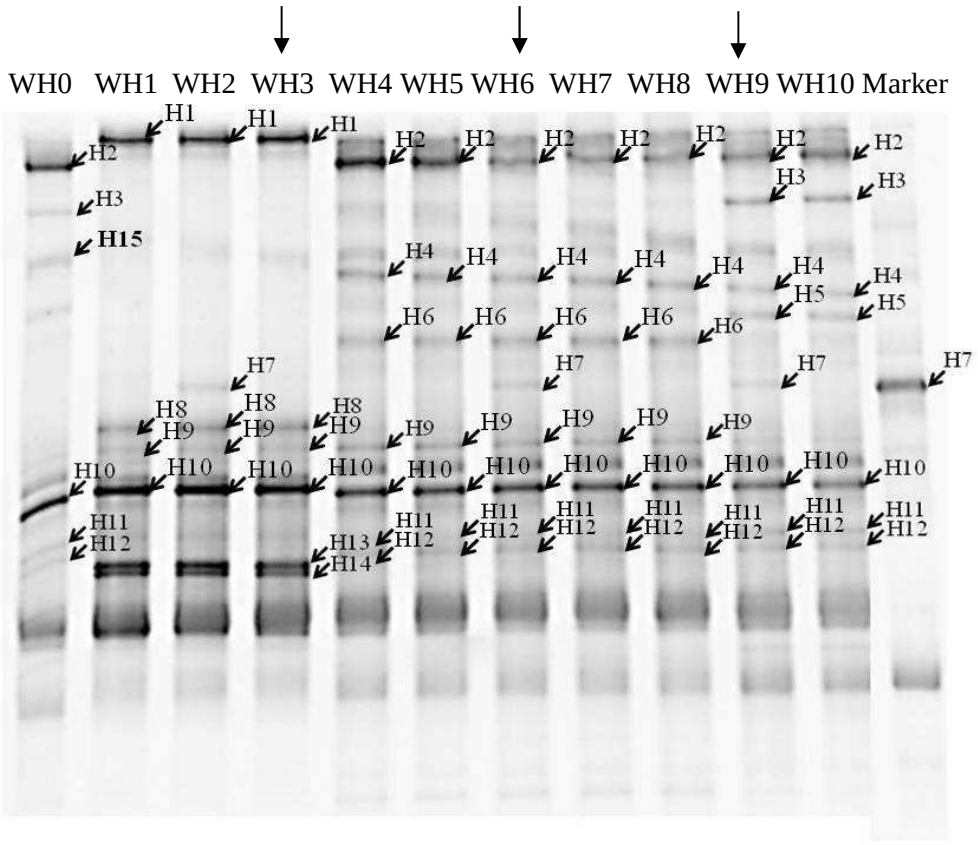

Figure 4.3 Microbial community in water column of Set 2 (co-cultured between *Spirulina* mat and shrimp). WH0-WH10 was microbial community in water column from day 0 to day 10 that reared shrimp from PL2 to PL12 in co-culturing with *Spirulina* mat condition. Marker was *Spirulina platensis.* The arrow shows the water exchanging and the water samples were taken before water exchanging.

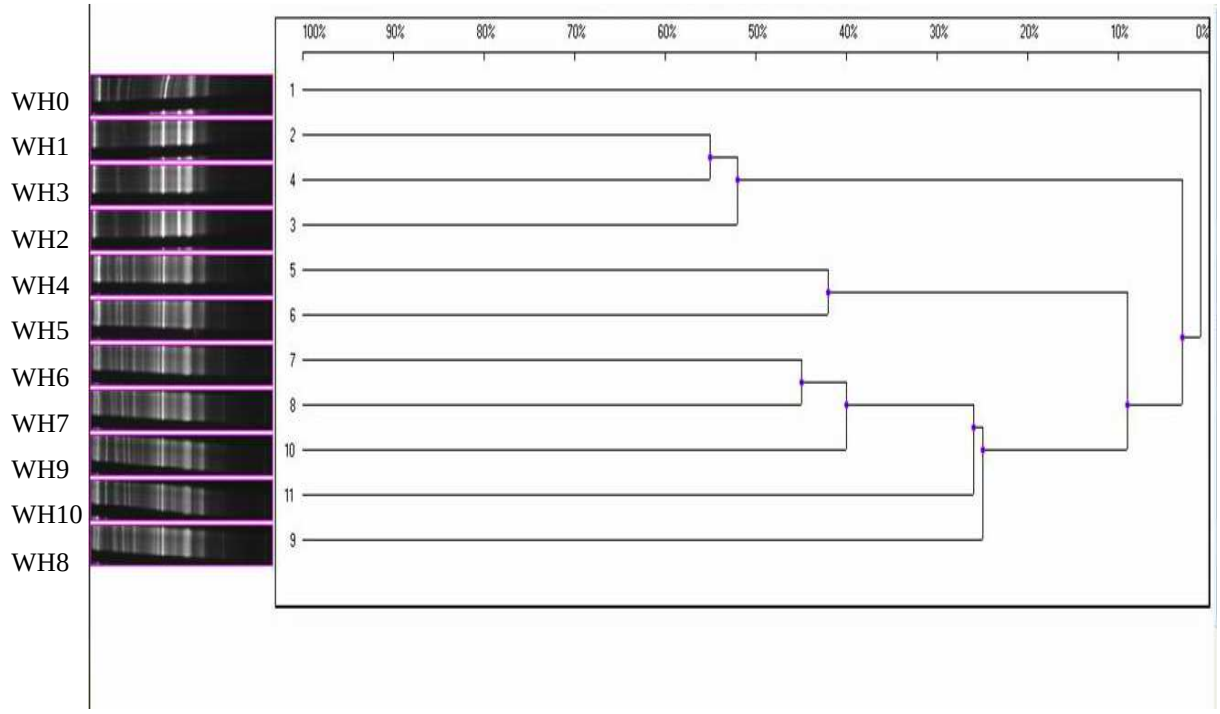

Figure 4.4 16S rDNA-based denaturing gradient gel electrophoresis (DGGE) profiles (Left) with cluster analysis or percentage of Nei and Li's similarity coefficients [87] (Right) of the bacterial community in water column of Set 2. Marker was pure culture of *Spirulina platensis.*

Table 4.3 Microbial community in water column of co-culturing with *Spirulina* mat based on partial 16S rRNA gene sequences of EUB domain and organisms with the best-matching sequences determined by BLAST searches microbial community

Band	Affiliation	Similarity (%)	Taxon	Accession Number
H1	Uncultured *Firmicute*	98	Firmicute	HQ 219323
H2	*Nitrosomonas marina*	96	β-proteobacteria	HQ 219324
H3	Uncultured Flavobacteriales *bacterium clone FX*	96	Bacteriodetes	HQ 219325
H4	*Pseudomonas* sp. *ITRI66*	98	γ-proteobacteria	HQ 219328
H5	*Marine bacterium SIMO-2515*	98	α-proteobacteria	HQ 219318
H6	Uncultured *Enterobacter* sp. *clone CA01014H02*	97	γ-proteobacteria	HQ 219319
H7	*Spirulina platensis*	98	Cyanobacteria	HQ 219314
H8	Uncultured *Flavobacteriaceae bacterium clone A1905*	97	Bacteriodetes	HQ 219320
H9	*Sphingomonas* sp. *PC5.28*	96	γ-proteobacteria	HQ 219312
H10	*Fusobacterium ulcerans strain KCTC5932*	98	α-proteobacteria	HQ 219330
H11	*Exiguobacterium arabatum*	95	γ-proteobacteria	HQ 219331
H12	*Exiguobacterium arabatum*	95	γ-proteobacteria	HQ 219332
H13	*Marine bacterium 30ORI8*	98	Bacteriodetes	HQ 219321
H14	*Marine bacterium 30ORI8*	98	Bacteriodetes	HQ 219322
H15	*Nitrobacter winogradskyi*	96	α-proteobacteria	HQ 219326

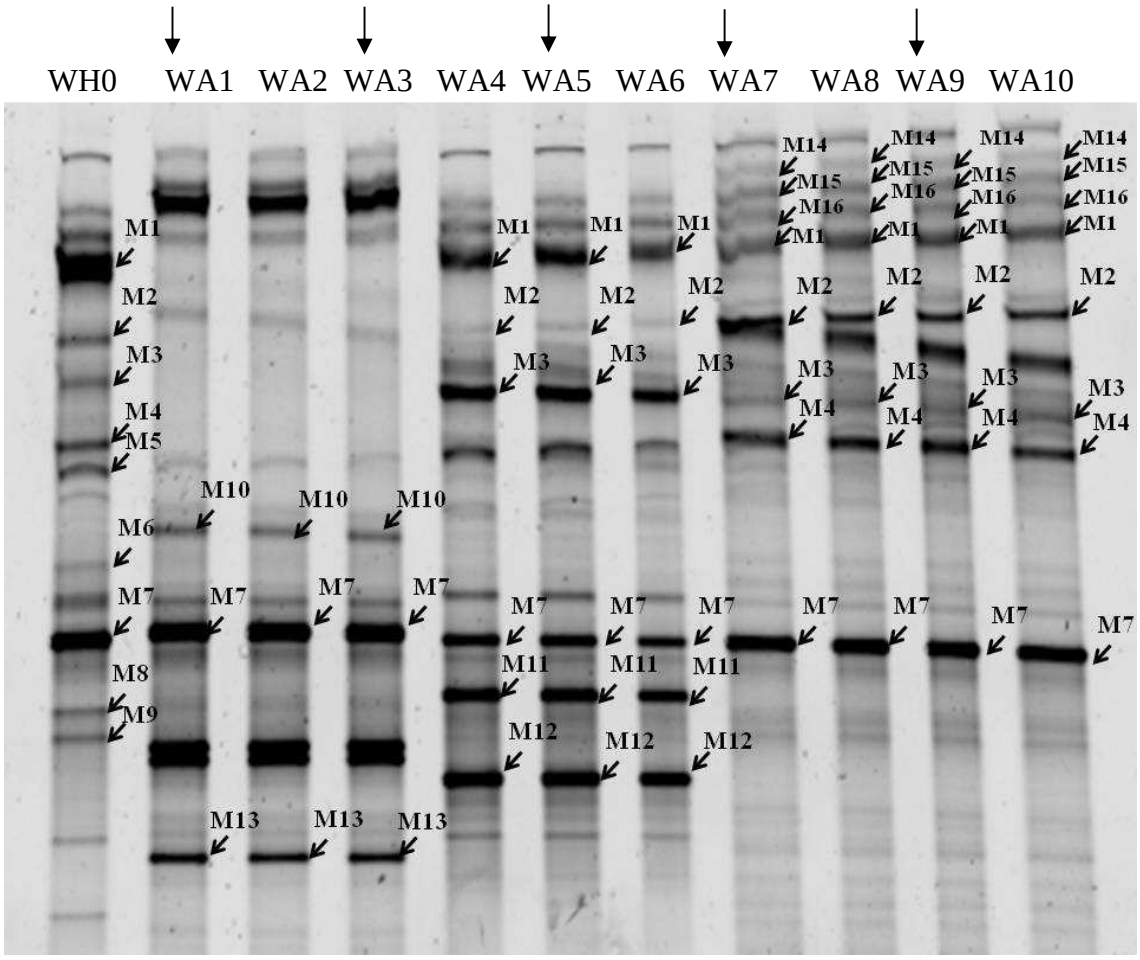

Figure 4.5 Microbial community in water column of Set 3 (co-cultured between artificial mat and shrimp). WA1-WA10 was microbial community in water column from day 1 to day 10 that reared shrimp from PL2 to PL12 in co-culturing with artificial mat condition. The arrow shows the water exchanging and the water samples were taken before water exchanging.

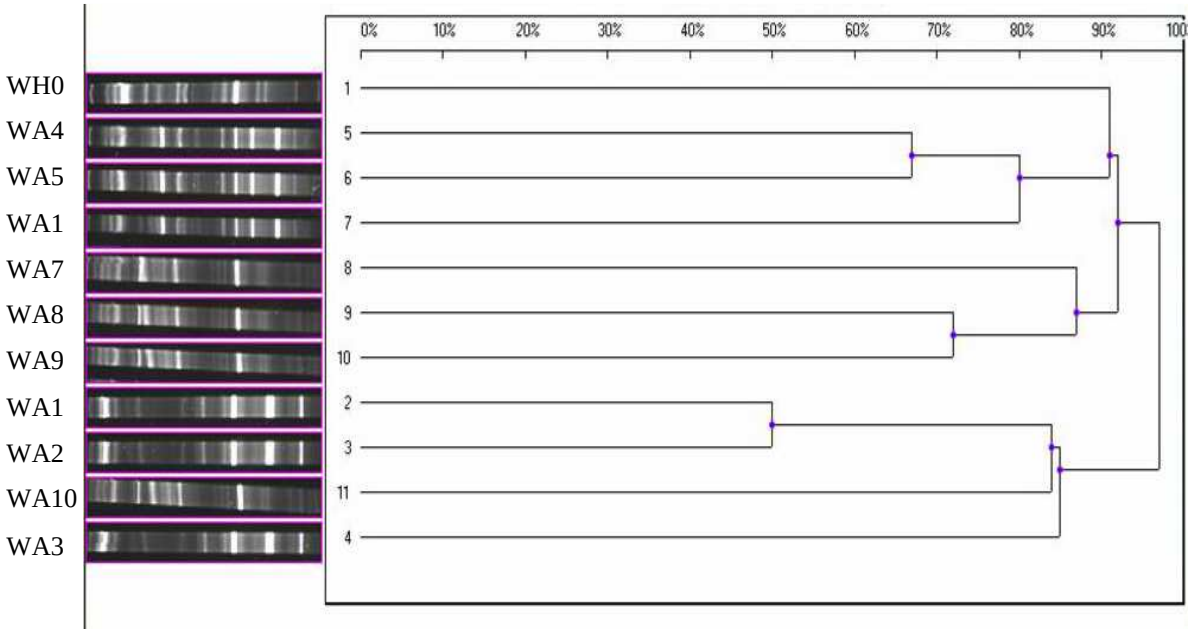

Figure 4.6 16S rDNA-based denaturing gradient gel electrophoresis (DGGE) profiles (Left) with cluster analysis or percentage of Nei and Li's similarity coefficients [87] (Right) of the bacterial community in water column of Set 3. Lane WH0 and WA1 to WA10 was bacteria community in water column in Set 3.

Table 4.4 Microbial community in water column of co-culturing with artificial mat based on partial 16S rRNA gene sequences of EUB domain and organisms with the best-matching sequences determined by BLAST searches microbial community

Band	Affiliation	Similarity (%)	Taxon	Accession Number
M14	Uncultured *Firmicute*	98	Firmicute	HQ 219323
M1	*Nitrosomonas marina*	96	β-proteobacteria	HQ 219324
M2	Uncultured Flavobacteriales *bacterium clone FX*	96	Bacteriodetes	HQ 219325
M5	*Pseudomonas* sp. *ITRI66*	98	γ-proteobacteria	HQ 219328
M7	*Fusobacterium ulcerans strain KCTC5932*	98	α-proteobacteria	HQ 219330
M8	*Exiguobacterium arabatum*	95	γ-proteobacteria	HQ 219331
M9	*Exiguobacterium arabatum*	95	γ-proteobacteria	HQ 219332
M3	*Nitrobacter winogradskyi*	96	α-proteobacteria	HQ 219326
M4	*Stenotrophomonas maltophilia*	98	γ-proteobacteria	HQ 219327
M10	*Vibrio* sp. P047C	95	γ-proteobacteria	JN 171848
M13	Uncultured Flavobacteriales *bacterium A830*	89	Bacteriodetes	-
M11	*Uncultured bacterium* clone B-10	91	β-proteobacteria	-
M12	*Uncultured bacterium* clone MTB3 16S	80	α-proterobacteria	-
M6	*Vibrio* sp. NH 89-11	99	γ-proteobacteria	-
M15	*Marine sediment bacterium* ISA-6371	99	Bacteroidetes	-
M16	*Salmonella enterica* sub sp. SL476	97	γ-proterobacteria	-

4.1.1.4 Microbial Community on *Spirulina* and Artificial mats

Changes of microbial community on *Spirulina* mats and on artificial mats were compared in Figure 4.7 and Table 4.5. The DGGE profile of 16S rDNA showed that bacteria community on *Spirulina* distinctly changed in the second day. There were at least 13 major bacteria species as indicated by dense DGGE bands at the initial day. At the second day, there were at least 11 new dominant bacteria (S1, S2, S4, S5, S7, S8, S9, S10, S11, S12 and S13). On day 0, the dominant species were *Spirulina* (S7), In addition, during the operation, the dominant communities on *Spirulina* mat including bands S2 and S5 was disappeared, while S4, S12 and S13 were died out gradually. The comparison of microbial community on *Spirulina* mat and in water column of Set 2 found that *Nitrosomonas marina* (H2, S1), Uncultured Flavobacteriales *bacterium clone FX* (H3, S2), *Pseudomonas* sp. *ITRI66* (H4, S5), *Spirulina platensis* (H7, S7), *Fusobacterium ulcerans* strain KCTC5932 (H10, S10), *Exiguobacterium arabatum* (H11, S11, H12, S12) and *Nitrobacter winogradskyi* (H15, S3) were found both on

Spirulina and in water column as shown in Table 4.5 The microbial community between *Spirulina* mat and artificial mat had similar profiling as shown in Figure 4.7. The differences were bands A1, A6 and A8, which were closely related to Uncultured *Firmicute*, Uncultured bacterium clone GZKB45 and Uncultured bacterium F1 clone 81, respectively.

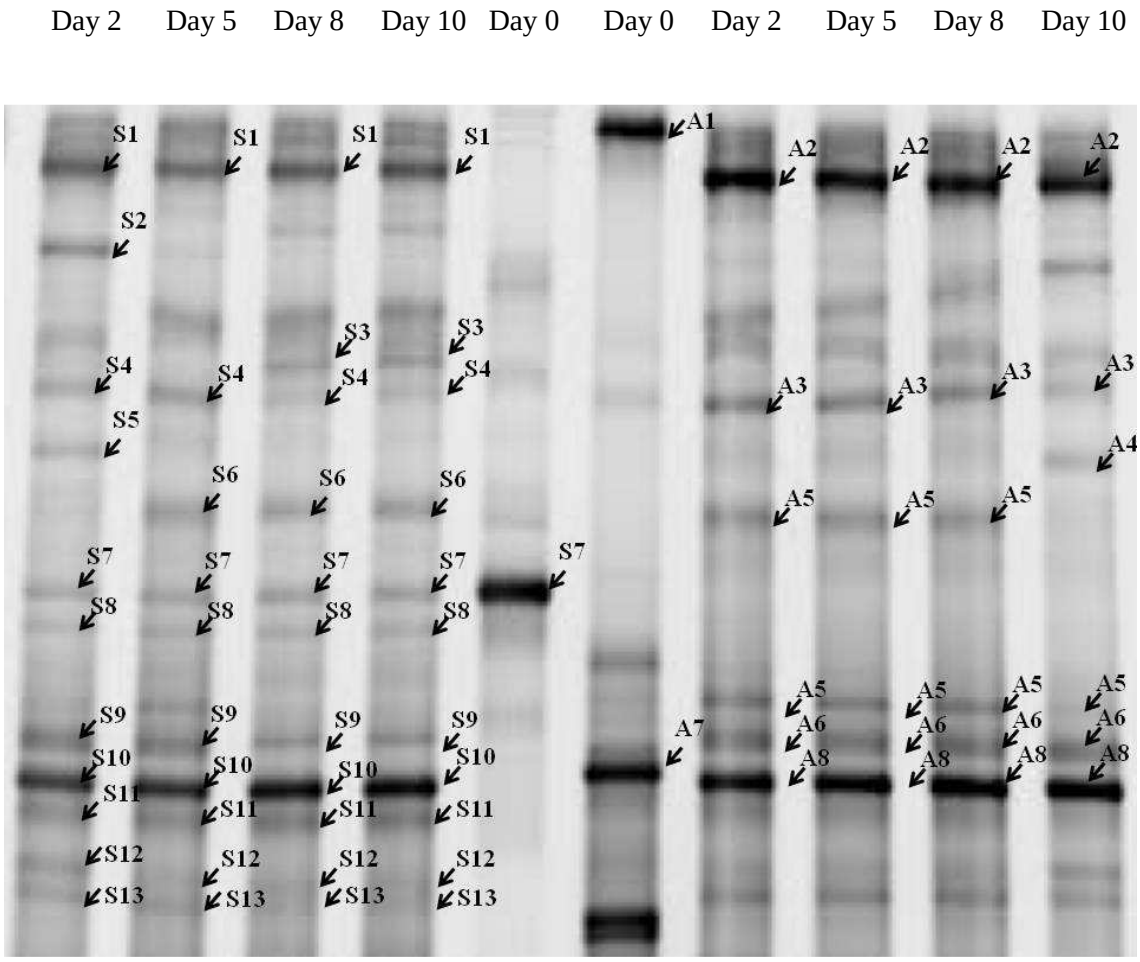

Figure 4.7 The comparison of microbial community on *Spirulina* mat (S) and artificial mat (A) on day 0, 2, 5, 8 and 10, respectively.

Table 4.5 Microbial community on *Spirulina* mats and artificial mats based on partial 16S rRNA gene sequences of EUB domain and organisms with the best-matching sequences determined by BLAST searches microbial community

Band	Affiliation	Similarity (%)	Taxon	Accession Number
A1	Uncultured *Firmicute*	98	Firmicute	HQ 219323
S1, A2	*Nitrosomonas marina*	96	β-proteobacteria	HQ 219324
S2	Uncultured Flavobacteriales *bacterium clone FX*	96	Bacteriodetes	HQ 219325
S5, A4	*Pseudomonas* sp. *ITRI66*	98	γ-proteobacteria	HQ 219328
S7	*Spirulina platensis*	98	Cyanobacteria	HQ 219314
S10, A9	*Fusobacterium ulcerans strain KCTC5932*	98	α-proteobacteria	HQ 219330
S12	*Exiguobacterium arabatum*	95	γ-proteobacteria	HQ 219331
S13	*Exiguobacterium arabatum*	95	γ-proteobacteria	HQ 219332
S3	*Nitrobacter winogradskyi*	96	α-proteobacteria	HQ 219326
S4, A3	*Stenotrophomonas maltophilia*	98	γ-proteobacteria	HQ 219327
S6, A5	Uncultured *bacterium clone SWB23*	98	γ-proteobacteria	HQ 219313
S8	Uncultured *Bacteroidetes bacterium clone CC85*	98	γ-proteobacteria	HQ 219315
S9, A7	*Vibrio* sp. *MSSRF64*	97	γ-proteobacteria	HQ 219329
S11	*Klebsiella* sp. *NAP_L*	95	γ-proteobacteria	HQ 219317
A6	*Unculture bacterium GZKB*	97	γ-proteobacteria	-
A8	*Unculture bacterium F1Clone81*	83	γ-proteobacteria	-

4.1.2 Microbial Community in the Co-culturing with *Spirulina* mats System

Changes in the bacterial community composition of each sample were observed over time, as demonstrated by changes in the dominant bands and the affiliated retrieved sequences. These results showed that the microflora of the system with *Spirulina* mats (15 H-bands, Figure 4.3) was less diverse than the system without *Spirulina* mats (19 C-bands, Figure 4.2) and with artificial mat (16 M-band, Figure 4.5), respectively. The results further identified two major microbial groups in both the water columns and on the *Spirulina* mats: gram-negative genera containing *Firmicutes, Flavobacteriales,* and *Fusobacterium ulcerans*; and *Stenotrophomonas maltophilia* and Proteobacteria, which included *Nitrosomonas marina, Nitrobacter winogradskyi, Pseudomonas* sp. ITRI66, and *Exiguobacterium arabatum*. The microflora was composed of distinct bacteria from the systems with and without *Spirulina* mats and on the *Spirulina* mats: *N. marina* (band H2, C1, S1; 96 % similarity), *Flavobacteriales* bacterium (H3, C2, S2; 98 % similarity), *Pseudomonas* sp. (H4, S5, C5; 98 % similarity), *F. ulcerans* (H10, S10, C7; 98 % similarity), and *E. arabatum* (H11, S12, C8; 95 % similarity). Conversion of ammonia to nitrite is accomplished by ammonia oxidizing bacteria (AOB), generally known as *Nitrosomonas* bacteria; nitrite oxidizing bacteria (NOB), which includes Nitrobacter bacteria, oxidize nitrite to nitrate. Incomplete nitrification occurs when a lack of NOB productivity is present, leading to increased concentrations of nitrite. The toxicity of nitrite is a function of the effects on the circulatory and immune systems of aquatic organisms [89]. In the current study, *N. marina* and *N. winogradskyi* were found to play major roles in the nitrogen cycle: the former can oxidize ammonia into nitrite and the latter can oxidize nitrite into nitrate, a nitrogen form that can be up taken by *Spirulina* [20]. This important role of the bacteria in nitrogen control via the nitrification process is essential to reduce the toxicity of nitrogenous wastes, such as excess feed or feces to shrimp PL in the cultivation systems [90]. In general, the nitrifiers prefer a free-cell form under unstressed conditions, possibly provided with sufficient energy to continue their independent mobility [91]. In environmental stress or sub-optimum conditions, the nitrifiers may prefer to conserve their energy by limiting movement, by attaching to surfaces of the forming microbial mats. Interestingly, *N. winogradskyi* (H15, S3, C3; 98 % similarity) was found only on the *Spirulina* mats. In contrast, we found that *Nitrosomonas* sp., the higher oxygen affinity species, occupied the inner layers of the biofilm where oxygen was limited,

while the lower affinity *Nitrobacter* sp. was found on the outer layers where oxygen was in higher supply [92], in addition to oxygen that was a by-product from *Spirulina* cells. Not only nitrifying bacteria found in aquaculture biofilters [39], *Pseudomonas* sp., *Vibrio* sp., *Aeromonas* sp., and *Flavobacterium* sp. were also the most common reported genera of gut microflora in aquatics [74]. The heterotrophic bacterium *Pseudomonas* sp. ITRI66 (represented by H4 or S5) in this study is a denitrifying bacterium that combines the processes of nitrification and de-nitrification [6]. This observation in agreement with Ayyasamy *et al.* [93], who achieved denitrification using aerobic *Pseudomonas* sp. in a groundwater environment. One study found that the highest survival and growth rates of Artemia cultures were obtained in *Pseudomonas* sp. [94]. In regards to beneficial bacteria, *Pseudomonas* sp., *Exiguobacterium* sp., and *S. platensis* were reported to support a good survival rate of shrimp. *Pseudomonas* spp. can produce a variety of bioactive compounds, including phenazine compounds, quinolones, and pyrrolnitrin [95]. Moreover, the bacterium also has diverse mechanisms of action, e.g., cell lysis or as an acetyl-CoA carboxylase synthesis inhibitor that can control *Vibrio* spp. with no harm to the shrimp [95]. In this study, most samples taken from the water column and the *Spirulina* mats were found to contain *Exiguobacterium* sp. (H11, S12, C8; 95 % similarity), which is located in many environments, including marine hatcheries. According to morphological examinations, physiological tests, and molecular techniques, *Exiguobacterium* sp. is similar to *Bacillus* sp. [96], producing lactic acid as probiotic bacteria property to improve the survival rate and development of aquaculture [97]. Consequently, the lactic acid bacteria might be able to protect the host from pathogens by blocking the integumental attachment sites (adhesion receptors) [98]. As *S. platensis* has the potential to control non-beneficial bacteria, particularly *Vibrio* sp. (represented by S9 and C12), this might be the cause of *Vibrio* diseases affecting the survival and growth of shrimp [46]. Tayag *et al.* [99] showed that both superoxide anions and phagocytic activity increased in *L. vannamei* fed with *S. platensis* extract. The report suggested that polysaccharides of S. platensis extract receptors exist in macrophages and hemocytes, and stimulate the activation of innate immunity in shrimp and fish. These might support our findings of higher shrimp PL growth and survival rates in the *Spirulina* mat condition.

Cytophaga sp. (band C16) and *Aeromonas bivalvium* (bands C13 and C14) were found only in conditions without *Spirulina* mats. These are important bacterial pathogens of

cultured aquaculture that typically cause external lesions in aquaculture and might cause infection in penaeid shrimp larvae. They are also associated with poor water quality [100]. It was reported that catfish grown with *Spirulina* sp. achieved the best growth performance and development of antibody levels against the bacteria *Aeromonas* sp. [101]. The *Spirulina* mat might have some effect on the disappearance of *Aeromonas* sp. The S7 or H7 band was found in all samples, due to the fact that it was *S. platensis* that detached from the *Spirulina* mat. During the 10 days of the experiment, there was a cell loss from the *Spirulina* mat of 6.72 % (detached 3.8 from 52.4 g dry wt m^{-2}). Causes of detachment include shear stress on *Spirulina* mats from circulation in the cultivation system, nutrient starvation on *Spirulina* mats [102], and shrimp nibbling.

4.1.3 Effect of *Spirulina* mats on Water Quality and Water Exchange Rate

During the PL stage of *P. vannamei* cultivation, dry pellet feeding resulted in a high concentration of inorganic nitrogen in the water column as TAN leached out of feces and uneaten feed [90]. Accumulation of ammonia can cause mortality [103]. Between the two forms of ammonia nitrogen, ionized (NH_4) and unionized (NH_3), the latter is very toxic to marine organisms. This unionized ammonia (UIA) can easily pass through gills, which are considered the main route of entry into aquatic organisms [104], to accumulate in hemolymph until shrimp death from pH [103]. Therefore, the recommended TAN concentration is lower than 2 mg L^{-1} so as to control the UIA level to levels lower than 0.1 mg L^{-1}, which is considered a safe level [105]. In this experiment, an increase in TAN was detected in both the PL co-cultured with and without *Spirulina* mats. The water was controlled by a 50 % dilution when TAN levels increased to the highest recommended concentration mentioned above, with averages of 1.93 and 1.98 mg L^{-1}, respectively (Fig. 4.8). The TAN accumulation rate in the PL co-cultured with *Spirulina* mats was slower than in cultures without the mats and cultures with artificial mat, at 4.28, 6.35, and 7.35 mg N day^{-1}, respectively (Table 4.6). In addition, since nitrification takes place in two sequential steps, conversion of ammonium into nitrite, followed by nitrite conversion into nitrate [106], the decrease in TAN level paralleled an augmentation in nitrite and nitrate levels. The concentration of the two nitrogen forms exhibited similar patterns to that of TAN. The concentration of nitrite and nitrate in the water of PL co-cultured with *Spirulina* mats, without *Spirulina* mats and with artificial mat was shown in Fig. 4.9 and Figure 4.10, respectively. Accordingly, the accumulation rate of the nitrate (Table 4.6) in the system with the mats

was lower (17.07 mg N day^{-1}) than that of the system without the mats (25.20 mg N day^{-1}) and with artificial mat (18.32 mg N day^{-1}), respectively. Water dilution in the PL co-cultured with *Spirulina* mats was prolonged on days 3, 6, and 9, in comparison with prolonged water dilution on co-cultures without the mats and with artificial mats on days 1, 3, 5, 7, and 9. As a result, the exchanged water with *Spirulina* mats as approximately 45 L, however, without *Spirulina* mats and with artificial mat had exchanged water approximately 75 L for the culture of PL (Table 4.6), respectively. These observations indicate that co-cultivation PL with *Spirulina* mats could reduce the volume of water exchange up to 40 %. Nevertheless, other water quality including pH, DO, and alkalinity of the two systems were not significantly different, in the range of 7.8–8.0, 5–6, and 100–120 mg L^{-1}, respectively. The TAN and nitrite concentration in water columns also affected shrimp food ingestion, growth, and survival rates [107]. Hence, water exchange is an important factor in controlling water quality and disease [9].

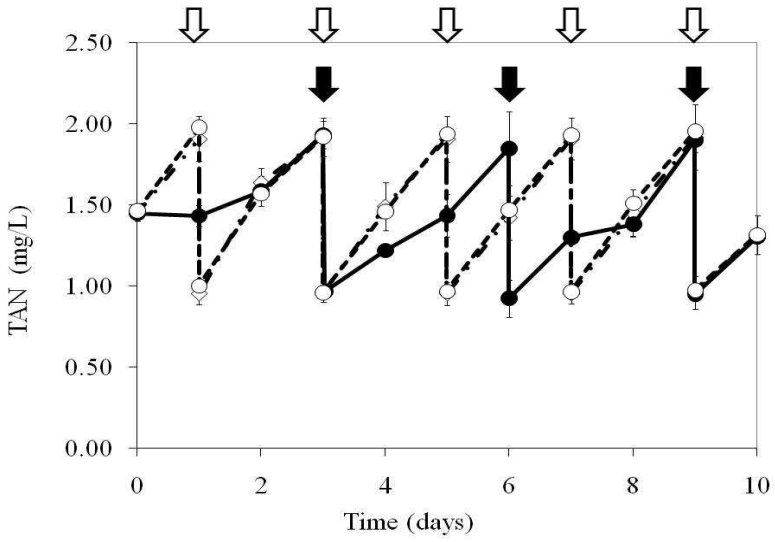

Figure 4.8 Changes of TAN in water column of Set 1, (○); Set 2, (●); and Set 3, (◊); respectively during PL2 to PL12 cultivation. The Set 1, 2 and 3 represented the only shrimp cultivation, shrimp co-cultured with *Spirulina* mat and shrimp co-cultured with artificial mat, respectively. White arrows indicate a 50% water exchange rate in Set 1 and Set 3 while Black indicates a 50% in Set 2, respectively.

Table 4.6 Summarized water quality and shrimp quality in the hatchery over 10 days

Parameter	**Shrimp cultivation condition**		
	Set 1	**Set 2**	**Set 3**
Water quality			
TAN accumulation (mg-N d^{-1})	6.35 ± 0.11^{bc}	4.28 ± 0.08^{a}	7.35 ± 1.28^{bc}
Nitrite accumulation (mg-N d^{-1})	0.45 ± 0.01^{bc}	0.42 ± 0.01^{a}	0.44 ± 0.01^{bc}
Nitrate accumulation (mg-N d^{-1})	25.2 ± 0.44^{c}	17.07 ± 0.32^{a}	18.32 ± 0.63^{b}
Water exchange rate (L)	75	45	75
pH	7.83 ± 0.01^{a}	7.85 ± 0.02^{a}	7.89 ± 0.02^{ab}
Dissolved oxygen	7.83 ± 0.01^{a}	7.85 ± 0.02^{b}	7.86 ± 0.02^{ab}
Alkalinity	108.50 ± 3.27^{abc}	107.30 ± 2.91^{abc}	106.40 ± 2.93^{abc}

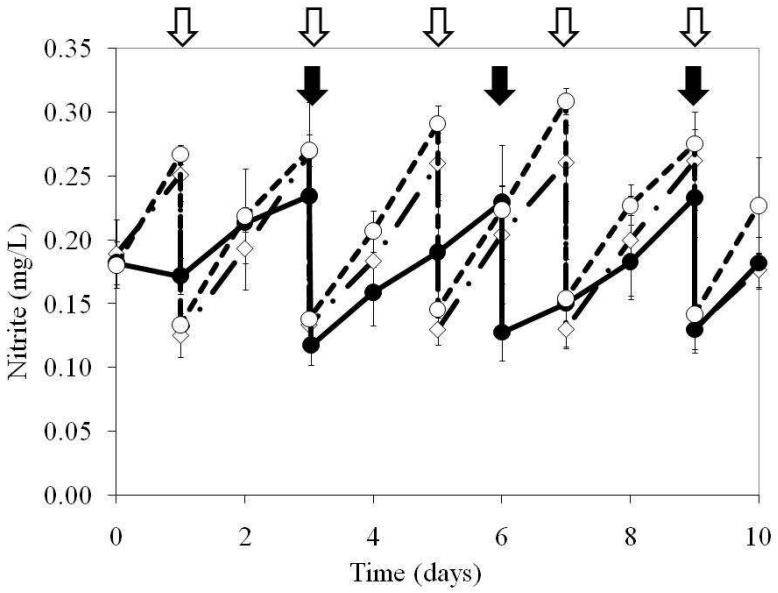

Figure 4.9 Changes in nitrite in water column of Set 1, (○); Set 2, (●); and Set 3, (◊); respectively during PL2 to PL12 cultivation. The set 1, 2 and 3 represented the only shrimp cultivation, shrimp co-cultured with *Spirulina* mat and shrimp co-cultured with artificial mat, respectively. White arrows indicate a 50% water exchange rate in Set 1 and Set 3 while Black indicates a 50% in Set 2, respectively.

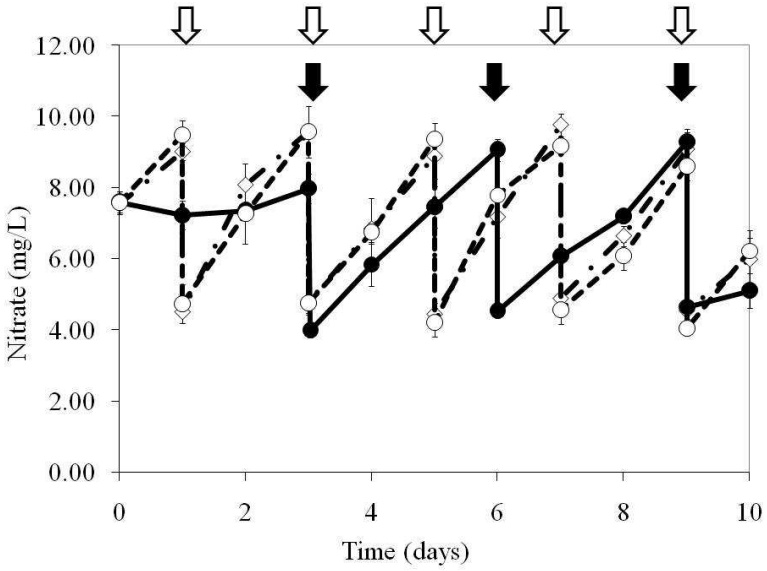

Figure 4.10 Changes in nitrate in water column of Set 1, (○); Set 2, (●); and Set 3, (◊); respectively during PL2 to PL12 cultivation. The Set 1, 2 and 3 represented the only shrimp cultivation, shrimp co-cultured with *Spirulina* mat and shrimp co-cultured with artificial mat, respectively. White arrows indicate a 50% water exchange rate in Set 1 and Set 3 while Black indicates a 50% in Set 2, respectively.

4.1.4 Effect of *Spirulina* mats on the shrimp PL quality

The results in Table 4.7 show that co-culturing with *Spirulina* mats enhanced PL with a larger body size, faster specific growth rate, and higher survival rate than those of PL in the cultures without Spirulina mats. On the starting (day 0), the length of PL2s in both condition was 4.32 ± 0.37 mm. One-way ANOVA analysis ($P \leq 0.05$) showed that the PL co-cultured with, without *Spirulina* mats and with artificial mat was on average 9.81 ± 0.47 , 8.69 ± 0.29 and 8.96±0.39 mm in length, respectively. Likewise, the survival rate of the PL co-cultured with the mat was higher at 72.32 ± 5.18 %, compared with PL without the mat at 61.32 ± 4.38 % and with artificial mat at 64.83±4.68% as shown in Figure 4.11. Moreover, the specific growth rate of PL co-cultured with mats was 0.55 day^{-1} compared with 0.44 mm day^{-1} in PL without the mats and 0.46 mm day^{-1} in PL with artificial mat. This could be due to the effects of the *Spirulina* on TAN and its effect as a supplement. Several microalgae have been reported to increase in growth from protein accretion, feed utilization, physiological activity, stress response, starvation tolerance, disease resistance, and carcass quality in aquaculture [108]. In addition to the nutrition that could be provided by *Spirulina* mats, its physical presence within cultures enlarged the area available for shrimp distribution. This is an

interesting feature, as several studies have shown an inverse relationship between stocking density and shrimp growth. The presence of artificial substrates delays the negative effects of overcrowding, contributing to the improved performance of *L. vannamei* reared in an intensive culture system [109] and the freshwater prawn *Macrobrachium rosenbergii* [110]. This suggests the importance of using substrates, mainly due to the availability of food provided by the biofilm formed on the substrates [109], [110]. Therefore, there may be a synergy between the physical and biological aspects of artificial substrates on *Spirulina* mats, similar to that found in *L. schmitti*, which show a survival rate as high as 82–87 % [21]. The survival rate of shrimp grown with *Spirulina* mats in our study was slightly lower than those observed in other studies.

Even though *Spirulina* was not fed directly to the shrimp, it played a major role in controlling the water quality and did not cause any damage to the cultured organisms [10].

Table 4.7 Summarized shrimp quality in the hatchery over 10 days

Parameter	**Shrimp cultivation condition**		
	Set 1	**Set 2**	**Set 3**
Shrimp quality			
PL length (mm)	8.69 ± 0.29^{a}	9.81 ± 0.47^{b}	8.96 ± 0.39^{c}
Specific growth rate (mm day^{-1})	0.44 ± 0.06^{bc}	0.55 ± 0.02^{a}	0.46 ± 0.06^{bc}
Survival rate (%)	61.32 ± 4.38^{bc}	72.32 ± 5.18^{a}	64.83 ± 4.68^{bc}

Remark: The initial PL2 length was 4.32 ± 0.37 mm.

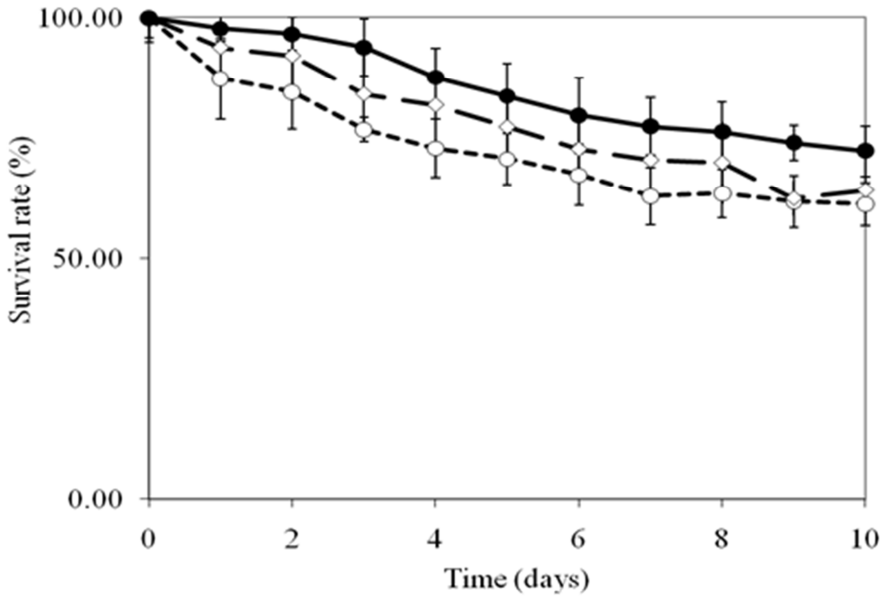

Figure 4.11 Survival rates of shrimp among Set 1, (○); Set 2, (●); and Set 3, (◊); respectively during shrimp rearing periods for 10 days.

Our findings clearly showed that co-culturing Pacific white shrimp (*P. vannamei*) PL with *Spirulina* mats in a closed re-circulating system reduced inorganic nitrogen levels, particularly ammonia and nitrate, and resulted in better water and PL quality. The lower ammonia concentration, in accordance with dynamic bacterial communities, subsequently decreased in water exchange rates by approximately 40 %. This was an environmentally friendly process, due to the reduction in discharge into environment and the seawater used during the shrimp PL production process, which can certainly decrease the costs of shrimp production. Therefore, the *Spirulina* mat could be suggested as an alternative green material for water quality control and management in aquaculture system, especially shrimp PL, as shown in the proposed scheme (Fig. 4.12).

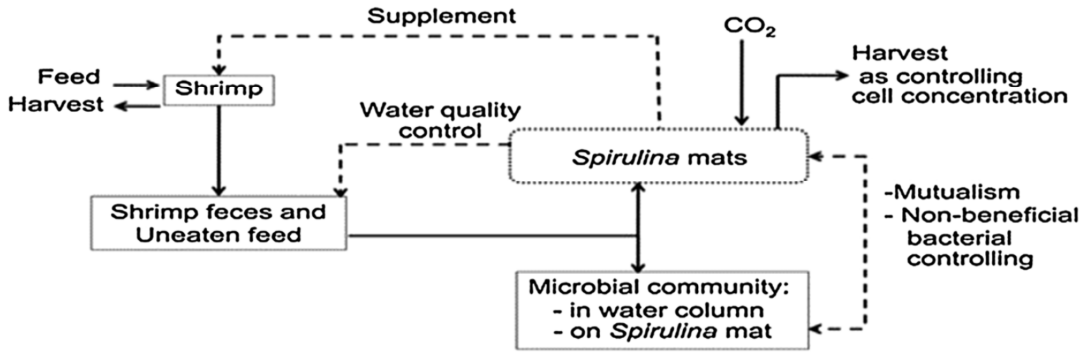

Figure 4.12 Schematic flow of the co-culturing in shrimp larvae (PL2-PL12) with *Spirulina* mat. Solid line indicates Nitrogen flow; dash line indicates the relationship between modules.

4.2 Dynamic of Microbial Community during Shrimp Growing Period

In this study, the shrimp culture in earthen pond was investigated. The correlation between shrimp quality, water quality and microbial community were studied. The water quality and microbial profiling during shrimp growing period were monthly investigated while the shrimp quality was investigated at the harvesting time.

4.2.1 Application of *Spirulina* mats in Shrimp Earthen Pond

Spirulina mats application was preliminary studied in shrimp earthen pond to investigate microbial community on the mat during shrimp cultivation. It was found that *Spirulina* mat had limitation to apply in earthen pond due to soil particle from earthen pond and suspended solid from shrimp's feces and microalgae in shrimp pond attached on *Spirulina* mat resulting photoshading. Consequently, *Spirulina* lost from mat area as shown in Figure 4.13. Therefore, microbial community on the *Spirulina* mat in this part did not study.

4.2.2 Microbial Community Profiling during Shrimp Cultivation Period

Microbial diversity and shifts in bacterial communities present in samples were estimated based on the DGGE patterns of the partial 16S rDNA gene PCR products as show in Figure 4.14. A total of four shrimp farm water samples were compared to correlate the relationship of ammonia, nitrite and nitrate concentrations and microbial community. W_0 represented a water sample taken before shrimp were released containing low nutrients. The water samples obtained after shrimp were released for one month, two month and three month was W_1, W_2, and W_3, respectively. It is known that

the ammonia within a shrimp pond is largely derived from the breakdown of protein added in the form of shrimp feed and subsequent excretion by shrimp during the cultivation period.

The analysis of DGGE band-patterns from water samples revealed a clear shift in microbial composition during the shrimp cultivation process (Figure 4.14). During the farming period, this shift was pronounced in bacterial community structure, as indicated by the comparison of the calculated similarity indices. Bacterial similarity between the water sample, W0 and the water samples, W_1, W_2 and W_3 were 18%, 12% and 18%, respectively, while the bacterial similarity between W_1 and W_2 was 81% (Table 4.8).

Table 4.8 Similarity of microbial community during shrimp growing period

Farm period	**W_0**	**W_1**	**W_2**	**W_3**
W_0	100%			
W_1	18%	100%		
W_2	12%	81%	100%	
W_3	18%	17%	16%	100%

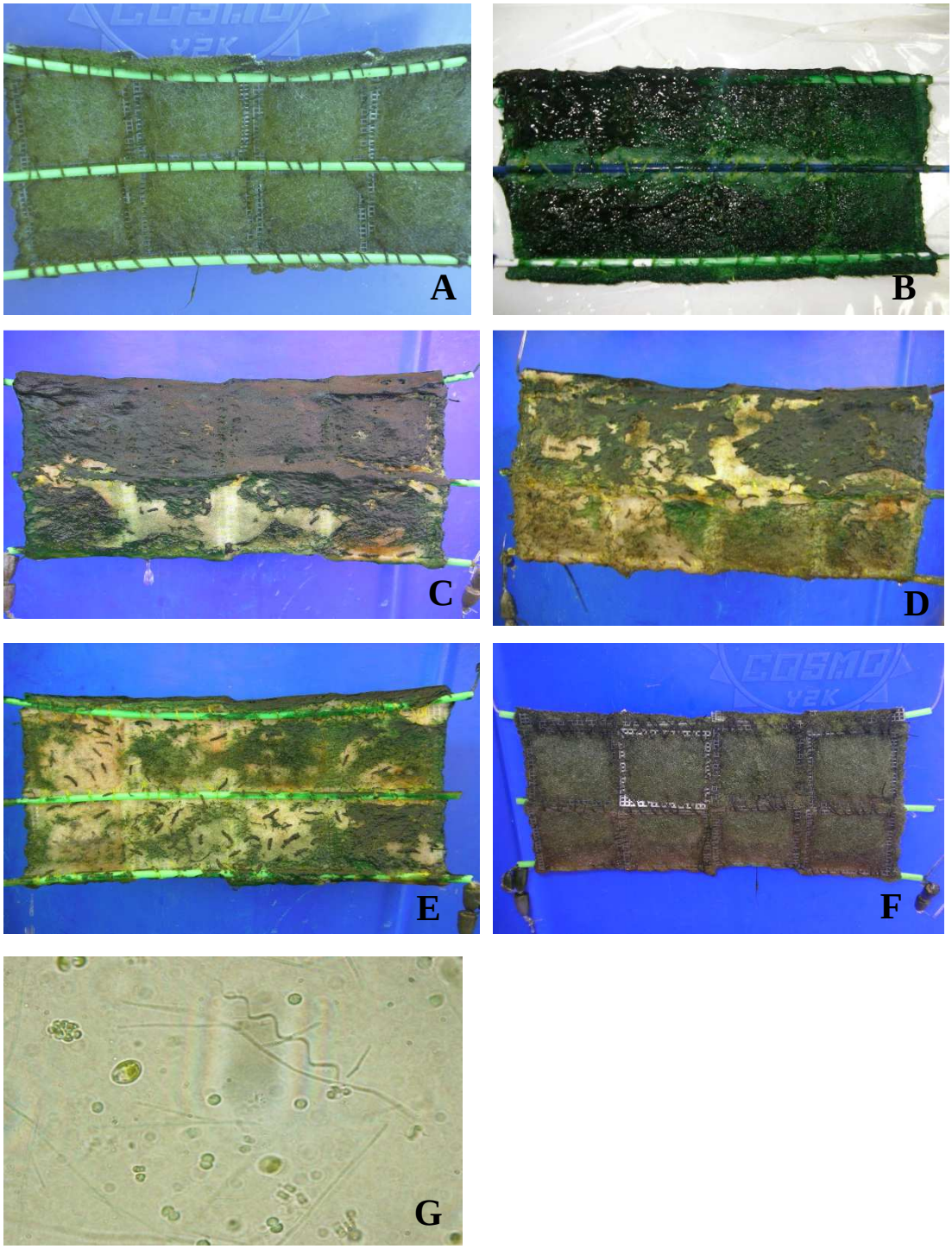

Figure 4.13 Using *Spirulina* mats during shrimp growing period in grow-out pond. A to F was *Spirulina* mat on day 0, 7 14, 16, 17 and 21, respectively. Figure G represented microbial community on *Spirulina* mat after 21 days under microscope.

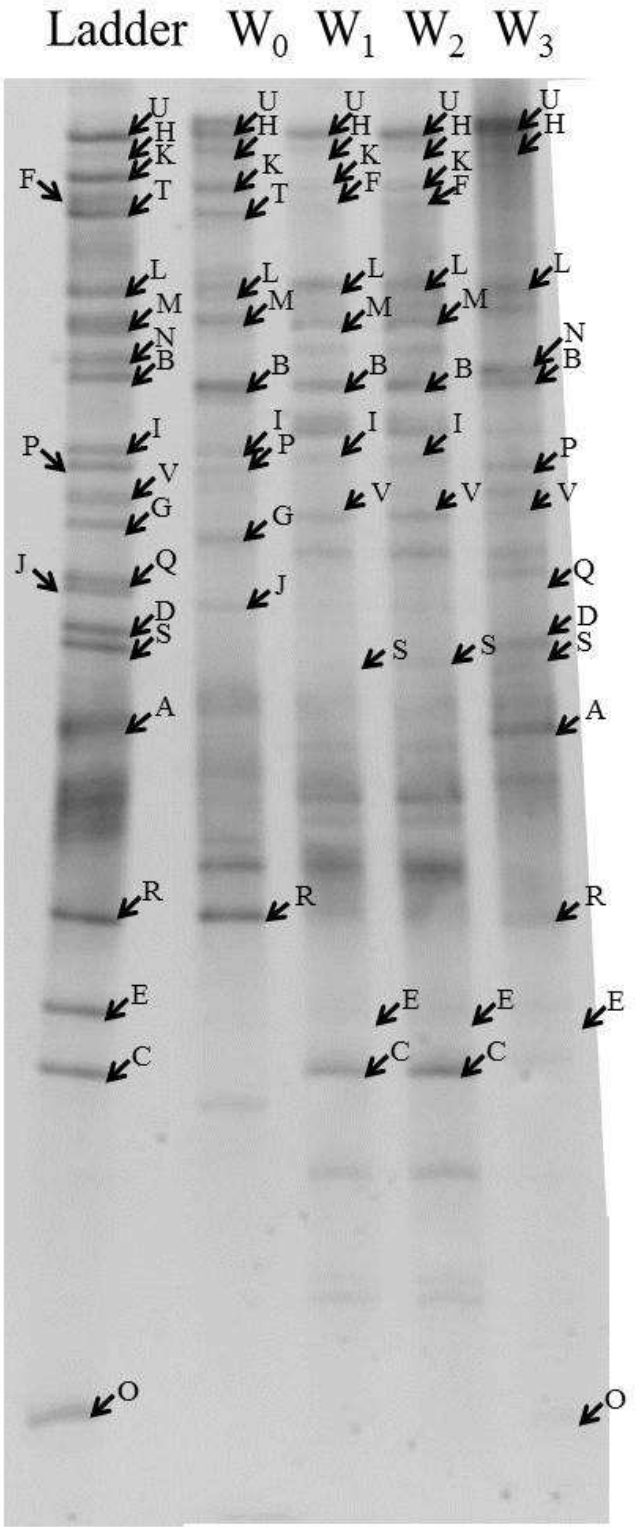

Figure 4.14 DGGE profile of bacterial amplicons from shrimp grow-out water; $W_{0,}$ microbial community before shrimp released to grow-out pond. $W_{1,}$ $W_{2,}$ and $W_{3,}$ after shrimp growing period 1, 2 and 3 months, respectively. Ladder was the 22 clones mixture.

4.2.3 Phylogenetic Analyses of Microbial Community of Water Column in Grow-out Pond

To assign the composition of microbial community visualized in the DGGE patterns, nearly full-length bacterial 16S rDNA gene fragments, retrieved from the four water samples, were used to construct 16S rDNA clone libraries. The microbial community during the shrimp growing periods consisted of 22 OTUs (Band A to V) as shown in Table 4.9. Phylogenetic affiliation of sequences was initially assessed by BLAST similarity searches, and confirmed by phylogenetic analysis (Table 4.9 and Figure 4.15 to Figure 4.17). The distributions of microbial community were in Beta- (Figure 4.15), Alpha- (Figure 4.16), Gamma- (Figure 4.16) proteobacteria, Cytophaga–Flavobacterium–Bacteroides (CFB; Fig. 4.16) and Cyanobacteria+Chloroplast (Fig. 4.17) at 41%, 20.5%, 16%, 4% and 18.5%, respectively. Four obvious DGGE bands were observed in all four samples, there were Clone B, H, L and U that closely related to *Exiguobacterium* CNJ771, *Euglena spathirhyncha*, *Exiguobacterium* SKPB5 and *Nitrosomonas eutropha*, respectively. *Nitrosomonas* sp. had major role to control ammonia in aquaculture system therefore it was found along grow-out period.

Other DGGE bands showed different distribution patterns; some were present in one or several sampling positions. Clone I, K and M was closely related to *Gammaproteobacterium*, *Fibrobacterium* and *Synechococcus* RS 9915, respectively were found in W_0, W_1 and W_2. Clone E, S and V was closely related to *Burkholderia* sp. WBF2, *Rheinheimera aquimaris* strain SW-369 and *Nitrobacter wingogradskyi*, respectively was found in W_1, W_2 and W_3. *Nitrobacter* sp. had major role to control nitrite in aquaculture system therefore *Nitrobacter wingogradskyi* was found in water after shrimp cultivation for 1 month. Clone G and T was closely related to *Alpha*-proteobateria and *Clostridium* sp., respectively was found in only W_0 while Clone A, D, N, O and Q was closely related to *Nitrosospira* sp., *Stenotrophomonas maltophilia*, *Alpha*-proteobateria, *Aquiflexum balticum* and Unclutured *diatom* clone HT2A8, respectively was found in only W_3.

Clone C and F was closely related to *Aquimonas* sp. and *Exiguobacterium undae*, respectively were found in W_1 and W_2. *Exiguobacterium undae* could grow in wide

range of temperature from –2.5 to 30 ^{o}C [111]. Although W_1 had the lowest temperature due to effect of winter, *Exiguobacterium undae* was also found.

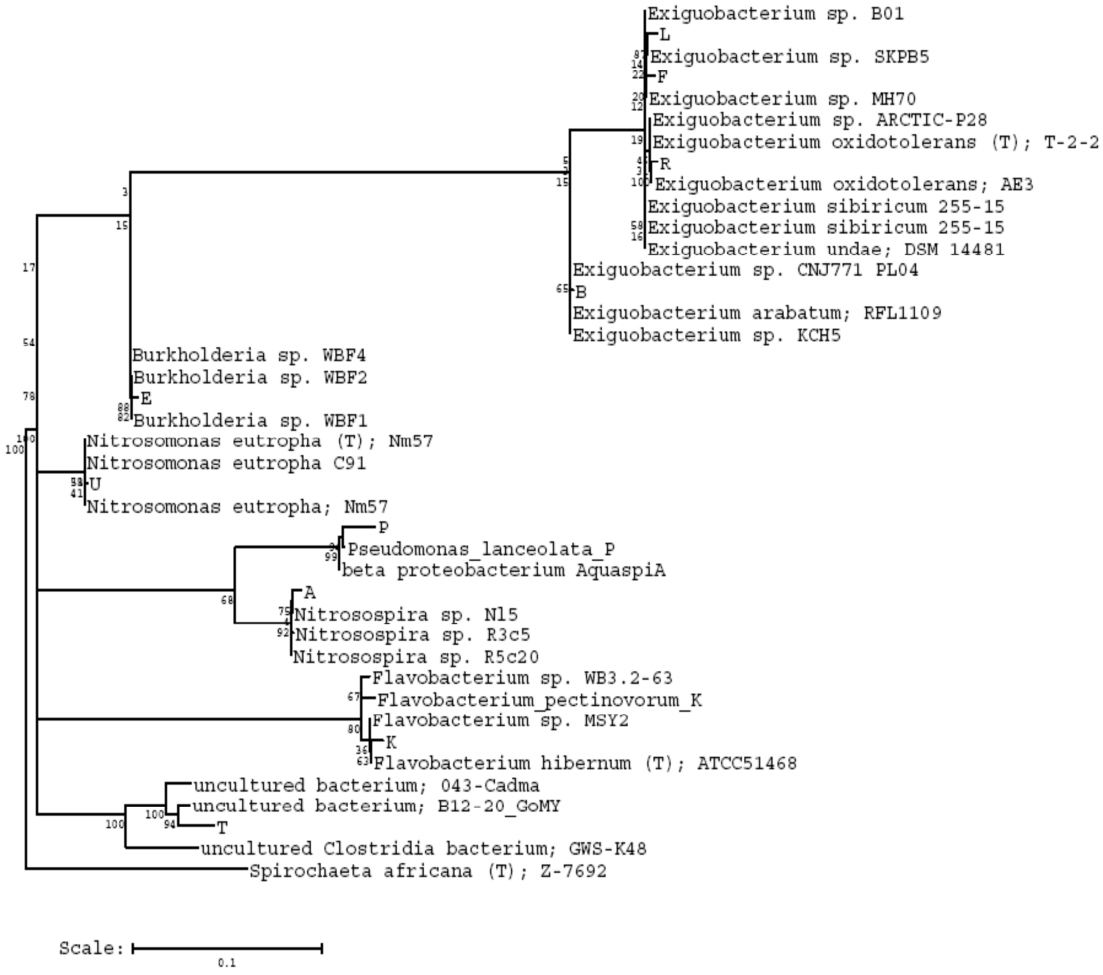

Figure 4.15 Phylogenetic tree constructed based on 16S rDNA 1.5-kb clone sequences recovered from water during shrimp growing period in *Beta-proteobacteria*. The scale bar length of 0.1 denotes the number of amino acid replacements per site validated with 1000 bootstraps. The number on the branches indicates the support proportion of each branch.

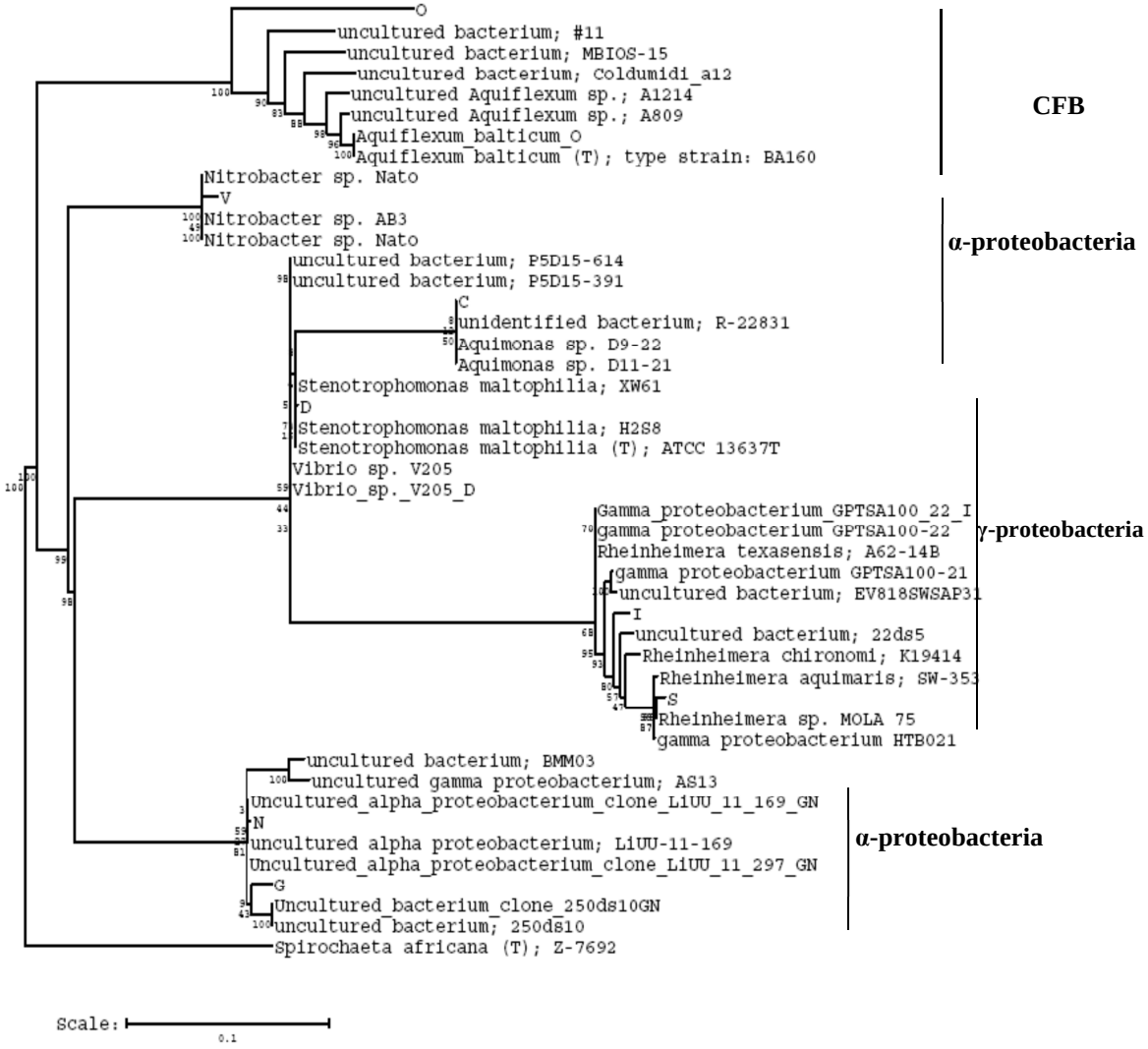

Figure 4.16 Phylogenetic tree constructed based on 16S rDNA 1.5-kb clone sequences recovered from water during shrimp growing period in *Alpha-*, *Gamma-*proteobacteria and *Cytophaga–Flavobacterium–Bacteroides* (CFB). The scale bar length of 0.1 denotes the number of amino acid replacements per site validated with 1000 bootstraps. The number on the branches indicates the support proportion of each branch.

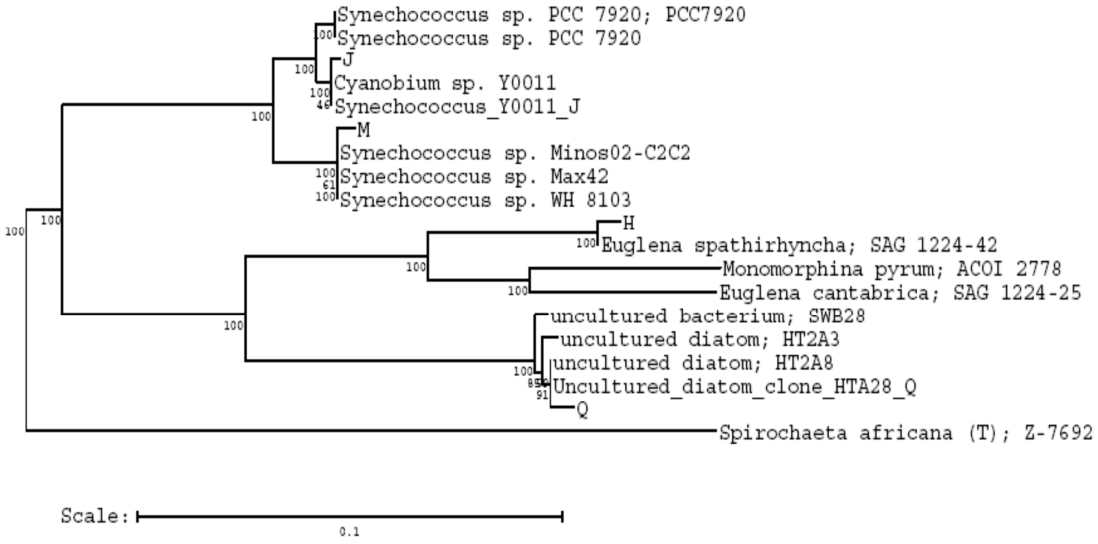

Figure 4.17 Phylogenetic tree constructed based on 16S rDNA 1.5-kb clone sequences recovered from water during shrimp growing period in Cyanobacteria and Chloroplast. The scale bar length of 0.1 denotes the number of amino acid replacements per site validated with 1000 bootstraps. The number on the branches indicates the support proportion of each branch.

Table 4.9 Affiliation of the clone library of microbial community in shrimp farming

Clone	Bacteria	No. of clone (%)	Identity (%)	Group
A	*Nitrosospira* sp.	12 (6)	93	β-proteobacteria
B	*Exiguobacteria* sp. CNJ771	19 (9.5)	99	β-proteobacteria
C	*Aquimonas* sp. D11-21	3 (1.5)	99	α-proteobacteria
D	*Stenotrophomonas maltophilia*	15 (7.5)	99	γ-proteobacteria
E	*Burkholderia* sp. WBF2	1 (0.5)	99	β-proteobacteria
F	*Exiguobacterium undae*	4 (2)	98	β-proteobacteria
G	Alpha proteobacteria clone LiUU 11-169	14 (7)	98	α-proteobacteria
H	*Euglena spathirhyncha* strain SAG 1224-42	11 (5.5)	99	Cyanobacteria and Chloroplast
I	*Gamma proteobacterium* GPTSA100-21	3 (1.5)	97	γ-proteobacteria
J	Synechococcus sp. Y0011	8 (4)	92	Cyanobacteria and Chloroplast
K	*Uncultured Flavobacterium* clone LiUU-11-73	7 (3.5)	84	β-proteobacteria
L	*Exiguobacterium* sp. SKRP 5	8 (4)	99	β-proteobacteria
M	*Synechococcus* sp. RS9915	8 (4)	99	Cyanobacteria and Chloroplast
N	Alpha proteobacteria clone LiUU 11-169 GN	14 (7)	97	α-proteobacteria
O	*Aquiflexum balticum*	8 (4)	92	Cytophaga–Flavobacterium–Bacteroides
P	*Pseudomonas Lanceolata*	7 (3.5)	99	β-proteobacteria
Q	Uncultured diatom clone HT2A8	10 (5)	99	Cyanobacteria and Chloroplast
R	*Exiguobacterium* sp.	7 (3.5)	97	β-proteobacteria
S	*Rheinheimera aquimaris* strain SW-369	14 (7)	99	γ-proteobacteria
T	*Clostridium* spp..	4 (2)	86	β-proteobacteria
U	*Nitrosomonas eutropha*	13 (6.5)	99	β-proteobacteria
V	*Nitrobacter winogradskyi*	10 (5)	99	α-proteobacteria

Clone P and R was closely related to *Pseudomonas lanceolata* and *Exiguobacterium* sp., respectively were found in W_0 and W_3. Clone J was closely related to *Synechococcus* sp. was found in W_0 and W_2. From 22 OTUs, microbial community was defining as; Bacteriological nitrification is the most practical method for the removal of ammonia from closed aquaculture systems and it is commonly achieved by setting of sand and gravel bio-filter through which water is allowed to circulate. Clone U was closely related to *Nitrosomonas* sp. that removed ammonia from the water.

The ammonia oxidizing bacteria are placed under five genera, *Nitrosomonas, Nitrosovibrio, Nitrosococcus, Nitrosolobus* and *Nitrosospira,* while nitrite oxidizing bacteria are placed under three genera, *Nitrobacter, Nitrococcus* and *Nitrospira.* There are also some heterotrophic nitrifiers that produce only low levels of nitrite and nitrate and often use organic sources of nitrogen rather than ammonia or nitrite. Nitrifiers in contaminated cultures have been demonstrated to nitrify more efficiently. Nitrification not only produces nitrate but also the pH slightly towards the acidic range, facilitating the availability of soluble materials [39].

Clone V was closely related to *Nitrobacter winogradskyi* that removed nitrite from the water. The ammonia and nitrite concentration were stabled and could be controlled by these bacteria. *Nitrosomonas* and *Nitrobacter* were commonly used for the commercially bioremediators for aquaculture [112]. Furthermore, Supamattaya *et al.* [113] reported water quality affect the immune system of *Penaeus* monodon, when *P. monodon* are maintained in an unsuitable environment over a prolonged period, or come under stress as a result of environmental change or imbalance, the immune system is depressed and the shrimp become more susceptible to disease. Shrimp farmers should take care to ensure good control over important water quality criteria such as DO [113].

Dissolved oxygen should be maintained above 4-5 mg L^{-1} throughout the day and night. A high DO supports good shrimp health, increased feed intake and growth as well as ensuring that other oxygen dependent pond processes are maintained. On the other hand, low DO levels, especially at night when phytoplankton photosynthesis stops, result in stress and greater vulnerability to disease. Oxygen is not the only factor, however, as other water and bottom soil quality factors also have an impact on the shrimp immune system and productivity [113].

The chlorophyll *a* content in shrimp water was increased along with time during shrimp growing period when *Synechococcus* sp. RS9915 represented by clone M that was found in W_0, W_1 and W_2. While, *Synechococcus* sp. Y0011 represented by clone J was found in W_0 and W_2.

Aquiflexum balticum (Clone O) was found only in W_3, which represented in the *Cytophaga–Flavobacterium–Bacteroides* (CFB). In marine and freshwater environments, a high abundance of CFB bacteria can occur, and the CFB bacteria are considered to be of high relevance for the degradation of organic matter, such as complex polysaccharides [114].

Clone D was closely related to *Stenotrophomonas maltophilia* that initially classified as *Pseudomonas maltophilia, S. maltophilia* , which represented in the genus *Xanthomonas* before eventually becoming the type species of the genus *Stenotrophomonas* in 1993 [115]. This bacterium had potential to degrade protein [116] and it might be nitrogen source of nitrifying bacteria.

Overall, there were 3 groups of microbial dynamics observed in this study. The first dynamic consisted of the microbes that were found at all the time points investigated: *Nitrosomonas eutropha*, *Exiguobacterium SKRP 5*, and *Exiguobacteria* sp. CNJ771. The ammonia oxidizing bacteria, *Nitrosomonas eutropha*, has been reported as typical of the flora in earthen shrimp ponds, due to the excess ammonia that is found in these ponds. The sources of excess ammonia had been from overfeeding, shrimp feces or the sediment for nitrite production [117]. Moreover, this bacterium also correlated with *Nitrobacter winogradskyi*, a nitrite oxidizing bacteria which can oxidize nitrite to nitrate [118] that was keeping nitrite concentrations at less than 0.05 mg L^{-1}, while nitrate concentrations increased over the growth period. The other ammonia-oxidizing bacteria, *Nitrosospira* sp., which was only found in W_3 sample, might be working with *Nitrosomonas eutropha* to control the ammonia concentration in the shrimp pond system. *Exiguobacterium* spp., another type of microflora found in aquacultures , was also found in *Artemia* cysts and gut rumen [94], salmon intestinal systems [119] and shrimp farming sediment [120]. Therefore, it was expected that *Exiguobacterium* spp. would also be found during this investigation. Moreover, *Exiguobacterium undae* (found in W_2 and W_3) and *Exiguobacterium* sp. (found in W_0 and W_4) were also

associated with the activity of *Exiguobacterium SKRP 5* and *Exiguobacteria* sp. CNJ771 during the shrimp growing period. The second group of microbial dynamics involved the replacement of *Flavobacteriales bacterium* with *Aquiflexum balticum*, both of which are bacteria involved in shrimp shell degradation. In the first two months (W_0 to W_2), *Flavobacteriales bacterium* was responsible for the degradation of shrimp shells. However, as the salinity gradually decreased to 2 ppt (to control pathogens in the shrimp pond) and the temperature rose to 28.6 $^{\circ}$C, the environmental conditions became optimal for *Aquiflexum balticum*; this finding might account for the observed replacement of *Flavobacteriales bacterium* with *Aquiflexum balticum*. The third group of microbial dynamics involved bacteria that were observed only at one time point, such as *Synechococcus* sp. *Y0011* (W_0), *Stenotrophomonas maltophilia* (W_3), or at several time points, such as *Pseudomonas lanceolata* (W_0 and W_3) and *Burkholderia* sp. WBF2 (W_1 and W_2). As previously mentioned, although this third group of bacteria was not found at all of the time points investigated, they have an important role in balancing the shrimp culturing system, which is needed for a successful shrimp cultivation.

The importance of the microbial community composition for water quality and shrimp production suggests the need for management strategies that promote beneficial processes, while controlling adverse processes. The interrelationships between various functional groups within the microbial community are not completely understood, and relationships between microbes, system inputs, water quality, and shrimp health are complex. Changes in the composition of the microbial community were significantly linked to temperature, salinity and nutrient (ammonia, nitrite and nitrate) concentrations. Future studies aimed at understanding the role of each micro-organism within the shrimp pond community will provide greater guidance not only for the development of more successful shrimp culturing systems but also for the application of these developments for sustainable environmental protections.

4.2.4 Water Quality during Shrimp Growing Period

In this study, water was collected from shrimp pond in Thung Kru, Bangkok. The water was collected during shrimp cultivating crop at approximately 3 months. Physical and chemical parameters of shrimp pond were measured during water sampling as shown in Table 4.10.

Table 4.10 Physical and chemical parameters (Mean±SD, N=3) of water column obtained during shrimp growing period (90 day-grow-out set)

Parameter	Water samples			
	W_0	**W_1**	**W_2**	**W_3**
pH	7.8±0.03	7.9±0.08	8.0±0.07	7.8±0.1
Temperature (^oC)	22.4±0.3	18.2±0.5	27.3±0.6	28.6±0.2
Salinity (ppt)	10	7	5	2
Chl *a* (µg L^{-1})	42.67±5.20	71.00±4.30	164.63±11.40	187.16±15.10
TAN (mg-N L^{-1})	0.26±0.01	0.21±0.03	0.02±0.00	0.05±0.01
Nitrite nitrogen (mg-N L^{-1})	< 0.05	< 0.05	< 0.05	< 0.05
Nitrate nitrogen (mg-N L^{-1})	2.72±0.06	3.17±0.01	3.61±0.07	3.98±0.06

4.2.5 Effect of Cultivation Period on Total Ammonia Nitrogen in Water Column

Changes of inorganic nitrogen compounds in water column were shown in Table 4.10. The highest of total ammonia nitrogen (TAN) concentration was found in W_0 at 0.262±0.014 mg-N L^{-1} and then slightly decreased. The nitrite concentration was not found during growing periods whereas nitrate concentration was lowest in W_0 and slightly increased; the highest nitrate concentration was 3.977±0.061 mg-N L^{-1} in W_3. The chlorophyll *a* concentration also had the similar profile of nitrate concentration; the highest was 187.16±15.1 µg L^{-1} in W_3. These concentrations were relatively intermediate compared with other regions where is cultured white shrimp in semi-intensive ponds: 33–79 µg L^{-1} in Gulf of Fonseca Honduras [121]; 13–93 µg L^{-1} in south of Sinaloa, Mexico [122]. The chlorophyll *a* concentration was also the indicator of phytoplankton in shrimp grow-out ponds [123].

The pH, temperature and salinity were in range 7.8-8.0, 18.2-28.6 ^oC and 2-10 ppt, respectively as show in Table 4.10. These were in the recommended range of water quality for white shrimp culture [124]. The relationship between these parameters had effect on water quality and shrimp health. For water quality, the correlation between TAN, nitrite and nitrate concentration show the possibility the role of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) [125]. The decreased

concentrations of ammonia in this study were observed between days 30 (W_1) and 90 (W_3), while the concentrations of nitrate were increased. The nitrate concentration were found to increase with days of culture with the highest of 3.977±0.061 mg-N L^{-1} at the end of growing period, W_3, while the nitrite concentration were not detect. The high nitrate concentration with low concentrations of ammonia and nitrite would suggest that nitrification process was established in the shrimp pond [126].

Nitrification takes place in two sequential steps: the first step, the conversion of ammonium into nitrite and the second step, the conversion of nitrite into nitrate [106]. However, the nitrate concentration still lower than standard value (less than 50 mg L^{-1}) due to nitrate uptake from phytoplankton during shrimp growing period [16]. The correlation between pH and toxicity of ammonia nitrogen, the occurrence of ammonia in alkaline condition *i.e.* pH 9.3, about 50% in form of ammonia can become a major problem in shrimp pond [127]. Increasing of ammonia in environment has been reported to reduce growth, enhance molting, decrease the value of immune parameters such as total haemocyte count (THC), prophenoloxidase (prpPO), superoxide dismutase (SOD), and peroxidase (POD), and even cause the death of penaied shrimp [128].

Moreover, type of feed affected ammonia and nitrite concentration in cultivation system; the different diets (artificial and natural) indicated that nitrogenous excretion was predominant in shrimps fed with artificial diets. Live food, such as algae, despite a high protein content, contributes to low nitrogenous excretion and hence has less adverse effects on water quality compared to artificial diets [129]. Temperature and salinity affected shrimp immunity, quality and growth [130], at low salinity will increase the virulence of *Vibrio* sp. in *P. vannamei*, however, increasing age in shrimp also improve their immunity [124] therefore shrimp was cultured in high salinity and then decreased during growing period.

The increasing in temperature over the range of 20°C to 32°C resulting in an increase of growth of *L. vannamei* juveniles had been reported [131]. Shrimp at 20°C were relatively inactive and exhibited low food consumption compared with hyperactive animals at 35°C. When offered unlimited food, shrimp maintained at 35°C had the highest rate of food consumption. These observations are consistent with those of Zein-Eldin *et. al* [132] in *P. azfecus.* Wyban *et al.* [133] suggested that temperature optima

for fastest growth are size-specific and decrease as shrimp size increases. For small shrimp (< 5 g), temperature optima may be greater than 30°C while for large shrimp; the optimum temperature is about 27°C [133].

In grow-out pond, a higher temperature may markedly shorten the culture period of shrimp [133]. This was fond in the results of previous study (i.e., the proportion of intake energy invested in growth (%C) appeared to decrease with increases of temperature from 20°C to 32°C, whereas total metabolic expenditure (respiration and excretion, %C) displayed inverse trends with temperature; this suggests that it is energetically expedient to grow *L. vannamei* juveniles at relatively lower temperatures [134].

4.2.6 Comparison of Nitrification Activity

Nitrifying bacteria, a beneficial bacterial community, naturally exist in aquaculture water column, although the nitrification activity in shrimp pond water is normally lower than that the sediment [135]. To determine the dynamics of the community, time course of the nitrification rate was studied. Figure 4.18 and Table 4.11 show ammonia oxidation rate among 4 water samples. W_0 represents water samples taken before shrimp were released into the earthen pond. W_1, W_2 and W_3 represent water samples taken one, two and three months, respectively, after the shrimp were released. Bacterial ammonia-oxidation rate was 0.0229, 0.0019, 0.0138 and 0.0202 mg-N L^{-1} h^{-1}, respectively.

The nitrite-oxidation rate, shown in Figure 4.719 and Table 4.11, had a similar trend with ammonia-oxidation (0.073, 0.0131, 0.0224 and 0.0241 mg-N L^{-1} h^{-1}). The result indicated that W_1 had the lowest activity of ammonia oxidizing bacteria and the lower nitrtite oxidation rate, corresponded to the temperature variation which was found as 22.4 ± 0.3 (W_0), 18.2 ± 0.5 (W_1), 27.3 ± 0.6 (W_2) and 28.6 ± 0.2 (W_3) °C, respectively. Since the temperature is a parameter affected growth of nitrifying bacteria [135]. At 20 °C, an increase in the nitrification rate of 1.1% for 1 °C increment under oxygen limitation conditions and 4.3% under TAN limitation conditions [106]. In addition, the result also revealed the existing of the ammonia oxidizing bacteria and nitrite oxidizing bacteria in the microbial community. Due to TAN and nitrite was decreased during activity test [135]. Caffrey J.M. *et al.* [136] reported that nitrifying bacteria are

sensitive to water quality and management strategies and the critical environmental variables known to affect nitrification rate include temperature [137], salinity [138], and NH_4^+ availability [137]. While abundance of AOB and nitrification rates in freshwater were greater than marine environment [138].

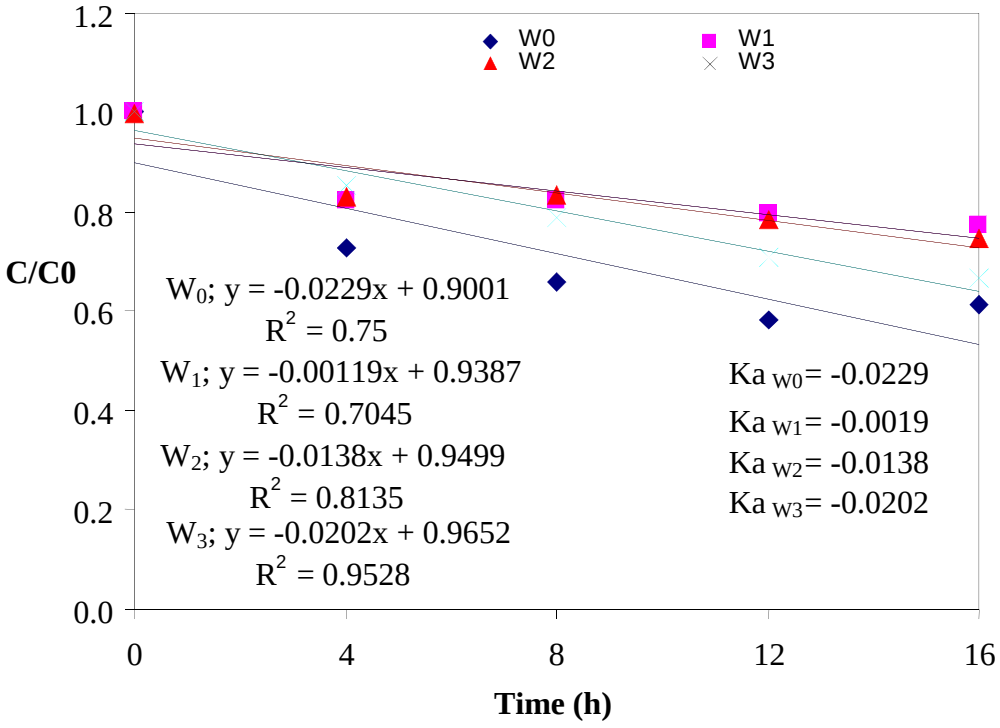

Figure 4.18 Ammonia oxidation rate: ammonia oxidation process with aerobic condition. W_0, the activity in water before shrimp growing period; W_1, W_2, and W_3, the activity in water after shrimp growing period 1, 2 and 3 months, respectively. The initial ammonia concentration was 10 mg-N L^{-1}.

Table 4.11 Nitrifying activity rate during shrimp growing periods

Activity process	**Water samples**			
	W_0	**W_1**	**W_2**	**W_3**
Ammonia oxidation rate (mg-N L^{-1} h^{-1})	0.0229	0.0019	0.0138	0.0202
Nitrite oxidation rate (mg-N L^{-1} h^{-1})	0.0073	0.0131	0.0224	0.0241

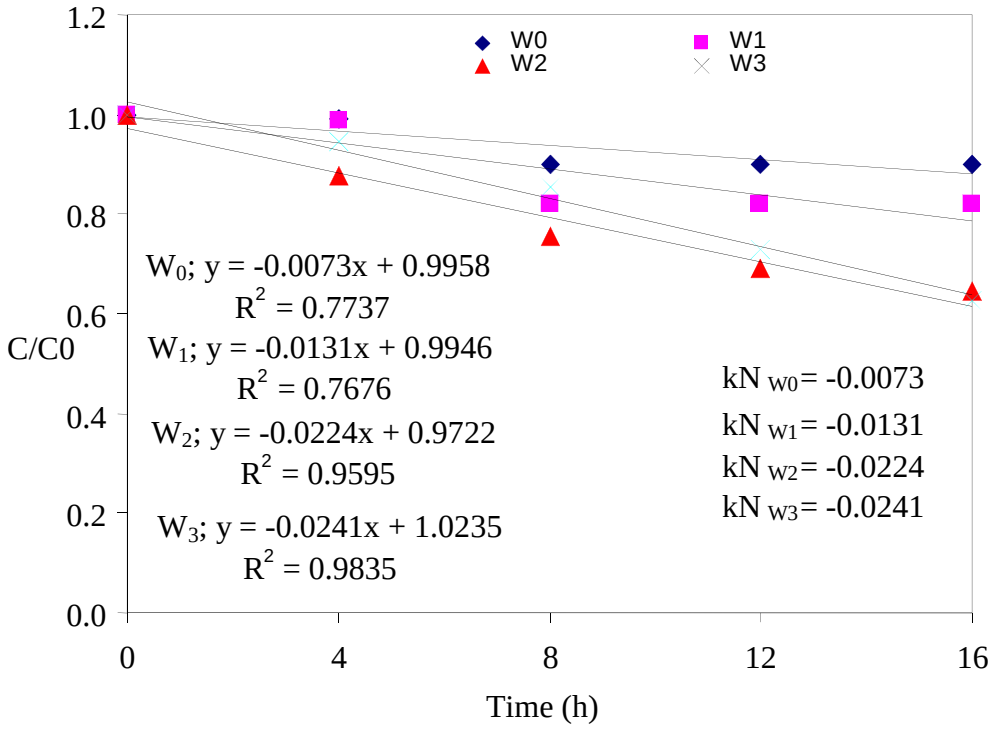

Figure 4.19 Nitrite oxidation rate: nitrite oxidation process with aerobic condition. $W_{0,}$ the activity in water before shrimp growing period; $W_{1,}$ $W_{2,}$ and $W_{3,}$ the activity in water after shrimp growing period 1, 2 and 3 months, respectively. The initial nitrite concentration was 5 mg-N L^{-1}.

4.2.7 Correlation between Microbial Community and Nitrification Oxidation Rate

In Figure 4.18-4.19 and Table 4.10-4.11, W_1 had the lowest ammonia oxidation rate, although W_1 (investigated in winter) had a similar microbial community to W_2. This indicated that temperature during the cultivation period might affect, corresponded to a report [139] that factors, salinity, pH, and ammonium concentration were important to the determination of the community structures of AOB (Ammonia Oxidizing Bacteria). Moreover, they found that the abundance concentration of AOA (Ammonia Oxidizing Archaea) in seawater aquarium biofiltration systems is slightly larger than those of AOB [139].

4.2.8 Shrimp Quality and Feed Conversion Ratios during Shrimp Cultivation

The farm operated under a semi-intensive condition (0.3–1 kg/m^2). The size of this earthen pond was 3 rai with shrimp production at 2,700 kg (0.59 kg/ m^2). The harvested shrimp was 60 shrimp/kg under 90-days-cultivation with 80% survival rate (Table 4.12).

Table 4.12 Characteristics of shrimp farming

Parameter	**Average**
Stocking density (PL/m^2)	42
Shrimp production (kg/m^2)	0.59
Survival (%)	80
Harvest size (g)	16.67
Food add (kg/rai)	1,350
Food Conversion Ratio (FCR)	1.5

Moreover, an important measure of how well the feed is utilized by the animals in the culture system is the Feed Conversion Ratio (FCR). The lower FCR value is the more efficiently the feed is being utilized. Generally, FCR less than 2.0 are good [140]. The FCR in this study (Table 4.12) was 1.5 that are in the acceptable range [140]. High FCR values have been resulting from nutritionally deficient feeds, overfeeding, poor water quality or crowding. Whenever high FCR are found, it is important to take a critical look at the feeding program and production process to try to identify the root causes.

4.3 Comparison of Water Quality and Microbial Community in Hatchery and Grow-Out (Earthen) Ponds

To elucidate how bacterial community in hatchery differs from shrimp grow-out pond, comparison of bacterial diversity in shrimp hatchery and earthen grow-out pond were studied using DGEE technique.

4.3.1 Comparison of Water Quality in Hatchery and Grow-Out (Earthen) Ponds

The water quality indicators measured in the hatchery and grow-out pond were summarized in Table 4.13. Temperature and salinity had effects on shrimp quality and growth [130]. During the experimental period, water temperatures were within the range of tremendous effects 22-29 °C (mean = 24.2 °C) in hatchery, while those in grow-out pond ranged between 18.2-28.6 °C (mean = 23.4 °C). The lowest temperature at 18.2 °C in grow-out pond was due to winter effect during growing period. The recommended ranges of the temperature were 27-33 °C [133], in hatchery and 25-32 °C, in grow-out pond. The increasing in temperature over the range of 20 °C to 32 °C resulting in an increase in growth of *P. vannamei* juveniles had been reported [131]. Shrimps at 20 °C were relatively inactive and exhibited low food consumption compared with hyperactive animals at 35 °C. When offered unlimited food, shrimps maintained at 35 °C had the highest rate of food consumption. These observations are consistent with those of Zein-Eldin *et al.* [132] in *P. azfecus.* Wyban *et al.* [133] suggested that the optimal temperature for fastest growth were size-specific and decreased as shrimp size increased. For small shrimp (< 5 g), the optimal temperature may be greater than 30 °C, while that of large shrimp, while that of large shrimp was about 27°C. In grow-out pond, a higher temperature may markedly shorten the culture period of shrimp [133].
Salinity also has an impact on shrimp health. In this study, the salinity in hatchery was between 26-32 ppt whereas in grow-out pond was between 2-10 ppt that was in the recommended salinity range. The recommended range of salinity in hatchery was 10-30 ppt, for hatchery [131] and 0.5-30 ppt; for grow-out pond [130]. The salinity in hatchery was higher than that of grow-out pond due to the rearing condition. The low salinity increases the virulence of *Vibrio* sp. in *P. vannamei*, resulting in significant mortalities [141]. One of the factor that affect immunity is age of shrimp [124] therefore avoiding the infection in hatchery, the juvenile shrimp should be reared at high salinity and decrease them during rearing periods.

Other water quality parameters total ammonia nitrogen (TAN), nitrite and Chlorophyll *a* were affected by sampling time. The highest TAN and nitrite concentration were 1.92 mg-N L^{-1} and 0.26 mg-N L^{-1} in hatchery (were in hatchery's recommended range). The recommended concentration of TAN and nitrite for *Penaeus* juvenile culturing was

3.7 mg L^{-1} and 3.8 mg L^{-1}, respectively, in hatchery [142]. The highest TAN and nitrite concentration were 0.262 mg-N L^{-1} and 0.002 mg-N L^{-1}, respectively, for grow-out pond. The toxicity of TAN levels range from 2.6-3.95 mg-N L^{-1} and nitrite critical concentration for *P. vannamei* was 6.1 mg-N L^{-1} in 15 ppt grow-out pond [143].

Although some differences were found during the experimental period in both hatchery and grow-out pond, all water quality parameters were within suitable ranges for normal marine shrimp survival and growth. Total ammonia nitrogen and nitrite concentrations in hatchery were higher than shrimp grow-out pond due to type of feed. Shishehchian *et. al* [129] examined ammonia and nitrite excretions by black tiger shrimp (*P. monodon*) when fed with different diets (artificial and natural). The experiment indicated that nitrogenous excretion was predominant in shrimps fed with artificial diets. Live food, such as algae, despite high protein content, contributes to Low nitrogenous excretion and hence has less adverse effects on water quality compared to artificial diets [129].

For Chlorophyll *a* (Chl *a*) concentration, it was found that in grow-out pond water body was higher than hatchery due to light is the most important factor affecting microalgal photosynthesis kinetics [123]. The Chl *a* concentrations in this study were 42.6-187.16 μg L^{-1} which were relatively intermediate compared with those of other regions where is cultured white Pacific shrimp in semi-intensive ponds: 33–79 μg L^{-1} in Gulf of Fonseca Honduras [121]; 13–93 μg L^{-1} in south of Sinaloa, Mexico [122] and 125–179 μg L^{-1} in Guayaquil, Ecuador [144].

Table 4.13 Water qualities from white shrimp hatchery and grow-out ponds

Parameter	**Source**	
	Hatchery	**Grow-out**
Temperature ($^{\circ}$C)	22-29	18.2-28.6
Salinity (ppt)	26-32	2-10
Ammonium (mg-N L^{-1})	0.92-1.94	0.023-0.262
Nitrite (mg-N L^{-1})	0.17-0.25	0.001-0.002
Chl *a* content (μg L^{-1})	N/A	42.6-187.16
pH	7.6-7.8	7.5-7.9

4.3.2 Comparison of Microbial Diversities between in Hatchery and Grow-Out Ponds

The microbial diversities in grow-out and hatchery water consist of bacteria as shown in Table 4.14, Table 4.15 and Figure 4.20, respectively. Nitrifying bacteria such as *Nitrosomonas* sp. and *Nitrobacter* sp. were found in both kinds of pond. This was probably due to shrimp feed having high nitrogen content but most of it (80%) was not retained as shrimp biomass [145]. As ammonia is the main excretory product of shrimp and heterotrophic bacteria (ammonifiers) convert organic nitrogen including protein in the sediments to ammonium or ammonia depending on pH level in the environment. In this study, pH was in the recommended range for good shrimp culture management [124]. The occurrence of ammonia in alkaline condition i.e. pH 9.3, with about 50% in the form of ammonia can become a major problem in shrimp pond. Consequently, nitrifying bacteria (*Nitrosomonas* sp. and *Nitrobacter* sp.) play an important role in the management of water quality by oxidizing ammonium to nitrite and nitrate, respectively [127].

Most bacterial infections result from extreme stress and common bacterial infection, in marine shrimp is *Vibrio* sp. whose infection often occurs following environmental stress. They exist in a high number in both the water and the sediment of shrimp pond especially in an intensive culture system [146]. In this study, *Vibrio* sp. was found in only hatchery, however, *Vibrio* sp. was not found in grow-out water body although the salinity was lower than hatchery water body. This might due to the effect of green water

that was indicated by the chlorophyll *a* content [147] in grow-out pond which was higher than hatchery. With the recent development of the green water culture of the tiger shrimp *P. monodon,* occurrence of infections attributed to Luminous *Vibrio* can be prevented [148].

The using this green water, water from tilapia (*Tilapia hornorum*) culture ponds is transferred to shrimp ponds as rearing water with or without tilapia stocked in cages in polyculture with shrimp. Phytoplankton showed that some of them secrete anti-luminous *Vibrio* substances that can effectively inhibit the multiplication of this pathogen [148]. *Synechococcus* sp. were found in the grow-out pond's community in this study, which was also isolated from shrimp pond [149] that was probably the partial of community in green water. In addition, the pathogen that was found in hatchery and grow-out pond was *Flavobacteria* which was found in soil and water in a variety of environments. Several species are known to cause disease in aquaculture. *Flavobacterium psychrophilum* causes the bacterial cold water disease (BCWD) on *salmonids* and the rainbow trout shrimp disease (RTFS) on rainbow trout. *Flavobacterium columnare* causes the cotton-wool disease on freshwater fishes. *Flavobacterium branchiophilum* causes the bacterial gill disease (BGD) on trout [150]. Both of *Vibrio* sp. and *Flavobacteria* sp. often cause opportunistic infections [151].

Table 4.14 Phylogenetic affiliations of the clone library of microbial diversity in white shrimp grow-out water

Clone	Bacteria	No. of clone (%)	Identity (%)	Group
A	*Nitrosospira* sp.	12 (6)	93	β-proteobacteria
J	*Synechococcus* sp. Y0011	8 (4)	92	Cyanobacteria and Chloroplast
M	*Synechococcus* sp. RS9915	8 (4)	99	Cyanobacteria and Chloroplast
U	*Nitrosomonas eutropha*	13 (6.5)	99	β-proteobacteria
V	*Nitrobacter wingradskyi*	10 (5)	99	α-proteobacteria

Table 4.15 Phylogenetic affiliations of microbial diversity in hatchery water

Band	Bacteria	% Identity	Group
H4	*Nitrosomonas marina*	96	β-proteobacteria
H5	Uncultured *Flavobacteriaceae* bacterium clone FX	96	Bacteroidetes
H7	*Nitrobacter wingradskyi*	96	α-proteobacteria
H8	*Pseudomonas* sp. *ITRI66*	98	γ-proteobacteria
H9	*Vibrio* sp. *UST061013-028*	80	γ -proteobacteria
H11	*Vibrio* sp. 2E5	94	γ-proteobacteria
H12	*Fusobacterium ulcerans*	90	γ-proteobacteria

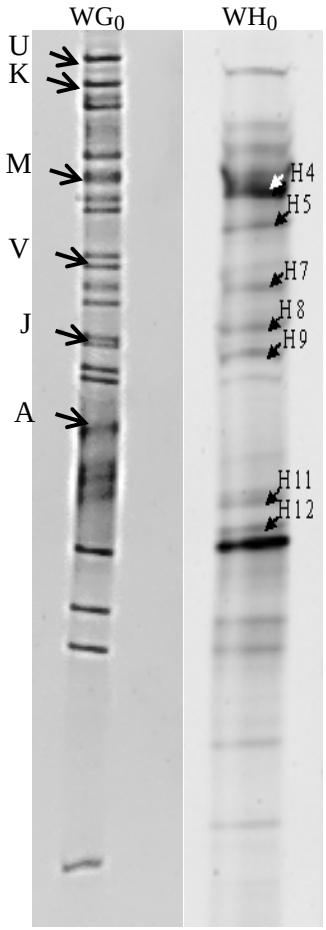

Figure 4.20 DGGE profile of bacterial diversity from the hatchery and shrimp grow-out pond water column. $WH_{0,}$ microbial community in hatchery water column and WG_0, microbial community in grow-out pond water column.

CHAPTER 5 CONCLUSIONS AND RECOMMENDATION

5.1 Conclusion

The results from this study provide important data to help us understand the inorganic nitrogen conversion and microbial diversity in the water column from shrimp cultivation processes and it can be concluded as following:

In hatchery, the findings clearly showed that co-culturing Pacific white shrimp (*P. vannamei*) PL with *Spirulina* mats in a closed re-circulating system reduced inorganic nitrogen levels, particularly ammonia and nitrate, and resulted in better water and PL quality. The lower ammonia concentration, in accordance with dynamic bacterial communities, subsequently decreased in water exchange rates by approximately 40 %. This was an environmentally friendly process, due to the reduction in discharge into environment and the seawater used during the shrimp PL production process, which can certainly decrease the costs of shrimp production. Therefore, the *Spirulina* mat could be suggested as an alternative green material for water quality control and management in aquaculture system, especially shrimp PL.

In grow-out pond, there were 3 groups of microbial dynamics observed in this study. The first dynamic consisted of the microbes that were found at all the time points investigated such as ammonia oxidizing bacteria. The second group of microbial dynamics involved the replacement such as *Flavobacteriales bacterium* was replaced with *Aquiflexum balticum*, both of which are bacteria involved in shrimp shell degradation. The third group of microbial dynamics involved bacteria that were observed only at one time point, such as *Synechococcus* sp. *Y0011* (W_0), *Stenotrophomonas maltophilia* (W_3), or at several time points, such as *Pseudomonas lanceolata* (W_0 and W_3) and *Burkholderia* sp. WBF2 (W_1 and W_2). As previously mentioned, although this third group of bacteria was not found at all of the time points investigated, they may play an important role in balancing the shrimp culturing system, which is needed for a successful shrimp cultivation.

The comparison of microbial communities between in hatcheries and grow-out ponds found that *Vibrio* sp. was found only in hatcheries. However, that they were not found

in grow-out ponds might be due to the effect of green water and low salinity in grow-out ponds. Moreover, nitrifying bacteria were found in both shrimp hatcheries and grow-out ponds. Some bacteria such as *Vibrio* sp., *Sphingomonas* sp. and *Pseudomonas* sp. were involved with shrimp health and nitrification in the system.

5.2 Recommendations

Spirulina mat could be applied in hatchery in case of shrimp quality improvement and water quality control (as microbial commensalism between *Spirulina* and beneficial bacteria such as nitrifying bacteria). For other application, *Spirulina* mat could be suggested as an alternative green material for water quality control and management in aquaculture system, especially shrimp PL. However, to increase the nutrient removal efficiency, the attached substrate for *Spirulina* mats would be developed for longer and/or sustained lifetime.

APPENDIX

Partial 16S rDNA Sequences of Microbial Community in Shrimp Culturing System

> H1, M14, A1
ACTCCTACGGGAGGCAGCAGTAGGGAATTTTCGGCAATGGAGGAAACTCTG
ACCGAGCAACGCCGCGTGAATGACGAAGTACTTCGGTATGTAAAGTTCTTTT
ATCAAGGAAGAATGACTTGCCTCCCGTAGGAGTAAGTACTTCGGTATGTAA
AGTTCT

> H3, S2, C2, M2
ACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGACGGAAGTCTG
AACCAGCCATGCCGCGTGCAGGAAGACTGCCCTATGGGTTGTAAACTGCTT
TTATATAGGAATAAAACCCTCAACGAGTTGTGGGGCTGTGATGTGCTATATG
AATATACACCGCGTATAACCCGTGTGACCAGCCGCGGTC

> S8
ACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGGCGGGAACCTG
ATCCAGCCATGCCGCGTGCAGGAAGACTGCCCTATGGGTTGTAAACTGCTTT
TGTACAGGAAGAAACACTCCCTCGTGAGGGAGCTGAGGTTGTAAACTGCTT
TTGTA

>H8
ACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGACGCAGGTCTG
AACCAGCCATGCCGCGTGCAGGAAGACTGCCCTATGGGTTGTAAACTGCTT
TTATACGGGAAGAAACCCCCCAACGAGTTGGGGGCTGACGGTACCGTAAGA
ATAAGGATCGGCTAACTCCGTGC

> H2, S1, C1, M1, A2
ACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTT
CAGTCGAGAAGAAAAGATTGTGATGAATAATCACAATTCATGACGGTATCG
ACAGAA

> C15, M13
ACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGACGGAAGTCTG
AACCAGCCATGCCGCGTGCAGGAAGACTGCCCTATGGGTTGTAAACTGCTT
TTATATAGGAATAAAACCCTCAACGAGTTGTGGGGCTGTGATGTGCTATATG
AATATACA

> H15, S3, C3, M3
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG
ATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTT
GTGCGGGAAGATAATGACGGTACCGCAAGAATAAGCCCCTAGGGTTGGTAA
ATCTTT

> S4, C4, M4, A3
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTG
ATCCAGCCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGTCCTTTT
GTCGGGGAGGAAGGGCATCTGGCTATACTCGGGTGGGTGACGTCCCCGGGA
ATATTG
> H4, S5, C5, M5, A4
ACTCCTACGGGAGGCAGCAGTTGGTAATATTGGACAATGGGCGCAAGCCTG
ATCCACCCATGCCGCGTGCATGAAGAAGCCCTTCGGGTTGTAAAGTGAATC
TGCGGGATGGTAACCATAAGTCTCATGGACGAACACATGTAAAGTGATGGA
CAATGGGC

> M6
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTG
ATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTC
AGCAGTGAGGAAGGTTCATACGTTAATAGCAGTATGGATTGACGTAAGCTG
CAGGAGGCGA

> C12, M10
ACTCCTACGGGAGGCAGCACTGCGGAATATTACACAATGCACCAATCCGGA
ATCTAGCCATGCCACGCGTCTGAAGAAAACTCCGGGTTGTAAAGCAATACT
AGCAGTGAACAAGGCGATATCGTCACTCGCGATACTAATATATTACACAAT
GCACCAATCCG

> H11, S12, C8, M8/H12, S13, C9, M9
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGACCAAAAGTCT
GATCCAGCAATTCTGTGTGCACGATGAAGTTTTTCGGAATGTAAAGTGCTTT
CACCTAGGAAGAAGTCAGTGACGGTACCAACAGAAGAAGCGAATGGACCA
AAAGTCTGATCCA

> H10, S10, C7, A9, M7
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAAATGTTACAAAGTCT
GTTGGAGCAACGCCGCGTGAACGATGAAGGCTTTCGGGTCGTAAAGTGCCT
GTTGTAAGGGAAGAACAAGTGCCGCAGGCAATGGCGGCGTTACAAAGTCTG
TTGGAGCAGTTGT

>M16
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTG
ATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTC
AGCGGGGAGGAAAGGAGTAAGGTTAATACCTTATGCATGAAGAAGGCCTTC
GGGTTGTAAAGTAC

> C10, C11
ACTCCTACGGGAGGCAAGTATCTCATTATGCTCCTTCAGAAAGAGTTACCAT
CGTTAGACTTCATCCTTCACGCGGCGTCGGTCCCTCTGCCTTTGGCGATTGG
GGAAGATTCCTCTCTGCTGCCTTGTGTATACTACCCGCTCCTTCAGAAAGAG
TTACCATCGTT

> C6
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTG
ATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTCTTTCA
GCCGTGAGGACGGTAATCGTTAATAGCGTAGGACGAGAATATTGCACAATG
GGCGCAAGCCTGA

> H9
ACTCCTACGGGAGGCAGCAGTGGGAAATATAGAAAAATGGGCGAAAGCCT
GATCCAGCAATGCCGCGTGAGTGATGAAGACCTTAGGGTTGTAAAGCTCTT
TTACCCGGGATGATAATGACAGTACCGGGAGAATAAGCTCCGGCTAACTCC
GTGCCAGCAGCCGTACCCG

> C17, M11
ACTCCTACGGGAGGCAACCGGTGTGTATTGTAGCAGCAGGGGGAAGAATCT
CACCATGCCAAACCCGAAGAAGACTCCGCGTGTGTGATGAAGGCTCCCGGG
TCGTAAAGCACTTCTGAAGGAAGAAACAAATGACTGTGTGTATTGTAGCAG
CAGGGGGAAGAATCTCA

> C13, C14
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAAGCCTG
ATGCAGCCATGCCGCGTGTGTGAAGAAGGCTTCGGGTTGTAAAGCACTTTC
AGCGAGGAGGAAAGGATGTTGGCTAATACCAGCATCTGGGAGGAAGAAGG
CTTCGAGCGAGGAAAAGCC

> C18, M12
ACTCCTACGGGAGGCAGCAGTGGTGATTCTAGGAGAAGCGGCACAAGCCAA
AACCACCCACCCCGCGTAATGAGGAGCACCAACGCCTCGTAAGGACGAAGC
CGCGGGTCGTAAAGCTCGTAGAGGAAGAAAGGACGAGGAAGAAAGGACGG
AAAGGACGAGGAAGAAAGA

> H5
ACTCCTACGGGAGGCACAGTGGGGAATATTGGACAATGGGCGCAAGCCTGA
TCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTCA
GTGGGGAGGAAAGGTTGGTGGTTAATACCCATCAGCGAAGAAGGCCTTCGG
GTTGCACTTCAGTGGGGA

> C19
ACTCCTACGGGAGGCAGCAGGGGGGACTACTACACGAGGCAGCAGTGGGG
AATATTGCACAATGGACGAAAGTATGATGCAGCAACGCCGCGTGGAGGATG
ACACATTTCGGTGCGTAAACTCCTTTATAGGTCAAGATATGCAGCAACGCCG
CGTGGAGGATGACACCATTT

> S6, A5
ACTCCTACGGGAGGCACAGCAGTGGGGAATATTGCGCAATGGAGGAAACTC
TGACGCAGCAACGCCGCGTGAGTGATGAAGGCCCTCGGGTCGTAAAGCTCT
GTTGGGAGGGAAGAAATTTGACGTACCTCCAGCAACGCCGCGTGGAGGATG
ACACGCAACGCCGCGTGG

> H7, S7

ACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGCAAGCCTG
ACGGAGCAAGACCGCGTGGGGGAGGAAGGCTCTTGGGTTGTAAACCCCTTT
TCTCAAGGAAGAACACAATGACGGTACTTGAGGAATAAGCCTCGGCTAACT
CCGTGCCAGCAGCC

> A6
ACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGGCGGGAGCCTG
ATCCAGCCATGCCGCGTGCAGGAAGACTGCCCTATGGGTTGTAAACTGCTTT
TGTACAGGAAGAAACACTCCCTCGTGAGGGACTCGGGTCGTAAAGCTCTGT
TGGGAGAAGCTCTGT

> A8
ACTCCTACGGGAGGCAGAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA
TGCAGCCATGCCGCGTGTATGAAGAAGGCCCTCGGGTTGTAAAGTACTTTC
AGCGGGGAGGAAGGTGTTGTGGTTAATAACCACAAGCTCTGTTGGGAAGCT
CTGTTGGGAAGCTCTG

> H6
ACTCCTACGGGAGGCAGCAGTTGGGAATATTGGACAATGGGCGCAAGCCTG
ATCCACCCATGCCGCGTGTCTGAAGAAGCCCTTCGGGTTGTAAAAGGAATC
TCGGGAGGAAGGCCATTAGGTCAAGAAGACACATGTGTCTGAAGAAGCCCT
TCGGGTTGTA

>H13/H14
ACTCCTACGGGAGGCAGCAGTTGGTAATATTGGACAATGGGCGCAAGCCTG
ATCCACCCATGCCGCGTGCATGAAGAAGCCCTTCGGGTTGTAAAGTGAATC
TGCGGGATGGTAACCATAAGTCTCATGGACGAACACAGACTGCCCTATGGG
TTGTAACTGC