CHAPTER 3
METHODOLOGY

This chapter describes study sites and methodology of ecological and diversity of mollusks. In terms of ecological aspects, physical and chemical parameters of water quality at each site were measured. Physical and chemical parameters consist of air temperature, water temperature, pH, conductivity, DO, BOD, ammonia nitrogen, nitrate nitrogen and orthophosphates. Heavy metals were analyzed by Inductively-Coupled Plasma Spectroscopy (ICP) technique. In addition, the impacts of heavy metals on mollusk tissues were investigated by Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM). Methodology of heavy metals accumulations are shown in schematic diagram of laboratory concept of analysis. Finally, statistical method was applied to experiment data.

3.1 Study Site

Bueng Jode wetland is located in Ban Gudnamsai, Nampong District, Khon Kaen Province. It is approximately 50 kilometers north of Khon Kaen city. The area of reservoir is approximately 300 rais (480 square kilometers) and heavily crowded by water hyacinth (Faculty of Engineering, 2003). The location via GPRS (Global Position Roaming System) of Bueng Jode wetland is located in UTM zone 48 at x: 259729, y: 1850899 and x: 260710, y: 1849874, latitude: 102.746-102.755, longitude: 16.719-16.728. It is adjacent to Pong River which is the principle river in this region, where Moon River is the continuation of this water-way into the lower northwest Esarn region. Bueng Jode wetland receives considerable amount of effluent from industry, local community and agricultures before flowing down to Pong River. Thus, any water pollution would have a combined effect on this effluent. People living in Loei, Nongbualamphu and Khon Kaen Provinces utilize water in this river for daily consumption and agriculture. Despite of reports of water pollution in the Pong River since 1986, there was still a major crisis of water pollution as reported in

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1992 (Faculty of Engineering, 2003). Sources of pollution have drained from several industries, signaling high impact on the Pong River. These industries are, for example, paper mill factory, combined gas turbine power plant, distillery plant, sugar factory and wood working plants. In addition, there are several small industries located within the vicinity along the Pong River. These must be major causes of heavy metals contamination in the Pong River coming from industries along with traces from pesticides, chemical fertilizers and other wastes from the community. Five sample collecting stations are defined as affected area in Bueng Jode wetland (Figure 1). Assessment of general environmental parameters is carried out from Station 3A which is 20 meters below Station 3. Study sites and environmental areas of five collecting stations are as shown in Figure 2. Collecting Station 1 is located directly next to the effluent from industry, local community and agricultures before flowing down to Pong River (Figure 2). As for Station 2, 3 and 4 is located in the general area of Bueng Jode wetland, Station 5 is located at the out flow into Pong River (Figure 2).

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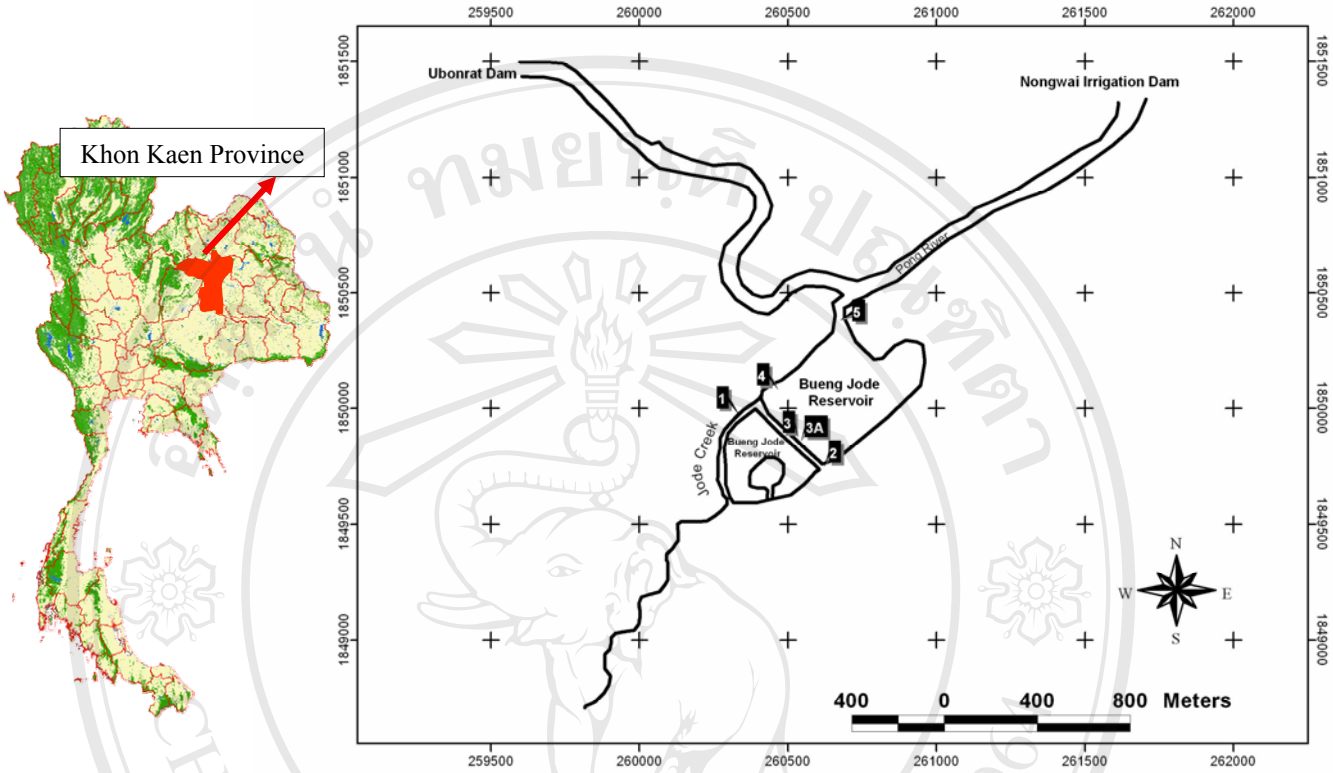

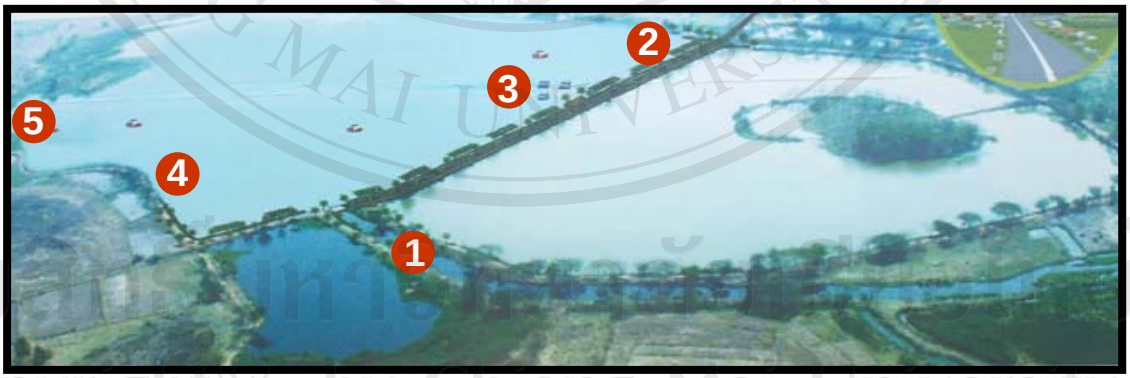

Figure 1. Overview of Bueng Jode wetland and location of five collecting stations as numbered.

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Station 1

Station 2

Station 3

Station 4

Station 5

Figure 2. Study Sites and the environmental areas of five collecting Stations

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3.2 Ecology and Diversity of Mollusk

3.2.1 Ecology

Samples of water, sediments and mollusks were collected from five sample collecting stations in three consecutive seasons, these were on Sunday 24 August 2006 (rainy season), Saturday 6 January 2007 (cold dry season), Saturday 1 April 2007 (summer season). The seasons in Thailand are divided into rainy season ranging from July to October, cold dry season from November to February and summer season from March to June. Water samples were collected using bottles with 20 centimeter depth for physical and chemical analysis.

The physical and chemical parameters of water quality at each station were analyzed directly in the field as follows (Golterman *et al.,* 1978);

1) Air temperature (^{0}C) was measured using a thermometer.

2) Water temperature (^{0}C) was measured by thermometer probe which was held 20 centimeters depth in the water until the mercury level stabilized.

3) pH, the microprocessor pH-meter was used to detect the pH level of water on each site at 20 centimeter depth.

4) Conductivity (μS./cm), electro-conductivity meter was used to detect the conductivity of the water level depth of 20 centimeters.

The following water quality parameters are analyzed in compliance with the American Public Health Association (Greenberg *et al*., 1992) procedures in the laboratory.

1) Dissolved oxygen (DO, mg/L), water samples from each site was collected very carefully using narrow-mouth, 300 ml. DO glass-stopped bottles and then measured according to the Azide Modification method (Greenberg *et al*., 1992)

2) Biochemical oxygen demand (BOD, mg/L), the BOD was measured according to the Azide Modification method (Greenberg *et al*., 1992).

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3) Ammonia nitrogen (NH_3-N, mg/L), the ammonia was determined according to the method described by Greenberg *et al*. (1992).

4) Nitrate nitrogen (NO_3^--N, mg/L), nitrate was examined by employin cadmium reduction method using Nitra Ver 5 Nitrate pillow and a HACH DR/2400 spectrophotometer.

5) Orthophosphates (*o*-PO_4^{3-}-P, mg/L), the Ascorbic Acid method, using a PhosVer 3 powder and a HACH DR/2400 spectrophotometer was used to determine phosphate.

3.2.2 Diversity of Mollusks

Mollusks were collected at each station and identified down to species level by Brandt (1974), Chithamvong (1992), Keawjam (1986), Klinhon (1989), Kijviriya (1990), Tarbsripair (1998), Upatham *et al*. (1983) and MRC (2006). These were collected manually and then each sample was bagged, labeled and kept frozen prior to analyze. Analyses were made within 10 days after collection.

3.3 Heavy Metals Analysis by Inductively-Coupled Plasma (ICP) Spectroscopy Technique

Sediment samples were homogenized, air dried and sieved through a 63 μm mesh. Homogenized samples were acid digested using a microwave digestion system (ETHOS touch control) following EPA method 3015 (UNEP, 1994). Concentrations of cadmium, copper, zinc, lead and mercury were analyzed by a PerkinElmer (Optima 4300 AV) Optical Emission Spectrometer.

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Mollusks collected in the field were identified to species. Each mollusk was homogenized and sub-samples were analyzed for cadmium (wavelength 228.802 nm.), copper (wavelength 327.393 nm.), zinc (wavelength 206.200 nm.), lead (wavelength 220.353 nm.) and mercury (wavelength 253.652 nm.). Homogenized tissue was digested using a microwave system (ETOS touch control) following EPA

method 3052 (UNEP, 1996) and analyzed for the same heavy metals as in sediment using the same procedure. Results were reported in dry weight basis.

Procedures for Sample Analysis

1) Calibration of Microwave Equipment

2) All digestion vessels and volumetric ware must be carefully acid washed and rinsed with reagent water. When switching between high solids (concentrated) samples and low solids (low concentration) samples all digestion vessels should be cleaned by leaching with hot (1:1) hydrochloric acid (greater than 80^{o}C, but less than boiling) for a minimum of two hours followed with hot (1:1) nitric acid (greater than 80^{o}C, but less than boiling) for a minimum of two hours, rinsed with reagent water, and dried in a clean environment. This cleaning procedure should also be used whenever the prior use of the digestion vessels is unknown or cross contamination from vessels is suspected. Polymeric or glass volumetric ware and storage containers should be cleaned by leaching with more dilute acids (approximately 10% V/V) appropriate for the specific plastics used and then rinsed with reagent water and dried in a clean environment. In addition, to avoid precipitation of silver, ensure that all HCl has been rinsed from the vessels.

3) Sample Digestion

(3.1) Weigh the fluorocarbon (PFA or TFM) digestion vessel, valve and cap assembly to 0.01 g prior to use.

(3.2) A 45 mL aliquot of a well shaken sample is measured in a graduated cylinder. This aliquot is poured into the digestion vessel with the number of the vessel recorded on the preparation sheet.

(3.3) A blank sample of reagent water is treated in the same manner along with spikes and duplicates.

(3.4) Add 5 mL of concentrated nitric acid to each vessel that will be used. Check to make sure the pressure relief disks are in the caps with the smooth side toward the sample and start the caps a few turns on the vessels. Finish tightening the caps in the capping station which will tighten them to a uniform torque pressure of l2

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ft-lbs. (16 N-m) or to the manufacturers recommended specifications. Weigh each capped vessel to the nearest 0.01 g.

CAUTION: Toxic nitrogen oxide fumes may be evolved, therefore all work must be performed in a properly operating ventilation system. The analyst should also be aware of the potential for a vigorous reaction. If a vigorous reaction occurs, allow to cool before capping the vessel.

(3.5) Evenly distributed the vessels in the carousel according to the manufacturer's recommended specifications. Blanks are treated as samples for the purpose of balancing the power input. When fewer than the recommended numbers of samples are digested, the remaining vessels should be filled with 45 mL of reagent water and 5 mL of nitric acid to achieve the full compliment of vessels. This provides an energy balance since the microwave power absorbed is proportional to the total mass in the cavity.

(3.6) Program the microwave unit according to the manufacturer's recommended specifications and, if used, connect the pressure vessels to the central overflow vessel with PFA-fluorocarbon tubes. The chosen sequence will bring the samples to 160 deg C ± 4 deg C in 10 minutes and will permit a slow rise to 165-170 deg C during the second 10 minutes. Start the turntable motor and be sure the vent fan is running on high and the turntable is turning. Start the microwave generator.

(3.7) At the end of the microwave program, allow the vessels to cool for at least 5 minutes in the unit before removal to avoid possible injury if a vessel vents immediately after microwave heating. The samples may be cooled outside the unit by removing the carousel and allowing the samples to cool on the bench or in a water bath. When the vessels have cooled to room temperature, weigh and record the weight of each vessel assembly. If the weight of the sample plus acid has decreased by more than 10% discard the sample.

(3.8) Complete the preparation of the sample by carefully uncapping and venting each vessel in a fume hood. Transfer the sample to an acid-cleaned bottle. If the digested sample contains particulates which may clog nebulizers or interfere with injection of the sample into the instrument, the sample may be centrifuged, allowed to settle or filtered.

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(3.8.1) Centrifugation: Centrifugation at 2,000-3,000 rpm for 10 minutes is usually sufficient to clear the supernatant.

(3.8.2) Settling: Allow the sample to stand until the supernatant is clear. Allowing a sample to stand overnight will usually accomplish this. If it does not, centrifuge or filter the sample.

(3.8.3) Filtering: The filtering apparatus must be thoroughly cleaned and prerinsed with dilute (approximately 10% V/V) nitric acid. Filter the sample through qualitative filter paper into a second acid-cleaned container.

(3.9) The concentration values obtained from analysis must be corrected for the dilution factor from the acid addition.

3.4 Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM)

3.4.1 Scanning Electron Microscope (SEM)

Scanning electron microscopy enabled the user to determine topography and morphology of a sample. An electron gun produced a stream of monochromatic electrons that was condensed by the first condenser lens. This lens was used to both form and limit the amount of current of the beam. It worked in conjunction with the condenser aperture to eliminate the high-angle electron from the beam. The second condenser lens forms the electrons into a thin, tight, coherent beam. A set of coils then “scan” or “sweep” the beam in a grid fashion, dwelling on points for a period of time determined by scan speed. The final lens, the objective, focused the scanning beam onto the part of the specimen desired. When the beam strikes the sample interactions occur inside the sample and were detected with various instruments. Before the beam moved to its next dwell point these instruments count the number of interactions and display a pixel on a screen, the intensity of which was determined by this number (the more reactions the brighter the pixel). This process was repeated until the grid scan was finished and then repeated, with the entire pattern being scanned around 30 times per second (Lyman *et al*., 1990).

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A number of different interactions can occur when the energized electrons in the microscope strike the sample. The production of backscattered electrons was relative to the atomic weight of the sample, with higher atomic number elements appearing brighter than those with a low atomic number. Secondary electrons assist with building a picture of the specimen's topography. Auger electrons and X-rays were emitted after secondary electrons were produced. Both have characteristic energies unique to the element from which they were emitted, allowing analysis of the elemental composition of a sample (Lyman *et al*., 1990).

Specimens were prepared by fixation, dehydration and coating with a very thin layer of either gold or carbon. They were mounted on a purpose-built stub, generally made of aluminium. In this study, the LEO 1450VP SEM was used to view and photograph specimens. The LEO 1450VP SEM is placed at Department of Biology, Faculty of Science, Khon Kaen University.

3.4.2 Transmission Electron Microscope (TEM)

The transmission electron microscope used a beam of electrons which passed through a thin section of a specimen, transmitting an image onto a screen. It was used to elucidate the morphology of specimens.

An electron gun at the top of the microscope produces a stream of monochromatic electrons. This stream was focused to a small, thin, coherent beam by the use of two condenser lenses. The first lens largely determined the "spot size", or the general size range of the final spot that strikes the sample. The second lens actually changed the size of the spot on the sample, changing it from a wide dispersed spot to a pinpoint beam. The beam was restricted by the condenser aperture, knocking out electrons far from the optic axis. The beam striked the specimen and parts of it were transmitted, then focused by the objective lens into an image. The image was passed down the column through the intermediate and projector lenses, being enlarged all the way. It then striked a phosphor screen and light was generated, allowing the user to see the image. The darker areas of the image represent those

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areas of the sample that fewer electrons were transmitted through, that is thicker or denser areas. The lighter areas of the image represent those areas of the sample that more electrons passed through (Lyman *et al*., 1990).

Specimens were prepared by fixing and embedding tissues in resin. Very thin slices, approximately 90-100 nm, are taken and placed on a very fine copper grid. The tissue slices may be stained using heavy metals to increase the contrast between elements of the cell. In this study, HITACHI H-600 transmission electron microscope was used to view and photograph specimens. The HITACHI H-600 transmission electron microscope is placed at Department of Anatomy, Faculty of Medicine, Khon Kaen University.

3.4.3 Tissue Analysis

Mollusks samples were identified to species level according to procedures introduced by Brandt (1974). Three mollusks were collected from each site of natural and polluted pond (Figure 2). Mollusk was killed immediately and the soft tissue was removed from the shell (shell length 3.3 – 3.6 cm). After removal of external shell, gross anatomy of mollusk soft tissue was studied and sketch as appeared (Figure 3). Three pieces of kidney were generally dissected from each mollusk. Small pieces of the kidney from each mollusks were fixed for 24 hour in 2% glutaraldehyde and 2% paraformaldehyde (Karnovsky's fixative). This was followed by washing in 0.1 M phosphate buffer and post fixation for 1 hour in 1% osmium tetraoxide in 0.1 M phosphate buffer. The tissues were then dehydrated through series of ethanol and embedded in pure plastic (Watt, 1997). For transmission electron microscope (TEM), ultrathin gold sections (90 – 100 nm.) were cut with a glass knife by Reichert Ultracut S type 654901, placed on copper grids, stained with urinyl acetate and lead citrate and examined using a HITACHI H-600 Electron Microscope. For scanning electron microscope (SEM), the tissues were dehydrated 15 min in amylacetate and using critical point dryer (CPD) machine. The dried tissues were fixed in stub and examined using a LEO 1450VP SEM.

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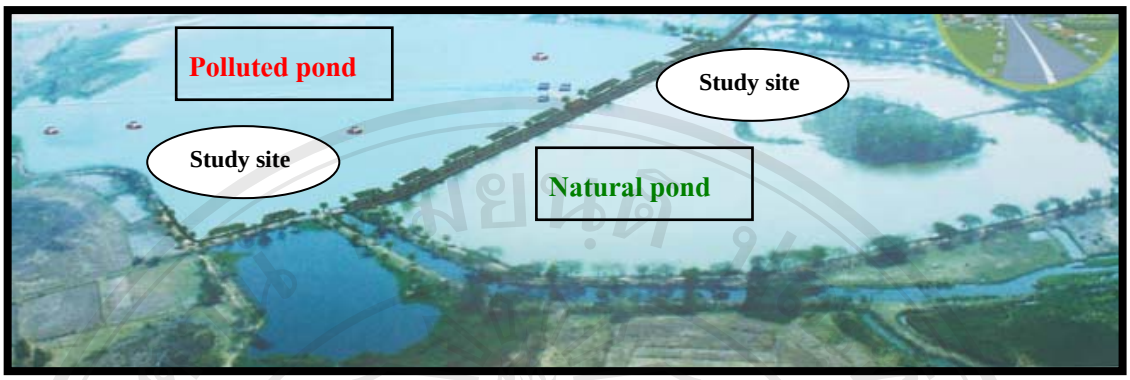

Figure 3. Overview of Bueng Jode wetland (natural and polluted pond) and study site (With complement of Phoenix pulp and paper Co., Ltd.)

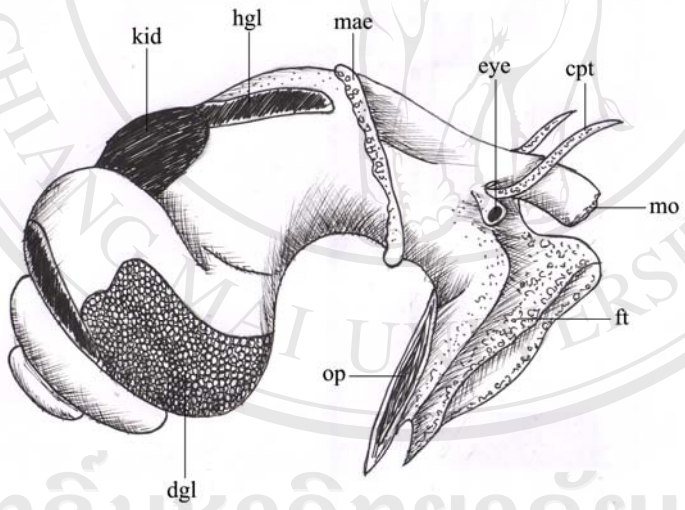

Figure 4. Gross anatomy of mollusk

Key to abbreviations : hgl = hypobranchial gland, kid = kidney, mae = mantle edge, cpt = cephalic tentacle, mo= mouth, ft = foot, dgl = digestive gland, op = operculum

(Modified from Environment Australia (1998a))

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3.5 Accumulation of Heavy Metal

3.5.1 Sampling and acclimatization of mollusk

Individuals of *Filopaludina martensi* similar size 3 - 4 cm were collected from unpolluted area. After cleaning the shell surfaces, all mollusks were transported to the laboratory. During a three-day acclimatization in the filtered deionized water mollusks were fed by fish food. The aquariums were aerated with air stones attached to an air compressor with daily refreshment of deionized water.

Virtually every type of feeding habit is exhibited by gastropods. There are herbivore, carnivores, scavengers, deposit feeders, suspension feeders and parasites. Despite great difference in feeding habits, it is possible to make a few generalizations (Barnes, 1986).

1) A radula is usually employed in feeding.
2) Digestion is always at least partly extracellular.
3) With few exceptions, the enzymes for extracellular digestion are produced by the salivary glands, esophageal pouches, the digestive diverticular or a combination of these structures.
4) The stomach is the site of extracellular digestion an the digestive diverticular are the sites of absorption and of intracellular digestion, if such digestion takes place.
5) As a result of torsion, the stomach has been rotated 180 degrees, so the esophagus enters the stomach posteriorly and the intestine leaves anteriorly.

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3.5.2 The heavy metal accumulation

The concept and experiment plan of laboratory is shown in Figures 4-5. After acclimatization, the mollusks were exposed to sublethal concentrations of lead (0.5 mg/L, 1.0 mg/L, 1.5 mg/L) and zinc (2.5 mg/L, 5.0 mg/L, 10 mg/L) for 30 days. The experiments were conducted on plastic aquaria size 30 x 30 x 25 cm, each containing

50 mollusks. At the end of each exposure period, 10 mollusks were randomly taken from each experimental group for metal content analysis. The initial of taking was carried out at day 0 for the analysis of back ground metals. Three replicates of each concentration were carried on at 3 exposed duration 10, 20 and 30 days. During the accumulation period the mollusk mortality rate was observed. Prior to the initiation of metal exposure, ten mollusks were randomly taken from each experimental group for analysis of background metals. The samples were analyzed for lead and zinc by ICP technique. In addition, control group were analyzed at days 0, 3 and 15**.** Soft tissues of *F. martensi* were disected in order to conduct enzyme analysis. The group of esterases (EST) enzyme and control group were analyzed at days 10, 20 and 30.

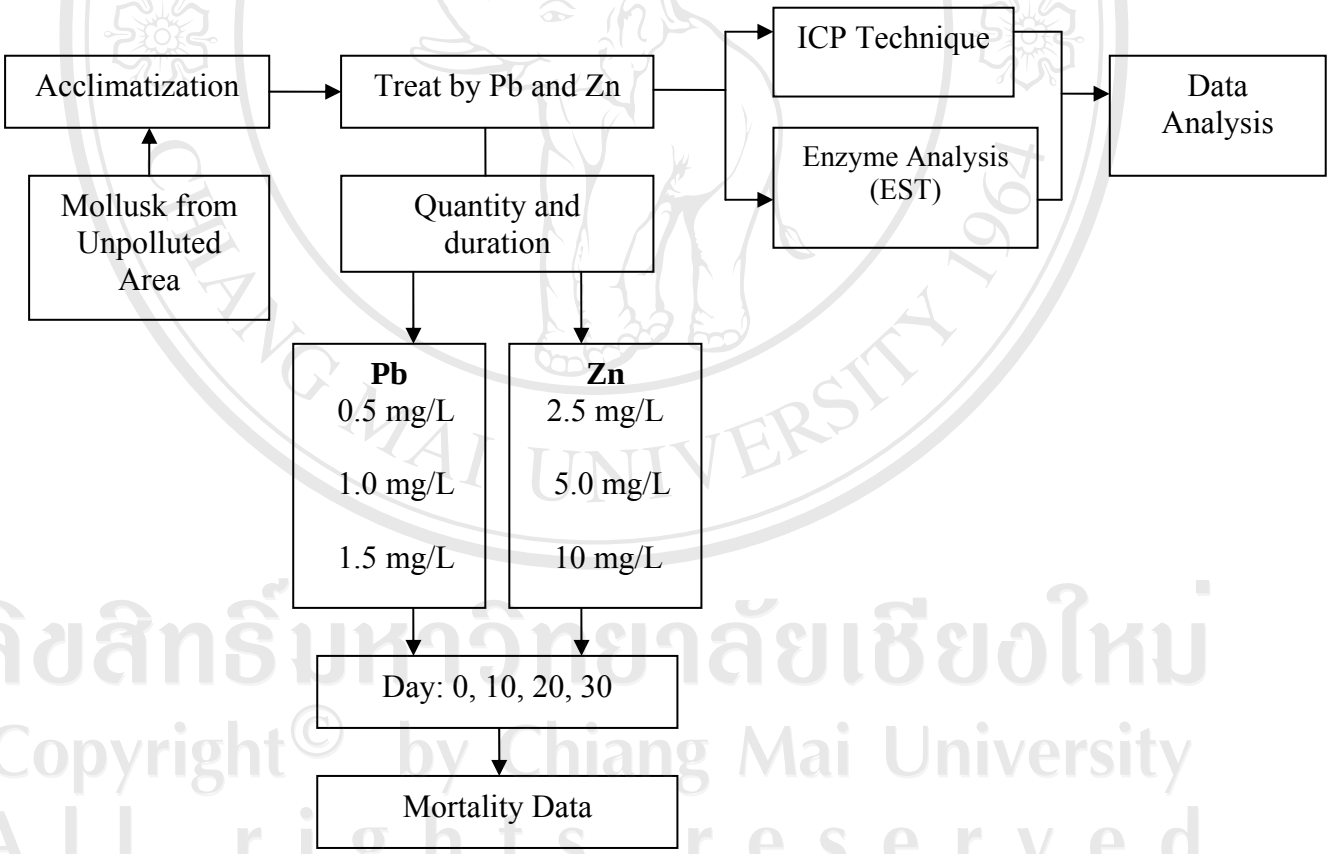

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Figure 5. Laboratory flow chart concept of heavy metals accumulation studies

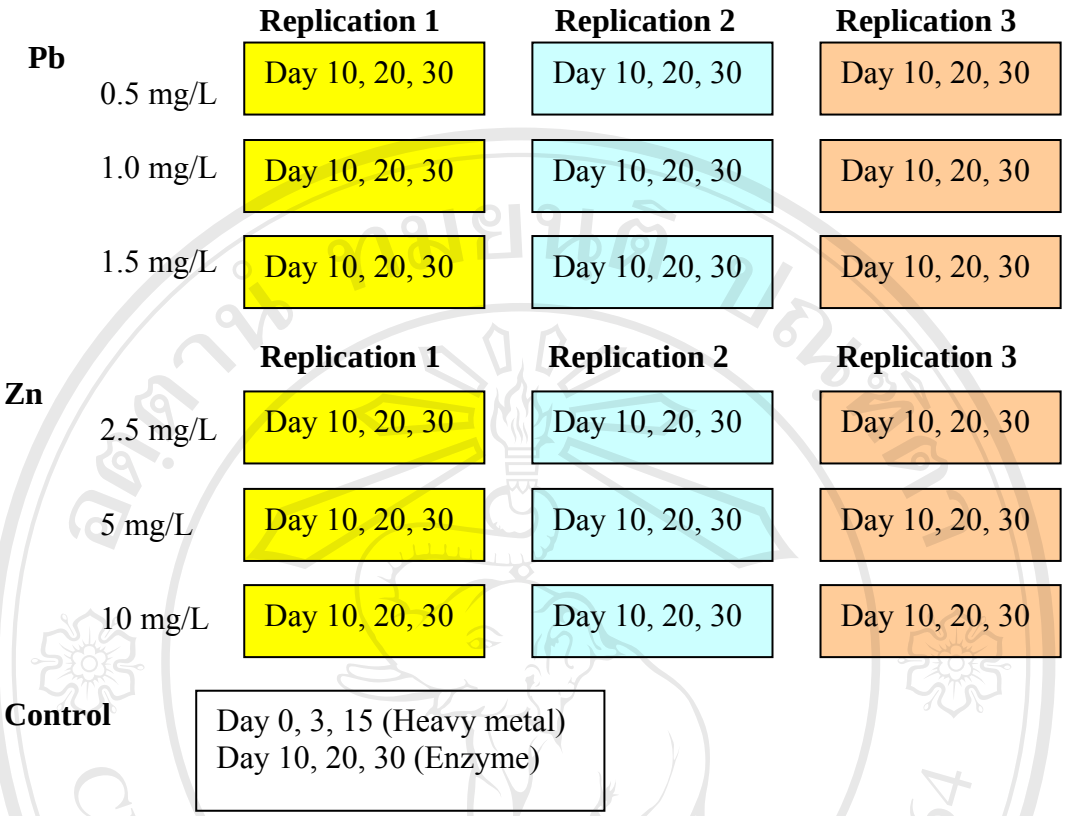

Figure 6. Experiment plan of heavy metals accumulated in mollusks

3.5.3 Analysis of esterases enzyme

1) Determination of esterase from tissue sample

Determination of protein concentrations in tissue homogenate samples were conducted according to Bradford Protein Assay using Quick Start™ Bradford Protein Assay which will be described below (Hercules, 2000; Bradford, 1976).

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Standard Protein Assay

Reagents:

(1) 1x Dye Reagent: 300 ml of 1x dye solution containing 350 mg of Serva Blue G, 100 ml 95% ethanol and 200 ml of 88% phosphoric acid.

(2) Bovine Serum Albumin (BSA) Standard, 2 mg/ml

Protocol: (Microplate Standard Assay)

(1) The standard protein assay protocol (standard BSA stock 2 mg/ml)

(2) Remove the 1x dye reagent from 4°C storage and allow to warm up to ambient temperature. Invert the 1x dye reagent a few times before use.

(3) Pipette 5 µl each standard and unknown sample solutions into 250 µl microplate wells, mix the samples using a microplate mixer. Alternatively, use a multi-channel pipette to dispense the 1x dye reagent. Depress the plunger repeatedly to mix the sample and reagent in the wells. Replace with clean tips and add reagent to the next set of wells.

(4) Incubate at room temperature for at least 5 min. Samples should not be incubated longer than 1 hour at room temperature.

(5) Set the spectrophotometer to 595 nm. Measure the absorbance of the standards and unknown samples.

(6) Create a standard curve by plotting the absorbance (595 nm) values (y-axis) versus standard BSA concentrations (and of unknown protein) in µg/ml (x-axis). Determine the unknown sample concentrations using values from the standard curve. If the samples are diluted, adjust the final concentration of the unknown samples by multiplying by the dilution factor.

(7) Example of Standard curve for the standard microplate assay procedure are listed in Appendix B.

(8) Calculate the protein concentration from protein samples (µg/ml) using a straight linear equation from standard graph as $y = 0.0003x+0.0628$, where y is absorbance value at 595 nm, which is the difference between the average absorbance of sample and the average absorbance of bank, and x is protein concentration (µg/ml). (Standard graph of absorbance with various standard concentration of BSA was shown in Appendix B).

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2) Protocol for the determination of esterase in tissue homogenates

The esterase determination method according to Sahgal *et al*., 1994 was as follow.

(1) Tissue homogenate samples were prepared in ice-cold 0.1 M Tris–HCl buffer (pH 7.8 at 25°C containing 1% Triton X-100)

(2) Homogenate samples were subsequently centrifuged in a refrigerated centrifuge (12000 rpm) for 20 min at 4°C and the supernatant was used for protein concentration assay using Quick Start™ Bradford Protein Assay.

(3) Appropriate amounts of tissue samples were added to 0.1 M Tris–HCl buffer (pH 7.8 at 25°C, containing 2 mM EDTA) and the final volume was adjusted to 990 µl.

(4) Samples were preincubated at 37°C for 10 min and the reaction was started by adding 10 µl of 50 mM stock *p*-nitrophenyl acetate solution in acetone (final concentration = 0.5 mM).

(5) Change in absorbance at 405 nm was recorded after 5 min against a reagent blank containing only substrate.

3.6 Data Analysis

3.6.1 Statistical analysis (one-way ANOVA) in sediment and mollusk samples

Statistical analyses were performed using SPSS program version 17. Mean and standard deviation (SD) values were shown. The t-test and one-way analysis of variance (ANOVA) were used to compare heavy metals in the sediment and mollusk data where the significance level was at *p* less than 0.05.

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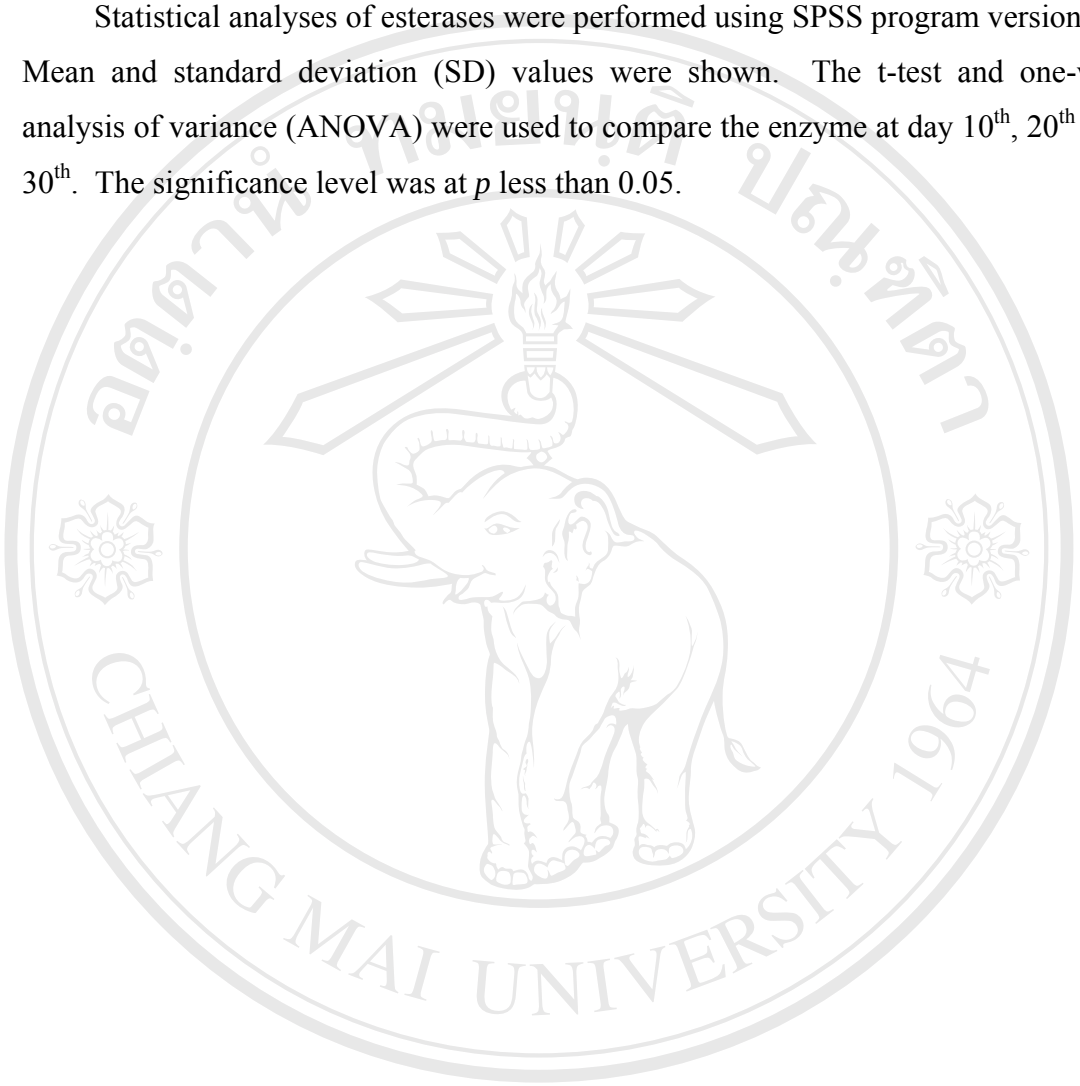
3.6.2 Statistical analysis esterases

Statistical analyses of esterases were performed using SPSS program version 17. Mean and standard deviation (SD) values were shown. The t-test and one-way analysis of variance (ANOVA) were used to compare the enzyme at day 10^{th}, 20^{th} and 30^{th}. The significance level was at *p* less than 0.05.



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