
CHAPTER THREE

MATERIALS & METHODS

3.0 MATERIALS AND METHODS

3.1 Study Site

Cape Rachado (Lat. 2° 31'N, Long. 101° 47 'E) is a small, rocky peninsula located in the district of Alor Gajah, Melaka, on the west coast of Peninsular Malaysia. The bay here, popularly known as Tanjung Tuan and sometimes even as the Blue Lagoon, has long been a popular holiday destination. The adjoining coast of the cape has been subjected to extensive land development in order to support the tourism industry, and is now lined with various resorts, private residences and a strip of beach-side stalls. The cape itself has been designated a Strict Nature Reserve since 1953.

The fringing coral reefs extend along the bay and around the cape, though it is not absolutely continuous. Three sites were delineated and studied in this survey, each identifiable with its exposure to a particular environmental stress (Figure 1). Site A lies in an area with heavy siltation caused by the removal of its coastal mangroves that straddle the zone between reef and beach. These mangroves were removed during the construction of a hotel and condominium complex in the mid-1980s. Site B lies in a popular picnicking and bathing area. Also noted at these two sites were sewerage pipes from the various resorts. Site C is the least exposed to anthropogenic disturbance as it is situated further out at the face of the promontory. The reefs of these sites extend approximately 50 - 140 m out into the Straits of Malacca before sloping down towards the reef edge.

In an earlier study of coral composition at sites B and C, live coral was estimated to form 32.9% of the reef cover (Goh & Sasekumar, 1980). Considered separately, the reef edge had a live coral cover of 59.6% and the reef flat 26.5%. Soft corals were most

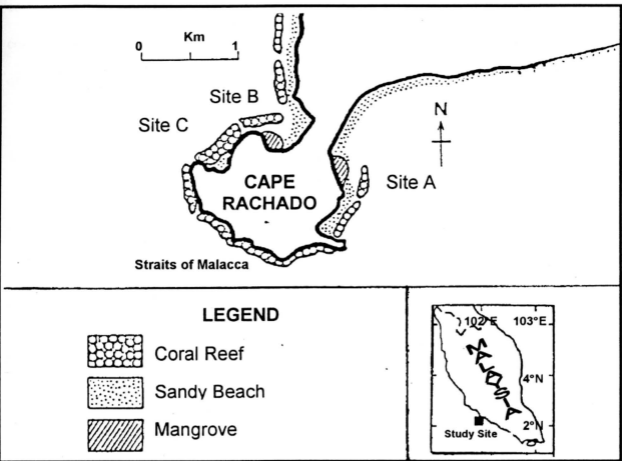


Figure 1 Study sites at Cape Rachado, West Coast Peninsular Malaysia

abundant on the reef flat whilst hard corals dominated the reef edge. Quantification of the coral community was not included in the present study, but it was noted that low coral cover still characterises the Cape Rachado reef community, especially on the inner portion of the reef flat. The abundance of live corals increases towards the reef edge.

Tides at Cape Rachado are semi-diurnal, with two high and two low tides each day. The mean difference between high and low waters at spring tide was 2.39 m, and 0.79 m at neap tide from April to September of 1998 (Hydrographic Branch, Royal Malaysian Army, 1998). The reef flats are completely exposed at less than 0.3 m low tide and on average, remain so for 3-5 hours depending on locality.

3.2 Field Measurements & Sampling

3.2.1 Seaweed Sampling

The line-transect and systematic quadrat-sampling method was selected primarily because this was used in the 1987/88 diversity study. Dimensions such as quadrat size and distance were also duplicated. This method was still deemed best for the current study because sampling was conducted on mixed stands and although each study site did not cover an unusually large area, working time was constrained by tidal movement. Sampling was done only at low tide, when the level was at approximately 0.3 m or less, thereby allowing a sampling time of at best, 2.5 hours. For logistical reasons, each site was sampled only once: Site B in May, Site C in July and Site A in September of 1998.

Firstly, the baseline was marked out on each reef, beginning at the start of the fringing reef structure and running parallel to the coastline. Three transects were then laid out at each



Figure 2 Site A, Cape Rachado, at low tide.



Figure 3 Site B, Cape Rachado, at low tide.



Figure 4 Site C, Cape Rachado, at low tide.

site, at an angle perpendicular to this baseline and at intervals of 40 m at sites A and B, and 60 m at site C. Quadrats were then placed systematically along each of the transects at a distance of 10 m apart. All algal material with holdfasts lying within the 0.09 m² (0.3 x 0.3 m) quadrats were collected and stored in plastic bags for identification in the laboratory.

3.2.2 Physicochemical Parameters

At each site, measurements were made at and water samples collected from five sampling points at 0, 20, 40, 60 and 80 m from the baseline, perpendicular to the shore. The following in-situ measurements were made:

<u>Parameter</u>	<u>Equipment</u>
pH	pH Meter Model 503
Salinity	ATAGO Hand Refractometer
Temperature	YSI Water Quality Logger Model 57
Dissolved oxygen	YSI Water Quality Logger Model 57

Water samples were collected in 500 mL polyethylene sample bottles for the analysis of ammoniacal-nitrogen (NH₃-N) and total suspended solids (TSS), whilst glass bottles were used for the water samples for orthophosphate (O-PO₄) analysis. All bottles were treated in a 10% H₂SO₄ soak for 24 hours before use. Seawater collected for NH₃-N and O-PO₄ analysis was filtered before freezing to remove particles larger than 0.45 µm. All water samples were maintained at a temperature below 4°C before analysis.

3.3 Laboratory Measurements

3.3.1 Chemical analysis

3.3.1.1 Ammoniacal nitrogen ($\text{NH}_3\text{-N}$)

The phenate method, modified from APHA (1989), was used. Principally, indophenol, an intensely blue compound, is formed by the reaction of ammonia, hypochlorite and phenol catalysed by a manganous salt.

The reagents were made up in the following manner: 10g of reagent grade phenol, $\text{C}_6\text{H}_5\text{OH}$, was dissolved in 100 mL of 95% ethyl alcohol, $\text{C}_2\text{H}_5\text{OH}$, to make the phenol-alcohol solution. For the sodium nitroprusside solution (5% w/v), 1 g sodium nitroprusside, $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO}\cdot 2\text{H}_2\text{O}$, was dissolved in 200 mL of water, storable for no more than a month. To make the alkaline citrate solution, 100 g of A. R. trisodium citrate, $\text{C}_3\text{H}_4\text{OH}(\text{COONa})_3\cdot 2\text{H}_2\text{O}$, and 5 g of A. R. sodium hydroxide, NaOH , was dissolved in 500 mL of water. The oxidizing solution consisted of 4 volumes of alkaline citrate solution and 1 volume of sodium hypochlorite, with the latter coming from a commercial hypochlorite solution, NaOCl .

The standard ammonia solution was prepared by dissolving 45.8 mg of ammonium chloride, NH_4Cl , in 1 L of distilled water, after which it was diluted ten times to give a working stock solution containing 1.2 μg of ammonia nitrogen per mL.

The standards comprised distilled water spiked with the standard ammonia solution of 0 mL, 0.25 mL, 0.5 mL and 1.0 mL to make up 50 mL of each (yielding concentrations of 0, 6, 12, and 24 μg $\text{NH}_3\text{-N}$ per litre). Triplicates of 50 mL seawater samples were made ready

100 mL conical flasks. To each standard solution and sample, 2 mL of phenol alcohol solution, 2 mL of sodium nitroprusside solution and 5 mL of oxidizing solution was added in sequence and mixed thoroughly. The blue colour of indophenol was allowed to develop at room temperature for at least one hour.

The extinction of standards was measured in a 10 cm cuvette against distilled water at 640 nm, using a 160 UV Shimadzu Spectrophotometer. Once a standard curve for the standards was produced, the extinction of each water sample was read and concentration obtained.

3.1.2 Dissolved orthophosphate (O-PO₄)

The ascorbic acid method (APHA, 1989) was used to determine dissolved orthophosphate, based on the principle that ammonium molybdate and potassium antimonyl tartrate react in an acid medium with orthophosphate to form a phosphomolybdic acid that is reduced to an intensely coloured molybdenum blue by ascorbic acid.

The paramolybdate solution was prepared by dissolving 15 g of A. R. ammonium paramolybdate, (NH₄)₆Mo₇O₂₄.4H₂O in 500 mL of distilled water. 140 mL A. R. concentrated H₂SO₄ was added to 900 mL of distilled water for the sulphuric acid solution. For the ascorbic acid solution, 5.4 g of high quality ascorbic acid, C₆H₈O₆, was dissolved in 100 mL of distilled water. The potassium antimonyl tartrate solution was prepared by dissolving 0.35 g of high-grade potassium antimonyl tartrate, KSbO₃.C₄H₄O₆, in 250 mL of distilled water. The mixed reagent was prepared by mixing 100 mL

ammonium molybdate, 250 mL sulphuric acid, 100 mL ascorbic acid and 50 mL potassium antimonyl tartrate solution.

The standard phosphate solution was prepared by dissolving 0.439 g of desiccated potassium dihydrogen phosphate, KH_2PO_4 , in distilled water, made up to 1 L. Chloroform was added prior to storage. A working solution of 1 L standard phosphate containing $1000 \mu\text{g PO}_4^{3-}\text{-P L}^{-1}$ was prepared by diluting 10 mL of the prepared stock.

Reference solutions containing 0, 2, 5 and 7 $\mu\text{g PO}_4^{3-}\text{-P}$ were obtained by taking 0, 2, 5 and 7 mL of the $1000 \mu\text{g PO}_4^{3-}\text{-P L}^{-1}$ standard solution and diluting to 100 mL with distilled water. The reference solutions were spiked with 0.5 mL mixed reagent each, and left for 30 minutes to allow colour development. Triplicates of 50 mL samples were measured out into conical flasks, to which 10 mL of mixed reagent was added. These were again left for 30 minutes to allow colour development.

The extinction of the four reference solutions was measured in a 10 cm cuvette against distilled water at a wavelength of 885 nm, using a 160 UV Shimadzu Spectrophotometer. Once the standard curve was obtained, the extinction of each sample was read and phosphate concentration determined.

3.3.1.3 Total suspended solids (TSS)

In accordance with APHA (1989) procedure, pre-weighed glass fibre filters were placed in a vacuum filtration apparatus and washed with three 20 mL volumes of distilled water. 100 mL samples were then filtered in triplicates and dried at 100°C for 72 hours, after which

they were left in the desiccator for 24 hours. These were then weighed and the drying process continued until a constant weight, or until a change of less than 0.5 mg was obtained. The increase in the weight of the filter was taken to be the total suspended solids content per 100 mL. The following calculation was used:

$$\text{mg TSS L}^{-1} = \frac{(\text{filter weight} + \text{residue, g}) - (\text{filter weight, g}) \times 1000}{\text{sample volume, mL}} \times 1000$$

3.3.2 Phycological analysis

3.3.2.1 Seaweed composition

All algal samples were identified up to species level. Where this was not possible, the specimens were accorded identification numbers (e.g. *Cladophora* sp. 1, *Cladophora* sp. 2). However, problems were anticipated to arise when making comparisons between the checklists of 1987/88 and 1998, since *Cladophora* sp. 1 in 1987/88 and *Cladophora* sp. 1 in 1998 may or may not be the same. In such cases, the two records of *Cladophora* sp. 1 were treated as separate species, and duly recorded as *Cladophora* sp. 1 (1987/88) and *Cladophora* sp. 1 (1998), respectively. The following references were used for taxonomic identification: Womersley (1984, 1987, 1994, 1996), Abbott (1988, 1992, 1994, 1995, 1997, 1999), Abbot & Norris (1985), Taylor (1967, 1969), Trono (1997) and Ismail (1995).

Algal species unique to one site were subsequently extracted from the species list and reported. Since taxonomic identification was performed down to the microscopic level, we assumed most dormant species to be reasonably accounted for.

3.3.2.2 Species richness of seaweeds

Species richness is defined as the absolute number of species found in a sampling unit or population. In this study, seaweed richness was extended to the rest of the taxa levels and hence, richness was also reported according to division, order, family and genus. In the statistical analysis, species richness alone was used.

3.3.2.3 Seaweed functional morphological groups

Each seaweed species was also classified according to its functional morphological growth form as described by Littler on the basis of photosynthetic production and susceptibility to grazing (Sze, 1998). Littler's description of each form is shown in Table 2.

Table 2 Functional forms of Littler

Functional-Form Group	External Morphology	Internal Anatomy	Texture
Sheet group	Thin tubular or sheet-like (foliose)	Uncorticated, one to several cells thick	Soft
Filamentous group	Delicately branched (filamentous)	Uniseriate, multiseriate, or lightly corticated	Soft
Coarsely branched group	Coarsely branched, upright	Pseudoparenchymatous or parenchymatous	Fleshy to wiry
Thick-leathery group	Thick blades and branches	Differentiated pseudoparenchymatous or parenchymatous, thick-walled	Leathery to rubbery
Jointed-calcareous group	Articulated, calcareous, upright	Calcified segments, flexible joints	Stony
Crustose group	Prostrate, encrusting	Calcified or uncalcified, compact row of cells	Stony, tough

Source: Sze (1998)

3.3.2.4 Total dry weight biomass

After identification, all seaweed samples from each quadrat were wrapped in pre-weighed aluminium foil and oven-dried at 100°C for 72 hours to determine total dry weight biomass. This was calculated in g m⁻² for each quadrat.

3.3.2.5 Diversity index (H')

Each species was assigned a proportion number, N , according to its percentage of the total wet biomass per quadrat (Table 3). These proportion numbers were then used as p in the diversity index.

Table 3 Distribution of seaweed wet biomass per quadrat according to the proportion number, N .

Percentage of biomass (%)	N
0 - 10	1
11 - 20	2
21 - 30	3
31 - 40	4
41 - 50	5
51 - 60	6
61 - 70	7
71 - 80	8
81 - 90	9
91 - 100	10

The selected diversity index was the Shannon and Weaver index (Phang, 1995), also popularly known as the Shannon index of diversity, H' , expressed as:

$$H' = - \sum_i p_i \log p_i$$

Where: p_i = proportion of species i in the sample

The base of the logarithm is arbitrary, but \log_{10} was used in this study to facilitate comparison to the indices of 1987/88. The H' index is zero when only one species is present, and increases with greater diversity. The maximum value of H' depends on the number of species present (species richness) and the distribution of relative abundance (evenness). When these two components are large, higher overall diversity is said to occur.

3.3.2.6 Evenness index (J)

By accounting only for the evenness component of overall diversity, the J index is an index of dominance, telling us whether there is an equitable spread of species in a community or not. The index lies within a 0 to 1 range, representing maximum and minimum dominance respectively. The evenness index (J) is expressed as (Cox, 1996):

$$J = \frac{H'}{\log S}$$

Where: H' = Shannon index

S = Number of species

3.3.2.7 Similarity quotient (S)

The similarity quotient may be used to compare the species diversities of two systems, particularly in the juxtaposition of an unpolluted site with a polluted one (Dash, 1994).

Sorensen's similarity quotient, S , is expressed as (Mueller-Dombois & Ellenberg, 1974):

$$S = (2C/A + B) 100$$

Where: A = number of species in one site
 B = number of species in another site
 C = number of species common to both sites

3.3.2.8 Frequency, dominance, and the importance value index (IVI)

The frequency of occurrence of a species is provided by the fraction of quadrats that contain the species, whilst dominance in this case is taken to be a species's fraction of the total wet biomass (from the N scores in Table 3). Like in the study of 1987/88, frequency and dominance were reported in the percentage (%) form:

$$\text{Frequency (\%)} = \frac{\text{Number of quadrats in which a species occurs}}{\text{Total number of quadrats sampled}} \times 100$$

$$\text{Dominance (\%)} = \frac{\text{Total wet biomass values for a species (N)}}{\text{Total wet biomass values for all species}} \times 100$$

Dominance was calculated as an overall value for each site, as shown above, and also in the form of intertidal dominance. In the latter, the dominance of each species was first calculated for each quadrat, after which the values of the three transects were pooled in order to obtain a picture of seaweed dominance stretching from the baseline to the reef edge.

The Importance Value Index (IVI), which was calculated as an average of relative frequency and relative dominance (Cox, 1996), required the expression of these values in the absolute (f & d) and relative (Rf & Rd) forms. As a combination of two or more measures of abundance (often, relative density is also included, though not in this study),

the IVI has been considered to make a more comprehensive estimate of the importance of a species in a stand than is given by any one of the measures of abundance (Greig-Smith, 1983). The following formulae from Cox (1996) were used:

$$\text{Frequency value } (f) = \frac{\text{Number of quadrats in which a species occurs}}{\text{Total number of quadrats sampled}}$$

$$\text{Relative frequency } (Rf) = \frac{\text{Frequency value for a species } (f)}{\text{Total frequency values for all species}} \times 100$$

$$\text{Dominance value } (d) = \frac{\text{Total wet biomass values for a species}}{\text{Area sampled}}$$

$$\text{Relative dominance } (Rd) = \frac{\text{Dominance value for a species } (d)}{\text{Total dominance values for all species}} \times 100$$

$$\text{Importance value index (IVI)} = \frac{\text{Relative frequency } (Rf) + \text{relative dominance } (Rd)}{2}$$

3.4 Statistical analysis

The statistical techniques applied to the data were the One-Way Analysis of Variance (ANOVA), Newman-Keuls Post Hoc Test, Simple Correlation, Regression analysis, Ordination, and Cluster analysis. All analyses were performed using Statistica Version 5 except for the Cluster and Ordination analyses, for which MVSP (Multi-Variate Statistical Package) version 3.1 for Windows was used.

A one-way analysis of variance (ANOVA) was performed to assess the between-site differences in water quality and biotic variables. The $\log_{10}(x + 1)$ transformation was used

to normalise data used in this analysis. The ANOVA was followed by the Newman-Keuls post-hoc test to identify the sites contributing most to the significant differences in means.

Principal components analysis (PCA) was performed separately on the water quality variables and biotic variables. Euclidean biplots were plotted to determine the relative contributions of the various variables to the variation in the data.

Simple Correlation was used to determine the relationships between and amongst water quality and biotic variables. Regression analysis was performed to identify the water quality variables that contributed most to the various biotic variables. Although correlation and clustering may seem redundant in the light of ordination, these were performed simply to quantify and verify variable relationships already detected in the Euclidean biplots of ordination analysis.

Clustering techniques were applied to identify different groupings of the sites based on water quality and biotic variables. The chosen technique was the Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) based on Gower's General Similarity Coefficient. Data for the variables were standardised prior to analysis.

Since it was necessary to address the issue of changes between the surveys of 1987/88 and 1998, a second statistical design was drawn up. First, two-way analysis of variance (ANOVA) was performed to compare changes in water quality and biotic variables between the surveys of 1987/88 and 1998, as well as to compare the effect of the sampling occasion (temporal) factor with the site (location) factor. The Newman-Keuls post-hoc

test was used to identify the sampling occasions contributing most to the significant differences in means. Correlation analysis was performed to determine relationships between the biotic and water quality variables, followed by Regression analysis, in which the water quality variables that contributed most to each of the biotic variables were identified. Finally, two-way ANOVA was performed on the pooled datasets of 1987/88 versus that of 1998 in order to determine significant changes specifically between the two surveys. This final step was essentially the main objective of the comparative analysis and therefore, much of the discussion has centred on the issue of changes between the two surveys.

Table 4 Summary of the statistical analyses.

Objective	Statistical test
1998 survey	
Differences in the water quality means between sites A, B and C.	One-way ANOVA followed by the Newman-Keuls post-hoc test.
Differences in the biotic variable means between sites A, B and C.	One-way ANOVA followed by the Newman-Keuls post-hoc test.
Depiction of relationship between water quality variables and sampling points.	Principal components analysis (PCA)
Depiction of relationship between biotic variables and sampling points.	Principal components analysis (PCA)
Associations between and amongst the water quality and biotic variables.	Simple Correlation
Identification of water quality variables that most affect a dependent biotic variable.	Regression Analysis
Comparative analysis between the surveys of 1987/88 and 1998	
Differences in the water quality means between sampling occasions (May 1987, Aug 1987, Nov 1987, March 1988 and 1998).	Two-way ANOVA followed by the Newman-Keuls post-hoc test.
Differences in the biotic variable means between sampling occasions (May 1987, Aug 1987, Nov 1987, March 1988 and 1998).	Two-way ANOVA followed by the Newman-Keuls post-hoc test.
Differences in the water quality means between the surveys of 1987/88 and 1998.	Two-way ANOVA

Table 4 Summary of the statistical analyses [Continued].

Comparative analysis between the surveys of 1987/88 and 1998	
Differences in the biotic variable means between the surveys of 1987/88 and 1998.	Two-way ANOVA
Associations between and amongst the water quality and biotic variables of 1987/88.	Simple Correlation
Identification of water quality variables that most affect a dependent biotic variable.	Regression Analysis