# CHAPTER 3

# METHODOLOGY

## 3.1 Location of research operation and data collection

The area where pesticides had been reportedly used and selected for the study sites was Ban Mae Sa Mai, Mae Rim district, Chiang Mai province, Thailand (Fig 3.1). This research focused on the impact of pesticides used in aquatic ecosystem so the Mae Sa Noi stream, main stream flowing through Ban Mae Sa Mai, was monitored. A stream on Doi Suthep-Pui in Doi Suthep-Pui national park was selected as a control stream. The control stream locates on the same mountain range with Mae Sa Noi stream (Fig 3.1). Both streams, at the same elevation have almost identical substrate and other geographical features.

### The study stream

The stream where passes through dense agricultural area. It was Mae Sa Noi stream (Latitude 18° 52′ N, Longitude 98° 51′ E). Three study sites in this stream were at 1050, 900 and 700 m above mean sea level (mAMSL) were seasonal monitored (Fig 3.1).

### The control stream

It was a small stream passing through Ban Doi Suthep (Latitude 18° 17′ N, Longitude 98° 55′ E) where there was not agricultural practice area. Three study sites at 1050, 900 and 700 m above mean sea level (mAMSL) were also seasonal monitored as was done in study sites (Fig 3.1).

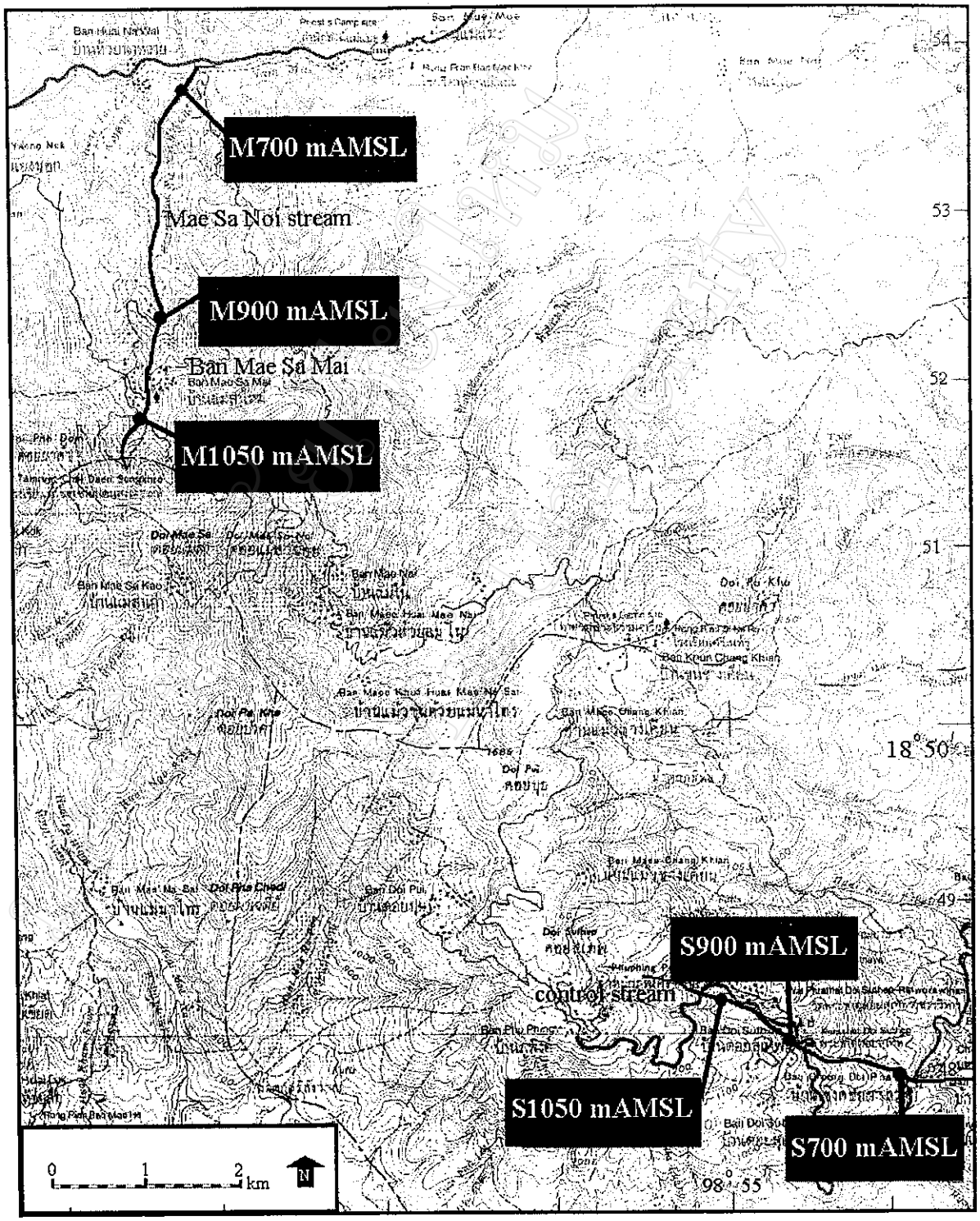


**Fig 3.1** The map of Mae Sa Noi stream and its control stream on Doi Suthep-Pui mountain, Chiang Mai, Thailand (Source: Royal Thai Survey Department)

## 3.2 Materials and Methods

The present study composed of 2 main works on monitoring the impact of pesticides. One was ecological work, which applied biological method in measuring of physico-chemical parameters was conducted and biological method was applied to monitor biological components. The another one was toxicological test in chironomids that sampled from both streams and assayed for cholinesterase (ChE) activity using Ellman method. The study anticipated the significantly different of ChE activity where the organisms in Mae Sa Noi had lower activity than the control. In case of non-significant between both streams *in vitro* inhibition study would be performed. These works are listed as follow.

3.2.1 Ecological works

3.2.1.1 Physico-chemical parameters of streams

3.2.1.2 Biological components

3.2.2 Toxicological tests

3.2.2.1 Measurement of ChE activity

3.2.2.2 *In vitro* Chironomids ChE activity inhibition test

The present work was conducted from April 1999 to February 2000.

### 3.2.1 Ecological works

Ecological work was composed of 2 major areas if the study; physico-chemical parameters and biological components. The primary target of this work was to monitor seasonally physico-chemical parameters in both streams

at the same season and elevation to assure both streams were comparable. Biological components for studying their changing might be correspond to physico-chemical parameters or other factors.

Since, the objective of the research proposed to find out the impact of pesticides used on living-organisms in Mae Sa Noi. Then a control stream, where there was no or less pesticides reportedly used, of similar physico-chemical parameters at the same season and elevation was selected. The physico-chemical parameters were measured and benthic macroinvertebrate of the study sites were sampled seasonally. Comparison of means with Mann-Whitney U test and cluster analysis (SPSS) were applied to reveal the factors affecting communities. Community characteristics macroinvertebrate samples were compared.

**3.2.1.1 Physico-chemical parameters**

Physico-chemical parameters that were seasonal monitored in each study site were stream velocity, temperature, conductivity, Total Dissolved Solid (TDS) and pH. Stream velocities were measured by using stopwatch and 50-ml plastic bottle tied with 2-meter string. This plastic bottle was released and flowed in stream for 2 meters while using stopwatch for timing that bottle spent. The distance; 2 meters, and the spent time were calculated for stream velocity in meters per second (m/s). Stream velocities were measured for 3 times each site. Temperature (°C), conductivities (µs/cm) and TDS (mg/l) were measured using conductivity meter (Ciba corning) for 3 times each site. pH of water was measured in water samples from each site which was

collected with 250-ml plastic bottle. Water samples were measured pH using pH meter (Beckman, BE SS-2) which was standardized with buffer solution (Beckman) in the laboratory and measured in 3 replications per site.

Means of physico-chemical parameters were compared between elevations, seasons and streams using Mann-Whitney U test (SPSS). Cluster analysis (SPSS) was applied for grouping physico-chemical parameters.

**3.2.1.2 Biological components**

Benthic animal; or benthos are organisms attached or resting on the bottom or living in the bottom sediments (Odum 1971), were collected and studied their communities. Although there were some problems in sampling such as non-random distribution following substrate and immigration (Wetzel, 1983), but in this research 10 replicates were taking in order to relief these problems (Fig A1). Surber sample was applied for sampling benthic animals as a quantitative study tool (Hynes, 1972). It is the twin quadrat compose crossing with another, one held nylon net cross stream flow and another one placed horizontal on the bed of stream (Brown, 1971). It is one of the most widely used technique to study on pesticide impact (Muirhead-Thomson, 1987).

Surber sampler was dropped into stream. Stirred substrate and scrubbed stone with hand in surber sampler's sampling plot entire its area (25 X 25 cm) to disturb benthic living-things moving out from their habitats and flow into the net of surber sampler. Collected objects in the surber net are put into plastic bag containing 10-15 ml 40% formaline, and adding water around

40-50 ml to dilute formaline down to 4%. Ten samples were collected from each site. These samples were cleaned and pour into petri-dish. They are studied under stereomicroscope (Olympus SZ30) to find macroinvertebrates and then identified and enumerated. Raw data of types and numbers of macroinvertebrates were calculated to community characteristics, i.e., richness index (Equation 3.1), Shannon's diversity index (Equation 3.2) and evenness (Equation 3.3), mean total population density and chironomid population density. Indices in details showed as following. Mann-Whitney U test was used to compare mean of total and chironomid population density between elevations, seasons and streams. Cluster analysis was applied to find out the similarity of macroinvertebrate community in all sites.

Equation 3.1 (eq 3.1) was richness index (R) (Margalef, 1958) (Ludwig and Raynolds, 1988). This index refers to number of species in the community.

$$R = \frac{S - 1}{\ln(n)} \qquad \text{eq 3.1}$$

**Where :** R = Richness Index (Margalef, 1985)

S = total number of taxa in community

N = total number of individual in community

Equation 3.2 and 3.3 (eq 3.2 and 3.3) were diversity index (H′) (Shannon, 1949) and evenness (E) (Ludwig and Raynolds, 1988). Diversity index is the incorporation of both species richness and evenness into a single value. Evenness refers to how the species abundances (e.g. the number of individuals, biomass etc) are distributed among the species.

$$H' = -\sum_{i=1}^{S}\left[\left(\frac{n_i}{n}\right)\ln\left(\frac{n_i}{n}\right)\right] \qquad \text{eq 3.2}$$

$$E = \frac{H'}{\ln(S)} \qquad \text{eq 3.3}$$

**Where :** H′ = diversity index (Shannon, 1949)

E = Evenness

S = total number of taxa in community

n = total number of individual in community

$n_i$ = total number of each taxa

### 3.2.2 Toxicological tests

The method described by Ellman was slightly modified and applied to measure ChE activity in chironomids for confirmation the use of OPs and CAs in highland agriculture at Ban Mae Sa Mai area. ChE activities in chironomids were used as biomarker to detect OPs and CAs using in study site that it might cause the changing of benthic communities. Chironomids were sampled by searching near the bank of the streams at all sites (1050, 900, 700 mAMSL in

rainy and cold seasons). Chironomids were immediately stored in an icebox container (~ 4°C) and transported to Toxicological Laboratory of Research Institute for Health Science and keep in the freezer at -20°C before assay. It was expected that ChE activity of chironomids sampled from Mae Sa Noi should be lower than those from the control stream. *In vitro* chironomid ChE activity inhibition test was also performed in order to clarify their susceptibility to specific pesticide exposure.

**3.2.2.1 Measurement of ChE activity**

The method used in the present study was modified from Ellman (1961). It composed of 3 processes shown in Fig 3.2. It started from chironiomid sampling and storage (A). Chironomid samples were homogenized in 1-ml polypropylene Eppendorf tube with 0.5-ml buffer solution and then centrifuged to separate debris and exoskeleton out from supernatant. Supernatant was aspirated into 2 parts, the first part was used in Chironomid ChE activity measurment (B) and the remaining; the second part, was used in protein determination (C). For protein determination, it was applied to standardize the ChE activity per unit weight of protein for prevention the high activity occurred from high amount of sample. Details of each process were shown as following.

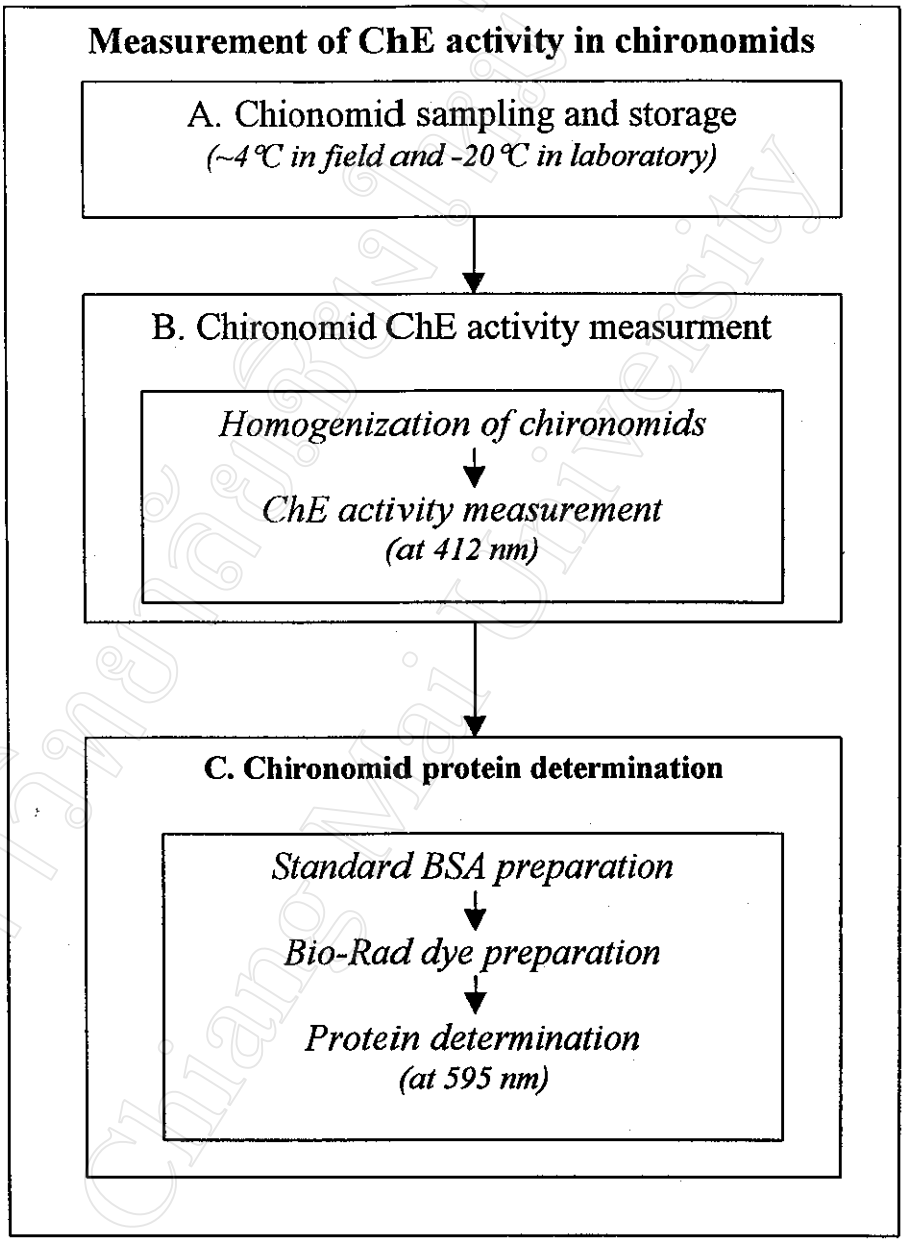


**Fig 3.2** Flow chart showed processes of chironomid ChE activity measurement (Modified from: Scaps *et al* (1997), Seto *et al* (1997))

## A. Chionomids sampling and storage

**Tools**

- Medium size pond net
- Stainless forceps
- Eppendorf Safe-lock tubes
- Ice box, foam box containing ice brick
- Glass dropper
- Freezer

**Methods**

At study site in area where have small particle of silt substrate with litter of leaf was found easy to find chironomids. They were found in the sediment near bank of stream. Pond net swept the sediment and find chironomids in it. Picking up ten chironomids in field and placed them in Eppendorf Safe-lock tubes. The samples were kept in the icebox with ice brick (~4°C). After that chironomid samples were transported to the lab and stored in the freezer (- 20 °C). Before freezing chironomid samples the water in the tube should be taken out using dropper.

## B. Chironomid ChE activity measurment

**Apparatus**

- Pyrex test tube (13 X 100 mm)
- pH meter; Beckman expandometic SS-2 and buffer solution
- Thermometer

- Shaking incubator; GCA/Precision Scientific, USA
- Autometic pipette and tip; Biohit proline (200 µl), Gilson pipetman (20 µl)
- Homoginizer; KREBS electric MFC, Co, INC, New York
- Centifuge; Biofuge A, Heraaeus sepatech, Germany
- Vortex shaker; Vortex-genie, Scienctific Industries Inc, USA
- Spectrophotometer; Cecil, CE292, England
- Refrigerator; Toshiba (GR-A2301)

**Chemical reagents**

- Homogenizing reagent (HGR); tris-HCL 0.1 M (MW = 157.64) pH 7.4 (HCL, NaOH adjust); GIBCO BRL ultrapure, USA
- Buffer reagent; tris-HCl 0.01 M (MW = 157.64) pH 7.4, diluted from HGR; GIBCO BRL, USA
- pH adjusting reagent; concentrated HCl and NaOH
- DTNB (5,5' dithiobis-(2-nitrobenzoic acid)); $[(NO_2C_6H_6SCOOH)_2]$ DTNB 0.5 mM in buffer (MW = 396.5), Sigma, USA
- Acetylthiocholine iodide (substrate); ($C_7H_{16}NOSI$) AChI 156 mM (MW = 289.2), Sigma, USA
- Eserine; Eserine 12 mM (MW = 273.35); $[(C_{15}H_{21}N_3O_2)_2H_2SO_4]$Sigma, USA. (The solution should be kept in dark container to prevent light degradation)

All solutions were kept in the refrigerator (< 8°C) and should be used in 1 month.

**Assay method**

*Homogenization of chironomids*

The method used was modified from Scaps et al (1997). Frozen samples were thawn in tab water. The samples were added homogenizing reagent for 100 µl and homogenated with motor-homogenizer with speed of 75% for 2-3 second, continuously done until Eppendorf tube had 0.5 ml of homogenizing reagent. After that, samples in Eppendorf tubes were centrifuged at 10,000 rpm for 10 minutes. Next, samples of chironomids from the same site were pooled into one test tube, mixed them with vortex shaker (Fig 3.3). Most of them were used for ChE measurement and the remains were used in chironomid protein determination.

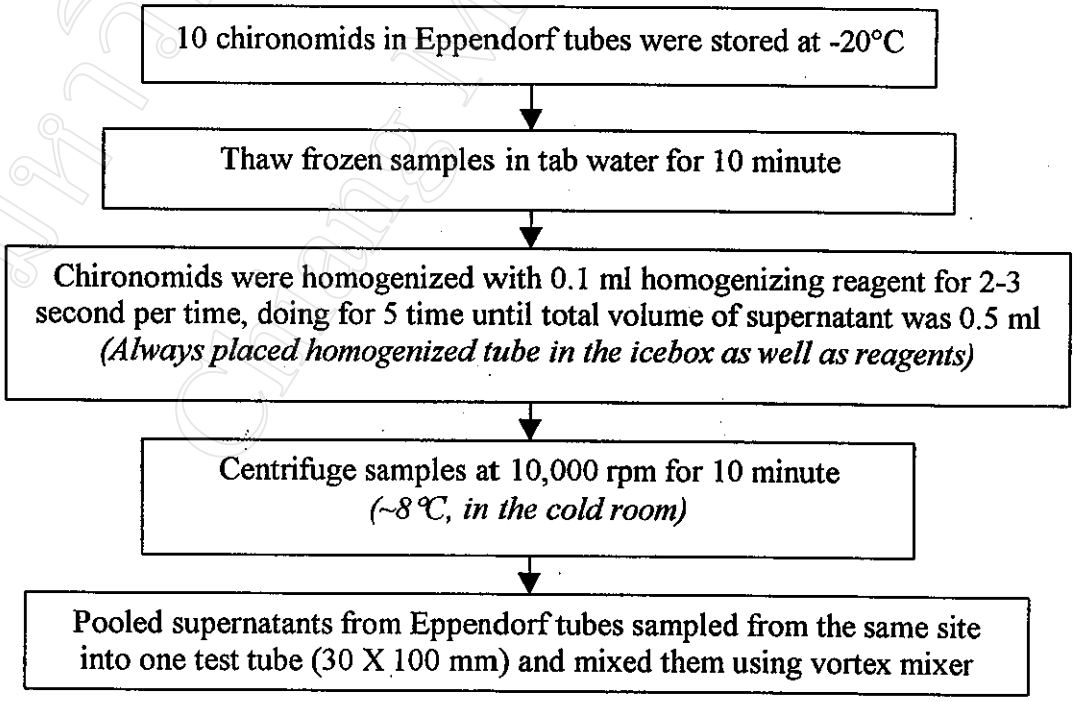


**Fig 3.3** Flow chart showed processing of chironomid homogenization (Modified from: Scaps *et al* (1997))

*ChE activity measurement*

The present work adapted the method described by Seto *et al* (1997) with slightly modified. The incubator with temperature at 20°C was warmed for 15 minutes. Test tubes (13 X 100 mm) were prepared in rack, in one treatment they were divided to 2 groups; blank (1 tube) and test (induplicate). Reagents; DTNB, AChI, supernatant of chironomids and eserine, were placed in icebox for keeping cool. The spectrophotometer wavelength at 412 nm was warmed and calibrated. All reagents were added according to following steps showed in flow chart in fig 3.4.

| Reagent/Solution | Blank (1 tube) | Test (2 tubes) |
|---|---|---|
| Add DTNB | 3.0 ml | 3.0 ml |
| Add chironomid supernatant | 0.1 ml | 0.1 ml |
| Add eserine | 0.05 ml | - |
| Mixed and incubated at 20°C, for 10 minute | | |
| Add substrate AChI | - | 0.01 ml |
| Mixed and incubated at 20°C, for 15 minute | | |
| Add eserine | - | 0.05 ml |
| Add substrate AChI | 0.01 ml | - |

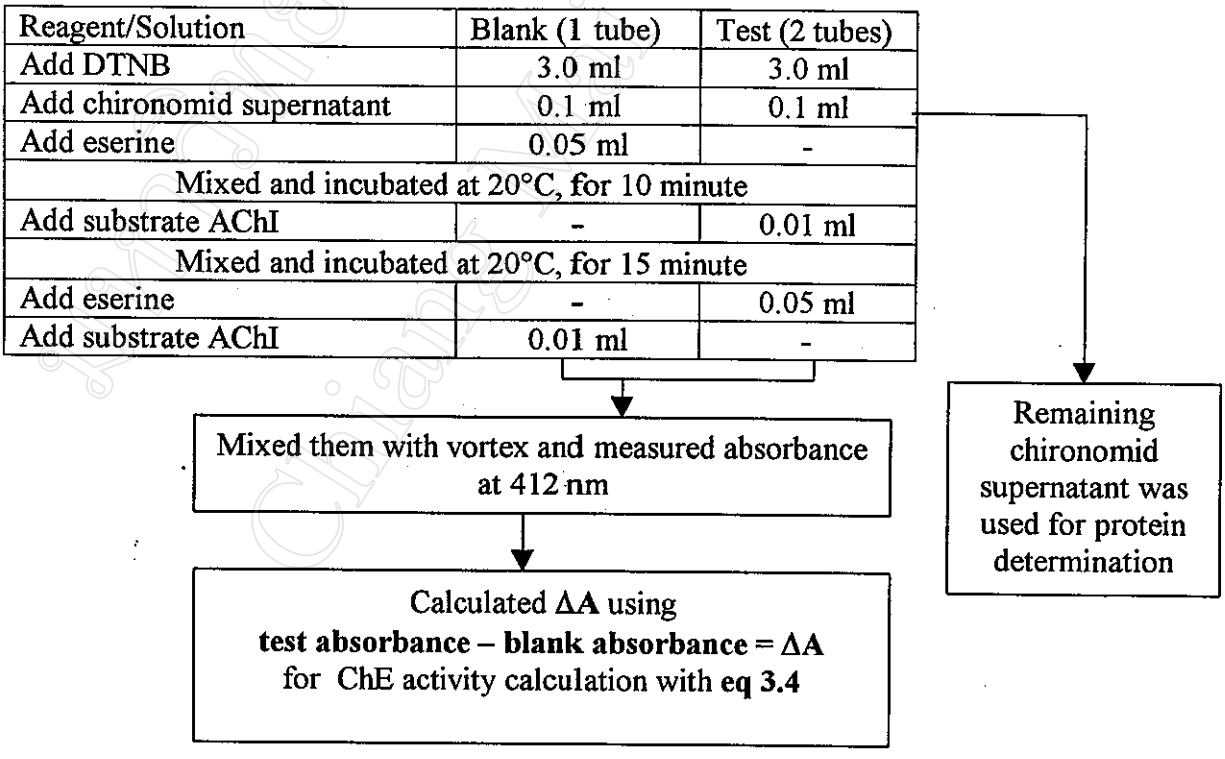


**Fig 3.4** Flow chart showed processes of chironomid ChE activity measurement (Modified from: Seto *et al* (1997))

## C. Chironomid protein determination

**Apparatuses**

- Tubes, microcentrifuge, Eppendorf Safe-lock tubes
- Automatic pipette and tip; Biohit proline, pipetman Gilson
- ELISA reader; CERES UV900 HDi, Bio-tek Instrument, INC.
- Microwell plates (96 wells, flatted bottom), MaxiSorp Surface, Nunc-Immuno plate
- Microwell plates shaker, Cooke laboratory Products, England

**Reagents**

- Bovine serum albumin (BSA) standard, Bio-Rad
- Protein dye, Bio-Rad
- Whatman filter paper No. 5

**Assay method**

*Standard BSA preparation*

BSA 5 mg was dissolved in 10 ml of distilled water to get 0.5 mg/ml concentration of protein standard. After that, it was diluted to 0.1, 0.2, 0.3 and 0.4 mg/ml BSA standard with distilled water and kept in the freezer (-20°C).

*Bio-Rad dye preparation*

Bio-Rad dye was mixed into water for 1:4 and filtrated it passing Whatman filter paper No. 5. This solution has brown color and was freshly used.

*Protein determination*

In each well of 96 microwell plate, add 300 µl of diluted Bio-Rad dye plus 10 µl of BSA standard protein or supernatant of chironomid and then incubated for 5 minutes. Before reading the absorbance with ELISA reader, the microwell plate was shaken with microwell plates shaker for 30 second. The dye was changed from brown to brilliant blue color more strong follow concentration of protein. Protein was determined with absorbance using ELISA reader at 595 nm. Using absorbance and concentration of BSA standard to draw standard curve, absorbance is Y-axis and X-axis is concentrations of protein. Protein concentrations of chironomid supernatant ($C_p$) were approximated from this standard curve. They were used in calculation of ChE activity (shown on eq 3.4).

$\Delta A$ and $C_p$ were used to calculate ChE activity with eq 3.4 that was modified from Ellman (1961).

$$R = \frac{\Delta A}{13600} \times \frac{3.16}{0.1} \times \frac{1}{C_p} \times \frac{1}{15} \qquad \text{eq 3.4}$$

Where : R = ChE activity (µmol/min/mg protein)

$\Delta A$ = Change in absorbance in 15 minute

$C_p$ = Chironimid protein concentration (mg/ml)

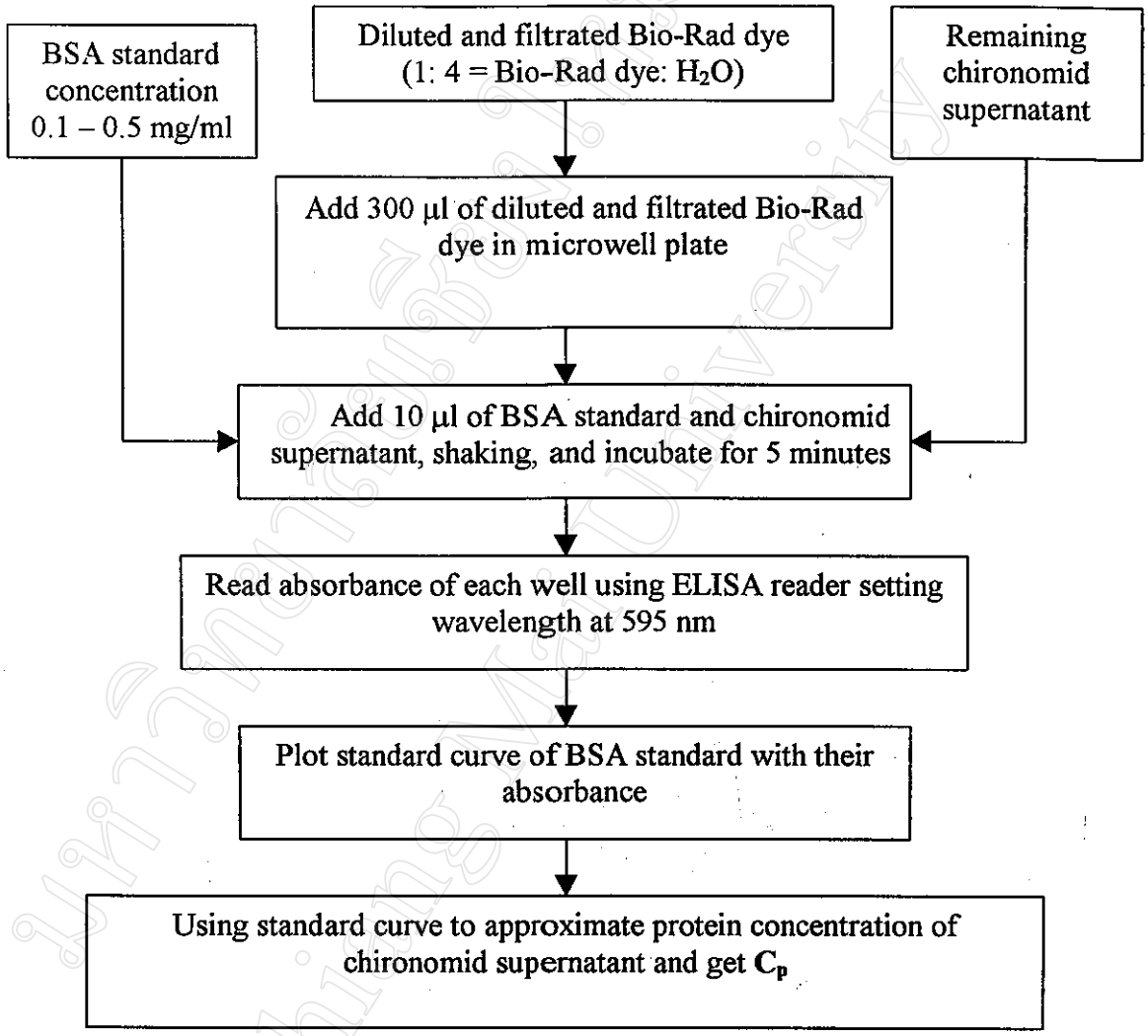


**Fig 3.5** Flow chart showed processes of chironomid protein determination

### 3.2.2.2 *In vitro* Chironomid ChE activity inhibition test

In this study, it was planed that the in vitro inhibition test would be applied if the result of chironomid ChE activity was non-significantly different between site to find out their susceptibility to a selected pesticide. The present study used methyl-parathion received from Dr. Ehrenstorfer, Augsburg, Germany, prepared concentration with dilution using methyl-alcohol 95% as solvent that shown in table 3.1 for adding into chironomid supernatant and measuring ChE activity. This test hypothesized that chironomids sampled from Mae Sa Noi stream accumulated some pesticides in their bodies, but not high until causing death comparing with chironomid sampled from the control stream where not reportedly sprayed of pesticides. Then after extra pesticides were added onto test tube % ChE inhibition of sampled collected from Mae Sa Noi might be higher than those sampled from the control stream. On the other hand, it mean chironomid from Mae Sa Noi would have higher susceptibility than the control stream.

*In vitro* Chironomid ChE activity inhibition test employed same method of chironomid ChE activity measurement. After incubation of chironomid supernatant for 10 minute in DTNB at 20°C. The pesticides (methyl-parathion) concentration was shown in table 3.1 were added for 0.1 ml onto all test tubes and continuously incubate for 15 min before adding substrate, the method was showed as following.

**Method**

After chironomid samples were homogenized s and incubated in DTNB phosphate at 20°C in incubator for 10 minutes, pesticides solutions (methyl-parathion) prepared concentration that shown on table 3.1; left column, were added for 100 µml

into these tubes. The concentrations of methyl-parathion were diluted which new concentrations are shown in table 3.1 right column. After that left pesticide incubated in test tubes for 15 minute. After that, test tubes were added substrate and waiting for 15 minute reaction and stopping reaction with eserine solution. Detect ChE activity with spectrometry method at 412 nm and calculation their activity with eq 3.5 and the method showed in Fig 3.6. Results were studied the tendency of activity to concentration of pesticides. The result of ChE activities were converted to %inhibition and find out the concentration of adding pesticide that cause % inhibition for 25%, 50% and 75%. The results were analyzed using Mann-Whitney U test and cluster analysis.

**Table 3.1** Concentrations of methyl-parathion for chironomid ChE inhibition test. In left column were concentrations of methyl-parathion, which they are prepared. After they were added onto test tubes for inhibition testing, they were diluted to concentrations that showed in the right column

| Concentration of methyl-parathion (μM) | |
|---|---|
| Preparing concentration | Testing concentration |
| 0 | 0 |
| 625 | 19 |
| 1250 | 38 |
| 2500 | 77 |
| 5000 | 153 |
| 7500 | 230 |
| 10000 | 307 |

| Reagent/Solution | Blank (1 tube) | Test (2 tubes) |
|---|---|---|
| Add DTNB for | 3.0 ml | 3.0 ml |
| Add chironomids supernatant | 0.1 ml | 0.1 ml |
| Add eserine | 0.05 ml | - |
| Mixed and incubated at 20°C, for 10 minute | | |
| Add methyl-parathion | 0.1 ml | 0.1 ml |
| Mixed and incubated at 20°C, for 15 minute | | |
| Add substrate AChI | - | 0.01 ml |
| Mixed and incubated at 20°C, for 15 minute | | |
| Add eserine | - | 0.05 ml |
| Add substrate AChI | 0.01 ml | - |

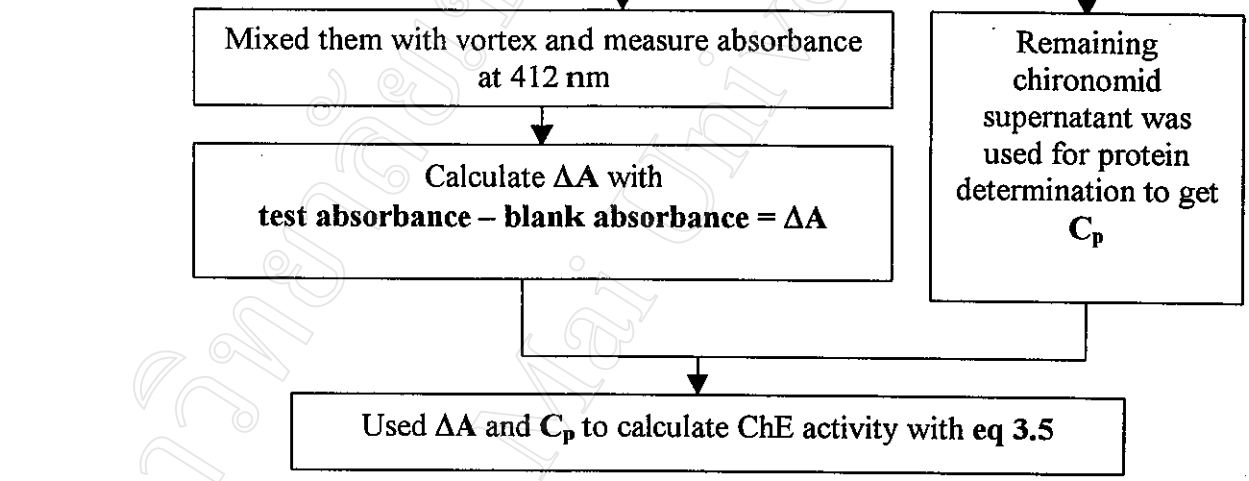


**Fig 3.6** Flow chart showed processing of *in vitro* Chironomid ChE activity inhibition test

Equation 3.5 (eq 3.5) slightly modified from eq 3.4 using for calculation of Chironomid ChE activity *in vitro* inhibition test.

$$R = \frac{\Delta A}{13600} \times \frac{3.26}{0.1} \times \frac{1}{C_p} \times \frac{1}{15} \qquad \text{eq 3.5}$$

Where : R = ChE activity (μmol/min/mg protein)

ΔA = Change in absorbance in 15 minute

$C_p$ = Chironimid protein concentration (mg/ml)

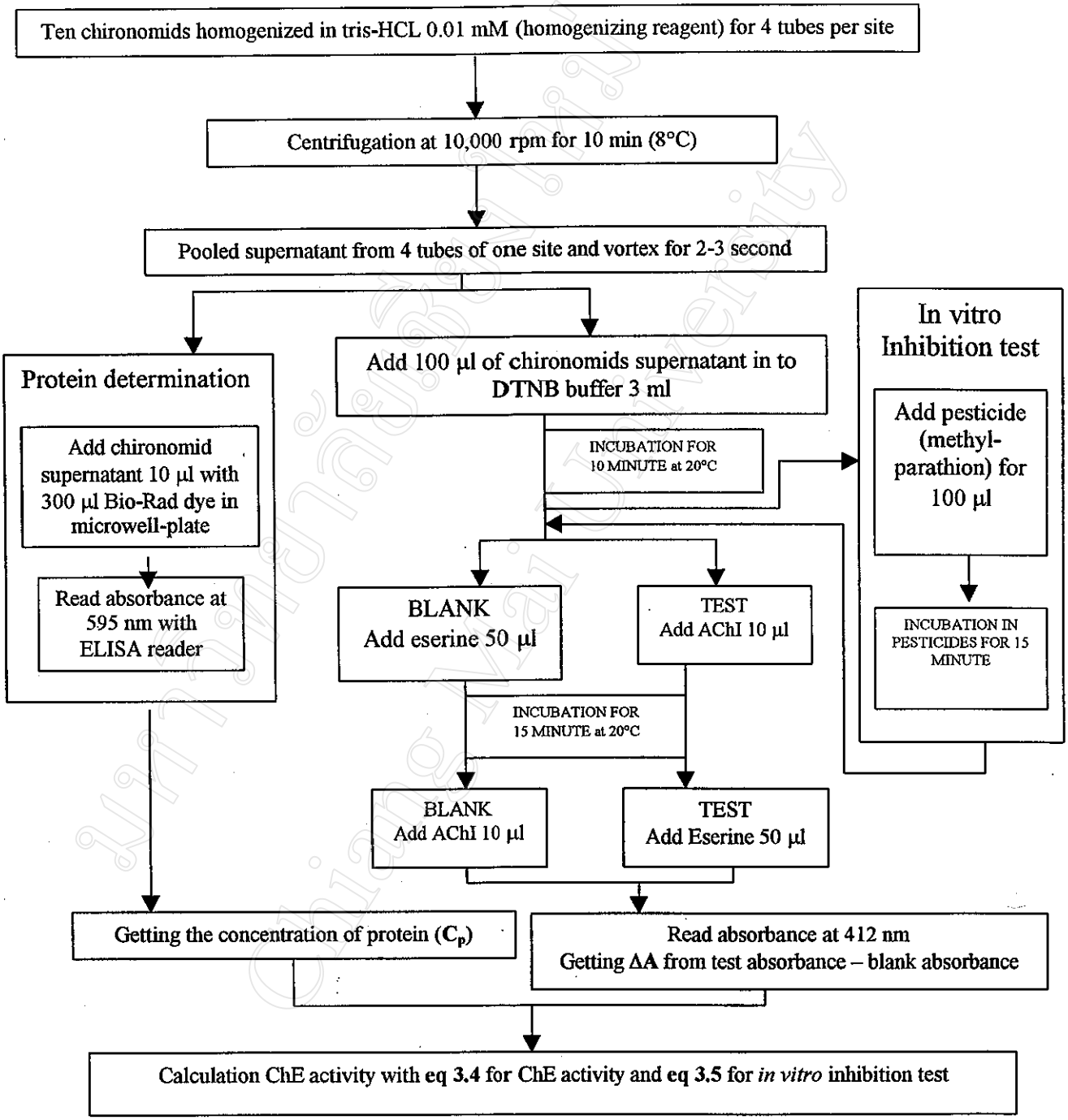


**Fig 3.7** Flow chart showed all processes of chironomid ChE activity measurement and *in vitro* inhibition test