DETAIL MORPHOLOGICAL STUDIES OF DIATOM SPECIES (BACILLARIOPHYCEAE) IN SARAWAK ESTUARIES USING TRANSMISSION AND SCANNING ELECTRON MICROSCOPY

FAREHA BINTI HAJI HILALUDDIN

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List of Abbreviations

Scanning Electron Microscope	SEM
Transmission Electron Microscope	TEM
Light Microscope	LM

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Fareha binti Haji Hilaluddin

Aquatic Resource Science and Management Program
Department of Aquatic Resource Science and Management
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

Abstract

A study was carried out to examine the detail morphological of diatom species in Malaysia. The sampling was conducted fortnightly at two selected estuarine water in Kuching, Sarawak; Santubong and Samariang Batu, from September 2008 till February 2009. Plankton samples were collected by using 20μm mesh-size plankton net. Samples then were kept in cool and taken to laboratory for further analysis. Samples were treated with acid wash (Hasle, 1970) before observed under light and electron microscope. The permanent slides were prepared for each sample for documentation. All samples were identified to species level based on TEM and SEM observation. Forty six species of diatom from 27 genera have been documented in this study. Diatom genera that have been represent in this study were including *Thalassiosira* sp., *Actinocyclus* sp., *Coscinodiscus* sp., *Azpeitia* sp., *Cyclotella* sp., *Bacteriastrum* sp., *Chaetoceros* sp., *Hemiaulus* sp., *Odontella* sp., *Ditylum* sp., *Triceratium* sp., *Amphiprora* sp., *Amphora* sp., *Surirella* sp., *Cocconeis* sp., *Frustulia* sp., *Petroneis* sp., *Navicula* sp., *Delphineis* sp., *Bacillaria* sp., *Cylindrotheca* sp., *Nitzschia* sp., *Lioloma* sp., *Thalassionema* sp., *Pleurosigma* sp., *Pseudo-nitzschia* sp., and *Diploneis* sp.. This represented one of the few studies of diatom using electron microscopy in Malaysian waters.

Key word: Detail morphological diatom, TEM, SEM, acid wash.

Abstrak

Satu kajian telah dijalankan untuk megenalpasti morfologi spesies diatom di Malaysia secara terperinci. Persampelan telah dijalankan dua minggu sekali di dua muara sungai Kuching, Sarawak; Santubong dan Samariang Batu, dari September 2008 sehingga Februari 2009. Pengumpulan sampel plankton telah dijalankan dengan menggunakan jaring plankton yang bersaiz 20μm. Sampel yang telah dikumpul kemudiannya disimpan di tempat sejuk dan dibawa ke makmal untuk proses analisis selanjutnya. Sampel dicuci menggunakan kaedah pencucian asid (Hasle, 1970) sebelum diperhatikan di bawah mikroskop elektron. Slaid kekal telah disediakan bagi setiap sampel untuk dokumentasi. Semua sampel telah dikenalpasti sehingga peringkat species berdasarkan pemerhatian di bawah TEM dan SEM. Empat puluh enam spesies diatom daripada 27 genera telah dikenalpasti dalam kajian ini. Genera diatom yang telah dikumpulkan dalam kajian ini termasuklah Thalassiosira sp., *Actinocyclus* sp., *Coscinodiscus* sp., *Azpeitia* sp., *Cyclotella* sp., *Bacteriastrum* sp., *Chaetoceros* sp., *Hemiaulus* sp., *Odontella* sp., *Ditylum* sp., *Triceratium* sp., *Amphiprora* sp., *Amphora* sp., *Surirella* sp., *Cocconeis* sp., *Frustulia* sp., *Petroneis* sp., *Navicula* sp., *Delphineis* sp., *Bacillaria* sp., *Cylindrotheca* sp., *Nitzschia* sp., *Lioloma* sp., *Thalassionema* sp., *Pleurosigma* sp., *Pseudo-nitzschia* sp., and *Diploneis* sp. *Kajian ini mewakili salah satu kajian diatom yang menggunakan mikroskop elektron di perairan Malaysia.*

Kata kunci: Morfologi diatom terperinci, TEM, SEM, pencucian asid.

1.0 Introduction

Phytoplankton is a microscopic single cell algae that photosynthesize at the surface of the water. They are the important component in the food webs as a primary producer. When they die, they sunk to the sea floor and provide a food source to deep-sea animals. They lack of swimming ability and move by water current. Many microalgae species also produce new compounds that show potent biological activities, especially secondary metabolites that have been identified to responsible for human illnesses.

Diatoms represent one of the most important groups of marine phytoplankton and are characterized by having a siliceous cell wall (frustule). They contribute up to 45% of the total primary production in the ocean (Mann 1999), or 20-25% globally (Werner 1977). The members of this class are mostly unicellular, with some member formed chain of cells and colonial aggregations. A significant number of fossil diatoms are also known, the earliest types being marine species date from the Cretaceous (Patrick and Reimer, 1966). Diatoms belong to phylum Bacillariophyceae with approximately 100,000 of known species. Most of them are photosynthetic micro-organisms, although some may live heterotrophically. Different species of diatom can be identified based on the structure of their cell walls feature.

Diatoms can be divided into three types based on ecological strategy which is euplanktonic diatoms, benthic (periphytic diatoms), and meroplanktonic (tycoplankton) diatoms (Kühn *et. al.*, 1981). Euplanktonic are permanent members of the plankton. In coastal waters and lakes, euplanktonic diatoms may survive between growing seasons as cysts that settle to the bottom and become inactive (Smetacek, 1985). In both freshwater and marine habitats, diatoms are often the initial algal colonizers on submerged substrates (Philip, 1993). Many diatoms move by means of their raphe system. Epiphytic diatoms are attached to their hosts permanently by a mucilaginous pad. The mucilage is derived from golgi vehicles and secreted through pores or a raphe (Blunn and Evans, 1981). Meroplanktonic diatoms are temporary members of the plankton. Pennate diatoms live without associated with substrate and during the period of turbulence which carried out into the open waters.

Diatoms have been well studied both in their natural habitat and in cultures by biologists and there is therefore a wealth of knowledge on their biology and ecology. The protoplast of diatoms consists of a cytoplasmic layer that lines the interior of the frustule and surrounds a large central vacuole, within the cytoplasmic layer there is a diploid nucleus and two to several pigment-bearing plastids (the site of photosynthesis) (Round *et. al.*, 1990).

Identification of diatoms usually must rely on the siliceous frustule. Shape, size, number, and arrangement of chloroplasts and the presence or absence of pyrenoids may also used for identification on the generic and specific level. With an increasing amount of information on details of the siliceous diatom cell wall, especially obtained with electron microscopy, a need for a generally accepted terminology became evident in the early 1970s. The first attempt along this line was published in 1975 as "Proposals for a standardization of diatom terminology" (Anonymous, 1975) followed by "An Amended Terminology for the Siliceous Components of the Diatom Cell Wall" (Ross et al., 1979).

Some new terms were introduced when the fine structure of pennate diatoms become more extensively studied (Mann, 1978; Cox & Ross, 1981; Williams, 1985, 1986). Terms specific to certain centric diatom families or genera, partly applicable to light microscopy, were also suggested (Hasle et al., 1983; Sundström, 1986; Rines & Hargraves, 1988). Factor such as the shape, pattern, size and structure of the cell wall in diatoms are highly species specific. Phytoplankton taxonomists rely on these differences of the frustule or the cell wall between species to identify the different species.

1.1 Gross Morphology

The gross morphology of the diatom frustule and structures more generally distributed within the class are defined below. The definitions of the terms may include elements that cannot be revealed under light microscopy. For example, the tubular parts of strutted processes may be visible in the light microscope, while the satellite pores are usually not observable.

Apical axis	: long axis of a bilateral diatom, axis between the poles of a frustule.
Pervalvar axis	: axis through the center point of the two valves.
Transapical axis	: third axis of a bilateral diatom.
Valvar plane	: Parallel to the valves-plane division.
Apical plane	: Perpendicular to the transapical axis.
Transapical plane	: Perpendicular to the apical axis.
Valve view	: Frustule seen from top or bottom.
Broad girdle view	: Frustule seen from broad side.
Narrow girdle view	: frustule seen from narrow side.

1.2 Diatom Classification

Diatoms may be classified into two major groups, the centric diatoms under order Centrales (or Biddulphiales) which are radially symmetrical and the pennate diatoms of the order Pennales (or Bacillariales) which are bilaterally symmetrical. The classification system developed by Simonsen (1979) and further developed by Round et al. (1990) is currently the most commonly accepted up to date. Centric diatoms including three sub-orders based primarily on the shape of the cells, the polarity and the arrangement of the processes. These are the Coscinodiscineae, with a marginal ring of processes and no polarity to the symmetry, the Rhizosoleniineae with no marginal ring of processes and unipolar symmetry, and the Biddulphiineae with a marginal ring of processes and bipolar symmetry.

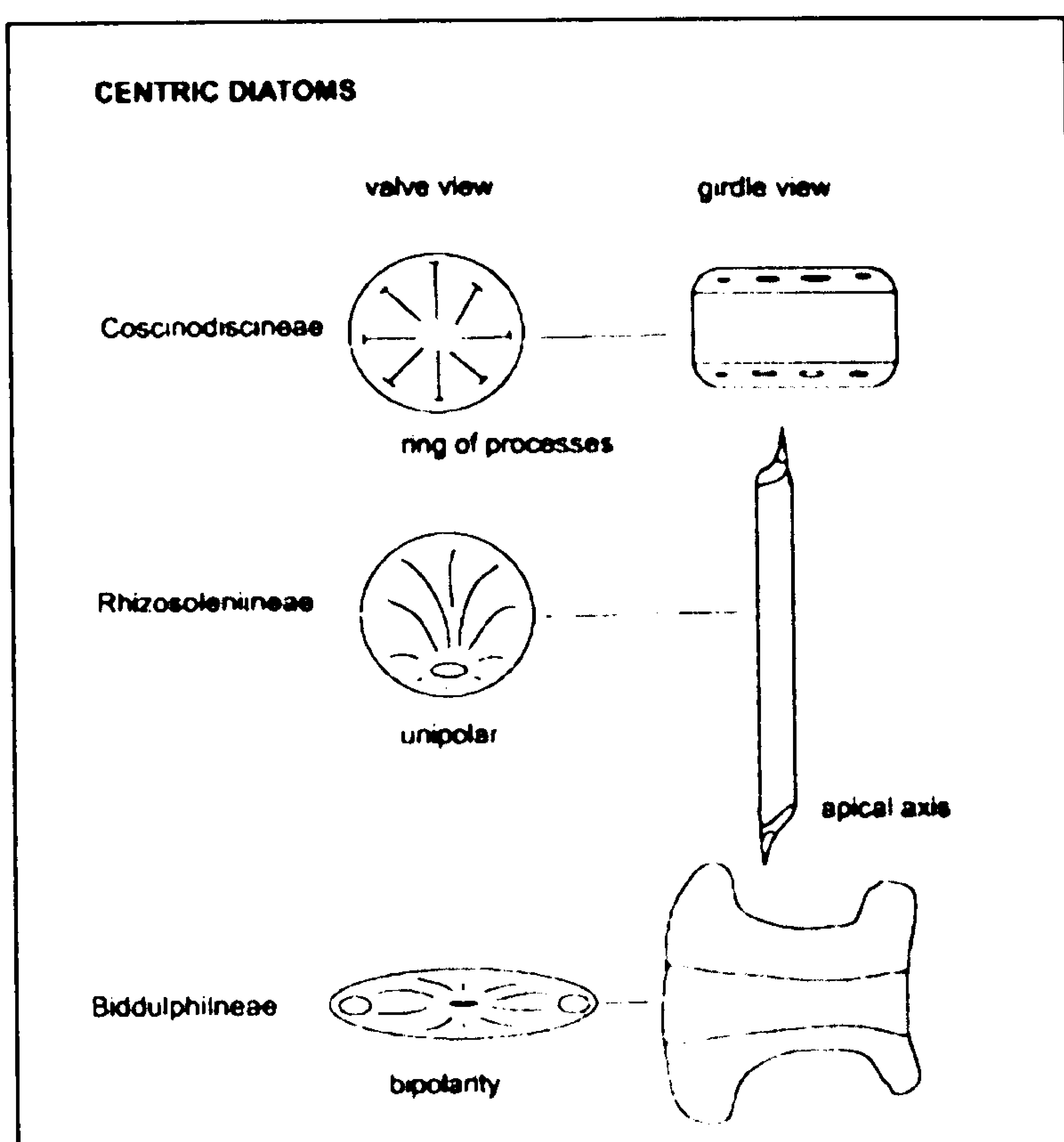

Figure 1.1 Schematic diagram of centric diatom suborders (Hasle & Syvertsen, 1997)

The pennate diatoms, which have ability for motility than centric are divided into two sub-orders, the Fragilariineae which do not posses a raphe (araphid) and the Bacillariineae which posses a raphe. The raphe (a silt-like channel) facilitates the secretion of a mucilaginous material which apparently aids movement of a diatom along a substrate.

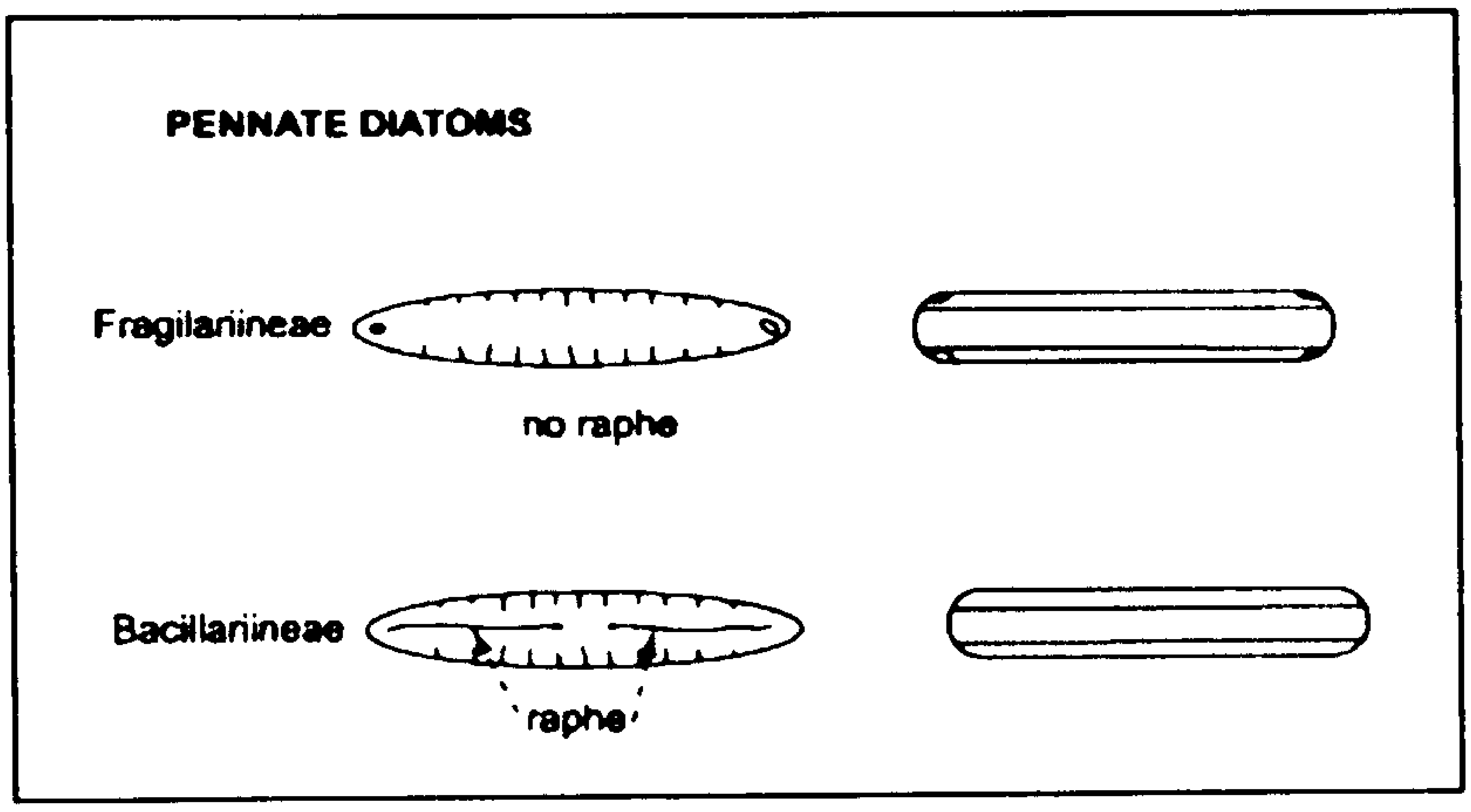

Figure 1.2 Schematic diagrams of pennate diatom suborders (Hasle & Syvertsen, 1997)

The diatom frustule is often likened to a pill-box or agar dish with an epitheca (larger upper valve), and a hypotheca (smaller lower valve). The vertical lip or rim of the epitheca is called the epicingulum, and the epicingulum fits over (slightly overlaps) the hypocingulum of the hypotheca. The epicingulum and hypocingulum with one or several connective bands make up the girdle. Many diatoms are heterovalvate with the two valves of the frustule are difference. This is most obvious within the family Achnanthaceae where one valve has a raphe and the other does not, and the Cymatosiraceae where one valve has a tubular process and the other does not. Chain-forming species with cells linked together by siliceous structures may, in addition, have separation valves. These valves are morphologically different from the valves within the chain. Therefore, one species may have four morphologically distinct types of valves.

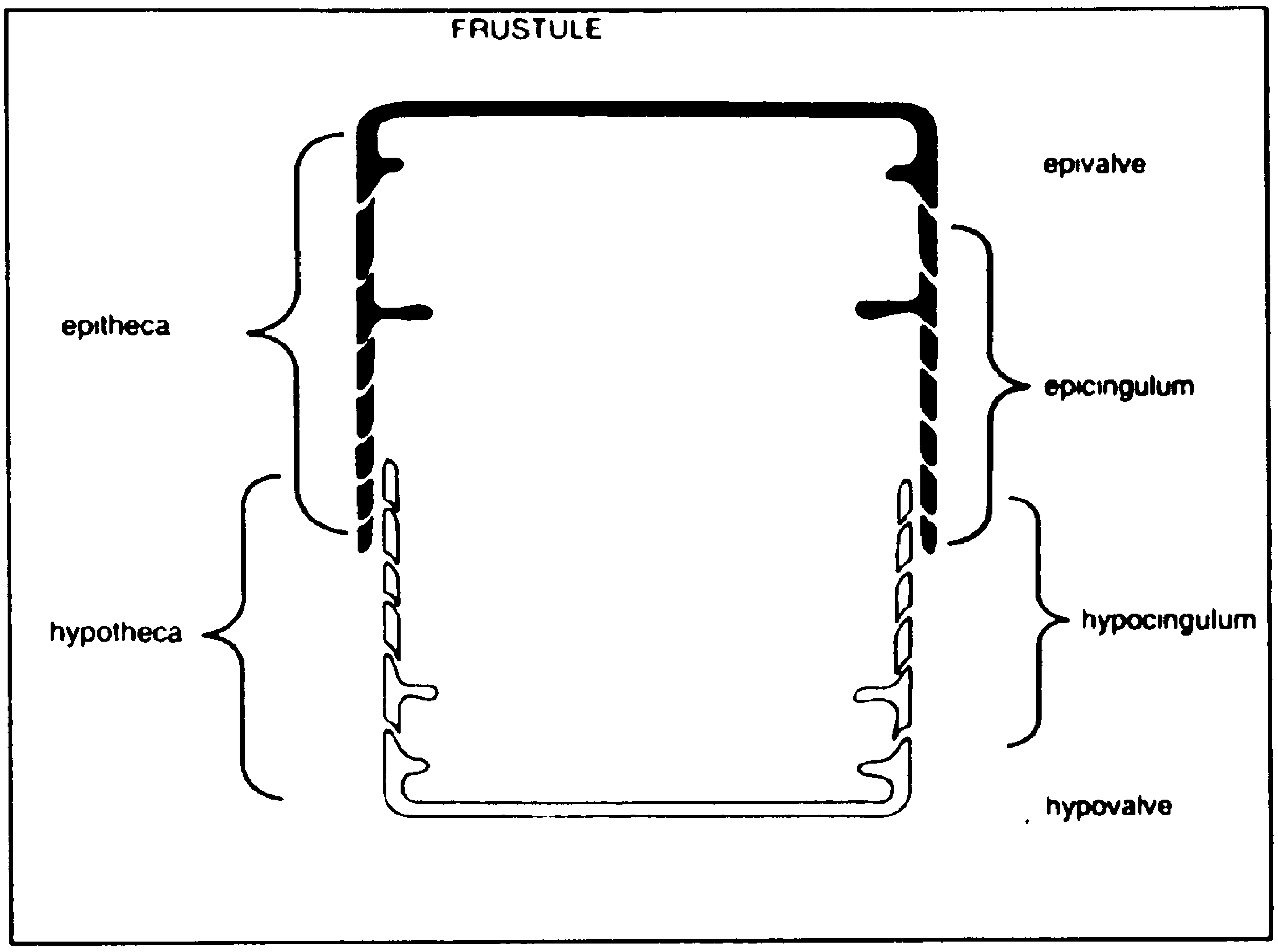

Figure 1.3 Diagrammatic section showing frustule's terminology

This study was carried out to examine the detailed morphological structure of diatom species that found in estuarine water of Malaysia. In Malaysia, the detail morphological studies of marine and brackish diatom are still very limited. Identification was carried out mostly to genus level by light microscopy. This study was carried out in estuarine water of Sarawak. Sampling was conducted at three selected estuarine water. The collected samples were brought back to laboratory and prepared accordingly for light microscope (LM) and electron microscope (EM).

2.0 Literature review

2.1 Taxonomy and Phylogeny of Diatom

Diatoms are the most species rich group of algae with tens of thousands of species (Mann 1999). Diatom taxonomy is in a state of revolutionary change involving splitting of genera, creation of new ones and revision of criteria for classification. With molecular genetics and breeding as added tools, taxonomy of diatoms has become a dynamic field of research. Diatoms have also been widely applied in palaeoecological reconstruction of past environmental conditions. Accurate identification of taxa with information on geographical distribution is essential for the use of diatoms as reliable indicators.

Diatom has been studied since the late eighteenth century. In the early twentieth, a century fossil diatom was first studied and became famous. Hustedt had produced a taxonomic and ecological study of diatoms which remains a key reference today. One of most complete references of diatoms was created by Round *et. al.* (1990). In the late twentieth, a taxonomic study on marine diatoms was carried out by M. De Stefano and D. Marino to characterize the epiphytic diatom communities on seagrass leaves.

A new species, *Cocconeis multiperforata*, and two new varieties, *C. neothumensis* var. *marina* and *C. scutellum* var. *posidoniae*, were proposed due to some ultrastructural characteristics of their valve areolation. Ultrastructural analysis also revealed new morphological characteristics in *C. britannica* Naegeli (Kützing) and *C. maxima* Grunow Peragallo & Peragallo, and confirmed observations of other authors for *C. molesta* var. *crucifera* Grunow, *C. pellucida* Grunow (Rabenhorst) and *C. stauroneiformis* (Rabenhorst) Okuno. Addition information on their distribution and their ecological habitat was also documented. Medlin and Kaczmarska (2004) have presented an alternative classification of Round *et. al.*, based on combined the molecular and morphological support.

2.2 Morphological and terminology of diatom

Occurrences of diatoms were behaved from the Jurassic. However, these records are uncertain because there were not enough references. A well preserved diatom from the Aptian-Albian stages of the Cretaceous was the earliest recorded in centric forms. Since these are quite diverse assemblages it is assumed diatoms have an earlier evolutionary history, perhaps lacking a relatively robust silica frustule. As with coccoliths, the earliest forms in the fossil record may reflect a biomineralisation event rather than an evolutionary appearance.

Study of diatom has major advancement in the field with the enhancement of microscope resolution in the 19^{th} century. Observations made with the light microscope have provided the foundation upon. The present knowledge of diatom wall structure was based the innovation of electron microscopy (Amspoker & Czarnecki, 1982). Such a complex wall structure has generated an abundance of terms in the description of the elements of the wall, and a revised terminology has been formulated in the hope that standardization of terms will result in greater clarity of information (Harold, 1978).

A requirement for a generally accepted terminology had became an evident in the early 1970s as the rising amount of information on details siliceous diatom cell wall that obtained from observation under electron microscopy. The earliest araphid (lacking a raphe) pennate diatoms appear in the Late Cretaceous, and raphid pennates in the Middle Eocene. When the fine structure of pennate diatom becomes more extensively studied, the new terms were introduced (Mann, 1978, 1981; Cox & Ross, 1981). The term that specific to definite centric diatom families and genera applicable that under light microscope was also suggested (Hasle *et. al.*, 1983; Sundstrom, 1986; Rines & Hargraves, 1988).

The origin of the diatom cell has been a subject of speculation. Cell of diatoms are uninucleate, the nucleus being either suspended in the centre by cytoplasmic strands or displaced to one side of the cell. The chloroplasts, whose shape and number show great variation, are the conspicuous feature of the living cell (Harold, 1978). They may be numerous discoid structures randomly distributed, such as in *Coscinodiscus* ; or in a stellate configuration, such as in *Striatella*; one or two platelike axile or peripheral structures, such as *Pinnularia*; or a single, lobed, H-shaped structure, such as in *Gamphonema* (Dawson, 1973a) and *Cocconeis* (Taylor, 1972).

2.3 Status of diatom studies in the coastal area of Malaysia

In the coastal area of Malaysia, the studies about plankton were very limited, moreover in diatom species. According to Chua & Chong (1973), the different water hydrography and the distribution pattern of plankton will dominate the distribution of pelagic fishes in the Strait of Malacca. Shamsuddin (1987), state that most of the diatom species that exist in the coastal area of Port Dickson are *Biddulphia sinensis, Coscinodiscus asteromophalus, Chaetoceros lorenzianus, C. coarctatus, Nitzschia closterium* and *Rhizosolenia styliformis*. Besides that, *Eucampia sp., Guinardia sp.* and *Bacteriastrum sp.* were also found at the coastal area. He had also documented most of the microplankton species that he found in coastal water were different than the species that exist in the open sea (Shamsuddin, 1990).

Shamsuddin *et. al.*, (1988) had documented the distribution of diatom in the coastal area of Sarawak. They had collected samples of microphytoplanktons from 16 stations where mostly from diatom species and green-blue algae. Diatom genera that found commonly along the coastal water of Sarawak were included *Chaetoceros, Rhizosolenia, Melosira, Thalassiothrix, Dactyliosolen* and *Guinardia*. However, many others species of diatom that also found were including *Bacteriastrum, Asterionella, Fragilaria, Nitzschia, Skeletonema, Coscinodiscus* and *Pleurosigma* (Shamsuddin *et. al.*, 1988). There were about 30 important species that have been documented along Sarawak coastal and were found in high distribution. The diatom from genus *Coscinodiscus, Chaetoceros* and *Rhizosolenia* were also found of distribution widely. Most of the diatom has been identified to genera level.

3.0 Materials and Methods

Sampling was carried out at two selected estuarine water in Kuching area fortnightly from September 2008 till February 2009 (Figure 3.1).

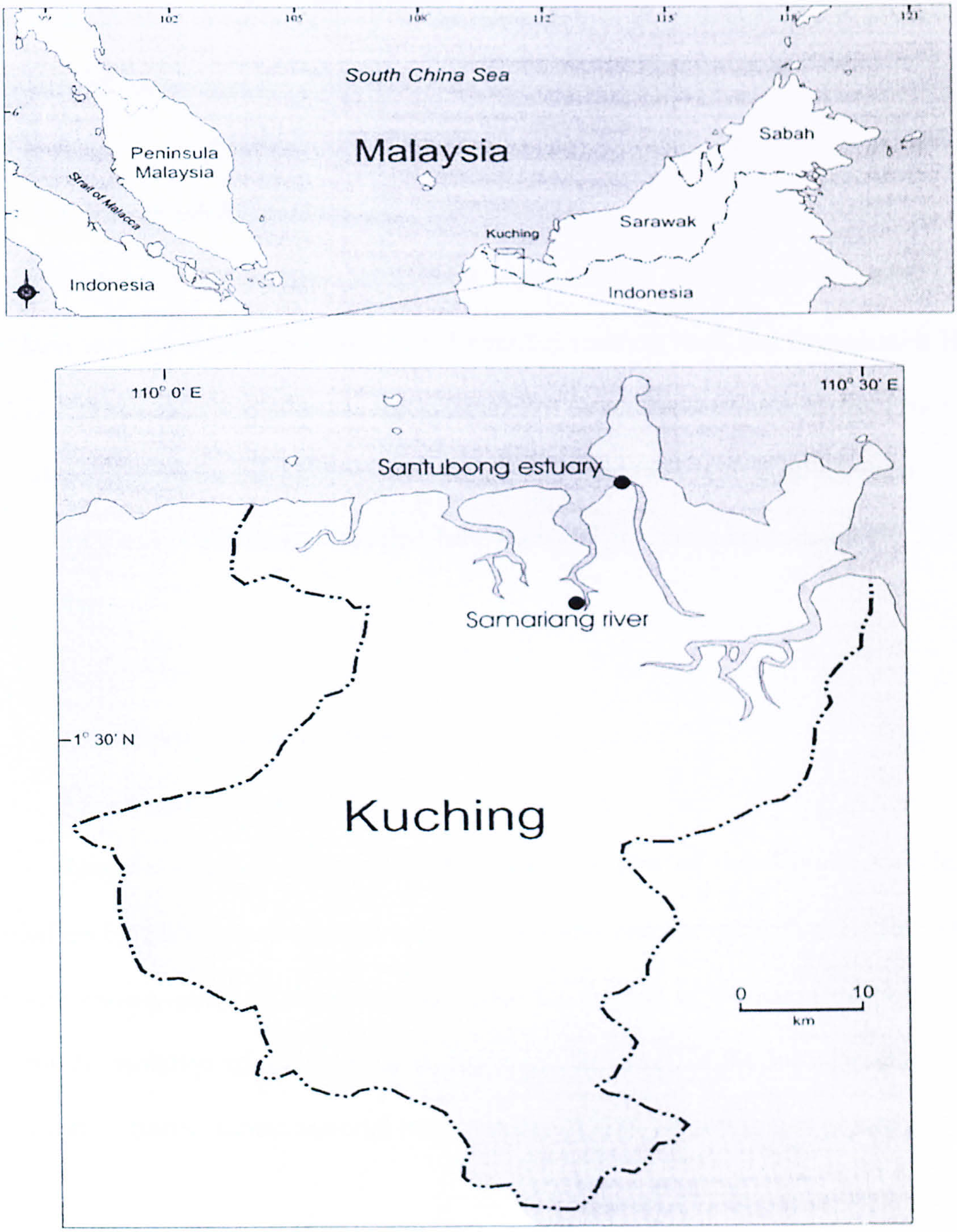

Figure 3.1 Sampling sites in this study, Santubong, and Samariang river

3.1 Sample collection

Samples were collected by using 20μm mesh-size plankton net. Two samples from each station were collected and kept in a 2L sample's bottle. The collected samples then were brought back to the laboratory. In the laboratory, acid wash methods by Hasle (1970) were carried out to clean the diatom for their qualitative purposed.

3.2 Acid Wash Methods (Hasle, 1970)

Samples were process using acid wash method according to Hasle, (1970). Plankton samples were transferred into 100ml Erlenmeyer flask and treated with H_2SO_4 (98%). $KMnO_4$ then was added to the sample until sample in the purple color. Oxalic acid was added to get the clear solution. The sample was then rinse with distilled water and was tested using pH paper until it become less acidic. Then it was centrifuged for 10min at 3000-4000rpm.

3.3 Laboratory identification

3.3.1 Light Microscope (LM)

Samples were observed under LM. Identification of the diatoms samples was carried on by using phase contrast optics. Compound microscope with a 10x objective is the best choice which allowing enough space for the use of sufficient magnification to control the isolation of the single cell. However, the study of the finer structures of the silica wall requires cleaned material and a 40x dry or 100x oil immersion objectives.

3.3.2 Transmission Electron Microscope (TEM)

For TEM, a drop of an aqueous suspension of cleaned material is put onto a foamvar-coated copper grid (Sournia, 1978, p.138). After air drying by silica gel, it was observed under TEM model JEOL JEM-1230 (Tokyo, Japan). The contrast between heavily silicified parts of the valves and less silicified structures can be observed. Finer details like vela are easily lost at the cost of coarser details that need a different printing. Wall is seen as one image, except when stereo pictures are used. This makes interpretation of the image demanding and considerable experience is often needed to arrive at the right conclusion.

3.3.3 Scanning Electron Microscope (SEM)

The same procedure can be followed for SEM, except that the acid wash sample is put onto a small coverslip. Samples were dried in a special manner that prevents them from shriveling. This is because the SEM illuminates them with electrons and they also have to be made to conduct electricity. Samples have to be prepared carefully to withstand the vacuum inside the microscope. After air drying, the material has to be coated using platinum under Auto Fine Coater model JEOL JFC-1600 to make sample become more conductive while observe under SEM model JEOL JSM-6390LA (Tokyo, Japan).

4.0 Results and Discussion

Diatom had represented as important group of plankton in the environment as a primary producer. Diatoms have been well studied both in their natural habitat and in cultures by biologists. Wealth knowledge on their biology and ecology had been document by biologists. However, the study about its morphology was still very limited moreover in Malaysia. The first references about diatom morphology had been documented by Round *et. al.* in his book *'The diatoms: biology & morphology of the genera'* which been published in 1990. In this book, the species of diatom was cannot been referred because this book only showed characteristic until genus level.

In the coastal area of Malaysia, the studies about morphology of plankton were very limited. Recent study about detail morphology of diatom species in Malaysia had been carried out by Shamsuddin (1990). In his study, he had collected diatom sample along the coastal zone in Malaysia since 1979. Shamsuddin had represented his result in his book *'Diatom marine di perairan Malaysia'* which had been published in 1990. This book had been the only reference that can be use by biologist and researcher to study about diatom in Malaysia. However, the picture of the species in his study was not clear and most of the samples were identified until generic level.

In this study, fourteen samples from seven sampling in different area over the eight month period been identified based on their detailed characteristic. Their detail process pattern, size, character of striae and aerolea, foramina, fibulae and poroid structure were observed in each specimen to examine their species. The specimen were shown below in two table based on their group; whether centric or pennate diatom.

The diatom species that had been found were showed in table below:

Table 1 Centric diatom species that had been found in Sarawak estuarine water

No.	Centric Diatom
1	*Thalassiosira pacifica*
2	*Thalassiosira angulata*
3	*Thalassiosira tenera*
4	*Thalassiosira gravida*
5	*Thalassiosira diporocyclus*
6	*Actinocyclus sagittulus*
7	*Coscinodiscus radiatus*
8	*Coscinodiscus marginatus*
9	*Coscinodiscus asteromphalus*
10	*Azpeitia nodulifera*
11	*Cyclotella litoralis*
12	*Bacteriastrum delicatum*
13	*Chaetoceros lorenzianus*
14	*Chaetoceros* sp.
15	*Hemiaulus hauckii*
16	*Odontella mobiliensis*
17	*Odontella sinensis*
18	*Ditylum sol*
19	*Ditylum brightwellii*
20	*Triceratium favus*
21	*Triceratium revale*