ผลของแสงอาทิตย์ต่อการเปลี่ยนแปลงประชากรจุลินทรีย์ในดินบริเวณพื้นบ่อเลี้ยงกุ้ง

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บทคัดย่อ

คุณภาพของน้ำและการควบคุมการเกิดโรค มีความสัมพันธ์ต่อกันและเกี่ยวข้องกับกิจกรรมการทำหน้าที่ของจุลินทรีย์ในระบบการเพาะเลี้ยงสัตว์น้ำ ซึ่งกระบวนการย่อยสลายสารอินทรีย์ของจุลินทรีย์ มีผลต่อคุณภาพของน้ำ อาทิ เช่น ปริมาณออกซิเจนละลายน้ำ แอมโมเนีย ไนไตรท์ และซัลไฟด์ นอกจากจุลินทรีย์จะมีประโยชน์ในการควบคุมคุณภาพน้ำแล้ว ยังมีจุลินทรีย์ที่สามารถก่อให้เกิดโรคต่อสัตว์น้ำได้ เป็นที่ทราบกันดีว่า รังสีอุลตราไวโอเลตจากแสงอาทิตย์สามารถฆ่าเชื้อจุลินทรีย์ได้ โดยจะทำลายส่วนของกรดนิวคลีอิกภายในเซลล์ ทำให้การทำงานและการแบ่งตัวของดีเอนเอผิดไปจากเดิม ส่งผลทำให้เซลล์หยุดการเจริญเติบโตและตายไปในที่สุด ในงานศึกษาวิจัยนี้จึงได้ทำการศึกษาผลของแสงอาทิตย์ต่อการเปลี่ยนแปลงชนิดและจำนวนของประชากรจุลินทรีย์ในดินบริเวณพื้นบ่อที่มีการเพาะเลี้ยงกุ้งขาวแบบ semi-intensive ในระหว่างช่วงระยะเวลาที่ทำการตากบ่อประมาณ 1 เดือน โดยใช้วิธีการเพาะเลี้ยงเชื้อ และเทคนิค PCR-DGGE จากผลการศึกษาวิจัยพบว่า แสงอาทิตย์มีประสิทธิภาพในการกำจัดเชื้อจุลินทรีย์ โดยพบว่าจำนวนของ heterotrophic bacteria ลดลงไป 10 เท่า หลังจากที่ทำการตากบ่อเป็นเวลา 2 สัปดาห์ นอกจากนี้แสงอาทิตย์ยังมีผลทำให้ชนิดของประชากรจุลินทรีย์ในดินมีการเปลี่ยนแปลงในระหว่างที่ทำการตากบ่อ ซึ่งผลที่ได้จากการศึกษาวิจัยสามารถนำไปใช้เป็นแนวทางในการปรับปรุง/พัฒนา สำหรับการเตรียมบ่อก่อนทำการเพาะเลี้ยงกุ้งให้มีคุณภาพที่ดีขึ้นได้

คำสำคัญ : แสงอาทิตย์ / ประชากรจุลินทรีย์ / ตะกอนดินในบ่อเลี้ยงกุ้ง / PCR-DGGE

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Effect of Natural Sunlight on Microbial Population in Shrimp Farming Sediment

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Abstract

Water quality and disease control is interdependent and linked to the microbial activities in aquaculture system. Microbial processes affect water quality factors such as dissolved oxygen, ammonia, nitrite and sulphide. It is known that aquatic bacterioplankton is sensitive to sunlight radiation, which causes cellular damage on different cell targets and lead to mutations, cell inactivation, and death. In this study, the effects of natural sunlight on microbial populations in semi-intensive shrimp pond sediment under a pond drying period were studied. During pond preparation, water was drained out and the pond sediment was left to expose to sunlight for 30 days. Microbial populations prior to exposure were compared to that after exposure using traditional methods for cultivating heterotrophic bacteria and denaturing gradient gel electrophoresis (DGGE) of 16S rRNA. Prior to sunlight exposure, the mean heterotrophic counts were 108 cfu/g of wet sediment. After 14 days of sunlight exposure, the mean heterotrophic counts of the sediment were declined 10-fold. Using DGGE, the composition of the microbial community in the sediment prior to exposure was compared to that after exposure to sunlight. The data on the effects of pond drying by exposure to sunlight on microbial populations would help improve the shrimp farming practice on the pond preparation process.

Keywords : Sunlight / Microbial Population / Shrimp Farming Sediment / PCR-DGGE

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1. Introduction

Shrimp farming industry all over the world has been frequently affected by diseases of infectious and non infectious origins. A major cause of infectious diseases in cultured shrimp farming is opportunistic bacterial pathogens which normally coexist with the host but under sub-optimal and stressful environmental conditions can take the advantage of ecological changes to cause mortalities [1-3]. Shrimp can be stressed by a range of factors including high population densities, excess or poor food quality, elevated water temperature and poor water quality [4]. Although many different microorganisms can induce mortalities, many microorganisms can carry out active decomposition of left over feed and metabolites to inorganic forms such as ammonia (NH_3), hydrogen sulphide (H_2S) and carbon dioxide (CO_2) through the process of mineralization. Microorganisms help not only in the production and breakdown of organic matter, but also in the nutrient recycling in aquatic ecosystem [5].

Farm management practices, including pond preparation are performed to improve pond conditions and water quality. During pond preparation, water was drained out and the pond sediment was left to expose to sunlight for a period of time. It is known that aquatic bacterioplankton is sensitive to sunlight radiation, which causes cellular damage on different cell targets and lead to mutations, cell inactivation, and death [6-8]. We present here results from an experimental designed to assess the effect of natural sunlight on microbial population in a semi-intensive shrimp farming sediments during a pond drying period. This study was carried out to investigate the total bacterial abundance using culture-dependent and culture-independent techniques and the microbial community structure using DGGE of 16S rDNA.

2. Materials and methods

2.1 Sample collection

Samples of shrimp grow-out pond sediment were collected from a semi-intensive shrimp farming pond culturing the white shrimp, *Penaeus vannamei*. This white shrimp farm was located in Thrungkru district, Bangkok, Thailand. The sediment samples were collected every week during the pond drying period for 1 month on April 2006. The samples were taken randomly from the top to 10 cm in depth and kept in sterilized plastic bags. All samples were transferred immediately for microbial analysis and stored at -20 °C until further used for DNA extraction.

2.2 Total viable count

Heterotrophic bacteria in each sample were enumerated by the spread-plating method. Five grams of sediment was mixed with 1% sterile normal saline, diluted to 50 ml volume and placed on a shaker at 160 rpm for 15 min. To ensure disassociate of bacterial flocs, the samples were sonicated on ice at an ultrasonic power of 2 W for 2 min using a 130-W Ultrasonic Processor (Daigger, IL). Aqueous suspensions of the sediment slurry were 10-fold serially diluted with 1% sterile saline before plating. Aliquots (0.1 ml each) of appropriate dilutions were spread in triplicate on to nutrient agar (NA) plates and incubated aerobically and anaerobically at 30 °C for 2 and 7 days, respectively. Results were enumerated by visually counting individual colonies and reported as colony forming unit (CFU) per gram of sediment. The results were calculated as the means of two determinations.

2.3 Total cell counts

4, 6-Diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR) stain was used to determine the total number of cells in the samples as described previously [9]. Briefly, the appropriate dilutions used in the plate counting were stained with DAPI at a final concentration of 5 μg/ml for 10-15 min at room temperature in the dark. The stained sample was filtered through a prewetted black polycarbonate membrane filter with 0.2 mm pore size (Millipore, Ireland) and then the filter was washed with 5 ml distilled water. Finally, the filter was placed on a glass slide with a drop of immersion oil and covered with a glass cover slip. The stained sample was viewed on an Olympus Microscope BX60 with a WU filter for DAPI (excitation 330–385 nm, dichroic mirror 400 nm, barrier 420 nm). Images were captured with an Olympus DP50 digital camera system using Viewfinder Lite 1.0 software (Olympus, Tokyo, Japan) and processed with Studio Lite1.0 software (Olympus). For each sample, 15 independent, randomly chosen microscopic fields across a filter were microscopically enumerated at 1,000-fold magnification. Results were calculated as the means of two determinations.

2.4 DNA extraction

DNA extraction was based on the method of Zhou et al. [10] using SDS with some modifications. Five grams of sediment was mixed with 13.5 ml of DNA extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM sodium EDTA, pH 8.0, 100 mM sodium phosphate, pH 8.0, 1.5 M NaCl, 1% CTAB) and 100 μl of proteinase K (10 mg/ml) in a 50 ml centrifuge tube by horizontal shaking at 200 rpm for 30 min at 37 °C. After shaking, 1.5 ml of 20% SDS was added and then the sample was incubated at 65 °C for 2 h with gentle inversions every 15-20 min. Supernatant was collected after centrifugation at 6,000 x g for 10 min at room temperature and transferred into a new 50 ml centrifuge tube. The sediment pellet was repeatedly extracted twice by adding 4.5 ml of the DNA extraction buffer and 0.5 ml of 20% SDS, mixed for 30 s, incubated at 65 °C for 10-15 min, and centrifuged as before. The supernatants from the three cycles of extraction were combined and mixed with an equal volume of chloroformisoamyl alcohol (24:1). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 h. The pellet of crude DNA was obtained by centrifugation at 16,000 x g for 20 min at room temperature, washed with 70% ethanol, and resuspended in sterile deionized water. The crude DNA was cleaned again using the Geneclean II kit per the manufacturer's instructions (Q BIOgene, Morgan Irvine, CA) and subsequently used for PCR amplification.

2.5 PCR amplification of 16S rDNA

In order to increase the sensitivity and to facilitate the denaturing gradient gel electrophoresis (DGGE) by analysis fragments of the same length, a nested PCR technique was applied. In the first round universal bacterial primers EUB8f (5'-AGAGTTTGATCCTGGCTCAG-3') [11] and U1492r (5'-GGTTACCTTGTTACGA CTT-3') [12], amplifying approximately 1,500-bp fragment of the bacterial 16S rRNA gene. PCR products for DGGE analysis were generated by reamplification of the 16S rDNA product using primers 968GCf (5'CGCCCGGGGCGCGCCCCGGGCGGGGCG GGGGCACGGGGGGAACGCGAAGACCT TAC-3') and 1401r (5'-CGGTGTGTACAAG ACCC-3') [13]. The PCR program for primer set

EUB8f/U1492r included an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min followed by a final extension at 72 °C for 7 min. The PCR program for primer set 968-GC-f/1401r included an initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 50 s, annealing at 60 °C for 30 s, and extension at 72 °C for 50 s followed by a final extension at 72 °C for 7 min. All PCR reaction was performed on a GeneAmp PCR System 9700 (Applied Biosystem). The 50 µl PCR reaction mixtures contained 50 mg of DNA template, 20 pmol of each primer, 200 µM of deoxynucleoside triphosphates (dNTP), 1X PCR buffer, 3 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (Qiagen, Germany). The size and amount of PCR products were visualized and estimated on a 1% agarose gel electrophoresis and ethidium bromide staining.

2.6 Denaturing gradient gel electrophoresis analysis

DGGE was performed as described by Muyzer et al. [14] using a DGGE-2000 system apparatus (CBS Scientific Company, Del Mar, CA). Briefly, samples containing equal amounts of PCR products were loaded onto 8% (wt/vol) polyacrylamide gels in 1X Tris-acetate-EDTA (TAE) with a denaturing gradient ranging from 35 to 60% denaturants (100% denaturant contains 7 M urea and 40% [vol/vol] formamide in 1X TAE). Electrophoresis was performed at 60 °C and at a constant voltage of 200V for 5 h. Following electrophoresis, the gel was stained with SYBR Green nucleic acid stain (Molecular Probes, Eugene, OR) for 30 min. The images were visualized on a UV transilluminator and captured using Biovision CN 1,000/26M (Vilber Lourmat, France).

2.7 Sequencing and phylogenetic analysis

To identify DGGE bands, individual DGGE bands were excised, resuspended in 20 µl of MilliQ water, and stored at 4 °C overnight. After this period the DNA has diffused out of the acrylamide gel and the solution can be used as template in a reamplification PCR. For sequencing, reamplification was performed using the primer set without GC-clamp, and sequenced by Macrogen, Korea. Sequences were compared with available databases by use of the BLAST search program from the NCBI to determine their approximate phylogenetic affiliations [15]. Phylogenetic trees were calculated and drawn using the Jukes-Cantor and Neighbor-joining program of PHYLIP in the Ribosomal Database Project II [16].

3. Results and discussion

3.1 Total microbial abundance during pond drying period

To determine the effect of sunlight on the abundance of microbial population in the shrimp pond sediments during the pond drying period, we used a culture-dependent method, plate count method and a culture-independent method, DAPI staining. Fig. 1 show the total microbial abundance during a pond drying period for 1 month by plate count on nutrient agar and direct count using DAPI staining. The average counts of heterotrophic bacteria were significantly decreased (mean and standard deviation) from $9.8 \pm 2.3 \times 10^7$ to $1.0 \pm 0.03 \times 10^6$ CFU/g sediment, after sunlight exposure for 1 month. The number of bacterial cells count as determined by DAPI were significantly decreased from $1.2 \pm 0.3) \times 10^9$ to $5.3 \pm 0.2 \times 10^6$ cells/g sediment. A similar trend of total population

decreasing was observed for both methods. After 14 days of sunlight exposure, the total microbial abundance of the sediments was declined about 10-fold. There was no significant decline of the microbial abundance after 3 weeks during the course of this study. Our results indicated that the exposure of sediment to natural sunlight can significantly affect on the abundance of bacteria population. Due to solar UV radiation cellular damage on different cell targets, including nucleic acids, proteins, and lipids, which may end up in mutations, cell inactivation, and death.

The enumerated values of standard heterotrophic plate counts of bacteria were about 1–2 orders of magnitude lower than the total bacteria counts determined by DAPI staining (Fig. 1). These results are accordance with previous studies on environmental samples that reported direct microscopic based total bacterial counts to be orders of magnitude greater than CFU or MPN estimated from the culture based methods [17-20]. It is estimated that less than 1% of bacteria are culturable with the present methods, hence using a culture based approach will inherently lead to an underestimation of microbial biodiversity [11, 21].

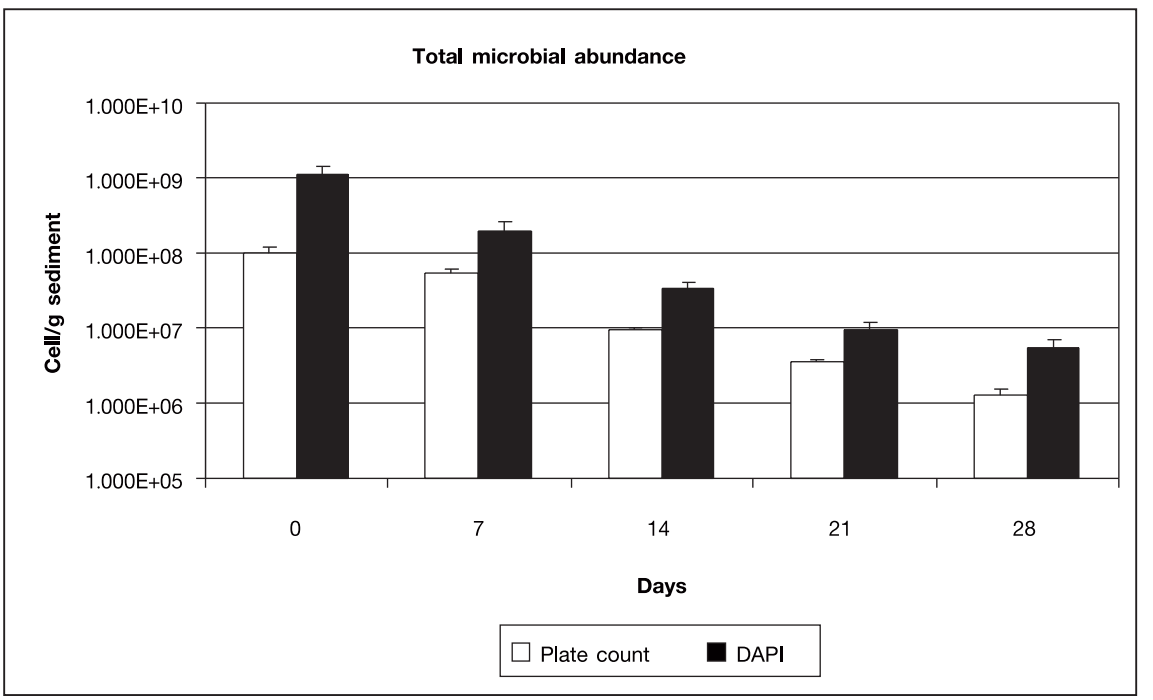

Fig. 1 Comparison of total microbial abundance during a pond drying period for 1 month by plate count on nutrient agar and direct count using DAPI staining. Data are the averages of values from duplicates samples. Bar represent standard deviation (SD).

3.2 Microbial community analysis using DGGE

In this study we have used a culture-independent approach based on the 16S rDNA sequence variability to analyze bacterial community. Following the DNA isolation from the sediment samples, PCR was performed to amplify the V6-to-V8 regions of 16S rDNA. The DGGE profiles were used to examine the overall effect of sunlight exposure on microbial structure during a pond drying period for 4 weeks are shown in Fig. 2. Nineteen bands were excised and subsequently sequenced to describe the phylogenetic diversity of the amplified 16S rDNA (Table 1 and Fig. 3). Our results indicated that the majority of bacteria identified from the sediment samples were *γ-Proteobacteria* (50% of the bands), and Gram-positive bacteria (40% of the bands) after exposed to sunlight. Only 10% of bands identified as delta/epsilon *Proteobacteria* were observed. No *Cytophaga-Flexibacter-Bacteroides* (CFB) group was retrieved in this study. Previous studies showed that members of the *γ-Proteobacteria* and CFB group are highly resistant to UV radiation, while *α-proteobacteria* are sensitive to UV [22-23].

Table 1 Phylogenetic identity of the dominant bands from DGGE of sediment samples collected from a semi-intensive shrimp farming

Band	% Identity	Closest relative	Taxonomic description
1	99	Unculture epsilon clone MC1	delta/epsilon *Proteobacteria*
2, 4	99	*Exiguobacterium* sp.	Gram-positive bacteria
3	99	*Pseudomonas* sp. fnd1	*γ-Proteobacteria*
5, 9	96	*Pseudomonas* sp. A322	*γ-Proteobacteria*
6, 10	95	*Pseudomonas* sp.	*γ-Proteobacteria*
7	99	Unculture bacterium clone BS124	Gram positive bacteria
8	96	*Clostridium* sp. FL2	Gram positive bacteria
11	94	*Vibrio vulnificus* strain MP-4	*γ-Proteobacteria*
12	98	Marine bacterium HP9	*γ-Proteobacteria*

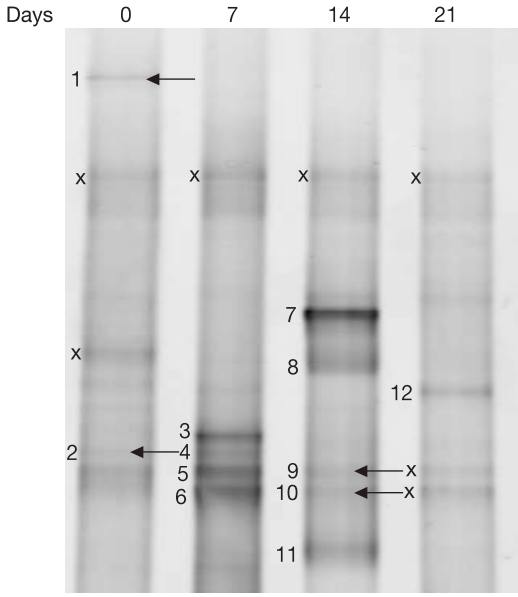

Fig. 2 DGGE profiles of sediment samples at days 0, 7, 14, and 21.
Total of 19 bands were excised from the DGGE gel and sequenced.
Crosses (x) indicate bands for which sequences were not successfully retrieved.

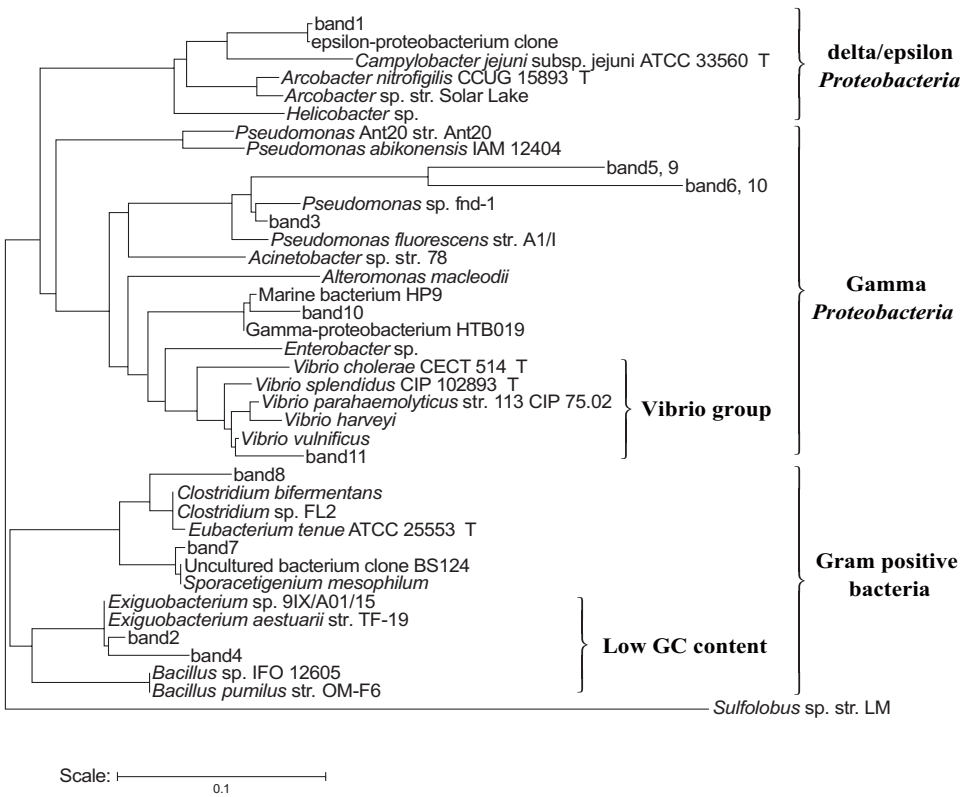

Fig. 3 Phylogenetic relationships between sequences retrieved from the DGGE bands obtained in this study (highlighted). Phylogenetic trees were calculated and drawn by using the Jukes-Cantor and Neighbor-joining program of PHYLIP in the RDP II.

Changes in the bacterial community composition of each week occurred over time as demonstrated by changes in the dominant bands and the affiliated sequences retrieved. The DGGE profiles of day 0 and day 21 did not display the dominant banding patterns as seen on day 7 and day 14. Sequences retrieved on day 0, were affiliated with an unculture epsilon clone MC1 (band 1) and an *Exiguobacterium* sp. (band 2) that was also present on day 7 (band 4). At day 7, three bands were dominant in the profile, band 3, 5 and 6 were identical and affiliated with three *Pseudomonas* sp.. By day 14, the major dominant band was affiliated with an unculture bacterium clone BS124 (band 7). Band 8 and 11 were affiliated with a *Clostridium* sp. FL2 and *Vibrio vulnificus* strain MP-4, respectively. Band 9 and 10 were affiliated with two *Pseudomonas* sp. and were identical to band 5 and 6, respectively found on day 7. Only one band on day 21 was successfully sequenced, band 12, which was affiliated with a marine bacterium HP9. Two bands were unsuccessfully sequenced with similar intensity and gel mobility to band 9 and 10, and therefore possibly the same sequences as band 9 and 10, was also observed at day 14 of the DGGE profile. Interesting sequence retrieved from the DGGE analysis came from band 2 and 4 at day 0 and day 7, respectively, that affiliated with an *Exiguobacterium* sp.. This organism is a gram-positive bacterium has been found in a marine hatchery [24], and was found to be haemolytic negative on blood agar plates. It was investigated further and would be a putative probiotic in the second phase of Schulze's study [24]. Throughout the pond drying period, we found the bands which were affiliated with several of *Pseudomonas* sp., which are commonly found in a wide range of terrestrial and aquatic habitats. It is generally known that these organisms are the predominant heterotrophic bacteria involved in denitrification during activated sludge treatment. Furthermore, *Pseudomonas* sp. (*P. fluorescens*) has been proposed and tested as probiotics in aquaculture [25]. Not only *Pseudomonas* are denitrifying bacteria, the *Clostridium* also are nitrate reducing bacteria, has been reported by Keith et al. [26]. We retrieved a sequence affiliated with *Clostridium* sp. (band 8) after exposed the shrimp pond for 2 weeks. Members of genus *Clostridium* are Gram-positive, spore-forming rods which are obligate anaerobic. Another interesting sequence observed on day 14 that was affiliated with *Vibrio vulnificus* (band 11). The *Vibrio* species are a Gram-negative, motile curved bacterium often found in marine and estuarine environments. They have been frequently isolated from oysters and other shellfish in warm coastal waters during the summer months [24, 27], and associated with disease in shrimp [27] as well as in humans.

Although the shrimp pond was exposed by natural sunlight, however, the bacteria produce spores as *Clostridium* sp. (band 8) was found after sunlight exposure for 2 weeks. There were some observations by Marshall Ward that spores were more susceptible to sunlight than the vegetative forms of bacteria and fungi. This would be related to their high fat content in spores and fats were known to be easily oxidized in the presence of oxygen and light [28]. Although Han Buchner showed that only 1 h of direct sunlight was sufficient to kill various kinds of pathogenic bacteria, such as *B. typhosus, B. coli* and *Vibrio cholera* in tap water [28]. But we also found the DGGE band that affiliated with *Vibrio* sp. (band 11, day 14), a septicemia-causing aquaculture pathogens. This might be due to the fact that *Vibrio*

may live in the deep of sediment, and that make them escaped from the UV light. Moreover the natural sunlight can also act as an energy source for some bacteria. Alonso-Saez et al. found that photosynthetic available radiation (PAR; 400-430 nm) significantly stimulated the activity of *Roseobacter* group compared to the dark treatment [23]. It is well known that some cultured members of this group are aerobic anoxygenic phototrophs and these bacteria derived energy from light with the use of bacteriochlorophyll *a* [29]. There are a large differences in sensitivity found among broad phylogenetic groups such as *Alpha- and Gammaproteobacteria* and *Bacteroidetes*, can act as a significant determinant of bacterial assemblage structure at high UV dose. The differential sensitivity to UV could be a relevant factor determining their relative dominating groups and biogeochemical roles in oceanic regions [23].

4. Conclusion

The dynamics of microbial community of semi-intensive shrimp pond sediments exposed to natural sunlight was monitored during a pond drying period by (i) microbial enumeration with spread-plating and DAPI staining, (ii) DGGE fingerprinting of total community DNA and identification of the bands by sequencing. Our results indicated that the natural sunlight has effect on the abundance and microbial community structure in the sediment samples. Direct exposure of sunlight for 3 weeks would sufficient for pond drying process to eliminated the undesirable in summer (April, 2006). Due to UV light cannot penetrate through opaque materials, possible resulting in an efficient suppression of UV-sensitive bacterial species in the bottom zone. Therefore, tilling of bottom sediment during pond drying will enhance efficiency of the exposure.

5. Acknowledgements

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