2: MATERIAL AND METHODS

2.1 Samples

Samples of freshwater or marine diatoms were obtained from a wide variety of habitats, (see Appendix 1). If possible, a sample was examined soon after collection while any diatoms present remained healthy and (in the case of raphid diatoms), motile. Studies of chromatophores and other cytological detail were made at this stage.

Sometimes, especially where the epipelon was to be studied, an attempt was made to separate the diatoms from their substrate, so as to facilitate acid cleaning and subsequent microscopic examination. Epipelic diatoms were separated from sediment using the techniques developed by Eaton & Moss (1966). In this case, three layers of lens tissue were placed on the sediment surface, but often only the upper two were removed for further examination and treatment; the separation of diatoms from sediment was often incomplete but this did not matter since quantitative estimates of species abundance per unit area were not required. A partial separation of epipelon and epipsammon was sometimes made, using the method of Round (1965).

Various permanent microscope preparations were also examined. These were obtained from the collections of Dr.F.E.Round; the Botany Department, University of Bristol; the British Museum; Naturhistorisches Museum Wien; the Hustedt Collection at Bremerhaven.

2.2 Cytological Studies

Wherever possible, diatoms were observed and drawn when alive, but this was often impractical because of their motility. Thus, some form of 'inactivating agent' often had to be employed, either a fixative or a compound known to affect movement without apparent adverse effect upon the cell.

2.2.1 Fixatives

Various fixatives were tried. Acetic acid, ethanol and acetic alcohol (1 part acetic acid : 3 parts ethanol) all gave poor preservation of the chromatophores and their use was discontinued. A 1.5% solution of potassium dichromate gave a lifelike preservation of the cell contents but solutions of formaldehyde and glutaraldehyde, (in concentrations of 1-4%), were found the most satisfactory. The formaldehyde was buffered at pH 7.2, using a phosphate buffer (see Jensen 1962). For marine diatoms the various aqueous fixatives were usually made up in sea-water.

Diatoms were observed and drawn while they remained in the fixative. Although this procedure was noxious and unpleasant where aldehydes were being used, it was considered preferable to the alternative process, viz. the preparation of permanent mounts, involving washing, dehydration, staining, etc., which would have introduced further distortion of the cell contents.

Aceto-carmine was used as a combined fixative and stain for nuclei. Diatoms were mounted in a drop of aceto-carmine on a slide, a cover-slip added and the preparation heated for a few seconds prior to microscopical examination. The chromatophores were much distorted by this procedure, but nuclear detail was preserved well.

2.2.2 Immobilisers

In a paper in which they discussed possible mechanisms involved in diatom motility, Drum & Hopkins (1966) noted that certain drugs inhibit locomotion without having much adverse effect upon other functions of the cell. Two of these compounds, Ephedrine and Caffeine (used in 0.1% solution), were therefore tested for their suitability in aiding the observation of chromatophores through their prevention of cell locomotion. Ephedrine was found to be quite effective,

although some cells 'woke up' too soon or showed signs of intracellular damage. It would seem that Ephedrine may be useful in checks upon results obtained from the study of fixed cells.

2.3 Preparation of cleaned samples

After samples had been examined as above, they were treated in such a way as to remove all organic matter, leaving only the diatom frustules and other siliceous material. Oxidation of the organic matter was achieved by various means which may be divided into 'wet' and 'dry' types.

2.3.1 Wet methods

These depend for the most part on the use of concentrated mineral acids to effect oxidation and often require some form of pre-treatment, for instance, to remove calcium salts if sulphuric acid is to be used subsequently.

2.3.1.1 Pre-treatments

a. Hydrogen peroxide.

Hot, approx. 28% ('100 volume') hydrogen peroxide was used to effect preliminary oxidation and hence to bring substances into solution or at least to reduce the mean particle size within the sample, thus increasing the available surface area of the sample and consequently increasing the efficiency of subsequent oxidative treatments. In general, however, the degree of oxidation achieved with hydrogen peroxide was disappointing and this compound was used usually only for the removal of epiphytic diatoms from their substrata.

b. Hydrochloric acid.

Concentrated hydrochloric acid (approx. 36%) was at first used in the manner of Hendey (1964: see also Buzer 1975, Cox 1975c) to bring calcium into solution. After treatment with this acid, most of

the calcium originally present in the sample could be removed by washing the residue repeatedly with distilled water and thus the formation of insoluble calcium sulphate, on subsequent treatment with sulphuric acid, could be prevented. The use of HCl was discontinued, however, since this acid contributes little to the process of oxidation, unlike nitric acid, which is also somewhat to be preferred in that fewer nitrates are insoluble than chlorides.

c. Nitric acid.

This was used either as the standard 70% solution or as the fuming product (95-98%).The latter, heated to its B.Pt., had a fairly rapid effect, oxidising much of the organic matter present and bringing calcium into solution: sometimes, further oxidative treatment was unnecessary after pretreatment with fuming nitric acid. Longer periods (several hours) of heating with 70% acid can also effect total oxidation but in general it has been found that nitric acid is most useful to begin the oxidative process, this being completed through the use of sulphuric acid.

Samples were usually placed in 100 ml. beakers and heated on a hot-plate. After any of the above treatments, the samples were washed repeatedly with distilled water until acid was undetectable in the supernatant. Washing was effected by successive decantings and resuspensions, at least 6 hours being allowed for the diatoms to sediment between decantings. This time interval allows most of the smaller diatoms to settle out, although some are lost (see Andrews 1972): longer settling times were considered to be impractical. Occasionally, centrifugation was employed.

2.3.1.2 Final acid treatments

a. 1:1 98% sulphuric acid : 70% nitric acid.

It was found that complete oxidation of calcium-free samples could be achieved using a boiling mixture of sulphuric and nitric

acids (see Lund 1945, Round 1953, 1955, etc.). With highly organic samples, however, oxidation was slow, many hours being required to complete the process.

b. 98% sulphuric acid + sodium nitrate or potassium dichromate.

Samples were boiled with conc. sulphuric acid for up to 30 min. and then crystals of either sodium nitrate (Hendey 1964), or potassium dichromate (Patrick & Reimer 1966), added until the supernatant became straw-coloured or orange respectively. After this, the samples were boiled for a further period (30-90 min.) until it appeared that all organic matter had been destroyed, when the sediment was white. Nitrate was preferred to dichromate since insoluble chromium salts were formed on a couple of occasions.

Following various experiments using the treatments outlined above, a * standard method was adopted, which is applicable to most samples. The time allowed for each stage varies with such factors as the amount of calcium carbonate present, particle size, organic matter content, etc. The method is as follows:

1. Place sample in a 100 ml. beaker and wash 2-3 times with distilled water. After the final washing, decant off as much as possible of the supernatant.
2. Slowly add approx. 30 ml. of cool, fuming nitric acid. (Caution: if there is much calcium carbonate present or if the sample is highly organic, the reaction may be vigorous).
3. When the reaction has subsided somewhat, place beaker on a (pre-heated) hot-plate and boil the sample until most of the acid has evaporated or until oxidation is apparently complete.
4. Wash to neutrality with distilled water.
5. After the final washing, decant off as much as possible of the supernatant and carefully add approx. 30 ml. concentrated (98%) sulphuric acid. Place beaker on a (pre-heated) hot-plate and boil the sample for 15-30 min.
6. Add small amounts of sodium nitrate (crystals) until the colour of the supernatant changes from black/brown to a light straw colour.
7. Boil further until the sediment appears white or for not less than 30 min. If the sediment is still not white after 90 min.,

remove from heat, examine sample microscopically (after washing!) and, if necessary, repeat process from step 5.

8. Wash to neutrality with distilled water.

9. Store samples under approx. 50% ethanol.

The fine silica plates closing the poroids of many raphid pennate diatoms are usually left intact at the end of this procedure, though, if the sample is composed almost wholly of diatoms, care must be taken that steps 3. and 7. are not prolonged unduly. Frustules are frequently left intact, allowing study of the cincture in situ.

2.3.2 Dry method

Some samples, after washing with distilled water to remove soluble salts, were heated on cover-slips to 550°C in a muffle furnace and maintained at this temperature for 20 min. (see Zoto et al.,1973). The carbon is thereby burnt off and the cover-slips may be mounted on slides in the usual way (see elsewhere). This method is simple and has a certain value in ecological studies, especially perhaps where the colonisation of glass slides is under investigation (see Patrick & Reimer 1966: the 'diatometer'), but frustules cleaned by this method tend to be distorted and appear less 'clean' than those prepared using 'wet methods'. Moreover, the frustules remain intact and this makes interpretation of their morphology difficult.

2.4 Ultrasonication

This study did not involve the preparation or examination of thin sections of fixed and embedded cells. It was found, however, that with regard to the siliceous elements of the cells, very satisfactory substitutes for these could be obtained through the use of an MSE Ultrasonic Disintegrator.

Thick aqueous suspensions of cleaned diatoms were subjected to short periods (usually less than 5 min.) of ultrasonication, which

proved sufficient to fracture most of the valves or frustules. Differential sedimentation was then employed to separate the larger fragments (which were retained) from the smaller. Because of the particular valve constructions found in most Nitzschiaceae and Epithemiaceae (and, indeed, in most pennate diatoms), there is a tendency for the valves of these diatoms to fracture along transapical planes, with the result that transapical 'sections' are often obtained by sonication, which may be studied with the SEM.

'Fracture-sections' have the advantage that they are easy to obtain and relatively easy to interpret. Conventional microtome thin sections, on the other hand, require much preparation and are often difficult to interpret, especially since the silica often shatters during sectioning, (see Dawson 1972). The latter have the advantage, however, that they may be examined with the TEM, which has a greater resolution than the SEM.

2.5 Microscopy

2.5.1 Light microscopy

For light microscope examination, small aliquots taken from a thoroughly mixed aqueous suspension of a cleaned sample were transferred to, and dried down onto, round cover-slips (18 mm. diameter, No. 0). The cover-slips were then mounted on slides using Naphrax (RI 1.74).

Initially, a Gillett & Sibert 'Conference' microscope was used, with x10 and x15 eyepieces and achromatic 4 mm. (NA 0.74) and 2 mm. (NA 1.3, oil immersion) objectives. It was found, however, that the illumination system of this microscope (Köhler type) did not allow the observation of the striae in, for example, the more delicate members of Nitzschia sect. Lanceolatae. Therefore, later an old Zeiss microscope was employed which did not, unlike the Gillett & Sibert,

have a built-in light source. The exterior, variable light source used with the Zeiss could be adjusted so as to produce a beam of light in which the rays were all parallel, i.e. with the lamp filament at the principal focus of the condensing lens, such as is necessary for the development of the Abbé-type of critical illumination (Allen 1940). Alternatively, by movement of the condenser lens relative to the filament, it was possible to focus an image of the filament in the plane of the microscope substage-condenser diaphragm, so that the Köhler modification of critical illumination could also be employed. The Abbé system was found to be superior for the finest work, whereas the Köhler system was used for those observations, e.g. of chromatophores, for which even illumination over the whole field and greater contrast were considered more desirable than the highest resolution.

The substage-condenser (NA 1.4) was separately mounted from the diaphragm; the latter could be decentred relative to the former when oblique illumination was required (for the observation of striae in finely-structured or lightly-silicified forms).

The objectives were each mounted on a 'sledge', those used commonly being the following:

a. 4.2 mm. (approx. x40) NA 0.65
b. 1.8 mm. (approx. x100) NA 0.90
} dry lenses

c. 3 mm. apochromat (approx. x 80) NA 1.3
d. 1.5 mm. apochromat (approx. x120) NA 1.3
} immersion lenses

Objectives b. and c. were employed mainly for observations of live diatoms, or where some factor (e.g. cover-slip thickness) precluded the use of the 1.5 mm. lens.

Various eyepieces were used in conjunction with the above, namely x10 and x15 'achromatics' and three compensating eyepieces, which complete the colour correction of the apochromatic objectives (see Allen 1940), which magnified approx. x10, x18 and x25, (in Zeiss

notation,'Kompens-Okular' 6, 12 and 18, respectively).

In order to develop the full aperture of the immersion objectives, it was necessary to oil the front lens of the condenser to the back of the microscope slide. This procedure was carried out only when absolutely necessary!

Diatoms were drawn with the aid of a Zeiss camera lucida attachment; an inclined drawing board was unnecessary since the microscope had a vertical, monocular tube.

Occasionally, a Zeiss photomicroscope was used, this being fitted with phase contrast and bright field systems. Photomicrographs were taken using the immersion objectives, (the condenser being immersed where the highest resolution was required), on Pan F film.

2.5.2 Scanning electron microscopy

Cleaned or uncleaned material was washed with distilled water and then dried down onto aluminium stubs. In order to prevent charging under the electron beam, it is necessary to coat specimens with thin layers of conducting material. At first, gold/palladium was used for this, the alloy being evaporated from a point source 10 cm. distant from the stub, under high vacuum. The stubs were rotated and tilted with respect to the point source in order that the Au/Pd coating might be as even in thickness and as complete as possible. Later, a sputter-coater was employed. This used a gold target, coating taking place under a low vacuum in an 'atmosphere' of argon. The conducting films of gold thus obtained proved more satisfactory than the Au/Pd coatings (as judged by the frequency of charging under the electron beam). Sputter-coating is also simple and quick. Damblon (1975), who worked on pollen grains, claimed that sputter-coated specimens allowed better resolution of detail, but in the present study other factors (e.g. accelerating voltage, condenser settings) were found to be more

critical in limiting resolution.

Stubs were examined using a Cambridge Mk. IVa Stereoscan at an accelerating voltage of 20 kV (rarely 10 or 30 kV). Photographs were taken on Ilford FP 4 film. The angle between the incident beam and the stub surface was usually 45^{o}, this being found, on average, to give the best signal : noise ratio.

2.5.3 Transmission electron microscopy

Cleaned material was washed several times with distilled water before being dried down (in a desiccator) onto formvar-coated, hexagonal-meshed grids. At first, the formvar was stabilised with carbon, but this was later found to be unnecessary.

The grids were viewed with an AEI EM 6G at an accelerating voltage of 60 kV and specimens photographed on Du Pont Cronar Cos-7 film.

2.5.4 The usefulness of various microscopical techniques in diatom studies

With the increasing use of electron microscopy, by diatom systematists and others, to elucidate details of frustule architecture, it has become necessary that an assessment should be made of the relative values of the various microscopical techniques now commonly available to the diatomist. More especially, it is pertinent to consider how the advent of the electron microscope has changed the worth of the light microscope with regard to the different branches of 'diatomology' - ecology, systematics, morphology etc.

Three factors may be distinguished which limit the usefulness of any microscopical system, namely -

its resolution

its practicality

& the 'visibility', by which is meant the detectability of the detail potentially resolvable by the system.

The theoretical resolution of the light microscope is given by the formula

$$d = \frac{0.612\lambda}{n \sin\alpha}$$

where λ is the wavelength of the light used.

n is the refractive index of the medium between objective and object.

α is half the total angular aperture of the front lens of the objective.

d is the minimum distance between two points which can be recognised as separate, (Robards 1970).

The quantity ($n \sin\alpha$) is termed the Numerical Aperture of the objective.

It is clear from the formula that, in order to increase the resolution (i.e. decrease d), λ must be decreased and/or the Numerical Aperture increased. Accordingly, for the finest LM work, blue light, which should be used in conjunction with apochromatic objectives since achromatics are corrected for use with green light, or ultra-violet must be used. Neither of these is very 'practical', however: blue light is unpleasant to work with, while ultra-violet requires the use of quartz lenses. Indeed, only a few studies of diatoms have been made with ultra-violet, the most notable being Köhler's (1929/30) investigation of valve structure in Pinnularia nobilis. At the same time, the Numerical Aperture must be raised as high as possible, to 1.3 or higher: in a successful attempt to resolve the striae of Nitzschia singalensis, in which he counted 115,200 striae to the inch (45 in 10 µm.), Merlin (1916) used a lens of NA 1.42. It was by the optimisation of the various factors controlling the resolution of their instruments that such workers as Müller (e.g. 1898) and Hustedt (e.g. 1928a, b, 1929) were able to make their extremely detailed observations of diatom structure which remained unsurpassed and largely unconfirmable until the recent introduction of the electron microscope.

It should be stressed, however, that the theoretical limit of resolution of the light microscope is only 200 nm. (using blue light) and that the actual resolution achieved is rarely above 250 nm. Two other matters should also be mentioned. Firstly, silica is transparent to visible light, which is both advantageous in that it is possible, for example, to study the internal structure of a living cell, and disadvantageous in that the images obtained through the use of bright field optics tend to be without contrast, making difficult the observation of fine, delicate structures: this problem may, however, be partially overcome through the use of phase contrast or Nomarski interference optics, although with the latter the resolution appears to be rather poor (unpubl. obs.). Secondly, the light microscope is simple to operate and the associated preparation techniques also fairly straightforward.

As is well known, the resolution of the TEM is far greater than that of the light microscope. This is because the wavelength associated with the moving electrons, (the value of λ being dependent upon the electron velocity and hence upon the accelerating voltage), is very much smaller than that of visible light, allowing a resolution of less than 0.5 nm. (Robards 1970). Silica is opaque to electrons and thus diatom valves, girdle bands, etc., appear in the TEM as silhouettes, little useful being discernible, therefore, except the velum or hymen structure (see 'Terminology') and the arrangement of the areolae. The SEM is more versatile, although its resolution is somewhat lower because of the difficulty of reducing the final beam diameter to less than 7 nm.; 'it is generally assumed that topographical features smaller than the beam cannot be resolved by the SEM' (Black 1974) and so 7 nm. is also the maximum resolution of this instrument. During the present study, the resolution achieved was probably never better than 20 nm.

Since cleaned diatom frustules consist almost entirely of hydrated

amorphous silica (Lewin 1962), then providing that the layer of coating metal is adequate to prevent charging and of relatively uniform thickness, specimen contrast in the SEM must be due solely to the topography of the specimen surface; (for discussion of the factors affecting e-emission, see Black 1974). Thus, for photographic purposes, it is often necessary to adjust the SEM settings to give greater contrast than is required during studies of most other biological material. The robust nature of the diatom valve also has the consequence that higher accelerating voltages may be employed and, therefore, potentially higher resolution achieved than is common in biological work. A compromise has to be reached, however, where resolution, depth of focus and specimen penetration are all at acceptable levels, (see Black 1974). In the present study, accelerating voltages of more than 20 kV gave rise to an unacceptable amount of specimen penetration, while voltages of less than 20 kV gave inadequate resolution.

The resolution of the SEM is insufficient to allow the study of all aspects of frustule structure. Thus, for instance, the hymen pores of Nitzschia species, each of which is approx. 5 nm. in diameter, are unresolvable with the SEM. Moreover, 'visibility' may often be limiting, especially in those species (e.g. of Nitzschia sect. Dubiae, Amphiprora, Tropidoneis etc.) where, because of the acute angling of the valve at the raphe, the escape of secondary electrons from the inside surface of the valve is effectively prevented. In these cases it is necessary to examine 'fracture-sections' (see 2.4) in order to determine fully the valve structure.

By the use of a combination of EM techniques, it is possible to determine wholly the morphology of the diatom frustule. Most features may be observed with the SEM, leaving the details of pore structure to be determined through TEM observations. It is rarely necessary to resort to the use of carbon replicas. The smallest diatoms and those

with lightly-silicified valves must be studied with the TEM alone, (see the studies of Cylindrotheca, the Chaetoceraceae and the Rhizosoleniaceae by Reimann & Lewin 1964, Hasle 1975 and Evensen & Hasle 1975, respectively).

For the study of diatom intracellular fine structure, the standard TEM techniques of sectioning and freeze-etching remain without substitute, (see for example, Crawford 1973, Manton et al. 1969a, b, 1970a, b, Pickett-Heaps et al. 1975, Tippit & Pickett-Heaps 1977, etc.).

What place then, if any, is there for the light microscope in contemporary diatom studies? There can be no doubt that it has little to contribute to morphological investigations of the frustule, except in so far as the transparency of silica to visible light may enable the determination of the spatial interrelationships of various frustule components, or in mensuration, where the light microscope may be preferred to the SEM because of its easier calibration. A pure exercise in the morphology of the frustule should not, therefore, be undertaken without the aid of the electron microscope. This said, it ought to be pointed out that a 'pure exercise in morphology' is a rare thing, since morphological studies are usually made as parts of wider investigations into systematics, phylogeny, etc.

With regard to studies of intracellular structure, the light microscope has a larger role to play, for while it is obvious that the structure of the chromatophores, nuclei, golgi apparatus, mitochondria, etc., cannot be apprehended fully without the use of TEM techniques, yet the disposition and numbers of these organelles are much more easily determinable from light microscope observations. The intracellular location of many biochemical compounds may be established by the use of various stains while for the observation of living cells and especially of processes such as cell division, auxospore formation

or cell locomotion, the light microscope is as yet without peer , (see, for example, Von Stosch et al. 1973, Geitler 1975a).

The development of a natural classification, i.e. one based on overall resemblance and, therefore, upon comparisons in as many characters as possible (Davis & Heywood 1964), in so far as it is dependent upon a knowledge of morphology and anatomy, is another field in which the light microscope has only limited application. But this is only one part of systematics: diatoms must be identifiable during ecological or physiological investigations and here light microscopy is essential. It would be extremely difficult, for instance, to gain accurate estimates of species abundance in a sample through the use of standard TEM techniques alone; random distributions of diatoms over formvar-coated grids would be almost impossible to obtain because of the non-planar nature of the film, surface tension effects, etc. With SEM techniques, the distributional problems could probably be overcome, (through sedimentation of diatoms onto small cover-slips and the subsequent transfer of these to stubs), but unless the sample was almost purely of diatoms, these being well separated from one another on the cover-slip and distributed evenly with no tendency to 'clump', then some diatoms would inevitably remain unseen and/or unidentifiable. Thus, although in the development of a natural classification of diatoms, the use of the electron microscope is now virtually obligatory, nevertheless taxa must as far as possible be identifiable using the light microscope. It is, therefore, necessary that the diatom frustule should remain the object of research using the most critical light microscopy. There is, of course, no a priore reason why species may not be separated on the basis of characters which are not determinable with this instrument, either because they are unresolvable or because they are non-visual. So far, however, we have no examples of such cryptic forms.

In summary, the advent of the electron microscope has not altered the need for the light microscope to be used to its full potential in studies of living and cleaned diatoms. There has been a change, however, in the use to which the information derived from such studies is put. Certainly, electron microscopy is essential in any investigation designed to elucidate frustule morphology or diatom classification.