CHAPTER 3: MATERIALS AND METHODS

Sample collection from Matang Mangrove

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MATERIALS AND METHODS

3.0 Introduction

This chapter describes the materials and the methodologies used for heavy metals assessment in the Matang mangrove system and the associated aquatic habitats. The study area is also described. The parameters measured, sampling methodology, methods of analysis are carefully described and explained.

3.1 Study Area

The Sepetang and Larut rivers flow through an important mangrove forest called the Matang Mangrove Forest Reserve before draining into the Straits of Malacca. The Matang Mangrove Forest Reserve (4° 50'N, 100°35'E) is located in the administrative districts of Krian, Larut/Matang and Manjung in the State of Perak, on the northwest coast of Peninsular Malaysia. It actually comprises of 19 indepently gazetted forest reserves and measures about 40,466 hectares excluding major waterways (Azahar *et al*., 2004). The reserve consists mostly of *Rhizophora apiculata* stands ranging in age from 1-30 years and represents about 40% of the total mangroves in Malaysia. The reserve forms a large, crescent-shaped embayment stretching for about 50 km along the coast bordering the Straits of Malacca and is 13 km in depth at its widest (Gan, 1995). Rainfall ranges from 2,000-2,800 mm per year. More than 85% of Matang mangroves are tidal swamp being flooded almost daily to being a wash only during the highest spring tide (Azahar *et al*., 2004). Tides are semi-diurnal with a mean high water springs of 2.65 m (Sasekumar *et al*., 1994).

The waterways of MMFR make up a total surface area of 8,653 hectares with a mean water depth of 5 metres (Sasekumar *et al*., 1994). These waterways form the important sites for fish cage aquaculture (Alongi et al., 2003). The mudflats in this area are important culture beds for cockles (*Anadara granosa*), other marine fauna, especially fish and prawns and baited trap catching of mud crabs (*Scylla serrata*). Furthermore, they also provide refueling and roosting sites for large numbers of migratory birds. The mangroves are habitants to 154 species of birds in which 49 species are migratory birds (Jamal Othman *et al*., 2004).

In this study, eight stations for sediment sampling in the Matang Mangrove Forest were selected. The coordinate was determined by Global Positioning System (GPS). The sampling station P1 was the sampling site nearer to the Kuala Sepetang whereas sampling station P4 was at the mouth of Larut River. Stations P5 and P6 were located 8.2 and 4.5km upstream, respectively whereas station P7 and P8 were located at about 2km offshore from the river mouth, respectively. (Fig 3.1). Four samplings were carried out between May 2008 to Mac 2009.

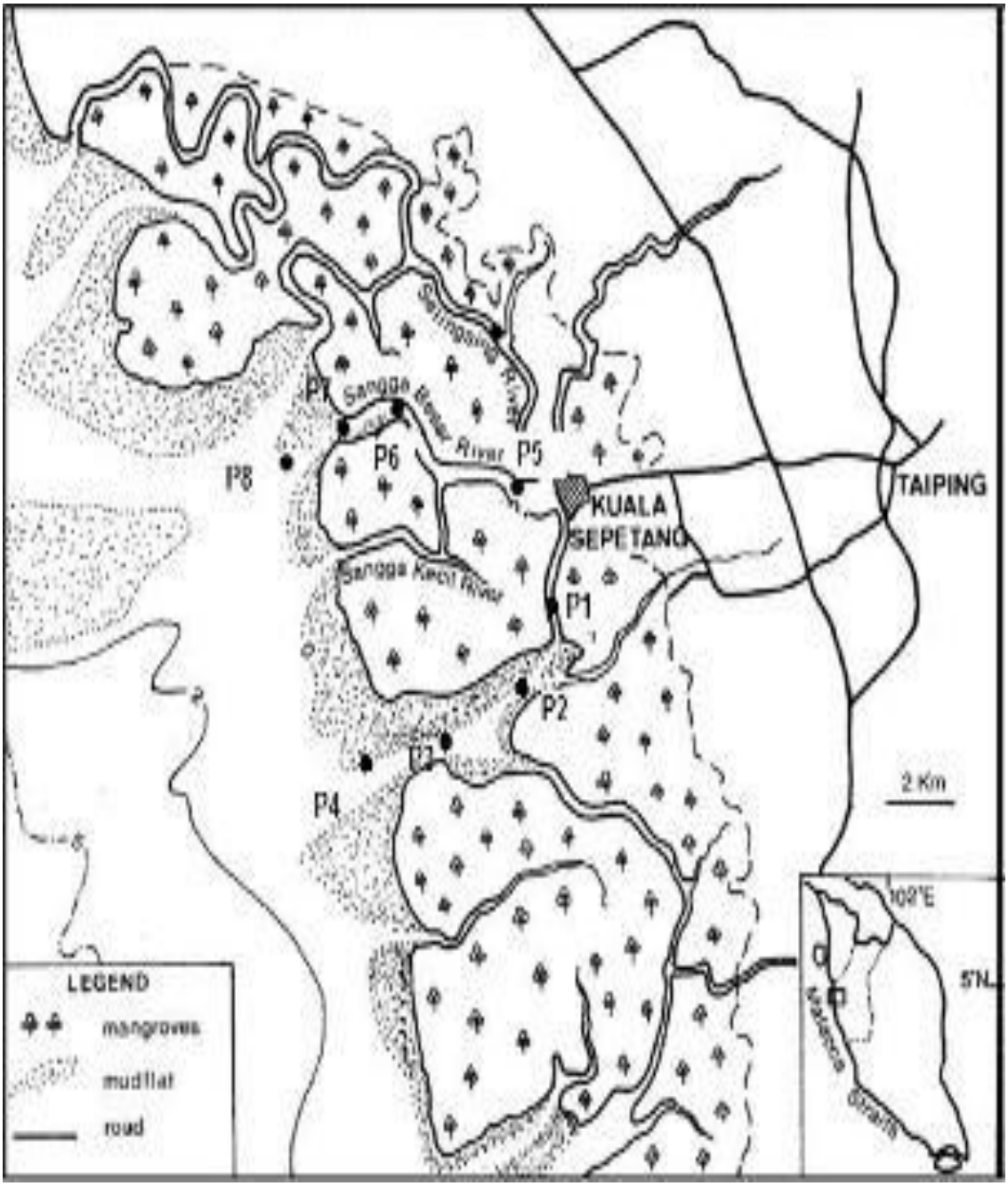

Figure 3.1: Sampling Location P1-P8 in Matang Mangrove

3.2 Sampling Methodology

Samplings were done four times starting from May 2008 to Mac 2009 (Table 3.1) at eight sampling points (Table 3.2). Each time samplings were commenced from the upper reaches of the river at Sepetang jetty to the river mouth. Sampling stations are

shown in Fig 3.1. All samples were collected and sent to the laboratory in ice (2-4°C) on the same day.

3.2.1 Water Collection

. Water samples were collected below the surface water at each sampling points. Prior to sample collection, all the one litre LDPE bottles were washed with 10% nitric acid followed by at least three times washing with distilled water. Before taking the water samples, the bottles were rinsed with the water to be collected, recapped and placed in a cooler filled with some ice for transport back to the laboratory. The sampling bottles were labeled with dates and sampling source. Temperature, conductivity, pH and salinity of the water samples were measured in-situ by using multi-probe hydrolab MS-5. In the laboratory, the water samples were filtered through a 0.45µm membrane filter before being acidified (pH <2) with concentrated nitric acid and were stored in a refrigerator at 4^{o}C until analysis. Samples directed to heavy metals determination should be immediately analyzed after collection. This is because element with low concentrations will decrease with time. When a quick analysis is not possible, samples should be stored away from any potentially contaminating sources. Samples should be preserved with the addition of ultrapure HNO_3 (pH less than 2) which prevents precipitation of metal hydroxides or adsorption of metal ions on the walls of the container. Samples is cooled down to 4°C in order to minimize microbial activity. All the analyses were carried according to the standard methods of APHA, (1998).

3.2.2 Sediments collection

The surface sediment samples were collected from eight sites in Larut River and Sangga Besar River (Fig 3.1). The sediment samples were collected using a 20 cm coring sampler. Then the sediments were sectioned at the depth interval of 0-5, 5-10,

10-15 and 15-20 cm. Samples were placed in polyethylene bags, refrigerated and transported to the laboratory immediately and stored at -20°C until analysis. The sediment samples were freeze dried for 18-24 hours using Labconco lyophiliser. Pulverization and homogenization were achieved by grinding the sediments with a Teflon mortar. The grind sediments were passed through 0.5µm sieve and stored in polyethylene bottles until analysis.

3.2.3 Aquatic organisms collection

The samples were caught using gillnets of graded mesh sizes. The species collected were separated and kept in the non coloured plastic buckets. The samples were placed in the ice box and transferred to the laboratory. Species were identified as *Scaptophagus argus* for fish, *Penaeus merguiensis* for prawns and *Anadara granosa for* cockles as shown in figure 3.2.

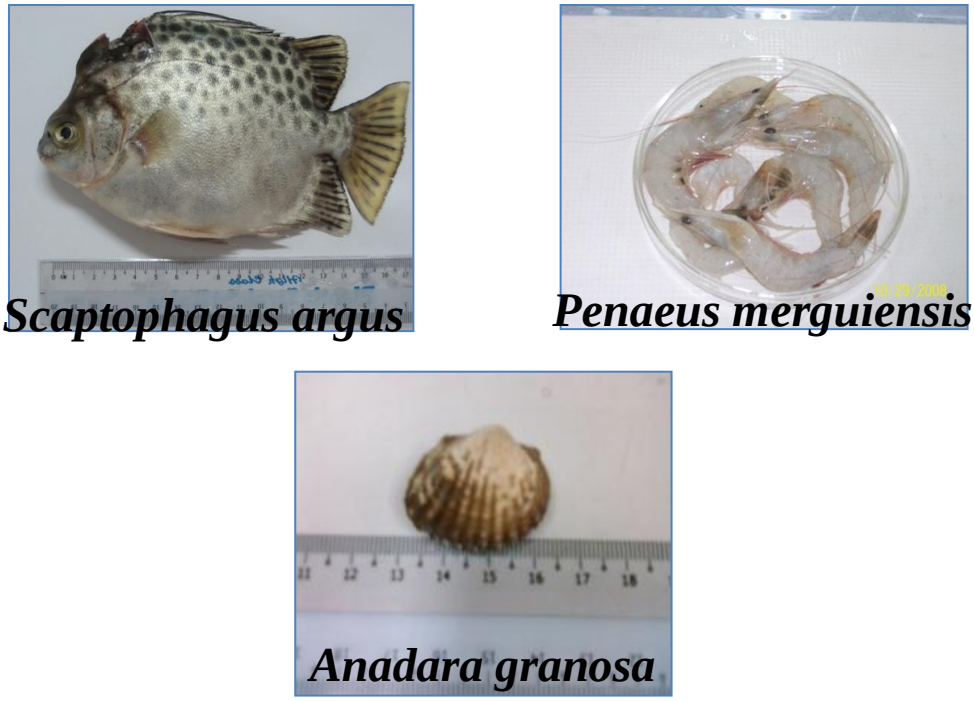

Figure 3.2: Estuarine organisms caught in the Matang Mangrove Forest

Table 3.1: Sampling locations in Matang Mangrove Forest

Sampling Locations	Description of Location	Coordinate
P1	Larut River	N 04º84’91.4’’ E 100 º 51’47.78’’
P2	Larut River	N 04 º 84’83.3’’ E 100 º 51’53.4’’
P3	Larut River	N 04 º 84’94.3’’ E 100 º 51’53.5’’
P4	Larut River	N 04 º 85’53.7’’ E 100 º 50’85.5’’
P5	Sangga Besar River	N 04 º 50’76.8’’ E 100 º 37’40.2’’
P6	Sangga Besar River	N 04 º 47’38.7’’ E 100 º 37’34.2’’
P7	Sangga Besar River	N 04 º 46’48.5’’ E 100 º 35’22’’
P8	Sangga Besar River	N 04 º 46’16.4’’ E 100 º 36’21.9’’

Table 3.2: Date of sampling carried out

Sampling	Date of sampling
1	1 May 2008
2	1 August 2008
3	14 Oct 2008
4	13 Mac 2009

3.3 Chemicals and Reagents

In these methods, all acids of ultra high-purity grade were used. Concentrated HNO_3 acid (specific gravity 1.41) and hydrochloric acid 35% (v/v) were obtained from-Merck, Darmstadt, Germany). The 30% (v/v) H_2O_2 of analytical reagent grades were purchased from Fischer Scientific, Canada. Deionized distilled water (DDW) was used for preparation of solutions, dilutions and for final rinsing of the acid cleaned vessels. Standard stock solutions of copper, cadmium, lead, chromium and zinc were prepared from Merck Titrasol (1000g/l). The working solutions were freshly prepared by diluting an appropriate aliquot of the stock solutions. In order to check on the purity of the chemical used, a number of chemical blanks were run. There was no evidence of any contamination in these blanks.

3.4 Glassware

All glassware was soaked overnight in 10% (v/v) nitric acid, followed by rinsing with double distilled water and dried before using.

3.5 Standard Reference Material

The Certified Reference Material (CRM) is a reference material, accompanied by certificate, one or more of whose property values are certified by a procedure which establishes its traceability to an accurate realization of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty statement as a stated level of confidence. CRMs are generally prepared in batches for which the property values are determined within stated uncertainty limits by measurements on samples representative for the whole batch.

The quality of metal analysis methodology in water was checked by using certified reference water of the Community Bureau of Reference (BCR):CRM 505 (trace elements in estuarine water). The quality of total acid digestion of the sediment was checked by using a certified reference material of the National Institute of Standard and Technology (NIST) 1646a (estuarine sediment). For tissues, the accuracy of the analytical method was evaluated through the analysis of the certified DORM-3-Fish protein from National Research Council Canada.

3.6 Preparation of Calibration Standard

The stock standard solutions of 1000mg/l obtained from Merck were prepared for all metals. The stock solutions were used to calculate the required volumes for concentrations of 0.05, 0.1, 0.5, 1.0, 3.0, 5.0 ppm in final volumes of 100 ml. The formula used was $C_1V_1 = C_2V_2$ where C_1 (standard concentration of 1000 ppm), V1 (the required volume to be calculated), C_2 (different concentrations of 0.05, 0.1, 0.5, 1.0, 3.0, 5.0 ppm), V_2 (volumetric flask used 100ml). After calculating the required volumes for each metal and pippeting this amounts into 100ml flasks, the flasks were filled with

deionised water. These calculated volumes were then used to set up a standard linear graph which was used to determine the concentration of metal.

3.7 Sample Preparation

3.7.1 Water Samples

The digestion of water samples was done using concentrated nitric acid according to Zhang (2007). 5 ml concentrated nitric acid was added to 50ml of water sample in a 100ml glass beaker. This was heated on a hot plate to boil until the volume was reduced to 20 ml. Another 5ml concentrated nitric acid was added and then heated for another 15 minutes and allowed to cool. About 5ml of nitric acid was used to rinse the beaker and the solution was transferred into a 100ml volumetric flask and made up to the mark with distilled water. A blank solution was similarly prepared following the same protocol. Total dissolve metals were determined by Inductive-coupled plasma-mass spectrometry. The average values of three replicates were taken for each determination.

3.7.2 Sediment samples

A weight of 0.3 g of the dried sediment samples was ashed at 500 – 550°C for 4 hours. The sediment samples were then placed in a Teflon vessel (100ml capacity) digested using a combination of HNO_3 (9ml) and HCl (3ml) acid using a CEM 2000 microwave digestion system according to the EPA Method 3051. The procedure used was that described for the digestion of samples in the CEM Digestion Application Manual. The vessels were then capped, sealed and heated in the microwave system with simultaneous temperature and pressure monitoring at 175°C for 10 minutes. The digested samples were then filtered through Whatman No.1 filter paper and diluted to 100 ml with double distilled water. The heavy metals for all prepared samples were

determined by using an air-acetylene flame atomic absorption spectrometer (AAS) Perkin-Elmer Model AAnalyst 400. The analytical reagent blanks were prepared together with each batch of digestion set and analyzed for the same element of the samples.

3.7.3 Tissue samples

Fishes were dissected into several parts of liver, skin, muscle and gills as shown in Figures 3.3 and 3.4. Tissue sub-samples were taken out quickly using stainless steel instruments on a clean glass working surfaces. Each sample of muscle tissues were separately in clean and labeled plastic container. Fish tissues samples, cockles and prawns were freeze-dried for 18-24 hours using Labconco Lyophiliser. The lyophilized samples were then ashed at 550 – 600 °C for 3-4 hours. Pulverization and homogenization were achieved by grinding the tissue samples with the exception of fish in a Teflon mortar. The powderized samples were analyzed for heavy metals. The Prawn and cockles tissues were digested according to the method of UNEP/FAO/IAEA (1982). The lyophilized tissue 0.15 g was heated with 10 ml of concentrated nitric acid (70-90 °C) till all tissue had been digested. The temperature was then gradually increased to 135 °C and drops of H_2O_2 were then added for further oxidation. After cooling, the solutions were diluted to 50 ml with double distilled water and filtered through a fiberglass filter paper, GFC. Metal concentrations of digested tissue samples were analysed by AAS. Blanks were also performed and measured for all metals following the same procedure.

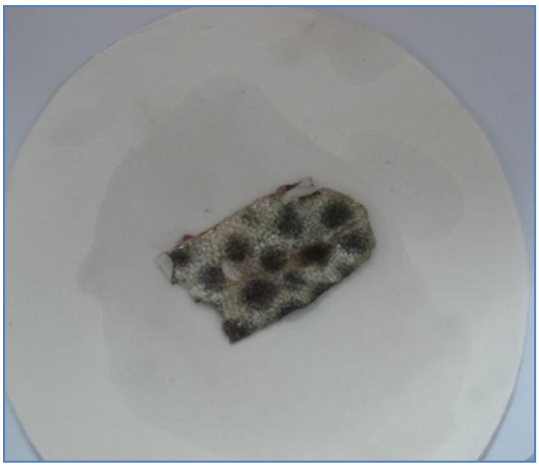

Figure 3.3: Skin tissue of fish (*Scaptophagus argus*)

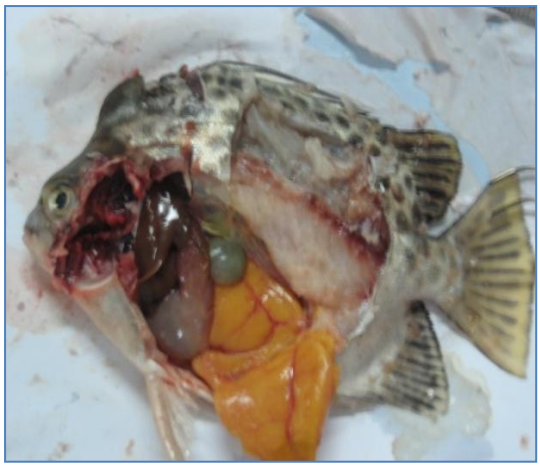

Figure 3.4: Dissection of fish (*Scaptophagus argus*)

3.8 Apparatus

In this experiment, concentrations of heavy metals were determined by Flame Atomic Absorption Spectrometry AAnalyst 400 (Perkin Elmer) and Inductive Coupled Plasma-Mass Spectrometry 7500 Series (Agilent). Digestion of sediments samples were done by microwave digestion system-closed vessel MarsXpress (CEM Corporation).

3.8.1 Atomic Absorption Spectrometry AAnalyst 400

A Perkin–Elmer AAnalyst 400 atomic absorption spectrometer with deuterium background corrector as shown in Figure 3.5 was used in this study. All measurements were carried out in an air/acetylene flame. The operating conditions adjusted in the spectrometer were carried out according to the Standard guidelines of the manufacturers. A 10 cm long slot-burner head, a lamp and an air/acetylene flame were used. An acetylene–air flame was used; the gas flow rates and the burner height were adjusted in order to obtain the maximum absorbance signal for each element. Argon 99.96% (v/v) was used as gas through the FAAS. Other instrumental parameters were set to the values shown in Table 3.3.

Table 3.3 Flame atomic absorption spectrometer operating conditions

Element	Wavelength (nm)	Slit width (nm)	Lamp current (mA)	Sensitivity (mg/l)	Flame
Cu	324.8	0.7	3.0	1.3	Air-C_2H_2
Zn	213.9	0.7	5.0	0.3	Air-C_2H_2
Cr	357.9	0.7	3.0	4	Air-C_2H_2
Pb	283.3	0.7	3.0	8	Air-C_2H_2
Cd	228.8	0.7	5.0	0.5	Air-C_2H_2

Figure 3.5: Atomic Absorption Spectrometry image

3.8.2 Inductive Coupled Plasma Mass Spectrometry

An Agilent 7500ce ICP-MS equipped with an octopole collision cell and autosampler was used to determine trace metal concentrations in the digests using the operating conditions in Table 3.4. The sample introduction system consisted of a concentric nebulizer and temperature-controlled spray chamber (S/C) that are connected

to an ASX 500 autosampler. Optimisation of the ICP-MS conditions was achieved by adjusting the torch position and tuning for reduced oxide and doubly charged ion formation with a standard tuning solution containing Li, Y, Ce, Co and Tl in 2% HNO_3 and 0.5% HCl in order to cover the entire mass region of interest.

Table 3.4: ICP-MS operating conditions

Tuning Parameter	
Plasma RF power	1,500 W
Reflected power	<15 W
Sampling depth	7.0-9.0 mm
Plasma gas flow	15 L min^{-1}
Carrier gas flow	0.8-1.0 L min^{-1}
Make up gas flow	0.1-0.3 L min^{-1}
Sampler and skimmer cones	Ni

Figure 3.6: Agilent ICP-MS Model 7500ce

3.8.3 Microwave Digestion System

A CEM *MARSXpress* closed vessel microwave system was used for microwave digestion (Figure 3.7). The operation of CEM MARS-X is based on the principle that combines the speed of microwave heating and closed vessel technology to achieve elevated temperatures under controlled conditions. The system is equipped with an inboard pressure and temperature control system for regulating sample extraction condition via magnetron power output controlled. Teflon PFA vessels 100ml capacity was used in the experiments. Before each digestion, vessels were washed with concentrated nitric acid and then thoroughly rinsed with deionized water. All the vessel components must be dry and free of particulate matter as drops of liquid or particles will absorb microwave energy causing localized heating. This may char and damage vessel components, leading to possible vessel failure.

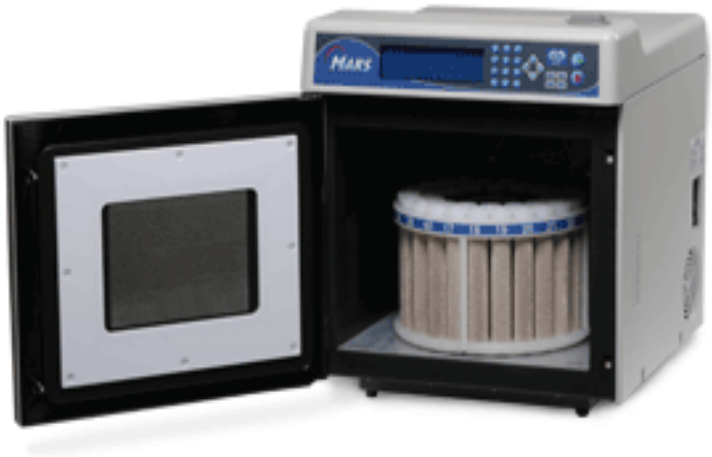

Figure 3.7: CEM-MarsX microwave digestion system