CHAPTER 3

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 SAMPLING STRATEGY

For sampling and environmental data collection, five stations were selected for each study site (B &C) of the study area (Figures 2a, 2b, 2c). The "stations" refer to the approximate location in each site where samples were collected at each sampling occasion. The stations were 50 m apart. Sites B and C refer to the original Site reference in Phang (1995). For a period of 14 months (November 1996 to November 1997), seven field trips were carried out at two month intervals.

The abiotic samples (sediment & seawater) and water environmental parameters were collected from each station at both sites. Biotic samples (seaweeds, soft corals) were collected as a pooled sample per study site, while sea cucumber samples were counted as individuals per sampling occasion. The pooled samples ensured that the final samples for analysis were homogenous. Seaweeds of different age as well as different parts of seaweeds are known to accumulate metals differently. Therefore at each sampling occasion five samples of water and sediment, one seaweed, one coral and five to seven sea cucumber were collected from each site.

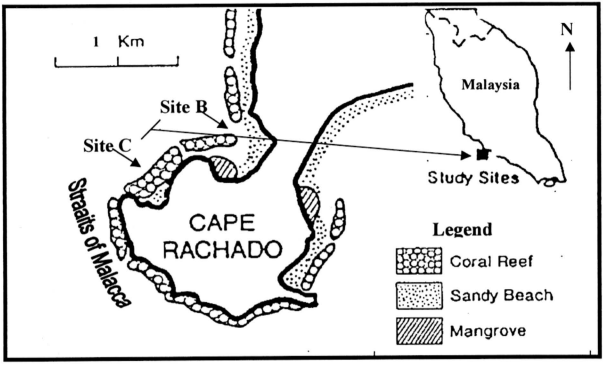

Figure 2a . Cape Rachado Study Area.

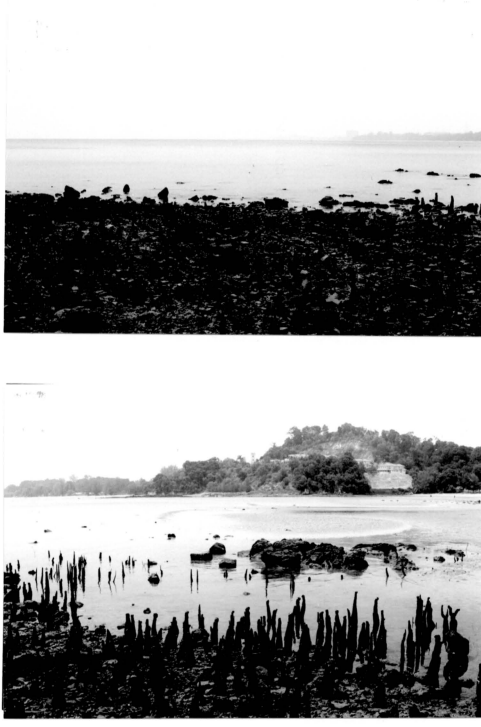

Figure 2b. Site B before and during the low tide.

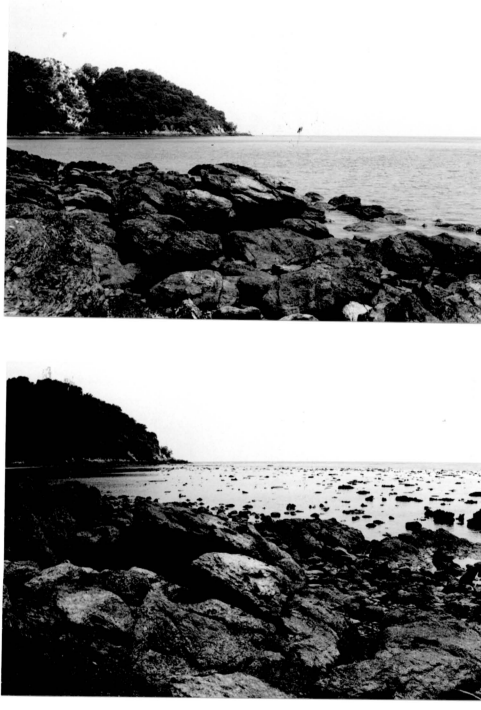

Figure 2c. Site C before and during the low tide.

3.2 COLLECTION OF SAMPLES

3.2.1 Water

Surface water samples were collected from each station of both study sites. Precleaned 2-L polyethylene bottles were used for water sampling. These bottles were again washed twice with seawater at each sampling station. The collected water samples were labelled and kept in an ice-chest and transported to the laboratory at the University of Malaya (2 hours away from the sampling area).

3.2.2 Sediment

During low tide, when the substratum was completely exposed, Samples of the top surface (2-5 cm) sediment were collected by hand from each station at both sites of the study area, labelled and kept in plastic bags. Samples were then kept in an ice-chest to be transported to the laboratory for further processing.

3.2.3 Seaweeds

The three species of brown algae (Phaeophyta) namely, *Sargassum baccularia* (Mertens) C. Agardh; *Padina tetrastomatica* Hauck and *Turbinaria conoides* (J. Agardh) Kuetzing were hand picked while snorkeling in the study sites. Seaweed samples were gently washed in seawater to remove the particles attached to the surfaces

of the seaweed specimens. The seaweeds from each site were pooled into one sample, labelled and kept in ice-chest and transported to the laboratory for further processing.

3.2.4 Soft Corals

Soft coral *Sinularia* sp. samples were collected while snorkeling in both study sites. Cut pieces from different coral colonies scattered in each site were pooled together to form a coral sample for each site. Samples were kept in labelled plastic bags and transported to the laboratory for further processing.

3.2.5 Sea Cucumber

Sea cucumbers were abundant and found only in Site C but were not available in Site B, although a previous survey by Goh and Sasekumar (1980) showed that they were available in both sites. This may indicate that the sea cucumber population has been decimated by harvesters for use in traditional medicine. Therefore sampling of *Holothuria atra* Jaeger was restricted to Site C only. Five to seven individuals of *H. atra* of almost similar total length (14 ± 1 cm) were picked by hand from Site C only. The specimens were kept in labelled plastic bags in an ice-chest and transported to the laboratory for further treatment.

3.3 ENVIRONMENTAL DATA COLLECTION

3.3.1 Water Quality

Water quality parameters were recorded during each field trip using calibrated portable field equipment. During sampling at each sampling station of both sites, water parameters such as dissolved oxygeñ (DO) and temperature (°C) were measured using a salinity compensated dissolved oxygen meter (YSI model 57). Salinity (S‰) was measured using an ATAGO hand refractometer. Hydrogen ion concentration (pH) value was measured using a ROYCE instrument, model 503 pH/CO_2 analyser.

3.3.2 Atmospheric Parameters

Atmospheric parameters such as air temperature (minimum and maximum in °C), mean sunshine hours per day, mean solar radiation (MJm^{-2}), and total rain fall (mm) for Malacca (2^0 16' N & 102^0 15'E) (the site nearest to the sampling location) were obtained from the Malaysian Meteorological Department, Selangor.

3.4 CLEANING OF APPARATUS

All plastic and glass wares used in this study were washed thoroughly using detergent (teepol) and tap water, then soaked in 3N nitric acid for a few days and washed again with distilled water. These items were soaked again in an another 3N nitric acid

basin over night. Before use, each item was washed thoroughly with deionised- distilled water. The same cleaning regime was applied each time.

3.5 SAMPLE STORAGE AND PRESERVATION

3.5.1 Water Samples

Seawater samples were used to determine the dissolved heavy metal concentrations. All water samples were filtered through 0.45μm size membrane to remove all the suspended particulates (Martin *et al.*, 1992). A glass Millipore filtration apparatus was used. Each sample was then acidified to pH < 2 using nitric acid (1.5 ml l^{-1}) (APHA, 1989; Martin *et al.*, 1992). The filtered acidified seawater samples were stored in the freezer until further chemical treatment and instrumental analysis (APHA, 1989). About 300 ml from each sample was removed before filtration and acidification for storage treatment, to be used for total solids, total volatile solids and total suspended solids determination.

3.5.2 Sediment Samples

Sediment samples were kept in labelled plastic bags in the freezer until further treatment.

3.5.3 Biotic Samples

Upon arrival at the laboratory, all biotic samples were sorted out and rinsed with seawater, distilled deionised water and kept in labelled plastic bags with air squeezed out of it and kept in the freezer until further treatment (Dybren, 1983; APHA, 1989).

3.6 CLEANING AND DRYING OF BIOTIC SAMPLES

3.6.1 Seaweeds

Seaweed samples of the three species were subjected to a second cleaning regime before drying. Distilled deionised water was used for washing the seaweeds in plastic bowls to remove all the associated sand grit and epiphytes. This was followed by further washing in distilled deionised water. Excess moisture was removed by standing each seaweed sample in a one-litter beaker for about one hour. The collected water was then discarded and samples were dried in the oven at 50-60 °C for three days or to a constant weight (Gnassia-Barelli *et al.*, 1995). The dried seaweeds were ground using an acid washed mortar and pestle.

3.6.2 Soft Corals

Soft coral samples were subjected to a further cleaning regime using distilled deionised water. Samples were dried in the oven at 80 to 100 °C for 24 to 48 hours or

to a constant weight. The dried soft coral samples were ground using an acid washed mortar and pestle. The samples were kept in labelled plastic vials for further treatment.

3.6.3 Sea Cucumber

Individuals of *H. atra* were gutted to remove all the viscera and sediment contents. *H. atra* muscle tissues were cleaned thoroughly using distilled deionised water. Each specimen was cut to very fine pieces using stainless steel scissors and dried in the oven at 100 °C for 24-48 hours or to a constant weight. Each dried sample was then kept in a labelled plastic vial for further analytical treatment.

3.7 ANALYTICAL PROCEDURES

3.7.1. Total Solids (TS)

Using a 50-ml measuring cylinder, 50 ml of homogenous unfiltered seawater was measured from each sample into a preweighed dry crucible. The seawater samples were then evaporated to dryness using a water bath. The dryness was completed by heating the samples in an oven at 100 °C to a constant weight. Weight of each crucible containing the dried sample was recorded after cooling in a dessicator. To obtain the total solids the following equation was used,

Total solids mg L^{-1} = W_2-W_1 x 1000/ V

Where

W2 = crucible weight containing dried sample

W1 = dry empty crucible

V = sample volume

3.7.2 Total Volatile Solids (TVS)

Rough estimation of the amount of organic matter in the sea water sample can be achieved by finding out the total volatile solids concentration (Zaid *et al*, 1980). After recording the final weight of the crucible containing the residues during the total solids determination, the residues were ignited at 550 °C for 3 hours in a muffle furnace, then cooled in a dessicator and weighed again. The TVS of each sample was calculated according to the following equation (APHA, 1989)

$$TVS\ (mg\ L^{-1}) = 1000\ (W1-W2)/\ V$$

Where:

W1 =weight of residue before ignition

W2 = weight of residue after ignition

V = sample volume

3.7.3 Total Suspended Solids (TSS)

From each sample a known volume (200 ml) were measured using a 250 ml measuring cylinder and filtered through a pre-weighed dried 0.45μm glass fiber filter membrane (Whatman GFC filter paper) using a Millipore filtration unit. The filter paper

was then dried in the oven at 100 °C overnight, then cooled in a dessicator. The weight of the filter paper and the residue was recorded. The TSS was then calculated according to the following equation,

$TSS\ (mg\ L^{-1}) = W2 - W1 \times 1000 / V$

Where

W2 = weight of filter paper and the residues

W1 = weight of the filter paper

V = sample volume

3.7.4 Heavy Metals

3.7.4.1 Water samples

Chelating resins for separation and preconcentration of heavy metals from seawater were used. A modification of the technique by Kingiston *et al.* (1978) involving Chelex 100 resin (200 to 400-mesh size) sodium form was applied for seawater analysis.

3.7.4.1.1 Column preparation and purification procedure

Columns were cleaned before use by soaking in diluted nitric acid (1 HNO_3: 4 H_2O) for few days and then washed with deionised distilled water. Chelex 100-resin of 200 to 400 mesh size (sodium form) was prepared in a slurry form. The slurry resin was

loaded into the pre-cleaned column. The resin was washed with 15 to 20 ml of 2.5 M HNO_3 in 3-ml portions to elute any trace metal contamination. The resin was washed with two 5-ml volumes of deionised distilled water to rinse the resin of excess acid. 10 to 15 ml of 2.0 M NH_4OH was added in 5-ml portions to transform the chelex resin to ammonium form (NH_4^+). The pH of the column effluent was checked in order to ensure basicity, then the column was rinsed with 10 to 15 ml of deionised distilled water to remove the excess NH_4OH.

3.7.4.1.2 Column preconcentration and separation procedure

Three replicates of 200ml of each seawater sample were measured using a precleaned 250-ml measuring cylinder and transferred into thoroughly cleaned beakers. Each replicate was subjected to a pH adjustment. Diluted nitric acid and an ammonium hydroxide were used for pH adjustment to pH 5.10 ± 0.01 with the assistance of a pH meter (METTLER TOLEDO 320). 0.5ml of 0.8 M ammonium acetate was used to aid in buffering the system of each replicate. A small amount of the seawater was added to the reservoir and column. This is to allow the resin to shrink to about half of it's original volume (2-3 minutes) as a result of ionic and pH changes. The remaining seawater sample was then added to the reservoir as needed to keep it filled and to pass through the column. The flow rate was approximately 0.8 to 1.0 ml min^{-1}. 40 ml of 1.0 M ammonium acetate was added to the column in 10-ml portions to selectivitly elute Na , K ,Ca , and Mg (major matrix) and replace them with NH_4^+. Then 10 ml of deionised distilled water was added to remove residual ammonium acetate. The transition metals

were then eluted by 7ml of 2.5 M HNO_3 in successive portions, and collected in 10-ml volumetric flasks. The volume was topped up to the mark by deionised distilled water. The pre-concentrated samples were kept in labelled plastic vials in the refrigerator for instrumental analysis.

3.7.4.2. Sediment

A modification of the techniques by McLaren *et al.*(1981); Din (1995); Solan (1995); Yuen (1996) was applied to the sediment samples as a wet digestion technique using a mixture of concentrated nitric (HNO_3), perchloric ($HCLO_4$) and hydrofluoric acids (HF).

Sediment samples were taken out of the freezer for a few hours, then dried in the oven at 80 °C for a few days or to a constant weight. During the drying period, sediment samples were taken out and mixed thoroughly. Dried samples were sieved by 2mm-nylon mesh, in order to remove gravel and large shell fragments. Sieved dried sediments (<2mm) were subjected to chemical analysis.

Three replicates of 0.5 g each for each dried sediment sample of both study sites for each sampling occasion were weighed using an analytical balance and transferred to Teflon beakers. To each replicate, 12 ml of nitric acid, 4 ml of perchloric acid (70%) and 2 ml of hydrofluoric acid were added. In order to enhance the digestion, the Teflon beakers of sediment samples were covered by Teflon lids and placed on a hot plate (120 °C) for four hours. Digestion was then continued openly after the lids were removed,

and until the solution volume was reduced to about one ml and just before complete dryness. The content of the beakers were then transferred to a 50 ml plastic volumetric flasks by washing the beakers many times using distilled deionised water and filtering through a Whatman filter paper. The volume was topped up to the mark by distilled deionised water. Samples were kept in the refrigerator until instrumental analysis

3.7.4.3 Biotic samples

A conventional wet digestion technique using concentrated acids (Tariq *et al.*, 1993; Moore *et al.*, 1993; Ramachandran *et al.*, 1995; Murugadas, 1997; Robledo and Pelegrin, 1997) were applied for biological sample digestion.

3.7.4.3.1 Seaweed samples

From each dried pooled sample of each of the three brown seaweed species, six to seven sub-samples were taken each site from each sampling occasion. Reflux digestion technique was applied to achieve the complete digestion of seaweed samples (Ramachandran, 1993; Ramachandran *et al.*, 1994; 1995; Murugadas, 1997). A mixture of nitric acid and hydrogen peroxide were used for seaweed digestion (Daffa, 1996; Fereletta *et al., 1996*; Robledo and Pelegrin, 1997). 0.4 g of each sub-sample was weighed using an analytical balance. Each seaweed sub-sample was transferred to a 100 ml Kjeldahal flask. 10 ml of nitric acid and 5ml of hydrogen peroxide were added to each sub-sample and the mixture subjected to the reflux digestion technique. A digester

unit with six heating wells in a fume-chamber was used for the reflux digestion of seaweed samples. Digestion was continued for about two hours until no more brown fumes were produced and a clear solution remained with no seaweed particles. This was digested further using an open digestion technique by heating the Kjeldahal flasks in the heating mantel, in the fume chamber after disconnecting the Kjeldahal condensers, until the volume was reduced to about 2 to 3 ml. The digest was then transferred quantitatively and filtered through a Whatman filter paper (No.4) into 25 ml-volumetric flask. The volume was then topped up to the mark using distilled deionised water and transferred to labelled plastic vials. All samples were stored in the refrigerator until further instrumental analysis.

3.7.4.3.2 Soft corals

Six to seven sub-samples of 0.8 g each were weighed from each pooled dried soft coral sample for each study site from each sampling occasion. The same chemical digestion treatment and storage procedure mentioned above for the seaweeds, was also conducted for the soft coral samples.

3.7.4.3.3 Sea cucumber

Three replicates of one g each were prepared from each sample of dried *H. atra* individual. The replicates were subjected to the same chemical digestion treatment and storage procedure mentioned above for the seaweeds.

3.8 ANALYTICAL QUALITY CONTROL PROCEDURE

Serious errors may be introduced at any stage of sample handling, either during sample collection, storage or during the various stages of laboratory analytical procedures. Therefore to ensure the reliability and accuracy of the analytical results obtained, appropriate quality assurance is necessary. As such, for all samples in this study thoroughly cleaned containers were used and recommended preservation and storage procedures for heavy metal studies were applied. In general during the analytical procedures, procedural blanks (solutions with no samples) with each batch of samples were treated in the same manner. Also three replicates for each sample analysis were used. For the recovery and accuracy assurance, certified standard reference materials and/or the standard addition method were used to verify the reliability and accuracy of the analytical results obtained.

For seawater, soft corals and sea cucumber analyses, standard addition method was applied where sample replicates were spiked with known metal concentrations and left a side for about one hour. Then the same entire analytical procedures were carried out for the spiked and non-spiked replicates of the same single sample. In sediment analysis, replicates of certified sediment reference material PACS-1 from the Division of Chemistry (Marine Analytical Chemistry Standards Program) at the National Research Council of Canada, was treated similarly to the replicates of the sediment samples. For seaweed analyses, Certified Standard Reference Material NIES No. 9 Sargasso and the standard addition method, were applied for accuracy assurance of the analytical procedure. The same entire analytical procedures for seaweed analysis were carried out

for the spiked and non-spiked replicates of the same single sample in addition to the certified reference material, in order to obtain the recovery of known addition and to verify the reliability and accuracy of the analytical results obtained. Two different known concentrations were used in the standard addition method, where the percentage of recovery of each analytical procedure used was calculated based on the spiked concentration of the same magnitude to the observed concentrations in the different components analysed.

3.9 PREPARATION OF CHEMICAL STANDARDS

Due to the large variation of the expected metal concentrations of the different studied metals in the various sample materials at the different sampling occasions, a wide range of multielement standards covering the expected range in all samples were prepared. ICP multielement standards solution (BDH, ARISTAR) of 100 mg L^{-1} in nitrate form in 5% nitric acid were used as stock standard solution for the preparation of the working standard solutions. Standards of 0.01, 0.1, 1, 2, 5, 10, 20 and 50 mg L^{-1} (ppm) were prepared from 100 mg L^{-1} multielement stock standard solutions using the conventional dilution method for preparation of standards.

3.10 INSTRUMENTAL ANALYSES

Metal concentrations (Cu, Fe, Zn, and Pb) in all sample replicates of the digested seaweeds, soft coral, sea cucumber, and preconcentrated seawater were analyzed using Inductively coupled Plasma-Atomic Emission Spectrometer (ICP-AES) model BAIRD

ICP 2000. The ICP-AES instrument used is a simultaneous multielement polychromator system with detection limits of 0.002 for Fe, 0.002 for Cu, 0.002 for Zn and 0.030 mg l^{-1} for Pb.

The operational procedure involved setting the operating conditions. The instrument is equipped a 46.68 MHz radio frequency (RF) generator where the RF power was set at 1100 wates. The coolant gas flow rate and the auxiliary gas flow rate were set at10.0L min^{-1} and 1.0 L $min,^{-1}$ respectively. The carrier gas flow rate was 0.6 L min^{-1}. Measurement integration time chosen was five seconds and the number of integration was three. After setting the operational conditions the operational procedure also, involved centering of the polychromator, running of blanks, measuring of standards, performing the calibration routine. In the collection of calibration data the average intensity of each of the working standard was considered. Curve set files were prepared for each studied element, using the polynomial calculation routine. The computer software of this instrument provides all the calculations (average intensities, dilution factor, and average concentration) including the RSD(relative standard deviation) of the three integrated instrumental analysis completed for each sample replicate. Curve coefficients were calculated automatically for each element on the bases of the standards data. Standard calibration plots were obtained for each element where a satisfactory curve correlation coefficient were performed (>0.999). Finally the ICP-AES is ready for running of the samples and to avoid carry-overs between samples the instrumental analytical procedure required 15 seconds rinse with UHQ water in between samples and 30 seconds pre-flush with the sample solution.

Sediment samples were digested with a mixture of acids including hydrofluoric acid (HF). Consequently it is not recommend to be analysed with this instrument (BAIRD ICP 2000) which is equipped with a glass made sampling system (concentric quartz plasma torch and nebulizer made of glass). Therefore sediment samples were analysed subsequently with ICP-AES equipped with HF resistant sampling system.

3.11 STATISTICAL ANALYSES

Different statistical techniques were applied in data analyses, including the Student t-test, Simple Correlation, Two-way Analyses of Variance (ANOVA), Newman-Keules Test as Post Hoc Test, Multiple Regression Analyses and Cross Correlation as Time Series Analyses (Table 7).

T-test was used to assess the difference between the means of the environmental parameters of the two study sites and to assess the difference between the means of metal concentration in each component of the two sites. The ANOVA was only conducted for the abiotic data and not for the seaweed and soft coral data because no field replicates were available for the two latter components. A two-way analysis of variance was used to assess: (i) difference in environmental parameters of the two different sites; (ii) difference in environmental parameters between different sampling occasions of the same site; (iii) difference in environmental parameters between the same sampling occasions of the different sites; (iv) difference between the ambient (seawater) metal concentrations of the two sites; (v) difference in the ambient metal concentrations between different sampling occasions of the same site; (vi) difference of

ambient metal concentrations between the same sampling occasions of the different sites. The Newman-Keules test was used to trace the significant difference resulting from the interaction of sampling occasions and sites for environmental parameters and for ambient metal concentrations.

Simple Correlation analysis was used to assess the concurrent association between metal concentrations in seawater, sediment, *S. baccularia; P. tetrastomatica; T. conoides; Sinnularia* sp. and *H. atra* with environmental parameters.

Cross Correlation was conducted in order to find out the degree of correlation between metal concentration in the biotic samples and the past exposure to the metals and environmental parameters.

Multiple regression analysis was only applied to find out which of the environmental parameters were more effective and contributed more to the accumulation of metals in the species studied (Jongman *et al.*, 1995; Dytham, 1999).

Data were log transformed to ensure the normality assumption as required for statistical analyses. Simple Correlation, Cross Correlation and Multiple Regression Analyses were applied on the bases of the combined data of both sites. All statistical analyses were performed using the statistical software Statistica Version 5.

Table 7 Summary of the statistical analyses

Objective	Statistical test
Difference between the means of environmental parameters the two study sites	Student t-test
Difference between the means of metal concentrations in each component* of the two study sites.	Student t-test
Difference in environmental parameters of the two study sites. Difference in environmental parameters between sampling occasions of the same site. Difference in environmental parameters between the same sampling occasions of the different sites.	Two-way Analysis Of Variance (ANOVA) followed by Newman-Keules test as Post Hoc Test
Difference in ambient (seawater) metal concentration of the two study sites. Difference in ambient metal concentration between sampling occasions of the same site. Difference in ambient metal concentration between the same sampling occasions of the different sites.	Two-way Analysis Of Variance (ANOVA) followed by Newman-Keules test as Post Hoc Test
Association between metal concentrations in the different studied components and environmental parameters.	Simple Correlation
Correlation between metal concentrations in the biotic species and the past exposure to metals and environmental parameters	Cross Correlation as Time Series Analysis
Contribution of different environmental parameters and metals to the accumulation of each metal in each biotic species	Multiple Regression Analysis

*component here refers to sea water; sediment; seaweeds; soft coral and sea cucumber