CHAPTER III

MATERIALS AND METHODS

Freshwater Mussels and Sediment Collection

The study area is at Khlong 7 canal of Rangsit agricultural area, at Pathumthani Province between Nong Sue and Khlong Luang District. Freshwater mussels and sediment were collected by hand every 3 months in the irrigated Khlong 7 during March 2006-2007. The sampling periods include period I (March-May 2006), period II (June-August 2006), period III (September-November 1006) and period IV (December 2006-March 2007). Three sampling locations along the 20-km stretch of the Khlong 7 were divided according to the flow direction of the canal into 1) upper stream, 2) middle stream, and 3) lower stream sites, respectively.

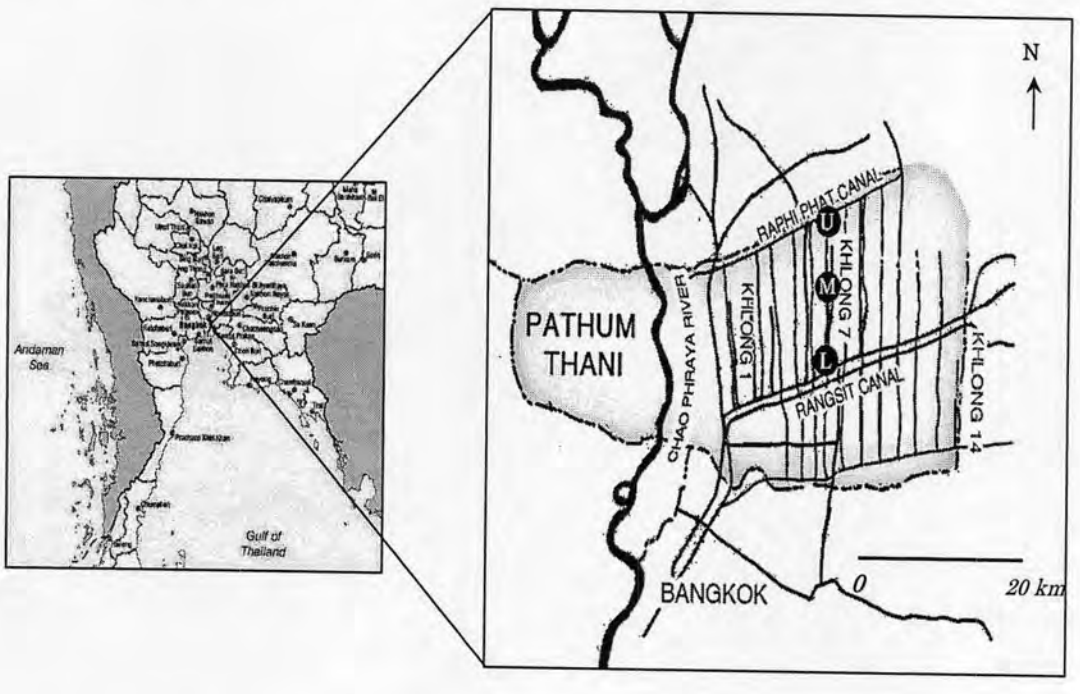

Figure 3.1 Map of Rangsit agricultural area, Pathum Thani Province. The study area is situated at Khlong 7 where (U) = upper stream, (M) = the middle stream, and (L) = lower stream

Sediment sample

Three sediment samples, each with 100 grams of surface sediment, were collected from each site. The samples were air-dried at room temperature for 5-7 days, then homogenized and sieved with mesh no.200 (SOP for USEPA Method 8081 A and 3541).

Freshwater mussel sample

At the 3 sampling sites along Khlong 7, the freshwater mussels including *Uniandra contradens ascia, Hyriopsis (Limnoscapha) desowitzi, and Pilsbryoconcha exilis exilis* were obtained by hand sampling. Live mussel samples were brought to laboratory at Chulalongkorn University and maintained in aerated dechlorinated tap water until autopsy. Whole body specimens were collected for OCP analysis. Gonads of mussels were dissected and kept for vitellogenin assay, and hepatopancreas of mussels were collected for glutathione s-transferase activity measurement. Specimens were kept in the -20 °C freezer until extraction.

Organochlorine Residue Analysis

Standard and Chemicals

EPA 8080 Pesticides Mix Catalog No. 47913 consisting of seventeen organochlorine pesticide (OCPs) standards for α-, β-, δ- and γ-HCH, aldrin, heptachlor, heptachlor epoxide, endosulfan I, endosulfan II, endosulfan sulfate, 4,4'-DDD, 4,4'-DDE, 4,4'DDT, endrin, endrin aldehyde, dieldrin, and methoxychlor were obtained from Supelco (Beollefonte, PA, USA).

A stock of the standard mixture containing 17 OCPs was prepared in 99% n-hexane at a concentration of 1,000 ng/mL and stored at -4 °C in a refrigerator.

Working standard solutions were prepared at the concentration of 0.001-100 ng/mL by volume and then diluted with 99% n-hexane.

Extraction and Clean-up of Organochlorine Pesticide in Sediment and Invertebrate Samples

Extraction

Each sediment sample had been well mixed and air-dried at room temperature without sunlight about 5-7 days. Dried samples were sieved with mesh no. 200 (500 μm) to remove coarse sands, stone and organic debris. Five grams of sediment samples were mixed with 5 g anhydrous sodium sulfate (1:1 w/w) and filled in a 34-mL vessel of accelerated solvent extractor (ASE-100, Dionex Canada, Oakville, ON, Canada) which was layered with activated copper powder, to remove sulfur contamination (Pan *et al.*, 2004), on the ASE filter paper and fill the vessel with the Ottawa sand. The sediments were extracted with 1:1 v/v 95% n-hexane: dichloromethane. ASE conditions were preheated for 5 min and extracted at 100 °C with pressure of 1,500 psi and static cycle of 10 min. Finally sample was purged with nitrogen for 60 sec.

In case of mussel samples, before extraction, the whole body of each group was homogenized by blender. Five grams of blended samples were mixed with 15 grams of anhydrous sodium sulfate (1:3 w/w). The mixtures were then placed into the vessel of ASE which was layered with filter paper and fill the vessel with the Ottawa sand. The operating conditions were similar to that of the sediment extraction

For both sediment and mussel extraction, the method was applied and validated according to Pan *et al.* (2004). After the extraction, sediment and

mussel samples were concentrated to 2 mL using Turbo-Vap under a gentle stream of nitrogen and then followed by clean up step.

Clean up

The extracted samples were cleaned up by the solid phase extraction (SPE) florisil (PR) with 1,000 mg cartridge (Alltech Associates Inc.). Because of the presence of pigments and sulfur in the eluted of sediment samples, it was necessary to remove the contaminants before injection to GC (Pan *et al.*, 2004). The extracted sediment samples were de-sulfur by laying 1.5 mg of activated copper powder in SPE. Then the extracted was eluted with 10 mL of 6%, 15% and 50% diethyl ether in petroleum ether, respectively. The eluted was then concentrated to 2 mL in Turbo Vap before further GC analysis.

Gas chromatography analysis

An Agilent 6890N GC equipped with micro Electron Capture Detector (μECD) was used for the quantification. Compound separation was completed using DB-35MS fused silica capillary column (30 m length, 0.25 mm i.d., and 0.25 μm film thickness) coated with 35% diphenyl polysiloxane (J&W Scientific). Sample quantification was performed using multiple external standards. One microliter of sample was injected into the GC on splitless mode with a 0.75 min vent delay. The injector and detector temperature were maintained at 260 °C and 300°C, respectively. The oven temperature was initially maintained at 100°C for 2 min, and then programmed to increase at 12 °C/min to 280°C and held for 10 min. Total run time was calculated to be 27.00 min. For optimum performance, the ultra-high-pure (UHP, 99.999%) of helium was used as carrier gas with flow rate at 2 mL/min linear velocity, and nitrogen (UHP) was set at 60 mL/min as make-up gas.

Quality control

Peaks and retention time of organochlorine pesticides (OCPs) were confirmed with DB-1701 fused silica capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) coated with 14 % cyanopropylphenyl and 86 % diphenyl polysiloxane (J&W Scientific). Calibration curve using the external mixed standard of 17 OCPs was performed for each compound to be quantified at concentrations of 5, 10, 20, 50, and 100 ng/mL. Calibration of standards was run at every 10 samples and measurements were performed in the ranges of linearity found for each compound. The methods were considered to be reliable for quantify the concentration of OCPs in all matrices following AOAC Peer Verified Methods Program (1993) including the limit of detection (LOD), the limit of quantitation (LOQ), the method detection limit (MDL), the relative standard deviations (RSD).

Following the SOP for AOAC method 983.21, performing blank analysis and assessing recovery from pre-extracted and fortified matrices were done in each sampling batch. Triplicate analyses of extracted samples from each sampling time were done to ensure that the measurement remained stable. The spiked recovery (%) and relative standard deviation (%) were calculated.

Method validation (US EPA, 2000)

Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) were defined as the peak height of analyte in standard solution that signaled significantly different from the peak height of noise. They were 3 and 10 times of signal per noise for LOD and LOQ, respectively. LOD and LOQ were verified. In

case of the OCP concentrations below the LOD, the results were described as ND or not detectable.

Spike recovery

Fortified samples were done in every sampling batch to ensure that the extraction efficiency would be under control (Kebbekus and Mitra, 1998). In addition, the acceptable recovery of the OCPs should be ranged from 70 to 130%, following SOP for USEPA METHOD 8081+3510 (waters). The recovery percentage can be calculated by the equation below,

$$\% \text{ Recovery} = \frac{\text{amount of OCPR determined}}{\text{amount of OC standard}} \times 100$$

Blanks

To avoid the effect of interferences, the set of blanks were done. The blanks included solvent blank, system blank, and fortified sample blank. These blanks were done every sample batch. The blanks must be free of contaminants, or the concentration of contaminated analyses must be at the lowest level.

Replications

The replications of samples were done to evaluate repeatability. The samples were extracted and analyzed in triplicate to be sure that the measurement remained stable. The % RSD was calculated from the equation as followed:

$$\%\text{RSD} = \frac{\text{standard deviation}}{\text{mean}} \times 100$$

Method detection limit (MDL)

The detection limit of the selected method was calculated based on the replicated determinations as following.

$$MDL = t_{0.95[n-1]} \times SD$$

Where t is the threshold value of student t – distribution at the degree of (n-1), n represents the number of replications, and SD represents the standard deviation. The confidence interval is 95% ($\alpha = 0.05$).

Vitellogenin Analysis

Protein extraction

For extraction of vitellogenin, 0.5 g of gonadal tissues from freshwater mussels was dissected on ice. The tissue was homogenized in 1 mL of extraction buffer (0.05 M Tris-HCl buffer pH 7.5 containing 0.2 M NaCl and protease inhibitors cocktail (ethylenediaminetetraacetic acid [EDTA], 4-[2-aminoethyl] benzenesulfonyl fluoride [AEBSF], bestatin, E-46, leupeptin and aprotinin)) for 1-2 minutes. The homogenate was centrifuged at 4 °C, 12,000 x g for 30 min to collect the final supernatant fraction (cytosolic fraction). The cytosolic samples were stored in aliquots and saved at -20 °C for further analysis.

Total protein concentration

The total protein concentration was determined from cytosolic fraction by the Bradford method using a microplate reader at 620 nm (Bradford 1976; Redinbaugh and Campbell, 1985), with bovine serum albumin (BSA) (Sigma, St. Louis, Mo) as a standard. The BSA standard and extracted gonad were diluted with phophate buffered saline (PBS) pH 7.2. The concentration of BSA standard ranged from 0.78125 to 25 µg/mL. The extracted gonad was diluted 1:100,

1:1,000, and 1:10,000. The standard curve between protein concentration and absorbance 620 nm was plotted with Microsoft Excel. The straight line with r^2 of more than 0.95 ($p<0.05$) was used as the standard curve. Once the concentration of protein was obtained, samples were diluted with PBS in order to normalize the total protein concentration in each sample at 1 mg/ml.

Determination of vitellogenin by enzyme-linked immunosorbent assay (ELISA)

Level of vitellogenin in gonad extract was carried out according to modified method of Won (2006). Vitellogenin standard and antibody were obtained from Professor Ian P. Callard (Boston University). The vitellogenin had been purified from freshwater mussel (*Elliption complanata*) living in the USA (Won, 2006) and the antibody was raised in rabbit against mussel vitellogenin (Babco, Berkeley, CA, USA). The vitellogenin standard and gonad extract samples were diluted with 50 mM carbonate-bicarbonate buffer pH 9.6 (15 mM Na_2CO_3, 35 mM $NaHCO_3$). The standard used in this assay ranged from 4.88 to 2,500 ng/mL. The samples and quality control were diluted to 1:100 and 1:1,000.

Triplicate wells in round bottom 96-well ELISA plates (Corning, Acton, MA) were coated with 100 µl of serially diluted purified mussel vitellogenin, diluted samples, quality control sample or buffer blank and incubated overnight at 4°C. After incubation, wells were washed 3 times with 0.02% Tween-20 in PBS (PBS-T), filled with 200 µl of 2% gelatin in PBS and incubated at room temperature for 2 hr to block nonspecific binding. Washing steps were repeated and 100 µl of primary antibody (1:4,000 in PBS) was added into each well and incubated for 2 h at 4°C. After washing 3 times with PBS-T, 100 µl of secondary antibody (goat anti-rabbit IgG-alkaline phosphatase conjugate, Sigma, St.Louis,

MO, 1:2,000 in PBS) was added into each well and incubated for 2 h at 4°C. Following washing steps, 100 µl of p-nitrophenyl phosphate solution (1 mg/ml in diethanolamine buffer containing 1 M diethanalomine, 0.5 mM $MgCl_2$, pH 9.8) was added to each well. After a 20-min incubation at room temperature, absorbance at 405 nm was measured by using a microplate reader (Multiskan EX, Thermo Labsystems). Using the absorbance values of serially diluted purified mussel vitellogenin, a standard curve was generated on a semi-log scale. The level of vitellogenin in the gonad extract was determined against the standard curve using Microsoft Excel.

Glutathione S-Transferase Analysis

Protein extraction

For glutathione-s-transferase assay, hepatopancreas of freshwater mussels collected from Khlong 7 were dissected on ice. The 0.5 g of hepatopancreas tissue was homogenized in 1 mL of extraction buffer (50 mM Tris-HCl, 0.15 M KCl, pH 7.4) for 1-2 minutes. The homogenated was centrifuged at 4 °C, 12,000 x g for 30 minutes. The supernatant of the cytosolic fraction were stored in aliquots and saved at -20 °C for further analysis.

Total protein concentration

The total protein concentration in hepatopancreas extract was determined in similar ways to the gonad extract in vitellogenin assay. Once the concentration of protein was obtained, samples were diluted with the extraction buffer to normalize the total protein concentration in each sample at 1 mg/ml.

Determination of GST activity

The activity of glutathione-s-transferase (GST) was determined spectrophotometrically using the method of Habig *et al.*, (1974). The reaction mixture consisted of 100 mM 1,2 chlorodinitrobenzene (CNDB) and 200 mM glutathione (Sigma, MO, USA) dissolved in 100 mM Tris-HCl pH 7.4. Twenty microliters of the hepatopancreas extract were added to 1 ml of reaction mixture. The activity of GST was determined at 340 nm at 25°C over 5 minutes after 1 minute of lag time. Data are expressed as µmol/min/mg of cytosolic protein.

The specific activity of GST was determined from the following equation:

$$\text{Specific enzyme acivity (µmol/mL/min)} = \frac{\Delta A340 \times V}{E_{mM} \times V_{ezm}}$$

$$\Delta A340 = \frac{\text{A340 (Final read)} - \text{A340 (initial read)}}{\text{reaction time (min)}}$$

V = the reaction volume (1mL)

E_{mM} = the extinction coefficient for CDNB conjugate at 340 nm ($9.6\ nM^{-1}$)

V_{ezm} = volume of enzyme (20 µL)

Statistical Analysis

All data were initially tested for normal distribution and homogeneity of variance before proceeding with parametric analysis. Data were present as mean ± standard error of the mean (S.E.M.). One-way analysis of variance (ANOVA) followed by Student-Newman-Keul's multiple comparison were used to compared mean of OCPs, enzyme activity and vitellogenin between site and sampling period. Pearson's correlation was used to determine correlation between OCP residues in sediment, OCP residues in mussel, vitellogenin level and glutathione-s-transferase activity in mussel. In all case significance difference is accepted at $p \leq 0.05$. Statistical analyses were performed using Sigma Stat, Version 2.0 (Jandel Scientific).