CHAPTER 3

METHODOLOGY

3.1 Ecological studies

3.1.1 Sample collection

Three sampling stations were selected in the study area. Two methods of quantitative sampling of ephemeropteran nymphs were used in this study. First was by wading through the rivers (approximately half a meter deep) and placing aquatic nets (Figure 3.1) in between large rocks (Figure 3.2) and boulders (where leaf litters tend to collect) in the stream for about two hours. After that period of time, the nets were collected and the contents were placed on a white enamel tray (Figure 3.3). Debris was then removed and specimens of mayflies that were found will be placed into vials containing 70% alcohol. Some species of nymphs that live under rocks buried in soft bottom (Heptageniidae) were sampled using visual-hand picking method by examining stones that were temporarily removed from its location. The stones and boulders were carried on to the steadily placed trays and rinsed gently with water from the stream. The nymphs clinging to the rocks will then fall into the white trays. The nymphs were then collected from the white trays and preserved in the field in vials containing 70% alcohol. Freshly collected nymphs were also placed into micro test tube (without alcohol), alive and transported to the laboratory in a

cold box. Within 24 hours the individuals were brought back to the laboratory, they were preserved at -20°C until analysis (for biochemical assay purposes). At each site five replicates (later pooled) were taken. Repeated sampling of ephemeropteran nymphs was carried out once every fortnight, only during the daytime (8.00 am to 4.00 pm) from August 2006 to July 2007 in all three river streams.

Figure 3.1. Aquatic net used for collecting ephemeropteran nymphs

Figure 3.2. Leaf litters trapped between rocks were collected using aquatic nets and sorted out on white enamel trays

Figure 3.3. Ephemeropteran nymphs were collected using the white enamel tray

3.1.2 Physical- Chemical Parameter and Water Quality Bioassessment

In-situ water quality data and water samples were collected once every fortnight (during sample collection) at every randomly selected location where the mayfly nymphs were collected from all three river streams to investigate the characteristics of the microhabitats that possibly influenced the distribution of the ephemeropteran nymphs in the forest stream. *In-situ* non-conservative and non-preservable environmental parameters such as illuminance, water temperature (°C), pH, dissolved oxygen (mg/L O_2), and conductivity (µS) were determined onsite using portable digital meters, LX-101, YSI-550A and YSI-EC 300. Water samplings were carried out by wading at mid-depths in the middle of the streams and were analysed within 8 hours at field laboratory. The Spectroquant NOVA 60 was used in the quantitative analyses of nutrients (nitrate, ammonical nitrogen, and phosphate). During these field studies, water-quality and ecological data were examined in an upstream-downstream order to identify spatial differences in water quality. Data from urban stream sites were compared to data from less-urbanized reference sites to evaluate the effects of urbanization on water quality. Data used to characterize water quality were collected every fortnight during the period from August 2006 to July 2007.

3.1.3 Identification

Mayfly nymphs obtained during sample collection were brought back to the laboratory for identification. The specimens were examined using dissecting microscope and are identified and classified with the aid of identification manuals from Khoo (2004) and Edmunds (1982). The number of specimen collected was also recorded.

3.1.4 Diversity Data Analysis

A variety of diversity indices were calculated to compare the ecological communities of ephemeropteran nymphs in three different streams in Ulu Gombak Forest Reserve. Species richness was calculated as the total number of species and abundance was calculated as the total number of individuals. Other diversity indices include Shannon's diversity index (H'), Shannon's Equitability (E_H), Simpson's Index of Dominance (D), Simpson's Index of Diversity (1-D), Simpson's Reciprocal Index (1/D), Simpson's Index of Evenness (E_S) and Margalef's richness index (d). The three biodiversity indices (diversity indices, evenness index and richness indices) gave better information about the environmental conditions under which the organisms lived (Gaufin, 1973; Hawkes, 1979; Teles, 1994) than a consideration of the individual taxa alone. An evaluation was made of the linear relationship between physical-chemical parameters and number of ephemeropteran nymphs using Pearson's Correlation to determine which environmental factors may be influencing nymphs' distribution in the selected sampling stations. Student t-tests were also performed, with significance reported at $p \leq 0.05$.

3.2 Biochemical Assay

Two genera of ephemeropteran nymphs were chosen for the biochemical assay. The *Baetis* sp. Nymphs used in this procedure were between 5mm to 7 mm while the *Campsoneuria* sp. Nymphs were between 8mm and 12 mm.

3.2.1 Preparation of Enzyme Homogenates

Ten whole mayfly nymphs of *Baetis* sp. and *Campsoneuria* sp. was homogenized with 2 mL of respective buffer for respective enzymes and 98 ml of distilled H_2O using Ultra Turrax T25 Basic. The crude homogenates were centrifuged at 8,000 rpm (Das and

Mukherjee, 2006) for 15 minutes using the MSE model. The resulting supernatants were used as the enzyme sources. As to prevent denaturation of protein samples, centrifugation was conducted in a cold room and supernatants were kept at -20^{0}C for one day to one week.

3.2.2 Protein Assay

Protein determination is crucial in measuring specific activity of an enzyme. The protein assay technique used in this study to measure the amount of protein in the sample was introduced by Lowry *et al.* (1951) and was later modified by Litwack (1960).

3.2.2.1 Preparation of Reagents for Protein Assay

3.2.2.1(a) Solution A

Ten grams of Na_2CO_3, 2.0g of NaOH, 0.8g of Sodium Potassium Tartrate ($NaKC_4H_4O_6.4H_2O$) and 5.0g of Sodium Dodecyl Sulfate (SDS) were diluted in 500 mL of distilled H_2O to give a solution with 2.0% of Na_2CO_3, 0.4% of NaOH, 0.16% Sodium Potassium Tartrate and 1.0% SDS.

3.2.2.1(b) Solution B

Solution B was prepared by dissolving 4.0g of $CuSO_4.5H_2O$ into 100mL of dissolved H_2O to give 4.0% of $CuSO_4.5H_2O$.

3.2.2.1(c) Reagent C

Reagent C consisted of 100:1 mixture of solutions A and B.

3.2.2.1(d) Folin Reagent

Folin Reagent was prepared by diluting Folin & Ciocolteu's phenol reagent in distilled H_2O by 1:1 ratio.

3.2.2.2 Testing procedures for protein determination

Table 3.1. Testing procedures for protein concentration

Reagent	**Blank Control (ml)**	**Blank Test (ml)**	**Test (ml)**
Enzyme	Distilled H_2O	0.25	0.25
Reagent C	-	0.83	0.75
Incubating at room temperature (25°C) for 10 minutes			
Folin	-	-	0.08

Solutions were once again incubated at room temperature (25°C) for 10 minutes. Samples were then transferred to semi-micro disposable cuvettes and absorbance values were read at 660nm using a spectrophotometer (Shimadzu, Model UV-1601). Protein concentration was determined by converting the absorbance into concentration based on a protein standard curve established from bovine serum albumin (BSA) based on Lowry *et al*. (1951) method (Figure 3.4).These values were then used to convert the enzyme activities. Average optical density and protein concentration, BSA (µg/ml) data shown in Appendix 3.1.

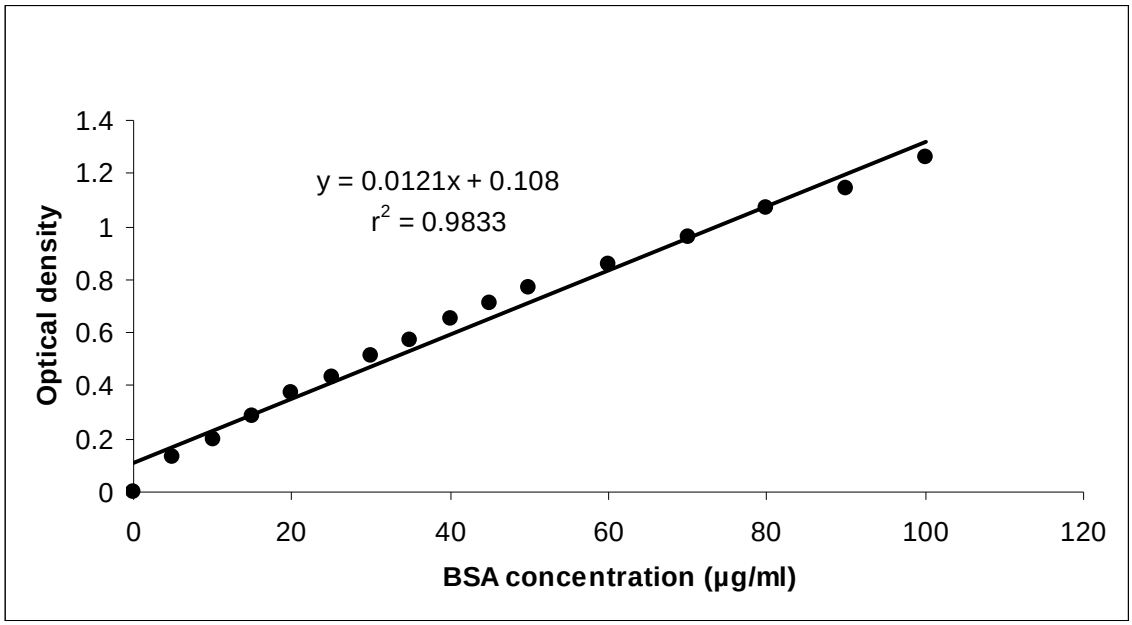

Figure 3.4. Protein Standard Curve based on Lowry *et al.* (1951) method

3.2.3 Preparation of Test Solutions

3.2.3.1 Non-specific esterases (EST)

Buffer

14.196g of Na_2HPO_4 and 15.601g of $NaH_2PO_4.2H_2O$ dissolved in 1000 mL of distilled H_2O to give 0.1M phosphate buffer pH 7.0. 400 mL of the buffer solution was then further diluted with 600 mL of distilled H_2O to give 0.04M phosphate buffer pH 7.0. All solutions were stored at 4°C.

Substrate

0.0112g of alpha naphthyl acetate dissolved in 1 mL of acetone and 199 mL of 0.04M phosphate buffer pH 7.0.

Coupling reagent

5.0g of sodium lauryl sulphate dissolved in 100 mL distilled H_2O to give 5% sodium

lauryl sulphate solution. 0.4g of fast blue salt (FBS) (tetrazotized *o*- dianisidine) dissolved in 40 mL of distilled H_2O to give 1% diazo-blue solution. Diazo-blue coupling reagent (DBLS) was freshly prepared by adding together 5% sodium lauryl sulphate and 1% diazo-blue according to the 5:2 ratio.

3.2.3.2 Acetylcholinesterase (AChE)

Buffer

14.196g of Na_2HPO_4 and 15.601g of $NaH_2PO_4.2H_2O$ dissolved in 1000 mL of distilled H_2O to give 0.1M phosphate buffer pH 7.0.

Substrate

0.0578g of acetylthiocholine iodide (ASChI) dissolved in 100 mL of 0.1M phosphate buffer pH 7.0.

Coupling reagent

0.012g of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) dissolved in 100 mL of phosphate buffer.

3.2.3.3 Glutathione-s-transferases (GST)

Buffer

0.1M phosphate buffer pH 7.0 was diluted in 200 mL distilled H_2O to give 0.08M phosphate buffer pH 7.0.

Substrate

0.2305g of glutathione was dissolved in 10 mL ethanol to give 5 x 10^{-3}M of reduced

glutathione.

Coupling agent

0.144g of 1-chloro-2,4 –dinitrobenzene (CDNB) was dissolved in 10 mL ethanol to give 5 x 10^{-4}M of CDNB.

3.2.3.4 Inhibitors for Non-specific esterases (EST) and Acetylcholinesterase (AChE):

Mercury chloride ($HgCl_2$), cadmium sulphate ($CdSO_4$), iron chloride ($FeCl_2.6H_2O$), plumbum acetate [$(CH_3COO)2Pb.3H_2O$] and copper sulphate ($CuSO_4$) were used as inhibitors for non-specific esterases (EST) and Acetylcholinesterase (AChE). A 10^{-3}M concentration was prepared by dissolving 0.8145g of $HgCl_2$, 0.208g of $CdSO_4$, 0.8109g of $FeCl_2.6H_2O$, 1.1379g of [$(CH_3COO)2Pb.3H_2O$] and 0.4788g of $CuSO_4$ in 100 mL of distilled H_2O. A serial dilution was then performed by diluting 10 mL of the 10^{-3} M concentration of the mentioned inhibitors in 90 mL of distilled H_2O to produce concentrations of 10^{-4}M, 10^{-5}M, 10^{-6}M, 10^{-7}M and 10^{-8}M. Pesticides used in this study were Malathion ($C_{10}H_{19}O_6PS_2$), Dichlorvos (DDVP) (2,2-dichlorovinyl dimethyl phosphate) and Fenitrothion ($C_9H_{12}NO_5PS$) with10^{-4} M, 10^{-5}M, 10^{-6}M, 10^{-7}M and 10^{-8}M concentration solutions.

3.2.4 Testing Procedures

3.2.4.1 Non-specific Esterase

Testing procedures for specific activity of non-specific esterase are as in Table 3.2.

Table 3.2. Testing procedures for specific activity and inhibition study of non-specific esterase

Reagent	**Blank Control(ml)**	**Blank Test (ml)**	**Control (ml)**	**Test (ml)**
Phosphate buffer 0.04M pH 7.0	1.5	1.0	0.5	-
Enzyme	0.5	0.5	0.5	0.5
alpha naphthyl acetate (substrate)	-	-	1.0	1.0
Inhibitors (heavy metal/pesticide)	-	0.5	-	0.5
Incubating at room temperature (37°C) for 30 minutes				
Diazo-blue coupling reagent (DBLS)	Distilled H_2O (0.5)	0.5	0.5	0.5

Three replicates were made each for both blank and test. A pinkish colour immediately appeared when diazo-blue coupling reagent (DBLS) was added to alpha naphthyl acetate (substrate). The pinkish colour however turned to blue after being incubated at room temperature for 30 minutes. This is due to the hydrolysis of alpha naphthyl acetate into α-naphthol which reacted with the fast blue salt (FBS), thus producing a change in the absorbance of the solution. The activity was measured spectrophotometrically after 30 minutes using a spectrophotometer (Shimadzu, Model UV-1601) at 590 nm. The intensity of the final colour indicates the presence level of non-specific esterases and the optical density (O.D.) value were obtained.

Specific activity is a term used in measuring enzyme kinetics (rate of reaction of an enzyme with a particular substrate). This term is defined as the amount of substance the enzyme converts (reaction catalyzed), per mg protein in the enzyme preparation, per unit of time (Nelson and Cox, 2000). Esterase specific activity was calculated based on the protein concentration obtained from the protein standard curve based on Lowry *et al.* method (1951) (Figure 3.4) and resulting optical densities (O.D.) were compared with standard

curves of O.D. for known concentrations of alpha naphthol (Figure 3.5) (data of alpha naphthol standard curve is shown in Appendix 3.2) to convert the absorbance to product concentrations. The enzyme specific activities were reported as alpha naphthol µM /min/µg protein.

$$\text{Specific activity of esterase} = \frac{\text{Total of alpha naphthol produced (µM)}}{\text{Incubation time (min) x total protein (µg)}}$$

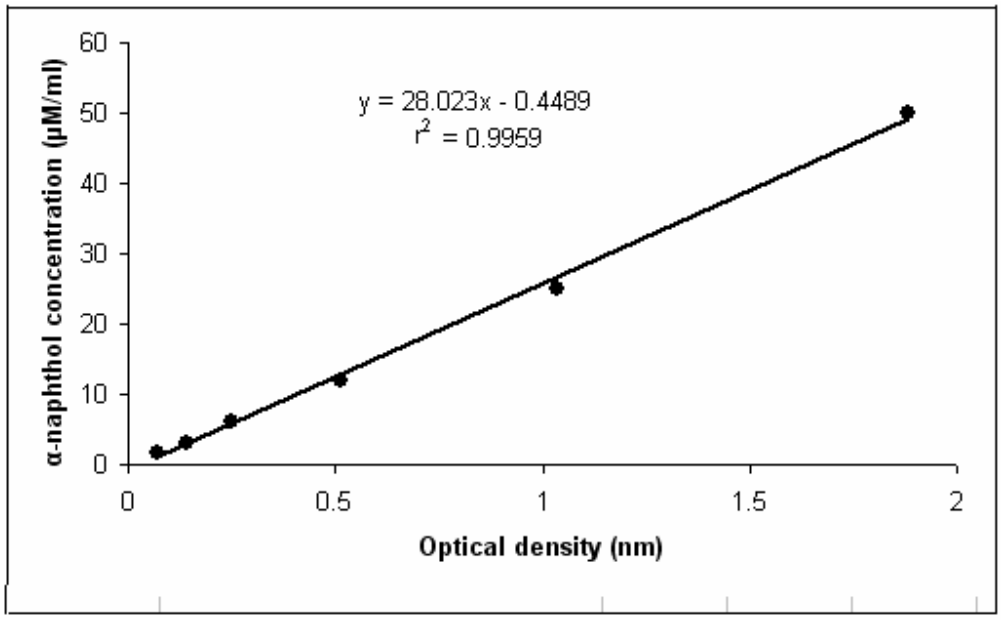

Figure 3.5. Standard curves of optical density for known concentrations of alpha naphthol

For inhibition study, the enzyme source was incubated with different concentrations of inhibitors ranging from 10^{-4}M to 10^{-7}M. 0.1 mL of each inhibitor (mercury, cadmium, lead, copper, iron, Malathion, Dichlorvos (DDVP) and Fenitrothion) was prepared in distilled H_2O. Each reaction tube containing enzymes was incubated with 0.1 mL of each inhibitor for 10 minutes to allow the inhibitors to react before the addition of substrate and coupling reagent. The activity was measured spectrophotometrically after 30 minutes using a spectrophotometer (Shimadzu, Model UV-1601) at 590 nm.

Percentage of inhibition in non-specific esterase was calculated using the equation:

$$\text{Percentage of Inhibition} = [(A_0-A_1)/A_0 \times 100],$$

where, A_0 is the absorbance of control and A_1 is the absorbance with the inhibitor samples. The median inhibition concentration (I_{50}) for each inhibitor was determined based on the percent inhibition of non-specific esterase versus log (inhibitor concentration) regression analysis.

This assay was performed on three replicates of extracts for each species. Results for specific activity were reported as mean±standard deviation and each inhibitor was analyzed in triplicate for every biochemical procedure. Student t-test was performed, with the significant difference reported at $p \leq 0.05$.

3.2.4.2 Acetylcholinesterase

Testing procedures for specific activity and inhibition study of acetylcholinesterase are as shown in Table 3.3.

Table 3.3. Testing procedures for specific activity and inhibition study of acetylcholinesterase

Reagent	**Blank Control (ml)**	**Blank Test (ml)**	**Control (ml)**	**Test (ml)**
Phosphate buffer 0.1M pH 7.0	1.9	2.8	1.8	1.7
Enzyme	1	-	1	1
Inhibitors (heavy metal/pesticide)	-	0.1	-	0.1
Acetylthiocholine iodide , ASChI (substrate)	-	-	0.1	0.1
Incubating at room temperature (37°C) for 30 minutes				
DTNB (coupling agent)	0.1	0.1	0.1	0.1

A yellowish colour or colourless solution was observed when DTNB (coupling agent) was added to ASChI (substrate). The reaction was further incubated at 25°C for 6 minutes. The colour intensities were read using a spectrophotometer (Shimadzu, Model UV-1601) at 412 nm and optical density values were obtained. Acetylcholinesterase specific activity was calculated according to the Beer-Lambert Law (Schwartz *et al.*, 2007) and expressed as ASChI µM /min/µg protein.

$$\text{Specific activity of AChE} = \frac{\text{O.D.}}{\varepsilon} \times \frac{1}{L} \times \frac{1}{t} \times \frac{1}{\mu\text{g protein}}$$

ε = molar extinction coefficient ($1.96 \times 10^{-4} M^{-1} cm^{-1}$)

L = optical path length (1 cm)

T = incubation time (minutes)

Percentage of inhibition in acetylcholinesterase was calculated using the equation:

$$\text{Percentage of Inhibition} = [(A_0 - A_1)/A_0 \times 100],$$

where, A_0 is the absorbance of control and A_1 is the absorbance with the inhibitor samples. The median inhibition concentration (I_{50}) for each inhibitor was determined based on the percent inhibition of acetylcholinesterase versus log (inhibitor concentration) regression analysis.

The K_i (bimolecular inhibition rate constant) value was determined from studies of the effect of inhibitor concentration on the activity of the enzyme under conditions of varying substrate concentration (Copeland *et al.*, 1995). The rate measurements were measured at substrate concentrations from 10^{-4} M to 10^{-8} M, and each measurement was

repeated three times. Activity was measured for 1 minute after addition of the enzyme to the mixture, and spontaneous hydrolysis of substrate was subtracted.

$$\text{Percentage of residual activity} = [(A_1/ A_0) \times 100]$$

The plot of the log of residual activity (AChE) against time was linear for a given inhibitor concentration. The bimolecular rate constant (Ki) was calculated by linear regression as described by Main and Iverson (1966). The gradient of the graph was obtained and the value of K_i was calculated based on the following formula:

$$K_i = m\ (2.303)$$

This assay was performed on three different batches of extracts for each species. Results for specific activity were reported as mean±standard deviation and each inhibitor were analyzed in triplicate for every biochemical procedure. Student t-test was performed, with the significant difference reported at $p \leq 0.05$.

3.2.4.3 Glutathione-s-transferases

Testing procedures for specific activity of glutathione-s-transferases are as shown in Table 3.4.

Table 3.4. Testing procedures for specific activity of glutathione-s-transferases

Reagent	**Blank Control (ml)**	**Control (ml)**
Phosphate buffer 0.08M pH 7.0	1.48	1.48
Reduced GSH (substrate)	1.0	1.0
CDNB (coupling agent)	0.02	0.02
Enyzme	(Distilled H_2O) 0.5	0.5

A yellowish colour was observed and the reaction was incubated at 35^0C for 10 minutes. The colour insentisities were read using a spectrophotometer (Shimadzu, Model UV-1601) at 344 nm and optical density values were obtained. GSTs activity was determined spectrophotometrically by monitoring the thioether formation at 344 nm using CDNB as the substrate (Habig *et al*., 1974). GST specific activity was calculated based on the protein concentration and expressed as CDNA-ηmol/min/µg protein.

$$\text{Specific activity of GST} = \frac{\text{O.D.}}{\varepsilon} \times \frac{1}{L} \times \frac{1}{t} \times \frac{1}{\mu\text{g protein}}$$

ε = molar extinction coefficient (10 $M^{-1}cm^{-1}$)

L = optical path length (1 cm)

T = incubation time (minutes)

This assay was performed on three different batches of extracts for each species. Results for specific activity were reported as mean±standard deviation and each inhibitor was analyzed in triplicate. Student t-test was performed, with the significant difference reported at $p \leq 0.05$.