CHAPTER III

MATERIALS AND METHODS

This study involved an investigation of bacterial diversity in shrimp pond sediment. The first part of this study (section 3.1) examined the sediment characteristics during shrimp cultivation in earthen shrimp ponds within the central part of Thailand. This was following by bacterial diversity analysis in sediment samples using both classical technique (plate count: section 3.2) and molecular technique (PCR-DGGE: section 3.3). In the last part (section 3.4), an additional experiment regarding nitrogen conversion by sediment from shrimp pond under laboratory condition was carried out.

3.1 Shrimp pond sediment characteristics

3.1.1 Study sites

Eight shrimp ponds in four locations were chosen for bacterial diversity study. The first two shrimp ponds were located in Nong Suea District, Pathum Thani Province. Both ponds were closed system farming with low salinity water. The Map and detail of each study sites are shown in Figure 3-1 and Table 3-1, respectively. One shrimp pond in Bang Khla District, Chachoengsao Province, was selected as the commercial shrimp farm. Two shrimp ponds in Ban Pho District, Chacheongsao, were represented the closed system shrimp pond that used ozone for water treatment. The last three ponds represented the extensive shrimp ponds in Bang Khun Thian District, south of Bangkok

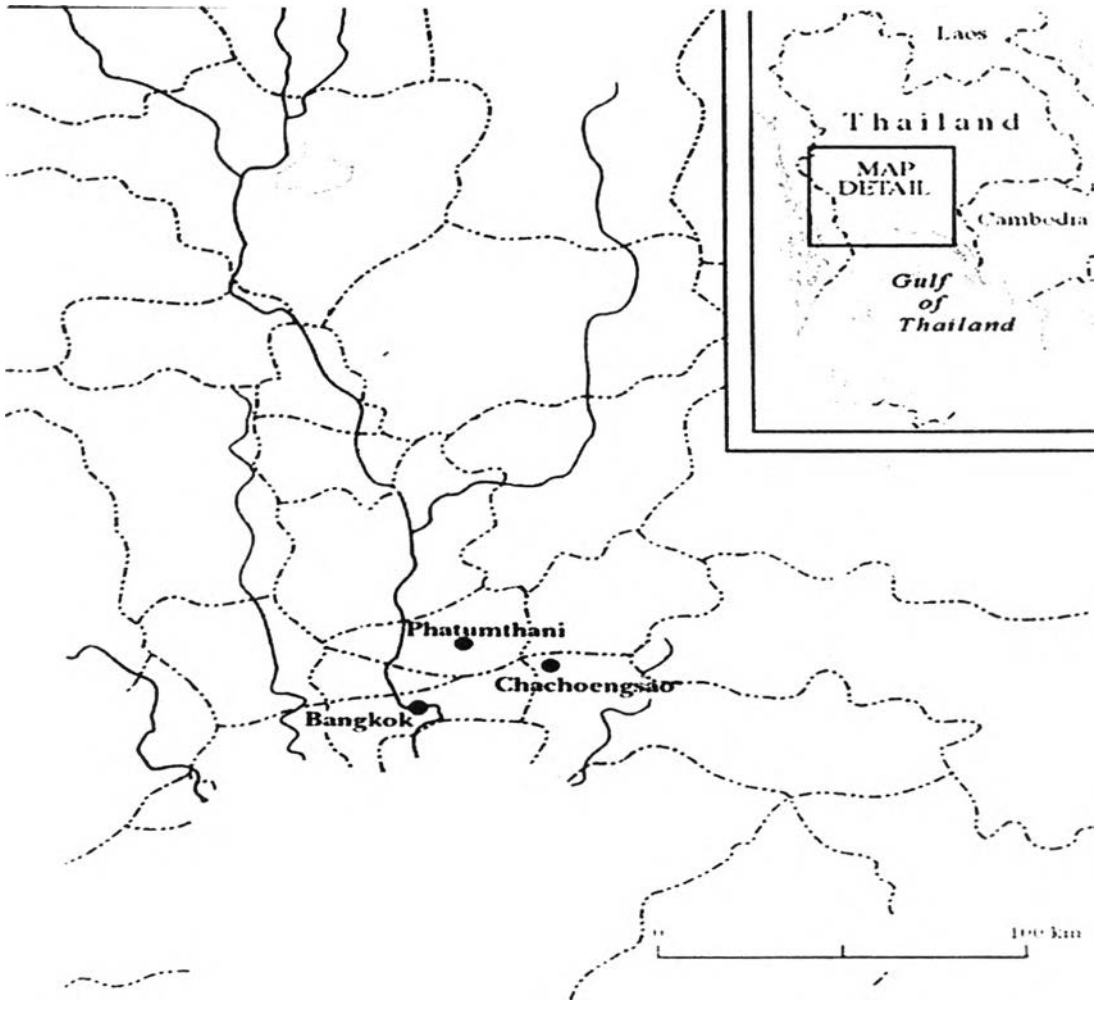

Figure 3-1: Location of the study sites.

Table 3-1: Location and characterization of shrimp ponds as investigated in this study.

Pond No.	Location	Type of culture system	Pond size (Ha (Rai))	Shrimp species	Remark
R1	Nong Suea	Intensive	0.08 (0.5)	*P. monodon*	Closed system
R2	Nong Suea	Intensive	0.08 (0.5)	*P. monodon*	Closed system
K1	Bang Khla	Intensive	0.16 (1)	*P. monodon*	New pond
P1	Ban Pho	Intensive	0.16 (1)	*P. monodon*	Closed system (with ozone)
P2	Ban Pho	Intensive	0.16 (1)	*P. monodon*	Closed system (w/o ozone)
T1	Bang Khun Thian	Extensive	8-(50)	*Penaeus merguiensis*	Opened system
T2	Bang Khun Thian	Extensive	15.2 (95)	*P. merguiensis*	Opened system
T3	Bang Khun Thian	Extensive	8.8 (55)	*P. merguiensis*	Opened system

3.1.2 Sediment sampling and preservation

Sediment samples from the pond bottom were collected every 2 weeks during shrimp cultivation except for the extensive ponds in Bang Khun Thian that were taken only once. Sediment was collected using core sampler with 5 cm in diameter. At least five cores were taken from each pond then the top 3 cm layer of sediment was mixed together and kept in sterile glass bottle before immediately transferred to the laboratory.

3.1.3 Sediment and water analysis

The sediment samples intended for the experiment was spread on a tray and place beneath the dryer overnight, or longer if necessary. Then, dry sediment was crumbled mechanically and passed through a sieve with a 2 mm mesh.

3.1.3.1 Organic matter

Organic matter in sediment analysis method used in this study was according to Loss on Ignition. The sample was air-dried at room temperature and following by oven-dried at 110°C for 2 hours. Dried soil sample was further burned at 700°C for 2 hours in exactly known weight ceramic crucible and cool down in desiccators. Finally, the organic matter can be calculated as the following:

%organic matter = the subtraction between the initiate and the later soil weight x 100 the initiate soil weight

3.1.3.2 Water content

The wet sediment was dried by oven-dried at 110°C for 2 hours in exactly known weight ceramic crucible and cool down in desiccators. The water content can be calculated as the following:

%water = the subtraction between the wet sediment and the dried sediment weight x 100 the initiate soil weight

3.1.3.3 Sediment pH

Method for the measurement of pH to all types of air-dried soils was performed with a combined glass electrode in a 1:2.5 (M/V) soil in 1 mol/L suspension.

3.1.3.4 Chlorophyll_*a*

The chlorophyll *a* analysis method used in this study was according to Strickland and Parson (1972). Phytoplankton cells in approximately 1 g of the dry sediment, chlorophyll-a pigment was then extracted using 9 ml. of 90% acetone in cool and complete darkness for 20 hours. After centrifugation, absorbance of acetone supernatant was measured at 630, 645 and 665 nm using spectrophotometer against 90% acetone blank. The concentration of pigments was calculated following this equation:

mg (or m-SPU) pigment/m3 = C/V

where C was a value obtained from the following equation

C (chlorophyll_*a*) =11.6665nm-1.31645nm-0.14630nm

and V was the volume of water filtered in liters.

3.1.3.5 Total ammonia

Ammonia was analyzed by phenol-hypochloride reaction method as modified from Strickland and Parson (1972). One gram of dry sediment was suspended by 9.0 ml of 2 N KCl. The mixture was vigorously shaken on a vortex mixer for 1 hour and then

centrifuged at 2,500 rpm for 10 minutes. One milliliter of sediment suspension sample was mixed with 0.2 ml of phenol solution (dissolve 20 g of crystalline analytical grade phenol in 200 ml of 95% (v/v) ethyl alcohol), 0.2 ml of sodium nitroprusside solution (1 g of $Na_2Fe(CN)5NO.2H_2O$ in 200 ml of deionized water), and 0.5 ml of freshly prepared oxidizing reagent (100 g of sodium citrate and 5 g of sodium hydroxide in 500 ml of deionized water mixed with sodium hypochloride 4:1 (v/v)) respectively. After mixing and stand for 1 hour, absorbance of the solution was measured at 640 nm using spectrophotometer (GENESIS®; model 10 UV scanning) against the blank (deionized water). Concentration of ammonium was calculated using the standard curve of ammonium solution (0.01-1.00 mg NH_4^+-N/L)

3.1.3.6 Nitrite

Nitrite was analyzed by sulfanilamide reaction as modified from Strickland and Parson (1972). One gram of dry sediment was suspended by 9.0 ml of 2 N KCl. The mixture was vigorously shaken on a vortex mixer for 1 hr. and then centrifuged at 2,500 rpm for 10 minutes. One milliliter of sediment suspension sample suspension sample was mixed with 0.1 ml of sulfanilamide solution (dissolve 5 g of sulfanilamide in a mixture of 50 ml of concentrate hydrochloric acid and about 300 ml of distilled water) and 0.1 ml of (1-Naphthyl)-ethylenediamine dihydrochloride solution (0.5 g of N-(1-Naphthyl)-ethylenediamine dihydrochloride in 500 ml of distilled water). After mixing and stand for 1 hour, absorbance of the solution was measured at 543 nm using spectrophotometer (GENESIS®; model 10 UV scanning) against distilled water blank. Concentration of nitrite was calculated using the standard curve of nitrite solution (0.007-0.224 mg NO_2–N/L)

3.1.3.7 Nitrate

Nitrate was analyzed by cadmium-conversion of nitrate to nitrite following by nitrite analysis using sulfanilamide reaction as described in nitrite analysis (3.1.3.6). Before analysis, 1 ml of concentrate ammonium chloride solution was added in 50 ml of filtered sediment solution sample. This mixture was poured in the glass column containing cadmium granules coated with copper sulfate. Flow rate of the cadmium column was set up at 50 ml per 5 minutes. With cadmium column, nitrate in water sample was converted to nitrite. Therefore, the correct nitrate-N concentration must be subtracted with nitrite-N concentration in the sample.

3.1.3.8 Total nitrogen

Total nitrogen was analyzed following by AOAC method (AOAC, 1980). One gram of dried sediment samples were digested in Micro-Kjeldahl flask with 100 ml of digestion mixture (Na_2SO_4 250 g, selenium 2.5 g in H_2SO_4 2,500 ml.). Digest the sample in a digestion unit which was preheated at 380°C for 4 hr. Distillation solution was titrated with 0.005 N H_2SO_4 or 0.005 N HCL. Blank prepared by distilled water with out nitrogen was titrated together

% nitrogen = (A-B)C x 1.4x5 / D

A= ml 0.005 N H_2SO_4 or 0.005 N HCL used for sediment samples

B = ml 0.005 N H_2SO_4 or 0.005 N HCL used for blank

C = concentration of H_2SO_4 or HCL

D = soil sediment weight

3.1.3.9 Phosphate phosphorus

Phosphate was analyzed by ammonium molybdate reaction (Strickland and Parson, 1972). Prior to analysis, 5 ml of sediment suspension was mixed with 0.5 ml of mixed reagent (mixture of 100 ml ammonium molybdate, 250 ml sulphuric acid, 100 ml ascorbic acid and 50 ml potassium antimonyl-tartrate solutions) was added in each sample tube. After mixing and stand for 1 hour, absorbance of the solution was measured at 885 nm using spectrophotometer (GENESIS®; model 10 UV scanning) against distilled water blank. Concentration of phosphate was calculated using the standard curve of phosphate solution (0.01-1.00 mg PO_4--P/L).

3.1.3.10 Total Phosphorus

Total phosphorus was analyzed by Olsen's method. The extraction of forms soluble phosphorus is performed in a sodium bicarbonate solution 0.5N with a sample: volume ratio of 1:20 (m/v). The phosphorus content is measured in a clear solution using spectrophotometer to analyze the blue color of the phosphomolybdic complex hot reduced by ascorbic acid (λ=660nm) (Olsen and Sommers, 1982)

3.1.4 Water analysis

Three parameters of water; temperature, salinity and alkalinity were measured. Temperature and salinity were measured in the ponds and alkalinity was measured in the laboratory.

3.1.4.1 Temperature

In every experimental pond, water temperature was measured by YSI D.O. meter at 10:00 am, 5 cm from water surface.

3.1.4.2 Salinity

Water salinity was measured by hand refractometer, at 10:00 am of sampling day.

3.2 Total aerobic bacteria counted and *Vibrio* counted

3.2.1 Total aerobic bacteria counted

One gram of soil sample was resuspended in 9.0 ml of 0.85% sodium chloride solution. The mixture was vigorously shaken on a vortex mixer and then allowed to settle. The supernatant were serial diluted before spreading on Plate Count Agar (PCA) (Oxoid, CA). Bacterial colonies were counted after incubation at 30°C for 3 days to calculate the number of all aerobic bacteria.

3.2.2 *Vibrio* counted

Supernatant from 3.2.1 was spread on TCBS (Thiosulfate-citrate-bile-sucrose) agar (Oxoid, CA). *Vibrio* colonies appeared green and yellow on TCBS agar after incubated at 30°C for 5 days.

3.3 Bacteria community analysis using PCR-DGGE methods

3.3.1 Total DNA extraction

Three grams of dry sediment was mixed with 27 ml of 0.1 M phosphate buffer (pH 4.5) and 0.6 g polyvinylpolypyrrolidone (PVPP) using blender for 1 minute and the mixture was cooled down in ice tub. The blending step was repeated for three times before mixing with 60 μL of a 20% sodium dedecyl sulfate (SDS) and vigorously shaking at 200 rpm for 2 hours. The extract was left at room temperature for 30 minutes to allow the precipitation of coarse particles. This was following by centrifugation (900 g for 10 minutes at 10°C). The supernatant containing released bacterial cells were collected and the extraction was repeated twice as previously described except the addition of PVPP and SDS were omitted. Thereafter, supernatant was mixed and centrifuged (10,000 g for 30 minutes at 10°C). Bacterial pellet was then washed twice with phosphate buffer (pH 4.5). DNA extraction of the pellet was performed by adding 2.0 ml of extraction buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate buffer (pH 8.0) with 3 mg lysozyme per ml, 2% (final concentration) SDS and 1 g glass beads (BIO 101, Vista, CA). The sample was transferred into the Multimix™ tubes and processed in the FastPrep® Instrument (FP120, BIO101) for 20 second at the setting speed of 5.5. The lysate was centrifuged at 600 g for 10 minutes in a sterilized tube and extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1). Nucleic acids were precipitated after adding 0.7 volume of isopropanol containing 0.3 M sodium acetate (pH 4.8) and 1 mM $MgCl_2$ and following by centrifugation (4°C) at 12,000 g for 10 minutes. The pellet containing nucleic acids was the washed with 70% ethanol, air dried and re-suspended in TE (Tris-EDTA).

3.3.2 PCR-DGGE Analysis

The V3 region of 16S rDNA gene from sediment samples was amplified by polymerase chain reaction (PCR) using PRBA338f (5'-ACTCCTACGGGAGCAGCAG-3') and PRUN518r (5'-ATTACCGCGGCTGCTGG-3') primers (Lise Øvreås *et al*, 1997) which correspond to position 338-357 and 518-534 in the *E. coli* 16s rDNA gene (GenBank accession no. j01695). Both primers complement with a conserved region of the domain bacteria. The GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGG GGGCACGGGGGG-3') described in Muyzer *et al*, (1993) was attached to the 5' end of the forward primer PRBA338f. The PCR complied with 3 µl of extracted DNA suspension, 0.5 µM of each primer, 15 µl of Taq DNA polymerase Master Mix (Quigen, CA) and sterile water to the final volume of 30 µl. The PCR condition was as follows: an initial denaturation step at 94°C for 2 minutes, followed by 40 cycles of a three-stage program with 1 minute at 94°C for denaturation, 30 seconds at 55°C for annealing, and 1 minute at 72°C for extension, and a final extension step run for 6 minutes at 72°C. The PCR products were checked by gel electrophoresis with 1% agarose and visualized under UV illumination after ethidium bromide staining.

Denaturing gradient gel electrophoresis (DGGE) was performed using the D-code system (Bio-Rad). Acrylamide gel (7.5% w/v of 38:2 acrylamide-N, N'-methylene-bisacrylamide) was prepared with 20-70% denaturing gradient in which 100% is defined as 7M urea and 40% (v/v) formamide. Electrophoresis was performed at 60°C for 6 h at a constant voltage of 130 V with TAE running buffer contained 40 mM Tris-acetate and 2 mM Na_2-EDTA. Then, gel was stained with 100 µg ethidium bromide in 1 L deionized water for 15 minutes and photographed under UV illumination. To identify the species of selected DGGE bands, they were excised, extracted from the gel and used as the template

for PCR re-amplification. The condition of PCR was as previously described except the forward primer PRBA338f did not include the GC clamp.

3.3.3 Cloning, Sequencing, and Identification of Bacterial Species

16S rDNA amplicons were cloned into *Escherichia coli* JM109 using the pGEM-T easy plasmid vector system (Promega, Madison, Wis.) in accordance with the manufacturer's instructions. The resulting transformants were plated on to Luria-Bertani (LB) plates containing ampicillin, IPTG and X-Gal. White colonies were selected. To ensure that plasmid DNA isolated from White colonies is recombinant plasmid DNA, plasmid DNA were digested with restriction enzyme *Eco*RI at 37°C overnight. The digested DNA was confirmed on agarose gel comparing to 200 base pairs ladder. Then, the ligation plasmid were cleaned using QIA Miniprep Kit (Qiagen,CA) and sequenced at the Bioservice Unit, National Center for Genetic Engineering and Biotechnology, Thailand. After acquiring DNA sequences, BLAST software (GenBank, www.ncbi.nlm.nih.gov.) was used to determine its identity and nearest gene neighbor.

3.3.4 Analysis of DGGE images

3.3.4.1 Bacterial Diversity Analysis

DGGE images were converted to digitized data using Scion Image program (Scion Corporation, Frederick, MD). The amounts of each bacteria population in a sample were deduced from the intensity of each DNA band on a DGGE lane. Consequently, it is possible to compare bacterial populations from different samples by aligning the digitized data from all samples (lanes) on Excel spreadsheet. Diversity indices were then calculated according to Eichner *et al.* (1999).

The Shannon-Weaver index of general diversity, *H'* was calculated using the function:

$$H' = - \Sigma Pi \log Pi$$

Where *Pi* is the importance probability of the bands in a DGGE lane. It was calculated as $Pi = ni/N$, where *ni* is the intensity of a DNA band and *N* is the sum of all band intensities in the DGGE lane.

3.3.4.2 Bacterial Community Analysis

To compare the community similarities between different samples, several DGGE images were combined with Adobe Photoshop 5.0 software package (Adobe systems Inc., San Jose, CA, USA), by referring to the synthetic marker, to amend the relative mobility between the different gels. Similarities of bacterial communities from sediment samples were analyzed from the digitized gel data by correspondence analysis using MSVP3.1 program (Kovach Computing Services, Wales, UK). The comparison was based on the incidence and abundance of DNA bands on DGGE images. Correlation between bacterial communities and sediment characteristic data were later analyzed by canonical correspondence analysis using MSVP3.1 program

3.4 Nitrogen conversion by sediment from shrimp pond under laboratory condition

Investigation of nitrogen conversion by the sediment taken from shrimp pond was performed in a plastic chamber under laboratory condition. The chamber, as shown in Figure 3-2, was made of acrylic plastic tube with 13.8 cm in diameter and 28 cm in height.

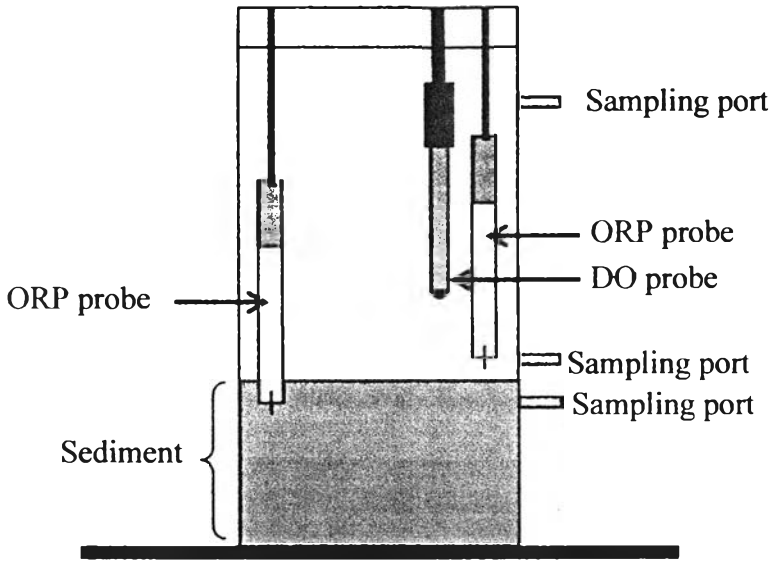

Figure 3-2: .The reactor for study nitrogen and phosphorus conversion in shrimp pond sediment in laboratory.

Sediment collected from shrimp pond at Nong Suea, Pathum Thani, was packed in the chamber at 8 cm height covering 0.0149 m^2 in surface area. The chamber was then filled with 2.5L of 6 PSU seawater and gently aerated with an air-stone. Temperature during the experiment was controlled at 25°C.

With this experiment, sterilized shrimp feed pellet was added into the chamber as the organic nitrogen source. Changed in inorganic nitrogen compounds including ammonia, nitrite and nitrate was monitored by water sampling through the sampling ports located at 2 cm below soil surface and 2 cm above soil surface. Ammonia and nitrite were analyzed by the standard method for seawater analysis as described in Strickland and Parson (1972). Nitrate analysis was the UV-screening method described in Greenberg *et al* (1992). Dissolved oxygen in the water column was continuously monitored using logging DO meter (HANNA HI91440) and oxidation-reduction potential

(ORP) at approximately 0.5-1 cm below soil surface was monitored using logging ORP meter (HANNA HI98140) respectively.